

HPLC Determination of Carnitine and Acylcarnitines in Human Plasma by Means of Fluorescence Labeling Using 2-(4-Hydrazinocarbonylphenyl)-4,5-diphenylimidazole

Naotaka KURODA,* Yuko OHYAMA, Kyoko NAKASHIMA, Kenichiro NAKASHIMA, and Shuzo AKIYAMA

School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852, Japan.

Received February 19, 1996; accepted April 12, 1996

Carnitine and acylcarnitines are important substances involved in the oxidation and metabolism of fatty acids. An HPLC method is presented for the quantitative analysis of these compounds. The method is based on the detection of fluorescent derivatives of carnitine and short- and medium-chain acylcarnitines labeled with 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI). The labeling of carnitine and acylcarnitines with HCPI was performed at room temperature for 1 h using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as a condensing reagent. The analytes prepared using cation-exchange cartridges were separated on an octadecylsilyl silica gel (ODS) column by a gradient elution system of acetonitrile/Tris-HCl buffer and determined using a synthetic internal standard. Fluorescence detection was performed at 475 nm with excitation at 340 nm. The detection limits for carnitine, acetyl-, propionyl-, hexanoyl- and octanoylcarnitine ranged from 0.24 to 1.97 nmol per ml human plasma, at a signal-to-noise ratio of 3. The within-day and between-day precision of the assay for carnitine and acylcarnitines in plasma samples had relative standard deviations (RSDs) lower than 10.3%. The concentration of free carnitine, acylcarnitines and total carnitine in human plasma could be successfully determined by the proposed method.

Key words fluorescent derivatization reagent; carnitine; acylcarnitine; human plasma; HPLC

Carnitine [3-hydroxy-4-(trimethylammonio)butanoate] is known to be essential for the mitochondrial β -oxidation of long-chain fatty acids.^{1,2)} The concentration and distribution of carnitine and its esterified derivatives (acylcarnitines) give information on the metabolism of organic acids. A sensitive, specific and reliable assay method for the determination of these is, therefore, important for clinical and biochemical investigations of metabolic disorders involving organic acids.

Several analytical procedures for determining carnitine and acylcarnitines in biological fluids have been developed including radiochemical-enzymatic assay,³⁾ FAB-MS,^{4,5)} GC-MS,⁶⁾ HPLC based on measuring coenzyme A esters released by enzymatically,⁷⁾ and HPLC with spectrometric^{8,9)} and fluorometric¹⁰⁾ detection after labeling with appropriate reagents. Among them, the radiochemical method has inherent disadvantages associated with disposal and safety, and the methods using MS require rather expensive and complex equipment. HPLC avoids these problems and, thus, is convenient for use in clinical laboratories.

The simultaneous determination of carnitine and individual acylcarnitine in urine was successfully performed by HPLC with spectrometric and fluorometric detection using 4'-bromophenacyl trifluoromethanesulfonate⁹⁾ and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone¹⁰⁾ as a labeling reagent, respectively, but fewer methods have been reported using plasma samples which are often more easily obtainable than urine samples in clinical laboratories.⁸⁾

The objective of this work was to develop a sensitive, reliable and selective HPLC method for the determination of carnitine and acylcarnitines in plasma using fluorescence detection. We have shown that 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI) is a useful fluo-

rescent derivatization reagent for carboxylic acids in HPLC.^{11,12)} In the present work, this reagent has successfully been applied to the HPLC assay of free carnitine, short- and medium-chain acylcarnitines, and total carnitine in human plasma. Figure 1 shows the scheme for the derivatization of carnitine and acylcarnitines with HCPI.

