ELSEVIER

Journal of Chromatography A, 667 (1994) 113–118

JOURNAL OF
CHROMATOGRAPHY A

Liquid chromatographic determination of carnitine by precolumn derivatization with pyrene-1-carbonyl cyanide

Kunihiro Kamata*, Misako Takahashi, Kiyosi Terasima, Motohiro Nishijima

Tokyo Metropolitan Research Laboratory of Public Health, 24-1 Hyakunincho, 3-chome, Shinjuku-ku, Tokyo 169, Japan

(First received August 2nd, 1993; revised manuscript received December 24th, 1993)

Abstract

A method is described for the determination of carnitine chloride in pharmaceutical preparations by precolumn derivatization and high-performance liquid chromatography (HPLC). Carnitine chloride is derivatized by reaction with pyrene-1-carbonyl cyanide (PCC) in dimethyl sulphoxide. The PCC derivative of carnitine is then used for quantitative HPLC analysis on a cation-exchange column with fluorescence detection. The calibration graph was linear over a sample concentration range from 2.0 to 20 μg/ml; the limit of detection was 0.5 μg/ml under the conditions studied. The proposed method was applied satisfactorily to the determination of carnitine chloride in pharmaceutical preparations.

1. Introduction

Carnitine [β-hydroxy-γ-trimethylaminobutyric acid] is one the most active biological substances known, being a mitochondrial fatty acid acyltransferase cofactor. Enzymatic and radioenzymatic methods are generally, used to determine carnitine in biological samples [1–6]. Carnitine has also been determined by various spectrophotometric methods based on the binding of its quaternary ammonium functionality and anionic chromophores such as periodide [7] and bromophenol blue [8,9].

It has recently been demonstrated that high-performance liquid chromatography (HPLC) is suitable for the determination of carnitine, and numerous applications of this technique to pharmaceutical preparations have been reported [10–12]. Carnitine is difficult to determine by conventional HPLC techniques because it does not have an adequate UV chromophore and must be measured with refractive index or low-wavelength UV detection. Neither of these detection methods is very sensitive or selective. Another method is based on the formation of UV-absorbing or fluorescent esters of carnitine and their determination by direct UV spectrophotometry or spectrofluorimetry, respectively [11,13–15]. The most appropriate method of determining carnitine is fluorimetry owing to its selectivity and sensitivity. Precolumn labelling with a fluorophore usually involves the carboxyl group of carnitine. Yoshida *et al.* [11] described a method using 9-anthryldiazomethane for the determination of carnitine by HPLC with fluorimetric detection. This procedure, however, has problems with the stability of the reagent.

In this paper, we report the precolumn re-

* Corresponding author.

0021-9673/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(93)E0078-9

action and fluorimetric detection of carnitine with pyrene-1-carbonyl cyanide and separation from the sample constituents by ion-exchange HPLC. The proposed method was applied to a profile analysis of carnitine in pharmaceutical preparations.

2. Experimental

2.1. Reagents and standards

DL-Carnitine chloride was obtained from Sigma (St. Louis, MO, USA). A stock standard solution was prepared by dissolving carnitine in 2% ammonia solution to give a concentration of 100 μg/ml. Working standard solutions were prepared by diluting the stock standard solution with 2% ammonia solution.

Pyrene-1-carbonyl cyanide (PCC) (used as a reagent for fluorescence labelling) was purchased from Wako (Osaka, Japan) and triamterene (used as an internal standard) from Sigma. The fluorescent reagent was prepared by dissolving PCC in dimethyl sulphoxide (DMSO) at a concentration of 0.2 mg/ml. PCC is stable in DMSO, but the solution was prepared freshly approximately every 3 weeks.

A stock standard solution of the internal standard was prepared by dissolving triamterene in DMSO to give a concentration of 5 mg/ml. A working standard solution was prepared by diluting the stock standard solution in acetonitrile to give a concentration of 50 μg/ml.

