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Quantitation of L-Carnitine, Acetyl-L-carnitine, Propionyl-L-carnitine and Their Deuterated Analogues by High-performance Liquid Chromotography Tandem Mass Spectrometry

Carlo Tallarico, Silvia Pace and Antonio Longo*

Department of Pharmacokinetics and Metabolism, sigma-tau Industrie Farmaceutiche Riunite s.p.a., Via Pontina km 30,400, 00040 Pomezia (Rome), Italy

A new simple and sensitive high-performance liquid chromatographic procedure with tandem mass spectrometry detection (HPLC/MS/MS), to determine L-carnitine (LC), D_3-L-carnitine (D_3-LC), acetyl-L-carnitine (ALC), acetyl-D_3-L-carnitine (D_3-ALC), propionyl-L-carnitine (PLC) and propionyl-D_3-L-carnitine (D_3-PLC) in plasma, was developed. Sample preparation consisted of only deproteinization of plasma samples with a mixture of acetone–methanol before injection in HPLC. The detection was achieved by multiple reaction monitoring. A chloro-derivative of L-carnitine was used as internal standard.

The limits of detection, calculated for a S/N ratio ~3, were 1 nmol/mL for LC and D_3-LC, and 0.1 nmol/mL for ALC, D_3-ALC, PLC and D_3-PLC. The limits of quantitation were 5 nmol/mL for LC and D_3-LC, 1 nmol/mL for ALC and D_3-ALC, and 0.25 nmol/mL for PLC and D_3-PLC. The recovery of the procedure was in the range 80–96% for the six analytes, 88.2% for the internal standard.

Validation was performed within the following concentration ranges: 5–160 nmol/mL for LC and D_3-LC, 1–32 nmol/mL for ALC and D_3-ALC, and 0.25–8 nmol/mL for PLC and D_3-PLC. Linearity was assessed during both intraday and interday sessions, and coefficients of regression were always higher than 0.99. Precision and accuracy were in the ranges 0.38–5.5% and 0.01–3.92%, respectively. The method is suitable for the quantitation of the three endogenous carnitines and their deuterated homologues in plasma in a single analysis. **© 1998 John Wiley & Sons, Ltd.**

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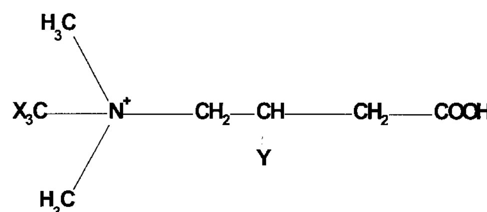
PLC is an endogenous ester of L-carnitine, and recently has been proposed to be supplemented as an effective drug in peripheral arterial disease. As for almost all endogenous compounds, the presence in the blood stream of basal levels of the compound and its biotransformation products, LC and ALC, presents an obstacle in studying and fully understanding the pharmacokinetics of administered PLC. Therefore, tri-deuterated PLC (D_3-PLC) was synthesized, such that the pharmacokinetics of the compound could be studied using the same approach as for other xenobiotics.

Several methods for the quantitative analysis of L-carnitine and its acylesters in biological fluids have been described. These include radio-enzyme techniques[1,2], HPLC with ultraviolet[3–5] or fluorimetric[6,7] detection, and fast atom bombardment tandem mass spectrometry (FAB-MS/MS).[8] However, either they cannot distinguish between deuterated and non-deuterated carnitine derivatives, or, as in the case of FAB-MS/MS, they are not suitable for routine analyses.

The aim of this study was to establish a single HPLC/MS/MS procedure, with adequate sensitivity, accuracy and reproducibility and with a fast sample preparation step. The method was designed to be suitable for the routine analysis of plasma samples obtained from animal and human pharmacokinetics studies, in which deuterated analogues of PLC, LC, or ALC are administered. The applicability of the method is assessed through some in-house validation studies.

*Correspondence to: A. Longo, Department of Pharmacokinetics and Metabolism, sigma-tau Industrie Farmaceutiche Riunite s.p.a., Via Pontina km 30,400, 00040 Pomezia (Rome), Italy.