Experimental

Reagents D,L-Carnitine hydrochloride, D,L-acetylcarnitine hydrochloride, D,L-hexanoylcarnitine hydrochloride and D,L-octanoylcarnitine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). D,L-Propionylcarnitine hydrochloride and D,L-cyclohexanoylcarnitine hydrochloride, as an internal standard (IS), were synthesized as described in the literature.¹³⁾ The yield of D,L-cyclohexanoylcarnitine hydrochloride was 64%; mp 159–160 °C. *Anal.* Calcd for C₁₄H₂₆ClNO₄: C, 54.63; H, 8.51; Cl, 11.52; N, 4.55. Found: C, 54.14; H, 8.28; Cl, 11.18; N, 4.58. Carnitine and acylcarnitines were dissolved in methanol to obtain

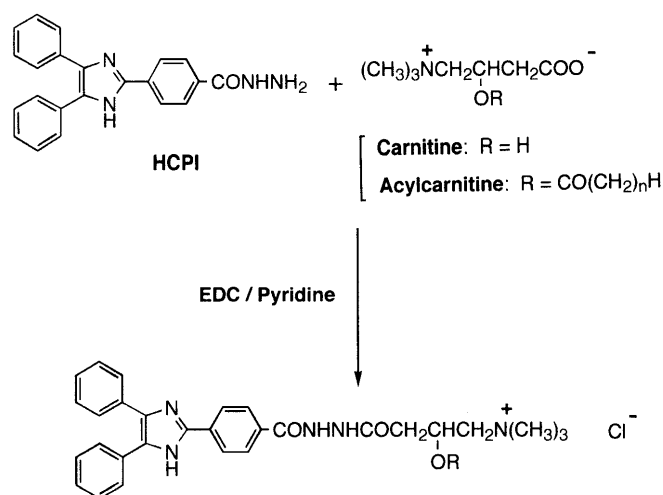


Fig. 1. Reaction Scheme of HCPI with Carnitine and Acylcarnitines

* To whom correspondence should be addressed.

appropriate concentrations for working solutions. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), used as a condensing reagent, was purchased from Nacalai Tesque (Kyoto, Japan). HCPI was prepared as described in a previous paper.¹¹⁾ The HCPI derivative with carnitine (HCPI-carnitine) was synthesized as follows: to 30 ml ethanol containing 2% pyridine and 1.5% *N,N*-dimethylformamide (DMF) were added HCPI (0.20 g, 0.56 mmol), EDC (0.11 g, 0.56 mmol) and D,L-carnitine hydrochloride (0.11 g, 0.56 mmol), stirred for 3 h at room temperature and then concentrated to *ca.* 5 ml with a rotary evaporator. The resultant material was treated with acetone at room temperature to give HCPI-carnitine chloride which was recrystallized from ethanol to give colorless crystals: yield, 48%; mp 263–264°C. *Anal.* Calcd for $C_{29}H_{32}ClN_5O_3 \cdot 1/4H_2O$: C, 64.68; H, 6.08; Cl, 6.58; N, 13.00. Found: C, 64.58; H, 6.14; Cl, 6.42; N, 12.70.

The cation-exchange cartridges used were LiChrolut SCX from Merck (Darmstadt, Germany) and Toyopak IC-SP S from Tosoh (Tokyo, Japan). Water was deionized and passed through a Pure Line WL21P water purification system (Yamato Kagaku, Tokyo) and the mobile phase was filtered through a membrane filter of 0.2- μ m pore size (Nihon Millipore, Tokyo) and degassed prior to use. All other reagents and solvents were of analytical grade.

Human blood samples were drawn from healthy volunteers in our department. Plasma specimens were obtained in the usual manner using EDTA and kept frozen at -20°C until use.

Apparatus Uncorrected fluorescence spectra and intensities were measured with a 650-10S fluorescence spectrophotometer (Hitachi, Tokyo) using 10-mm quartz cells with 10 nm bandwidths for both the excitation and emission monochromators.

The HPLC system consisted of two pumps (LC-6A, Shimadzu, Kyoto), a 7125 injector with a 20- μ l loop (Rheodyne, Cotati, CA, U.S.A.), a Daisopak SP-120-ODS column (250 \times 4.6 mm i.d., 5 mm, Daiso, Osaka, Japan), a Shimadzu RF-550 fluorescence spectrophotometer, a Hitachi 561 recorder and a Shimadzu SCL-6A system controller.

