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Determination of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine in human plasma by high-performance liquid chromatography after pre-column derivatization with 1-aminoanthracene

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Abstract

A new sensitive high-performance liquid chromatographic procedure for the determination of L-carnitine (LC), acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC) in human plasma has been developed. Precolumn derivatization with 1-aminoanthracene (1AA), performed in phosphate buffer in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) as catalyst, is involved. The fluorescent derivatives were isocratically separated on a reversed-phase column (C_{18}). The eluate was monitored with a fluorimetric detector set at 248 nm (excitation wavelength) and 418 nm (emission wavelength). Because of the presence of endogenous carnitines, the validation was performed using dialyzed plasma. The identity of the derivatized compounds was assessed by mass spectrometry and the purity of the chromatographic peaks was confirmed by HPLC–tandem mass spectrometry. The limits of quantitation were 5 nmol/ml for LC, 1 nmol/ml for ALC and 0.25 nmol/ml for PLC. The recovery of the extraction procedure was in the range 82.6%–95.4% for all 3 compounds. Good linearity ($R \approx 0.99$) was observed within the calibration ranges studied: 5–160 nmol/ml for LC, 1–32 nmol/ml for ALC and 0.25–8 nmol/ml for PLC. Precision was in the range 0.3–16.8% and accuracy was always lower than 10.6%.

Keywords: Carnitine; Acetylcarnitine; Propionylcarnitine; 1-Aminoanthracene

1. Introduction

L-Carnitine is a highly polar compound, widely distributed in nature. It plays an important role in oxidative metabolism and ketogenesis [1] as a cofactor for the transfer of long chain acyl groups from acyl-CoA derivatives across the inner mitochondrial membrane [2,3].

Within the body L-carnitine (LC) is in equilibrium with its acyl esters, among which acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC) are of particular interest. In fact, these short chain esters are

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under investigation as therapeutic agents for neurological (ALC) and cardiovascular (PLC) diseases, as shown in a growing number of pharmacological and clinical reports [4–10].

A large number of published methods for the analysis of LC and its acetyl-ester in biological fluids involve the use of high-performance liquid chromatography (HPLC) with ultraviolet absorbance [11–13] fluorimetric [14] and fast-atom bombardment tandem mass spectrometry (FAB-MS–MS) [15] detection. Conversely, PLC, because of its low concentration in biological fluids, could only be quantitated by continuous-flow FAB-MS–MS [15]. Other HPLC–fluorimetric methods have been established for carnitine [16–18] and acyl-carnitines [18] in standard and pharmaceutical preparations, but they are not suitable for the quantitation of these compounds in biological fluids.

We now describe a new sensitive method for the simultaneous determination of LC, ALC and PLC in human plasma. The method is based upon the synthesis of a fluorescent derivative of the carnitines, suitable for HPLC analysis. Two internal standards were used: methansulfonyl-L-carnitine, at high concentration, for LC and ALC, and isobutyryl-L-carnitine at low concentration, for PLC. Plasma is purified by solid-phase extraction prior to derivatization, which is catalyzed by a carbodiimide. The experimental conditions for the derivatization were optimized checking the effect of temperature and time on the reaction yield. Carbodiimides are widely used as activators of carboxylic groups in the synthesis of amides (Fig. 1).

The most interesting carbodiimide for our purpose is 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) [19,20]: in fact, like the carnitines, it is soluble in aqueous media, allowing the reaction to be carried out in a homogenous phase.

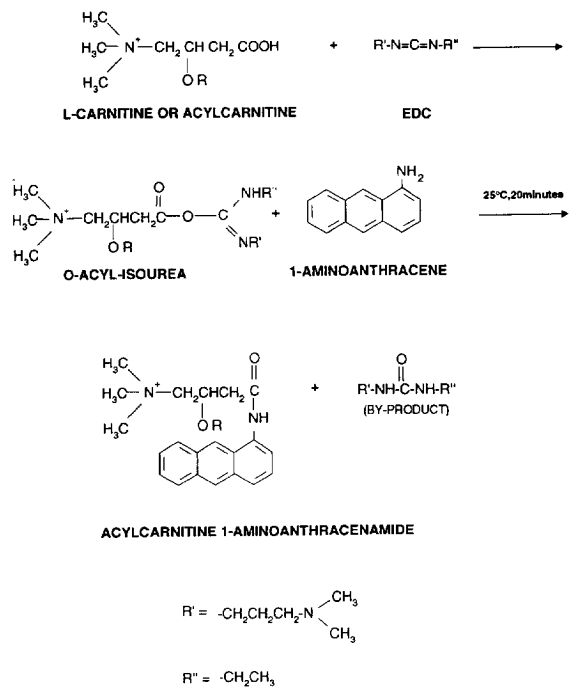

Fig. 1. Derivatization of acylcarnitines with 1AA using EDC as a catalyst.