X = H, Y = OH	L-CARNITINE	M.W. = 162
X = D, Y = OH	D_3-L-CARNITINE	M.W. = 165
X = H, Y = CH_3-CO_2	ACETYL-L-CARNITINE	M.W. = 204
X = D, Y = CH_3-CO_2	ACETYL-D_3-L-CARNITINE	M.W. = 207
X = H, Y = CH_3-CH_2-CO_2	PROPIONYL-L-CARNITINE	M.W. = 218
X = D, Y = CH_3-CH_2-CO_2	PROPIONYL-D_3-L-CARNITINE	M.W. = 221
X = H, Y = Cl	I -(–)-4-TRIMETHYLAMMONIUM-3CHLORO-BUTYRIC ACID (I.S.)	M.W. = 180

Figure 1. Chemical structure of LC, ALC, PLC, D_3-LC, D_3-ALC, D_3-PLC and internal standard.

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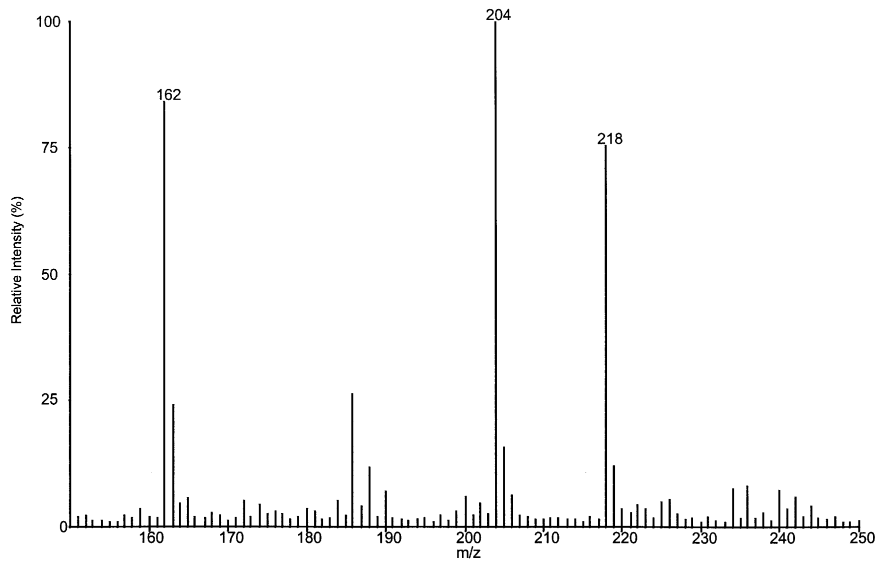

Figure 2. Single quadrupole mass spectrum of a mixture of LC, ALC and PLC.

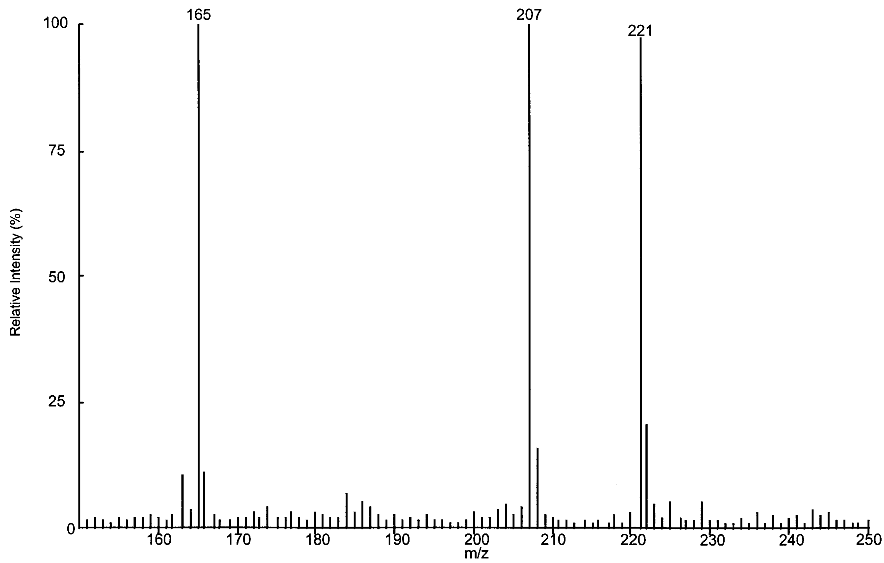

Figure 3. Single quadrupole mass spectrum of a mixture of D_3-LC, D_3-ALC and D_3-PLC.