The centrifugal evaporator used was from Yamato Kagaku.

HPLC Conditions Gradient elution using solvent A (acetonitrile) and B [acetonitrile/200 mM Tris-HCl buffer (pH 7.0), 1:4, v/v] was used for the separation of HCPI derivatives with carnitine and acylcarnitines: elution was started at 25% A (75% B) from 0 to 12 min and changed linearly to 45% A (55% B) from 12 to 22 min. Then, solvent A was changed to 100% (0% B) from 22 to 27 min and maintained at 100% from 27 to 37 min. The flow-rate was set at 1 ml·min⁻¹ at ambient temperature and the eluate was monitored at an excitation wavelength of 340 nm and an emission wavelength of 475 nm.

Preparation of Plasma The procedure for the determination of carnitine and acylcarnitines was as follows: to 50 μ l plasma in a test tube were added 10 μ l cyclohexanoylcarnitine in water (4×10^{-4} M) as IS and 1 ml 25 mM NaH_2PO_4 solution (pH adjusted to 1.0 with phosphoric acid). After mixing, the mixture was applied to a column containing 100 mg LiChrolut SCX, which was prepared by washing with 2 ml water before use. The column was washed with 5 ml water, and then eluted with 1 ml 0.15 M aqueous pyridine-2-propanol (1:1, v/v). The eluate, in a screw-capped reaction vial (amber-colored glass, 3.5 ml; Pierce, Rockford, IL, U.S.A.), was evaporated using a centrifugal evaporator and the resultant residue was then subjected to fluorescence labeling.

For the determination of total carnitine, plasma was pretreated as follows: to 50 μ l plasma was added 10 μ l 1 M KOH and the mixture was allowed to stand at 37°C for 30 min. After the addition of NaH_2PO_4 solution (1 ml) and IS solution (10 μ l), the mixture was applied to the ion-exchange column and treated as described above.

A standard mixture (50 μ l) of carnitine and acylcarnitines was evaporated and reconstituted in plasma to obtain "spiked" plasma.

Derivatization Procedure for HPLC To the evaporated residue of the eluate from plasma 20 μ l each of 2.5 mM HCPI in DMF and 100 mM EDC in DMF were added followed by 10 μ l pyridine. After vortex-mixing, the mixture was allowed to stand at room temperature for 1 h and then 1 ml DMF-water (2:3, v/v) was added. To remove excess reagents, the resultant solution was applied to the Toyopak IC-SP S cartridge prewashed with 2 ml water. The cartridge was then washed successively with 1 ml DMF-water (2:3, v/v), 10 ml water and 100 μ l 50% methanol in 0.6 M KCl. Elution was carried out with 200 μ l 50% methanol in 0.6 M KCl, and the resultant solution was injected into the HPLC.

To investigate the derivatization conditions, 50 μ l of a standard mixture of carnitine (50 nmol·ml⁻¹) and acylcarnitines (25 nmol·ml⁻¹ each) was evaporated and used as a sample.

Results and Discussion

Fluorescence Properties of HCPI-Carnitine The fluorescence characteristics of HCPI derivatives were examined using HCPI-carnitine as a representative derivative. Fluorescence excitation maxima (λ_{ex}), emission maxima (λ_{em}) and relative fluorescence intensities (RFI) at various ratios of acetonitrile/200 mM Tris-HCl buffer (pH 7.0) mobile phase are shown in Table 1. Both the excitation and emission maxima of HCPI-carnitine were red-shifted compared with HCPI. The emission maxima for both HCPI and HCPI-carnitine had a tendency to red-shift with increasing buffer content. The RFI for HCPI-carnitine showed a maximum in 75% acetonitrile and then decreased with increasing buffer content. Excitation at 340 nm and emission at 475 nm were selected as the monitoring wavelengths by considering the practical solvent ratio for the separation of HCPI derivatives on HPLC.