2. Experimental

2.1. Chemicals

Isobutyryl-L-carnitine, methansulfonyl-L-carnitine, L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine (reference standards) were synthesized by Sigma-tau Industrie Farmaceutiche Riunite (Pomezia, Rome, Italy). The chemical structures of the above compounds are shown in Fig. 2.

1-Aminoanthracene (1AA) and EDC were purchased from Aldrich (Milan, Italy). Acetic acid, acetone, acetonitrile, ammonium acetate, diethyl ether, hexane, heptane, chloroform, methanol and 37% hydrochloric acid were purchased from Merck

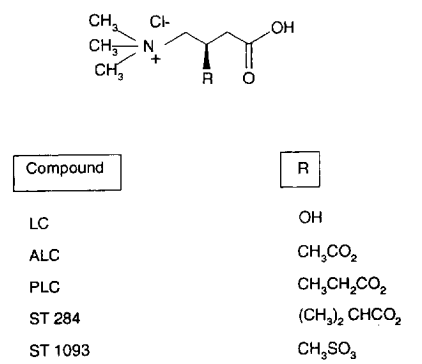

Fig. 2. Chemical structure of L-carnitine (LC), acetyl-L-carnitine (ALC), propionyl-L-carnitine (PLC), isobutyryl-L-carnitine (ST 284) and methansulfonyl-L-carnitine (ST 1093).

(Milan, Italy). Orthophosphoric acid, sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate were purchased from Carlo Erba (Milan, Italy).

2.2. Equipment

The chromatographic equipment used consisted of a Model PU980 solvent delivery pump, a Model 851-AS autosampler and a Model 821-B spectrofluorimeter photometer (Jasco, Hachioji, Tokyo, Japan). A JCL 6000 (Jasco) chromatographic data collection station was used. The mass spectrometer was an API III (PE Sciex, Toronto, Canada) equipped with an ion-spray interface.

2.3. Chromatographic conditions

The solid-phase extraction was performed on SAX-Isolute cartridges (100 mg) (Stepbio, Bologna, Italy). The HPLC mobile phase was prepared by mixing 700 ml of 0.1 *M* ammonium acetate pH 3.5 with 300 ml of acetonitrile; the solution was then filtered through 0.45-μm HV Millipore filters and degassed in an ultrasonic bath at room temperature for 10 min.

Chromatographic separation was performed at a flow-rate of 1.3 ml/min, using a Kromasil C_{18}, 250×4.6 mm I.D., 5 μm column (Saulentechnik Knauer, Berlin, Germany).

The excitation and emission wavelengths of the spectrofluorimeter were 248 and 418 nm respectively. The detector gain was set at 100. Mass spectrometry conditions were as follows: nebulizer gas pressure (air), 0.34 MPa; curtain gas flow (N_2), 1.8 l/min; collision gas thickness (argon), 400×10^{12} molecules/cm^2; ion-spray voltage, 5000 V; orifice voltage, 40 V.

2.4. Sample preparation

2.4.1. Dialysis of plasma

Human plasma was dialyzed using a membrane model Spectrapor MW CO:6-8000 dialyzer (Spectrum Medical Industries). The dialyzing buffer was a Krebs–Ringer phosphate solution containing: 78.1% of 0.154 *M* NaCl, 2.3% of 0.11 *M* $CaCl_2$–$2H_2O$, 3.1% of 0.154 *M* KCl, 0.8% of 0.153 *M* $MgSO_4$–$7H_2O$ and 15.6% of 0.1 *M* phosphate buffer pH 7.4. The plasma, closed in the membrane, was exposed to the buffer solution (1:25, v/v) for about 36 h at 4°C; during this period, the buffer solution was changed at least 3 times, at intervals of about 8–10 h.

2.4.2. Standard solutions

PLC stock solution was prepared dissolving 6.4 mg of the compound in 10 ml of water; LC and ALC stock solution was prepared dissolving 8.2 mg and 2.5 mg, respectively, in 100 ml of water: 1 ml of the PLC solution was added to the LC–ALC solution to obtain a mix solution containing 0.5 m*M* LC, 0.1 m*M* ALC and 0.025 m*M* PLC. This solution was stored at 4°C for 1 month. ST 284 stock solution was prepared dissolving 1.6 mg of the compound in 100 ml of water: this solution can be stored at 4°C for 1 week. ST 1093 stock solution was prepared dissolving 4.4 mg of the compound in 10 ml of water: this solution needs to be prepared daily. A mix solution of the 2 internal standards was prepared transferring 1 ml of both ST 284 and ST 1093 solutions into a 25 ml flask and diluting with water to volume.