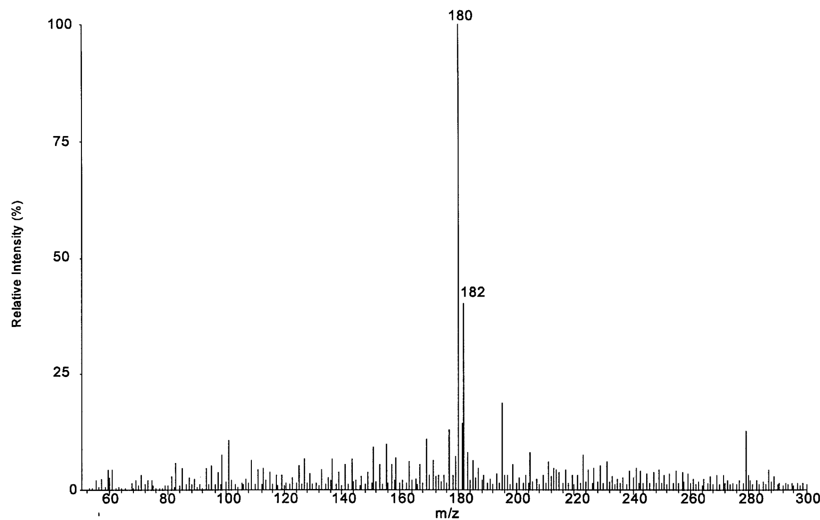

Figure 4. Single quadrupole mass spectrum of the internal standard.

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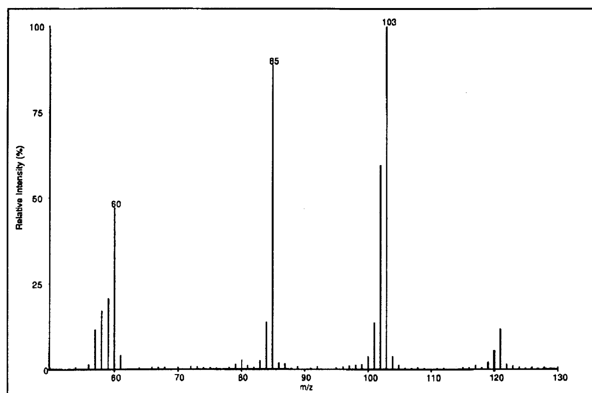
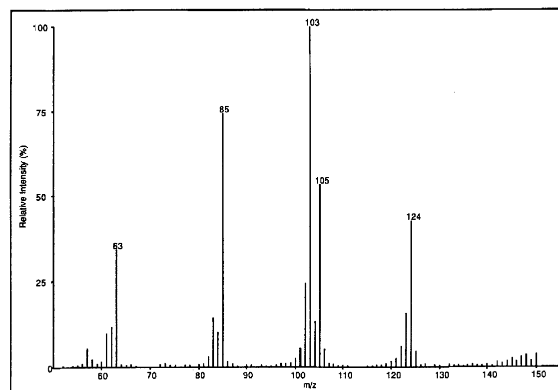
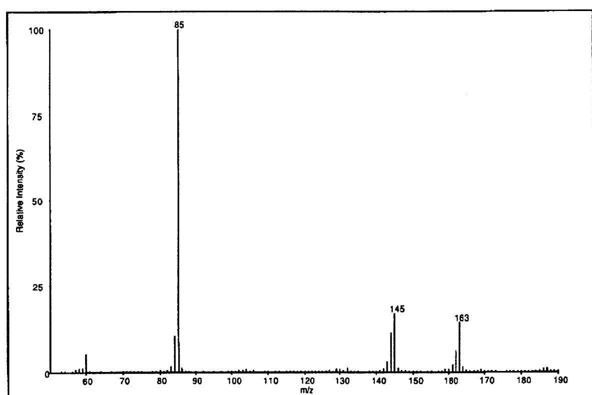
LC