Fluorescence Derivatization The effect of the concentration of HCPI was examined over the range 0.5–5.0 mM; 2.5 mM gave a maximum peak height for each derivative and, thus, was selected for further investigation. As shown in Fig. 2, EDC gave maximum and constant peak heights at 100–150 mM; 100 mM was chosen as optimum. The effect of the concentration of pyridine, which acted as a catalyst, on the reaction yield was examined using DMF containing 5, 25, 50, 75 and 100% (v/v) pyridine. Almost maximum and constant peak heights were observed with 50% pyridine or more; 10 μ l pyridine was used.

The water content of the reaction mixture affected a reaction yields. The yields decreased with increasing water content. For instance, a *ca.* 30% decrease in peak height was observed for acetylcarnitine when there was 20%

Table 1. Fluorescence Properties of HCPI and HCPI-Carnitine

Solvent ratio (Acetonitrile/Tris-HCl buffer ^{a)})	HCPI			HCPI-carnitine		
	λ_{ex} (nm)	λ_{em} (nm)	RFI ^{b)}	λ_{ex} (nm)	λ_{em} (nm)	RFI
100/ 0	335	430	100	345	460	309
75/ 25	335	455	270	340	470	352
50/ 50	335	455	301	340	475	308
25/ 75	335	460	261	340	480	214
0/100	330	470	99	340	490	53

a) 200 mM Tris-HCl buffer (pH 7.0). b) RFI of HCPI in acetonitrile was arbitrary taken as 100.

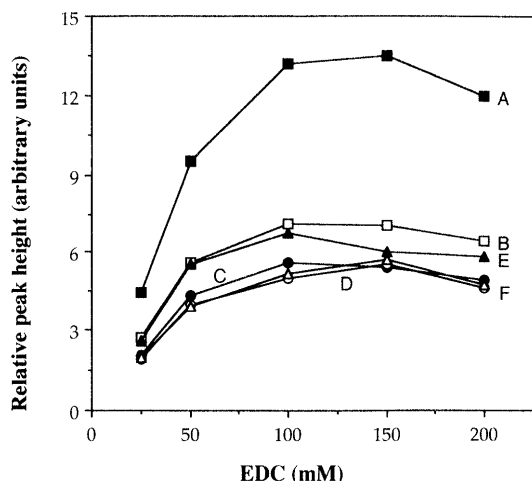


Fig. 2. Effect of EDC Concentration on the Fluorescence Derivatization of Carnitine and Acylcarnitines with HCPI

A 50- μ l aliquot of the standard mixture was treated as described in the procedure. Sample: A, carnitine; B, acetylcarnitine; C, propionylcarnitine; D, hexanoylcarnitine; E, octanoylcarnitine; F, cyclohexanoylcarnitine.

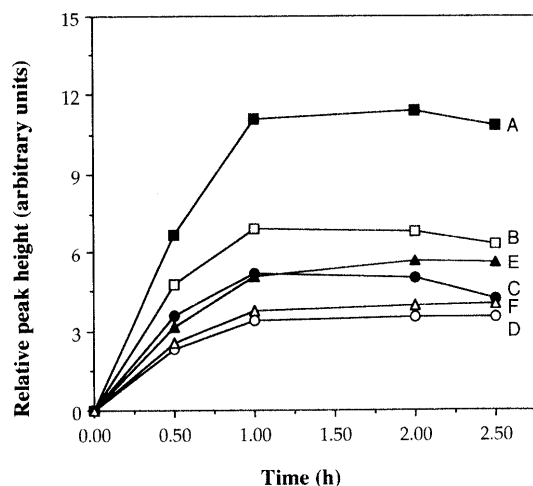


Fig. 3. Effect of Reaction Time on the Fluorescence Derivatization of Carnitine and Acylcarnitines with HCPI

A 50- μ l aliquot of the standard mixture was treated as described in the procedure. Sample: A, carnitine; B, acetylcarnitine; C, propionylcarnitine; D, hexanoylcarnitine; E, octanoylcarnitine; F, cyclohexanoylcarnitine.

water in the reaction mixture. Therefore, DMF dried with Molecular sieve 5A was employed as a solvent for dissolving EDC and HCPI.