2.4.3. Calibration curve

A six-point calibration curve was prepared by adding LC, ALC and PLC to dialyzed plasma to obtain the following concentrations: LC: 5, 10, 20, 40, 80 and 160 nmol/ml; ALC: 1, 2, 4, 8, 16 and 32 nmol/ml; PLC: 0.25, 0.5, 1, 2, 4 and 8 nmol/ml. Each calibration plasma sample was processed as described below.

2.4.4. Extraction procedure

To samples of 100 μl of plasma, containing the amounts of LC, ALC and PLC needed for the validation, 30 μl of the internal standards mix solution, corresponding to 0.075 nmol of ST 284 and 1.9 nmol of ST 1093, and 370 μl of water, were added. The samples were then loaded onto a SAX cartridge, previously conditioned with 0.5 ml of methanol and 1 ml of water. After elution, the cartridges were washed with 0.5 ml of 0.01 *M* phosphate buffer pH 3.5 and eluates were collected. The so-obtained samples were used for the derivatization reaction.

2.5. Derivatization

1AA (fluorescent reagent) was dissolved in acetone (16 mg/ml) and EDC in 0.01 *M* NaH_2PO_4–H_2O pH 3.5 (160 mg/ml). A 20-μl volume of 1 *M* HCl, 100 μl of the 1AA solution and 100 μl of the EDC solution (in 20 μl aliquots) were sequentially added to the sample with continuous vortex-mixing. The mixture was incubated at 25°C for 20 min and the excess reagent was removed by washing the sample with 5 ml of diethylether. A 300-μl aliquot of the aqueous phase was then transferred to a plastic tube, 700 μl of 0.01 *M* Na_2HPO_4–$2H_2O$ pH 9.1 was added to adjust the pH of the samples to about 7 and the mixture was washed with 5 ml of chloroform to eliminate the interference of amino acids. At this pH value the amides resulting from the reaction of amino acids with 1AA are highly soluble in chloroform, whereas the solubility of the derivatives of carnitines in chloroform is negligible. Aliquots (500 μl) of the final aqueous phase were diluted with 500 μl of 0.01 *M* NaH_2PO_4–H_2O pH 3.5 and 20 μl of this solution was injected into the HPLC system.

3. Results

3.1. Characterization of derivatized carnitines

The reaction of LC, ALC and PLC with 1AA gives rise to single compounds, with high fluorescence properties. The identity and structure of the 3 derivatized carnitines were confirmed using tandem mass spectrometry. The mass spectrum of each compound was obtained by injecting a 5 μmol/ml solution directly into the spectrometer, by means of an infusion pump. A single quadrupole acquisition was performed by scanning from 200 to 500 amu, with 1 amu steps and 10 ms dwell time. In this way a very simple spectrum was obtained, containing only the peak corresponding to the quasi-molecular ion (QMI) with mass $[M]^+$, since no fragmentation occurs under these conditions. The spectra of derivatized LC, ALC and PLC are shown in Fig. 3; the corresponding QMI were 337, 379 and 393 amu, respectively: all were in agreement with the expected values. With the same injection technique, the fragmentation spectrum of each of the above compounds was obtained by MS–MS mode: a daughter scan of each QMI was performed within the range 100–500 amu. The fragmentation spectra of each compound are shown in Fig. 4. The main fragment coming from LC weighed 278 amu, most probably formed through the loss of trimethylamine; the main fragment coming from ALC and PLC weighed 260 amu, possibly formed by the loss of trimethylamine and of the acyl group, followed by dehydration of the carnitine skeleton. A very simple scheme of these fragmentation pathways is shown in Fig. 5.

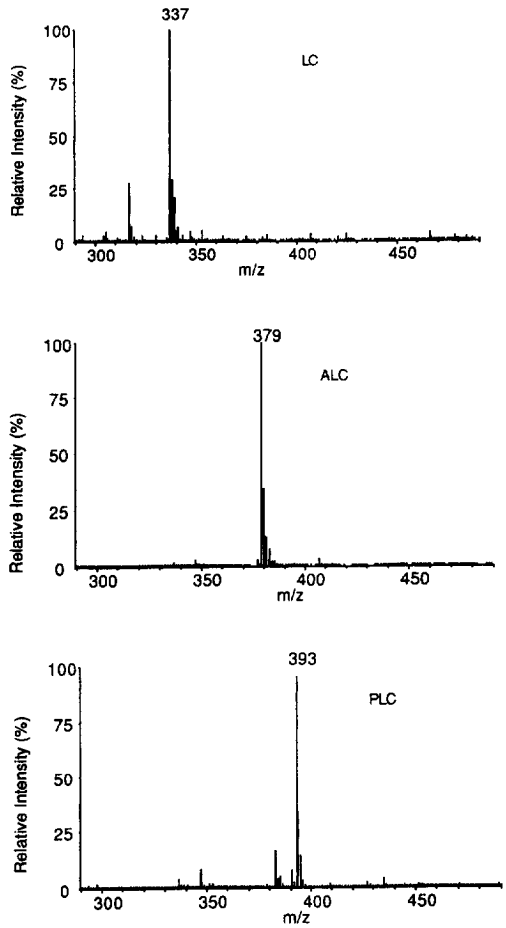

Fig. 3. Mass spectra of derivatized LC, ALC and PLC. The experimental conditions are described in the text.