Amberlite CG-120 (100–200 mesh, Na^+ form) cation-exchange resin was purchased from Fluka (Buchs, Switzerland). Acetonitrile, $(NH_4)_2HPO_4$, DMSO, ammonia solution and phosphoric acid were obtained from Nacalai Tesque (Kyoto, Japan). All reagents and solvents were of analytical-reagent grade. Water was purified with a Milli-Q II water purification unit (Nihon Millipore, Tokyo, Japan).

2.2. Derivatization

A 0.5-ml volume of carnitine solution (2.0–20 μg/ml) and 0.5 ml of internal standard solution were placed in a 5-ml reaction vial and the solvent was evaporated to dryness at 50°C under reduced pressure in a rotary evaporator. To the residue, 1 ml of a DMSO solution of PCC (200 μg/ml) was added and the reaction vial was loosely stoppered and heated for 30 min at 80°C. The reaction solution was then analysed by HPLC under the described conditions.

A calibration graph for carnitine of peak-height ratio of carnitine to the internal standard *versus* concentration was established over a sample concentration range of 2.0–20 μg/ml. All standard solutions were analysed in duplicate to construct the calibration graph.

2.3. Chromatography

The HPLC apparatus consisted of a JASCO Model BIP-1 pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne (Berkeley, CA, USA) Model 7125 injector equipped with a 1-μl loop, a JASCO Model 860-CO column oven, a JASCO Model 821-FP fluorescence detector set at excitation and emission wavelengths of 355 and 420 nm, respectively, and a Chromatopac CR-3A digital integrator (Shimadzu, Kyoto, Japan). The HPLC column used was a TSKgel SP-2SW (Tosoh, Tokyo, Japan) (250 × 4.6 mm I.D.; 5 μm). The mobile phase was acetonitrile–0.01 *M* $(NH_4)_2HPO_4$ (adjusted to pH 7.5 with phosphoric acid) (25:75, v/v). Before use it was filtered through a Millipore (Bedford, MA, USA) membrane filter (0.45 μm) followed by degassing using sonication under vacuum. The eluent was pumped at a flow-rate of 1.0 ml/min at a column oven temperature at 40°C.

2.4. Sample preparation

A suitable amount of the carnitine preparation was placed in a 50-ml volumetric flask and about 40 ml of water were added. If the dosage form was tablet composites, a representative number of tablets (usually 20) were accurately weighed and finely powdered. After sonication for 20 min, the flask was cooled and the solution was made up to volume. The mixture was then centrifuged at 2000 rpm (1300 *g*) for 10 min. The

final carnitine concentration was 25–250 μg/ml. Exactly 2.0 ml of the sample solution was transferred into a 15 × 1 cm I.D. column of cation-exchange resin (2 g) and the column was washed with 25 ml of water, discarding the washings. The receiver was changed to a 25-ml volumetric flask, carnitine was eluted with 20 ml of 2% ammonia solution and the eluate was made up to volume with water. A 0.5-ml volume of this solution was subjected to derivatization and HPLC as described above.

3. Results and discussion

Goto *et al.* [16] applied 1-anthroyl nitrile as a fluorescence labelling reagent, which is effective for secondary hydroxyl groups on bile acids. Kudoh *et al.* [17] also used 1-anthroyl nitrile as a fluorescence labelling reagent for lauryl alcohol ethoxylate and nonylphenol ethoxylates. We found that carnitine was also derivatized quantitatively into fluorescent compounds by 1-anthroyl nitrile. Three fluorescence labelling regent having a carbonyl nitrile group (9-anthronyl cyanide, 1-anthroyl nitrile and PCC) were tested for the determination of carnitine. The extent of each reaction product using these labelling regents was calculated from the fluorescence intensity. The following relative fluorescence intensities (%) were obtained: PCC, 100; 9-anthronyl cyanide, 10; 1-anthroyl nitrile, 18. Hence the best sensitivity was obtained with PCC. The reaction is probably as shown in Fig. 1.