The derivatization reaction proceeds at room temperature. As shown in Fig. 3, the reaction was almost complete in 1 h under the conditions used; 1 h was selected as a convenient reaction time. The reaction yield for carnitine, calculated by comparing the peak height with that of authentic HCPI-carnitine, was 84%.

HPLC-Fluorescence Detection of HCPI Derivatives of Carnitine and Acylcarnitines When the reaction mixture of the standard solution with HCPI was injected into the HPLC, without the extraction step involving a Toyopak IC-SP S cartridge, large peaks resulting from the reagents interfered with the determination of HCPI-propionylcarnitine. On the other hand, pretreatment with the cartridge effectively reduced these reagent peaks. The HCPI derivatives retained on the cartridge were successfully eluted with 50% methanol in 0.6 M KCl; 200 μ l was

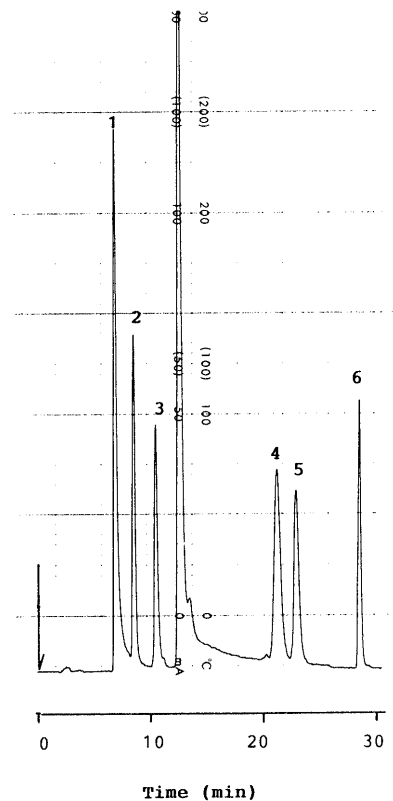


Fig. 4. Chromatogram of HCPI Derivatives of Carnitine and Acylcarnitines

A 50- μ l aliquot of the standard mixture was treated as described in the procedure. Peak: 1, carnitine; 2, acetylcarnitine; 3, propionylcarnitine; 4, IS; 5, hexanoylcarnitine; 6, octanoylcarnitine.

collected and used for HPLC analysis.

As shown in Fig. 4, the separation of HCPI derivatives of carnitine, four acylcarnitines and IS was achieved in 30 min with a reversed-phase column, Daisopak SP-120-ODS, using the gradient elution described in the Experimental section. Retention times for HCPI derivatives of carnitine, acetyl-, propionyl-, hexanoyl-, and octanoylcarnitine were 7.3, 8.7, 10.6, 22.8 and 28.0 min, respectively.

A linear relationship between the concentration of carnitine, acylcarnitines and peak height was observed over the range 5×10^{-8} – 5×10^{-5} M (50 pmol \cdot ml $^{-1}$ –50 nmol \cdot ml $^{-1}$; $r \geq 0.996$; $n=7$ in each). About 35 pmol \cdot ml $^{-1}$ carnitine and acylcarnitines could be detected at a signal-to-noise ratio of 3. The RSD of the peak heights for five replicate measurements of carnitine and acylcarnitines (25 nmol \cdot ml $^{-1}$ each) ranged from 4.7 to 7.8%.

Determination of Carnitine and Acylcarnitines in Plasma The cation-exchange column, LiChrolut SCX was used for the extraction of carnitine and acylcarnitines from plasma. Without this procedure, the peak corresponding to propionylcarnitine unfortunately overlapped with peaks from plasma components; the extraction step was carried out as described in the literature.¹⁰⁾ Although the Toyopak IC-SP S cartridge used for standard solutions gave almost the same results as LiChrolut SCX, the latter was chosen.