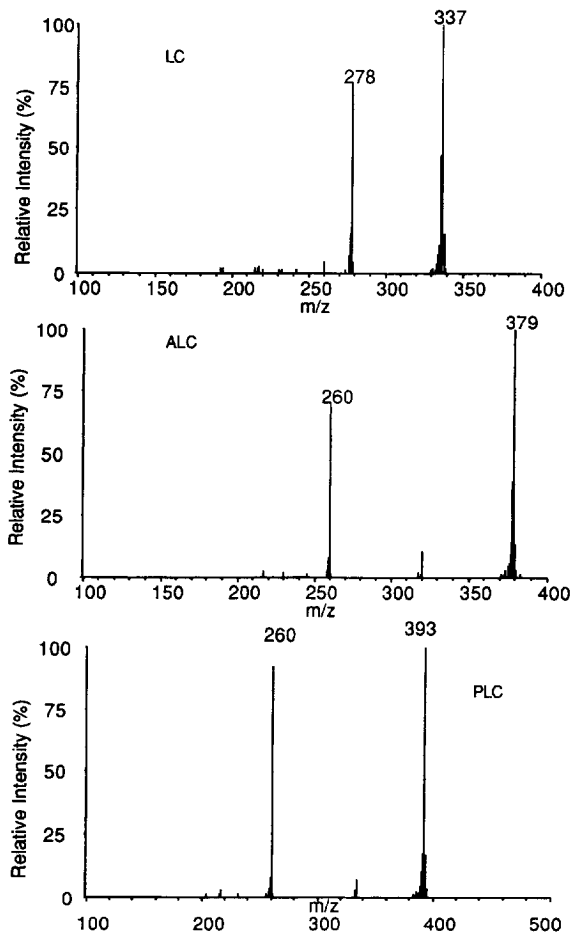

Fig. 4. Daughter scan spectra of derivatized LC, ALC and PLC. The experimental conditions are described in the text.

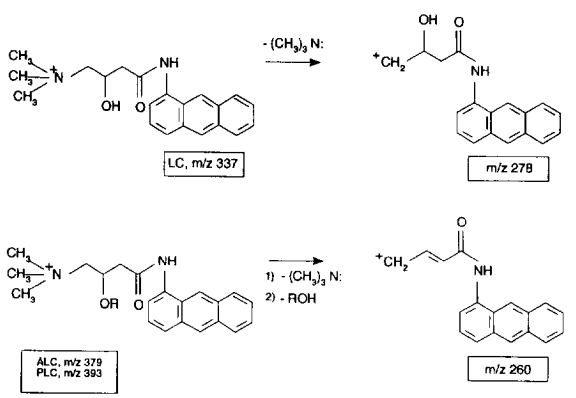

Fig. 5. Fragmentation pathway of derivatized LC, ALC and PLC.

3.2. Optimization of the derivatization procedure

The effect of temperature and time on the reaction yield was studied. Temperature within the range 25–55°C and reaction time within the range 0–60 min were checked. In Fig. 6 and Fig. 7 the results of temperature and time effect on PLC derivatization are reported; as shown, the highest yield is achieved at 25°C, in 20 min. The same study with LC and ALC gave similar results.

3.3. Selectivity

A typical chromatogram of a dialyzed plasma sample following extraction and derivatization is shown in Fig. 8; no interfering peaks were observed at the retention times corresponding to the compounds of interest. Fig. 9 shows a chromatogram of a

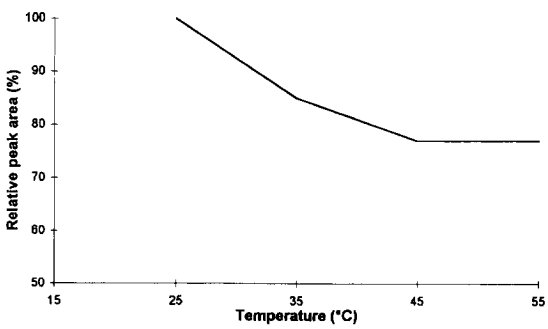

Fig. 6. Temperature effect on PLC derivatization.