The optimum conditions for the fluorescent derivatization of carnitine with PCC were determined. First, seven organic solvents (acetone, acetonitrile, DMSO, chloroform, hexane, tetrahydrofuran and N,N-dimethylformamide) were tried in order to determine the differences in the reaction system with each solvent. An aprotic solvent, DMSO, was selected as the solvent for the samples because it gave the highest fluorescence intensity.

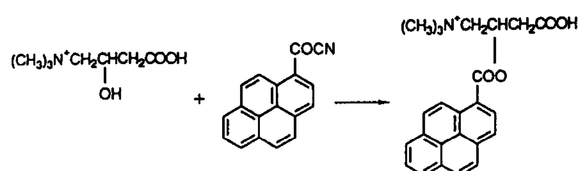

Fig. 1. Fluorescence derivatization of carnitine with PCC.

The effect of the PCC concentration was studied in the range 10–500 μg per 10 μg of carnitine. Constant peak heights were obtained above 100 μg of PCC. The amount of PCC chosen was 200 μg.

Fig. 2 shows the effects of reaction temperature and reaction time on the derivatization of carnitine with PCC. The peak heights of the PCC derivative of carnitine obtained increase in proportion to the increase in reaction temperature and time. As shown in Fig. 2, the optimum reaction temperature and time were adopted 80°C and 30 min, respectively.

Fig. 3 shows the yield of carnitine ester as a function of the water content in the reaction mixture. The yield of carnitine ester obtained decreased in proportion to the increase in water content. Therefore, the sample solution was evaporated to dryness before reaction.

The separation of carnitine has usually been performed by reversed-phase HPLC. The separation of the carnitine ester was therefore first examined using reversed-phase (C_{18}) HPLC. However, the retention time of carnitine ester was very close to that of impurities in the PCC

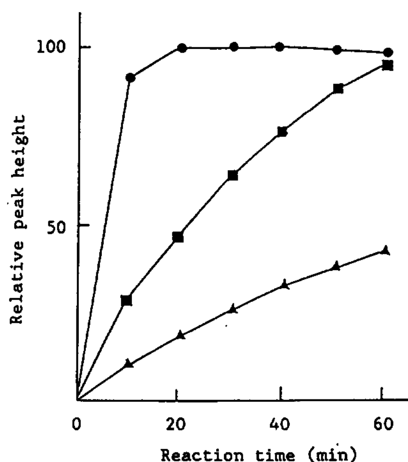

Fig. 2. Effect of reaction time and reaction temperature on the reaction yield of carnitine–PCC ester. ● = 80°C; ■ = 60°C; ▲ = 40°C.

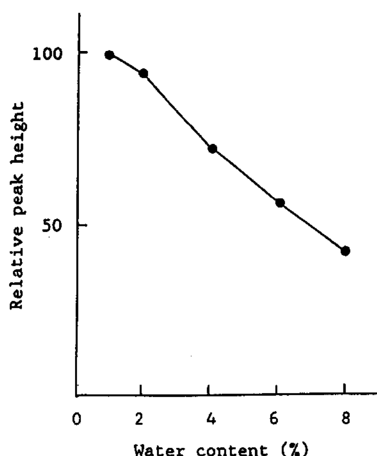

Fig. 3. Effect of water content in the reaction mixture on the reaction yield of carnitine–PCC ester.