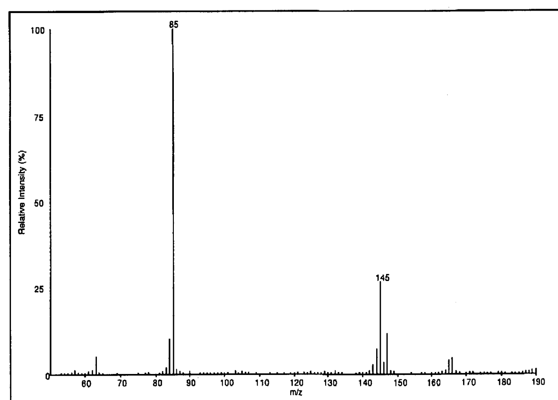
D_3-LC



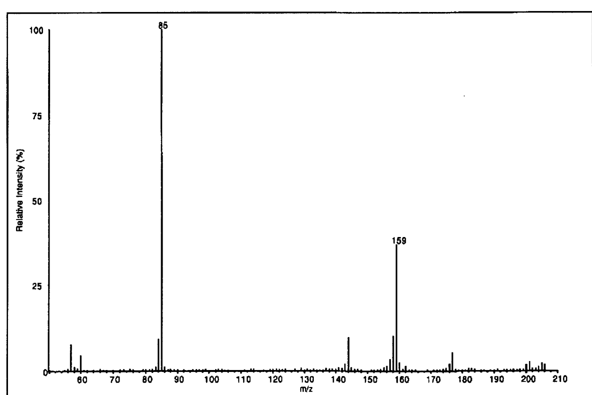
ALC



D_3-ALC



PLC



D_3-PLC

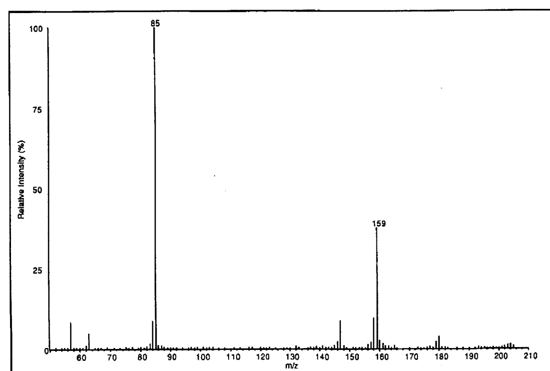


Figure 5. Fragment ion spectra of molecular ions of LC, ALC and PLC.

Figure 6. Fragment ion spectra of molecular ions of D_3-LC, D_3-ALC and D_3-PLC.

EXPERIMENTAL

Chemicals

L-carnitine inner salt and the hydrochloride salts of acetyl-L-carnitine, propionyl-L-carnitine, and the internal standard (see Figure 1), were synthesized by sigma-tau Industrie Farmaceutiche Riunite s.p.a., (Pomezia, Rome, Italy). Deuterated carnitines were synthesized by Academy of Wissenschaften (Berlin, Germany). The chemical structures of all compounds are shown in Fig. 1. Acetonitrile and ammonium acetate, analytical grade, were purchased from Merck (Milan, Italy).

Equipment

The chromatographic equipment consisted of a Perkin Elmer solvent delivery system Model 250 (Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a Varian 9095 autosampler (Varian, Sunnyvale, California, USA). The mass spectrometer was an API III (Perkin Elmer Sciex,

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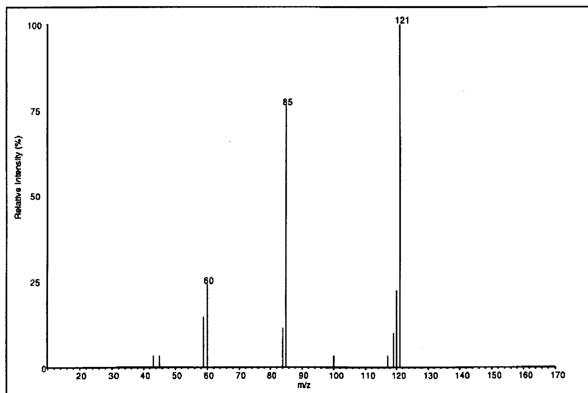

Figure 7. Fragment ion spectrum of the molecular ion of ST 1085 (internal standard).