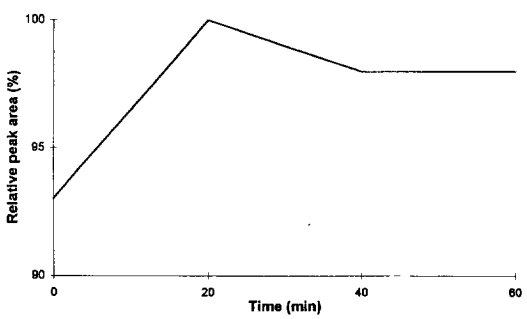

Fig. 7. Time effect on PLC derivatization.

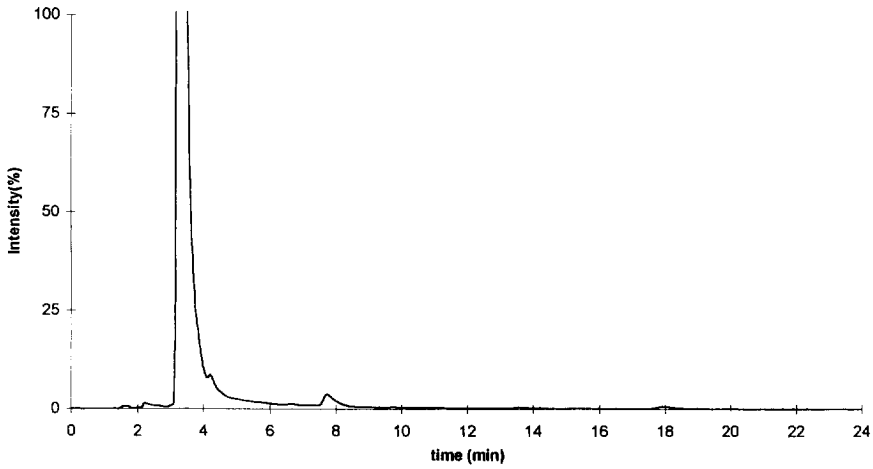

Fig. 8. Typical chromatogram of a blank dialyzed plasma sample.

dialyzed plasma sample spiked with LC (20 μ*M*), ALC (4 μ*M*), PLC (1 μ*M*), ST 1093 (19 μ*M*) and ST 284 (0.75 μ*M*).

3.4. Sensitivity

The limits of quantitation of the method are 5 nmol/ml for LC, 1 nmol/ml for ALC and 0.25 nmol/ml for PLC which correspond to the lowest points of the calibration curve for the 3 analytes; these concentrations are lower than the basal levels of LC, ALC and PLC in human plasma.

In Fig. 10, a chromatogram of basal samples coming from a pharmacokinetic study in healthy volunteers is reported; it contained 30 μ*M* LC, 1.1 μ*M* ALC and 0.4 μ*M* PLC.

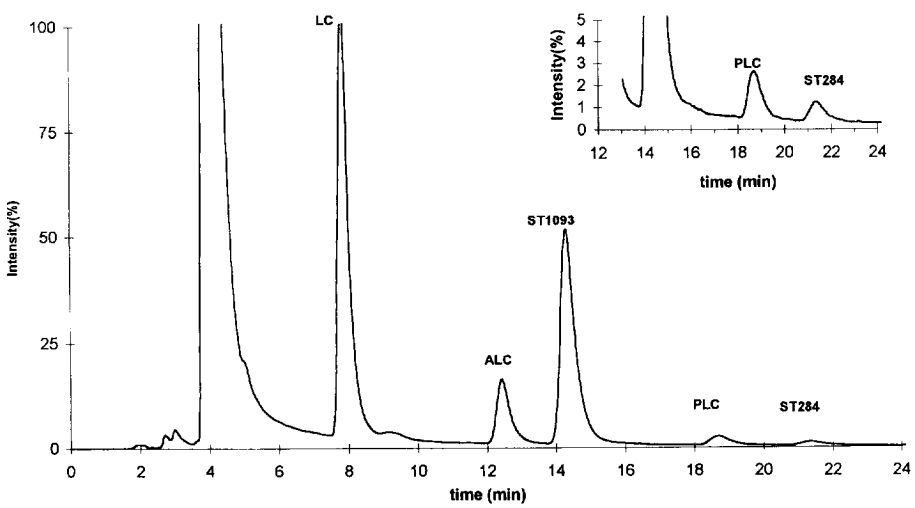

Fig. 9. Chromatogram of a dialyzed plasma sample spiked with LC (20 μ*M*), ALC (4 μ*M*), PLC (1 μ*M*), ST 1093 (19 μ*M*) and ST 284 (0.75 μ*M*).