The conditions for the hydrolysis of acylcarnitines were examined to determine total carnitine using different concentrations of potassium hydroxide at various tem-

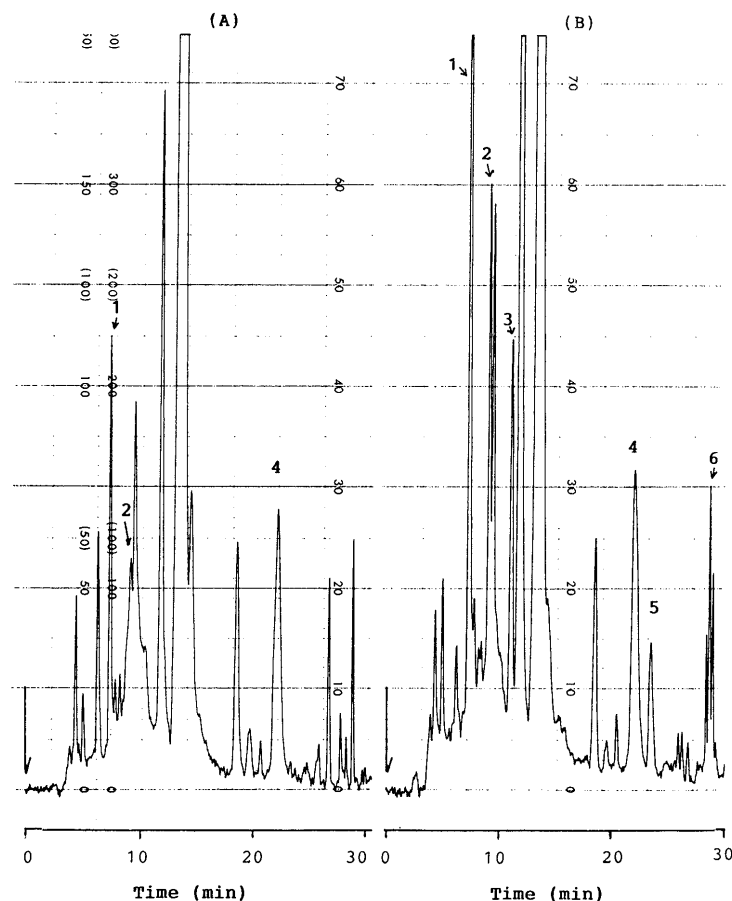


Fig. 5. Representative Chromatograms Obtained with (A) Normal Human Plasma and (B) Plasma Spiked with Carnitine and Acylcarnitines ($25 \text{ nmol} \cdot \text{ml}^{-1}$ Each)

A $50\text{-}\mu\text{l}$ aliquot of a plasma sample was treated as described in the procedure. Peak: 1, carnitine; 2, acetylcarnitine; 3, propionylcarnitine; 4, IS; 5, hexanoylcarnitine; 6, octanoylcarnitine.

Table 2. Concentrations of Free Carnitine, Acylcarnitines and Total Carnitine in Normal Human Plasma

Age	Sex	Concentration (nmol/ml)			
		Free carnitine	Acetylcarnitine	Propionylcarnitine	Total carnitine
47	M	34.74	5.23	1.10	46.74
36	M	31.52	6.61	1.04	44.57
25	M	39.04	ND	ND	42.40
24	F	35.18	5.53	1.51	43.53
23	F	35.74	2.74	ND	39.68
22	F	34.28	6.57	0.82	50.46

ND: not detected.

peratures and reaction times. As a result, hydrolysis with 1.0 M KOH at 37°C for 30 min was chosen as optimum.

Figure 5 shows typical chromatograms obtained with normal human plasma and plasma spiked with carnitine and acylcarnitines. Favorable separation of HCPI-derivatives was achieved without interference from other plasma components, with the exception of the peak for acetylcarnitine.

Working curves were prepared using spiked plasma with known concentrations of carnitine and acylcarnitines. The peak-height ratio of each HCPI-derivative to IS ($4 \times 10^{-4} \text{ M}$) was linear from 25 to $100 \text{ nmol} \cdot \text{ml}^{-1}$ ($r = 0.996$ – 0.998 , $n = 3$).