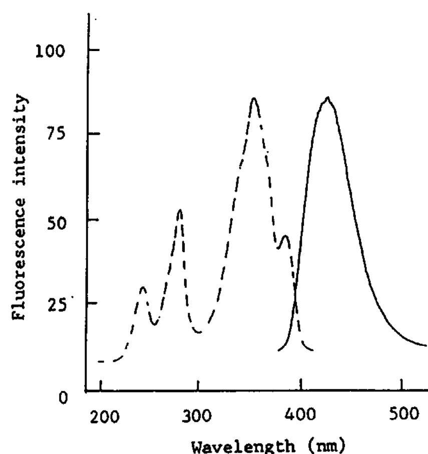

Fig. 4. Fluorescence excitation (solid line) and emission (dashed line) spectra of carnitine–PCC ester.

derivatives. In this study, the carnitine ester was separated by cation-exchange HPLC with a mobile phase consisting of acetonitrile and $(NH_4)_2HPO_4$ solution, because its structure containing quaternary ammonium ion. The composition of the mobile phase (percentage of water, salt concentration and pH) had a strong influence on the separation of the carnitine ester. The optimum mobile phase composition was chosen for each condition to give the best separation of the carnitine ester from impurities in the PCC derivatives. The influence of acetonitrile concentration, $(NH_4)_2HPO_4$ concentration and mobile phase pH on the chromatographic characteristics of carnitine ester was systematically examined. The retention time increased with decreasing concentration of acetonitrile and with increasing pH and was not sensitive to the concentration of $(NH_4)_2HPO_4$. The optimum concentration of $(NH_4)_2HPO_4$ for separating carnitine ester was 0.01 *M*. From the results, the solvent system finally chosen was acetonitrile–0.01 *M* $(NH_4)_2HPO_4$ (adjusted to pH 7.5 with phosphoric acid) (25:75, v/v).

When carnitine ester was eluted in the flow-through cell of the detector, the flow-rate of the mobile phase was stopped and both the excitation and emission wavelengths were measured (Fig. 4). The maximum excitation and emission wavelengths were found to be at 355 and 420 nm, respectively.

Fig. 5 shows chromatograms of (A) a derivatized standard solution and (B) the reagent blank. The carnitine ester peak was clearly separated from the impurities in the PCC derivatives.

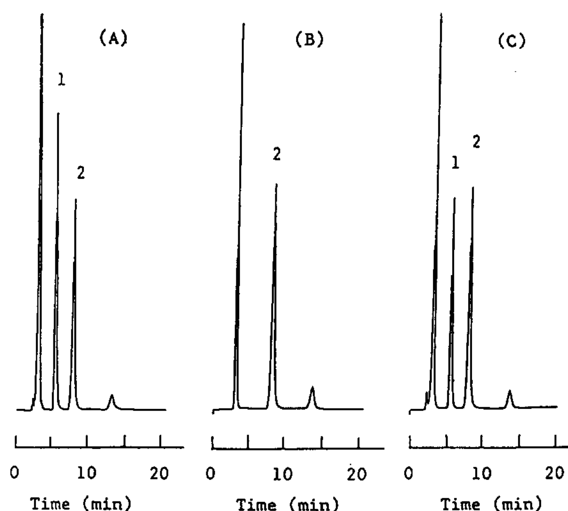

Fig. 5. Chromatograms of (A) a derivatized standard solution, (B) a reagent blank and (C) a derivatized commercial tablet sample. Peaks: 1 = carnitine–PCC ester; 2 = triamterene (internal standard).

The calibration graph of carnitine chloride to triamterene peak-height ratio *versus* sample concentration was found to be linear over the sample concentration range 2.0–20 μg/ml. The linear regression coefficient was 0.999, the slope was 0.199 and the intercept on the ordinate was −0.015. The reproducibility of this procedure was also adequate; the relative standard deviation (R.S.D.) for 10 μg/ml of carnitine chloride was 1.12% ($n = 5$). The detection limit of carnitine chloride under the conditions adopted was about 0.5 μg/ml with a 1-μl injection (signal-to-noise ratio = 3).

The determination of carnitine chloride in pharmaceutical dosage forms is markedly influenced by the clean-up procedure employed. For this purpose Amberlite CG-120 resin was chosen, based on previous work [12]. Several sources of interferences in the reaction of PCC such as water-soluble vitamins, crude extract and sugar were eliminated by the column clean-up procedure. The results obtained with the clean-up of pharmaceutical dosage forms were satisfactory with respect to reproducibility and recovery.