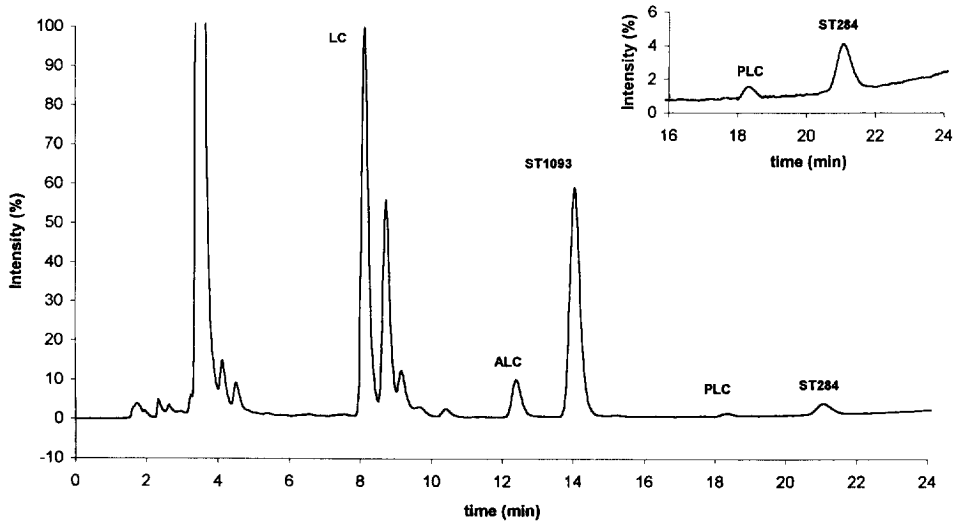

Fig. 10. Chromatogram of a basal plasma sample containing LC (30 μ*M*), ALC (1.1 μ*M*) and PLC (0.4 μ*M*).

3.5. Specificity

In order to assess the purity of the chromatographic peaks corresponding to LC, ALC and PLC three basal plasma samples were analyzed in four replicates both with HPLC–FL and with HPLC–MS–MS. The latter method is very specific and each compound can be assayed by monitoring one single fragment coming from its corresponding quasi-molecular ion. The results obtained with the two methods were compared and the Student's *t*-test was used to determine if there were any differences between methods (Table 1): as shown, there was no significant difference in the results obtained from the 2 methods ($p>0.05$).

Table 1
Specificity of the analytical method for LC, ALC and PLC

Sample	HPLC–FL		HPLC–MS–MS		*t*-Test
	Mean (nmol/ml)	S.D.	Mean (nmol/ml)	S.D.	
LC					
1	40.42	3.627	43.86	7.153	$p>0.05$
2	53.28	1.315	51.21	3.792	$p>0.05$
3	53.19	3.925	57.40	2.283	$p>0.05$
ALC					
1	2.70	0.299	3.04	0.044	$p>0.05$
2	2.06	0.354	2.48	0.046	$p>0.05$
3	3.15	0.467	3.09	0.045	$p>0.05$
PLC					
1	0.34	0.024	0.36	0.013	$p>0.05$
2	0.38	0.024	0.39	0.026	$p>0.05$
3	0.23	0.013	0.24	0.012	$p>0.05$

3.6. Linearity

The linearity of the assay was determined over the concentration ranges of 5–160 nmol/ml, 1–32 nmol/ml and 0.25–8 nmol/ml for LC, ALC and PLC, respectively, using the weighing factor $1/y$. Each calibration curve contained six concentrations for the three analytes. Calibration parameters obtained with four different curves were reported in Table 2. Good linear responses, expressed in terms of a square linear regression coefficient (r^2), and slope reproducibility, were observed for each compound.

3.7. Precision and accuracy

Intra-assay and inter-assay precision and accuracy were assessed by replicate determinations of the three analytes in dialyzed plasma on the same day and on different days, respectively, within the whole

Table 2
Reproducibility of four calibration curves

Compound	Slope (m)	Intercept (b)	r^2
L-Carnitine	0.112	0.031	0.9968
	0.113	0.022	0.9982
	0.118	0.269	0.9900
	0.159	0.538	0.9907
Mean	0.125	0.215	0.9939
S.D.	0.0225	0.2438	0.0042
C.V. (%)	18.0	–	0.4
Acetyl-L-carnitine	0.099	0.012	0.9975
	0.104	0.016	0.9974
	0.121	0.034	0.9949
	0.153	0.077	0.9895
Mean	0.119	0.035	0.9948
S.D.	0.0244	0.0297	0.0037
C.V. (%)	20.5	–	0.4
Propionyl-L-carnitine	2.005	0.079	0.9830
	1.745	0.124	0.9986
	2.085	−0.005	0.9946
	1.654	0.120	0.9862
Mean	1.872	0.579	0.9881
S.D.	0.2055	0.9439	0.0049
C.V. (%)	11.0	–	0.5

calibration range. The results obtained are shown in Table 3 and Table 4. Precision, expressed as C.V.%, was in the range 0.3–16.8%. Accuracy, expressed as error%, was always lower than 10.6%.