The detection limit for carnitine, acetyl-, propionyl-,

hexanoyl- and octanoylcarnitine was 0.24, 0.49, 0.80, 1.97 and $1.21 \text{ nmol per ml plasma}$, respectively, at a signal-to-noise ratio of 3.

The recovery (mean value of duplicate assay) of HCPI-carnitine and acylcarnitines from spiked plasma ($25 \text{ nmol} \cdot \text{ml}^{-1}$ each) was 107.5% (carnitine), 89.1% (acetylcarnitine), 90.5% (propionylcarnitine), 79.5% (hexanoylcarnitine) and 99.2% (octanoylcarnitine). The precision was established by repeated assays using plasma spiked with $25 \text{ nmol} \cdot \text{ml}^{-1}$ each of carnitine and acylcarnitines. The precision obtained for carnitine, acetyl-, propionyl-, hexanoyl- and octanoylcarnitine was 10.3, 8.6, 5.6, 4.5, 3.6% (within-day, $n = 4$), and 7.6, 10.3, 2.8, 3.0, 10.2% (between-day, $n = 3$), respectively, expressed as RSDs of

the peak-height ratio.

The results of the determination of free carnitine, acylcarnitine and total carnitine in plasma from healthy volunteers are summarized in Table 2. Hexanoylcarnitine and octanoylcarnitine were not detected in the plasma samples using the proposed method. These results in human plasma were in good agreement with those obtained by other workers.^{3,9,14)}

Conclusions

This study provides a sensitive and reliable HPLC method for the fluorescence detection of carnitine, short- and medium-chain acylcarnitines in blood plasma. This method might be applicable to long-chain acylcarnitines with minor modification of the HPLC elution profile and plasma sample size. The proposed method has adequate sensitivity for determining carnitine and acylcarnitines in only 50 μ l plasma. This sample size is 2–10 times smaller than that reported by others.^{3,9)} The fluorescence derivatization reaction of carnitine and acylcarnitines with HCPI gives intensely fluorescent derivatives under mild conditions; this may have the advantage of preventing racemization during the derivatization of enantiomers of carnitine and acylcarnitines. In conclusion, the proposed

method should be useful for clinical and biochemical studies of carnitine and its esters in plasma samples.

References

- 1) Bremer J., *J. Biol. Chem.*, **237**, 3628–3632 (1962).
- 2) Fritz I. B., Yue K. T. N., *J. Lipid Res.*, **4**, 279–288 (1963).
- 3) Rossle C., Kohse K. P., Franz H. -E., Furst P., *Clin. Chim. Acta*, **149**, 263–268 (1985).
- 4) Montgomery J. A., Mamer O. A., *Anal. Biochem.*, **176**, 85–95 (1989).
- 5) Millington D. S., Norwood D. L., Kodo N., Roe C. R., Inoue F., *Anal. Biochem.*, **180**, 331–339 (1989).
- 6) Huang Z.-H., Gage D. A., Bieber L. L., Sweeley C. C., *Anal. Biochem.*, **199**, 98–105 (1991).
- 7) Dugan R. E., Schmidt M. J., Hoganson G. E., Steele J., Gilles B. A., Shug A. L., *Anal. Biochem.*, **160**, 275–280 (1987).
- 8) Minkler P. E., Hoppel C. L., *J. Chromatogr.*, **613**, 203–221 (1993).
- 9) Minkler P. E., Hoppel C. L., *Anal. Biochem.*, **212**, 510–518 (1993).
- 10) Kamimori H., Hamashima Y., Konishi M., *Anal. Biochem.*, **218**, 417–424 (1994).
- 11) Nakashima K., Taguchi Y., Kuroda N., Akiyama S., *J. Chromatogr.*, **619**, 1–8 (1993).
- 12) Kuroda N., Taguchi Y., Nakashima K., Akiyama S., *Anal. Sci.*, **11**, 989–993 (1995).
- 13) Böhmer T., Bremer J., *Biochim. Biophys. Acta*, **152**, 559–567 (1968).
- 14) Penn D., Schmidt-Sommerfeld E., *Metabolism*, **32**, 806–809 (1983).