The proposed procedure was applied to the determination of carnitine chloride in pharmaceutical preparations. Table 1 reports the results obtained in the analysis of commercial samples of carnitine chloride and Fig. 5C shows a chromatogram of the PCC derivative of carnitine from a commercial tablet sample. Neither the derivatization nor the chromatographic separation was influenced by other compounds; for example, the major components contained in these samples are water-soluble vitamins (vitamins B_1, B_2 and B_6, nicotinamide and pantothenol), caffine, ethanol, inositol, sucrose, glucuronolactone and crude extract (gentian, ginseng, glycyrrhiza, cinnamom bark and coptis rhizome). The results indicate that, in each instance, the values are within acceptable limits, with a minimum recovery of 93.4%. The R.S.D. % is within 3.54% ($n = 5$). The chromatograms of all the other samples also showed a sharp peak for carnitine ester with no interference from other substances. The results indicate that this method is suitable for the determination of carnitine chloride in pharmaceutical preparations.

This work has shown that PCC is a very promising fluorescent reagent for determining carnitine chloride in commercial drugs and should be applicable for the analysis of other biological samples with low concentrations of carnitine chloride. Studies are in progress on the determination of carnitine chloride in human urine.

Table 1
Determination of carnitine chloride in commercial preparations

Sample	Declared	Found (%)[a]	R.S.D. (%)
A (tablet)	40 mg per 3 tablets	98.7 ± 2.0	2.02
B (capsule)	50 mg per capsule	93.4 ± 2.1	2.20
C (syrup)	100 mg/ml	101.4 ± 3.6	3.54
D (drink)	100 mg/ml	96.9 ± 3.3	3.36
E (drink)	80 mg/30 ml	95.1 ± 2.9	3.04

[a] Each value is the mean ±S.D. of five measurements.

4. References

[1] N.R. Marquis and I.B. Fritz, *J. Lipid Res.*, 5 (1964) 184.
[2] J.D. McGarry and D.W. Foster, *J. Lipid Res.*, 17 (1976) 277.
[3] E.P. Brass and C.L. Hoppel, *J. Biol. Chem.*, 253 (1978) 2688.
[4] A. Daveluy, R. Parvin and S.V. Pande, *Anal. Biochem.*, 119 (1982) 286.
[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] J.S. Wall, D.D. Christianson, R.J. Dimler and F.R. Senti, *Anal. Chem.*, 32 (1960) 870.
[8] S. Friedman, *Arch. Biochem. Biophys.*, 75 (1958) 24.
[9] M.A. Mehhlman and G. Wolf, *Arch. Biochem. Biophys.*, 98 (1962) 146.
[10] A. Fujita and R. Yamamoto, *Katei Kenkyuu*, 7 (1988) 40.
[11] T. Yoshida, A. Aetake and H. Yamaguchi, *J. Chromatogr.*, 445 (1988) 175.
[12] M. Takahashi, K. Nakayama, S. Uehahara, K. Kamata, T. Hagiwara and K. Akiyama, *Annu. Rep. Tokyo Metrop. Res. Lab. Public Health*, 41 (1990) 51.

[13] P.E. Minkler, S.T. Ingalls, S. Kormos, D.E. Weir and C.L. Hoppel, *J. Chromatogr.*, 336 (1984) 271.
[14] J. Gorham, *J. Chromatogr.*, 361 (1986) 301.
[15] P.E. Minkler, S.T. Ingalls and C.L. Hoppel, *J. Chromatogr.*, 420 (1987) 385.
[16] J. Goto, M. Saito, T. Chikai, N. Goto and N. Nambara, *J. Chromatogr.*, 276 (1983) 289.
[17] M. Kudoh, H. Ozawa, S. Fudano and K. Tsuji, *J. Chromatogr.*, 287 (1984) 337.