3.8. Extraction recovery

Recoveries of LC, ALC and PLC were assessed at the lowest and the highest concentrations of the calibration range; each sample was analyzed in six replicates and the results were compared with those obtained analysing standard aqueous samples containing the same concentrations of the analytes; the recovery ranged between 82.6% and 95.5%. In the same way, recoveries of ST 284 and ST 1093 at the concentrations of 0.75 nmol/ml and 19 nmol/ml, respectively, were assessed: they were 82.7% and 92.3%, respectively (Table 5).

3.9. Application

This method has been used to assay PLC, ALC and LC in plasma samples coming from a phar-

Table 3
Intra-day precision and accuracy

Compound	Theoretical concentration (nmol/ml)	Concentration found (mean±S.D.) (nmol/ml)	C.V. (%)	Accuracy (%)
L-Carnitine	5	4.96±0.03 (6)[a]	0.6	0.8
	10	9.65±0.03 (3)	0.3	3.5
	20	19.74±0.93 (6)	4.7	1.3
	40	43.62±1.35 (3)	3.1	9.1
	80	83.09±6.60 (3)	7.9	3.9
	160	158.05±9.57 (6)	6.1	1.2
Acetyl-L-carnitine	1	1.07±0.03 (6)	2.8	7.0
	2	1.91±0.01 (3)	0.5	4.5
	4	3.76±0.20 (6)	5.3	6.0
	8	8.00±0.09 (3)	1.1	0.0
	16	16.82±0.80 (3)	4.8	5.1
	32	32.10±3.07 (6)	9.6	0.3
Propionyl-L-carnitine	0.25	0.26±0.03 (6)	11.5	4.0
	0.5	0.55±0.02 (3)	3.6	10.0
	1	0.98±0.05 (6)	5.1	2.0
	2	1.94±0.08 (3)	4.1	3.0
	4	3.70±0.11 (3)	3.0	7.5
	8	8.21±0.15 (6)	1.8	2.6

[a] In brackets the replication numbers are indicated.

Table 4
Inter-day precision and accuracy

Compound	Theoretical concentration (nmol/ml)	Concentration found (mean±S.D.) (nmol/ml)	C.V. (%)	Accuracy (%)
L-Carnitine	5	4.47±0.75 (5)	16.8	10.6
	10	10.18±0.68 (5)	6.7	1.8
	20	20.91±1.24 (5)	5.9	4.6
	40	42.04±2.46 (5)	5.9	5.1
	80	81.69±3.05 (5)	3.7	2.1
	160	156.75±3.37 (5)	2.1	2.0
Acetyl-L-carnitine	1	0.94±0.12 (5)	12.8	6.0
	2	2.04±0.05 (5)	2.5	2.0
	4	4.04±0.16 (5)	4.0	1.0
	8	8.22±0.37 (5)	4.5	2.8
	16	16.85±0.43 (5)	2.6	5.3
	32	31.11±0.68 (5)	2.2	2.8
Propionyl-L-carnitine	0.25	0.26±0.03 (5)	11.5	4.0
	0.5	0.51±0.04 (5)	7.8	2.0
	1	1.00±0.06 (5)	6.0	0.0
	2	1.95±0.12 (5)	6.2	2.5
	4	3.98±0.08 (5)	2.0	0.5
	8	8.17±0.13 (5)	1.6	2.1

macokinetic study in healthy volunteers following single intravenous administrations of PLC at the doses 1, 2, 4 and 8 g over a 2-h infusion. The results, expressed as mean plasma concentration/time curves are reported in Fig. 11. This method allows us to analyze ca. 30 samples per day.

4. Discussion

This paper describes a new and sensitive HPLC method for the determination of carnitines in plasma, based upon the synthesis of fluorescent derivatives. It involves the activation of carnitine carboxylic groups with EDC and subsequent reaction with 1AA; thus LC, ALC, PLC and (potentially) other carnitines can be identified simultaneously. Each derivatized carnitine was characterized by tandem mass spectrometry. The derivatization yield was optimized in terms of temperature and reaction time. In order to eliminate endogenous carnitines without changing the matrix, dialyzed plasma was used for the validation. Plasma samples were cleaned by solid-phase extraction before the derivatization. The specificity of the chromatographic peaks was assessed by cross-validation with HPLC tandem mass spectrometry. The method can be widely used for the determinations of LC, ALC and PLC in human plasma. Moreover, in comparison with the previously published methods [11–15], our method offers many advantages: (a) a very simple and fast sample preparation allowing routine analysis of many samples per day; (b) the possibility to perform the reaction in an aqueous medium; (c) high sensitivity, allowing the quantitation of LC, ALC and PLC even at concentrations lower than the basal levels. The linearity, reproducibility, extraction recovery, selec-

Table 5
Extraction recovery of LC, ALC, PLC, ST 284 and ST 1093

Compound	Concentration (nmol/ml)	Recovery (mean±S.D.) (%)	C.V. (%)
LC	5	82.6±4.3 (6)	5.2
LC	160	84.8±2.9 (6)	3.4
ALC	1	93.3±8.8 (6)	9.4
ALC	32	95.5±3.0 (6)	3.1
PLC	0.25	91.0±10.0 (6)	10.9
PLC	8.0	85.3±2.0 (6)	2.3
ST 284	0.75	82.7±6.3 (6)	7.6
ST 1093	19	92.3±6.5 (6)	7.0

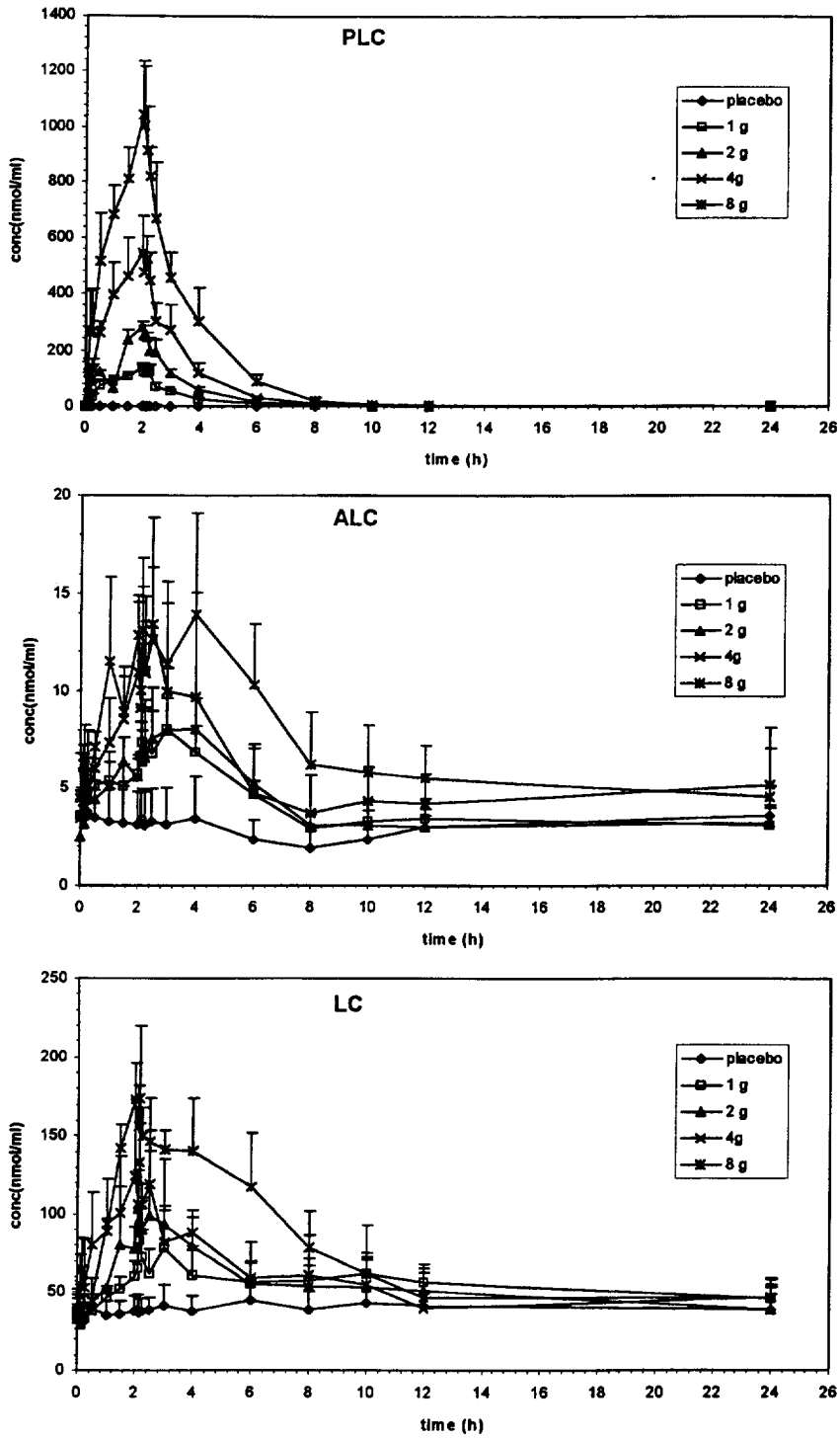

Fig. 11. Mean plasma concentration–time curves of PLC, ALC and LC in healthy volunteers treated with PLC by i.v. route at doses of 1, 2, 4 and 8 g, over a 2-h infusion.

tivity and accuracy make this method suitable for pharmacokinetic investigations in humans.

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