Toronto, Canada) equipped with an ionspray® interface. A Savant model RVT 4104 refrigerator vapour trap was used to dry samples after protein precipitation.

Chromatographic conditions

Chromatographic separation was achieved with a μBondapak™-NH_2 column (300 × 3.9mm) (Waters Chromatography Division, Millipore Corporation, Milford, MT, USA). The mobile phase was prepared by mixing 700 mL of acetonitrile, 300 mL of water and 5 mL of 1M ammonium acetate; the solution was filtered though 0.2 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 1.5 mL/min, at room temperature. A post-column split valve, with a split ratio of 1:25, was inserted between the column and the mass spectrometer.

Table 1. Test of the specificity of the analytical method for LC, ALC and PLC

		HPLC-FL		HPLC/MS/MS		
Analyte	Sample	Mean (nmol/mL)	SD	Mean (nmol/mL)	SD	t-Test
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	3.295	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	$p > 0.05$

Mass spectrometry conditions

The experimental conditions were as follows: nebulized gas pressure (air) 50 PSI, curtain gas flow (N_2) 1.8 L/min, collision gas thickness (Ar) 250 × 10^{12} molecules/cm^2, ion spray voltage 5500 V, orifice voltage 50 V.

Mass spectra study

The mass spectra of the seven carnitines were obtained by direct infusion of 10 μg/mL aqueous solutions of each

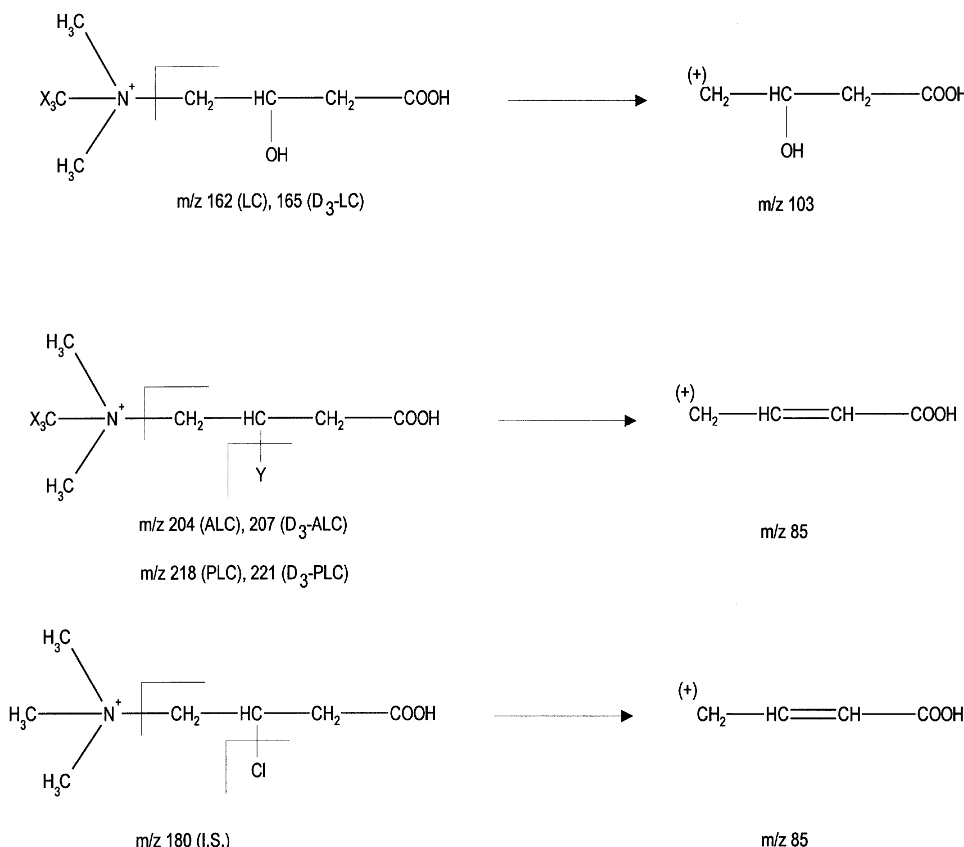

Figure 8. Main fragments of LC, ALC, PLC, D_3-LC, D_3-ALC, D_3-PLC and internal standard.

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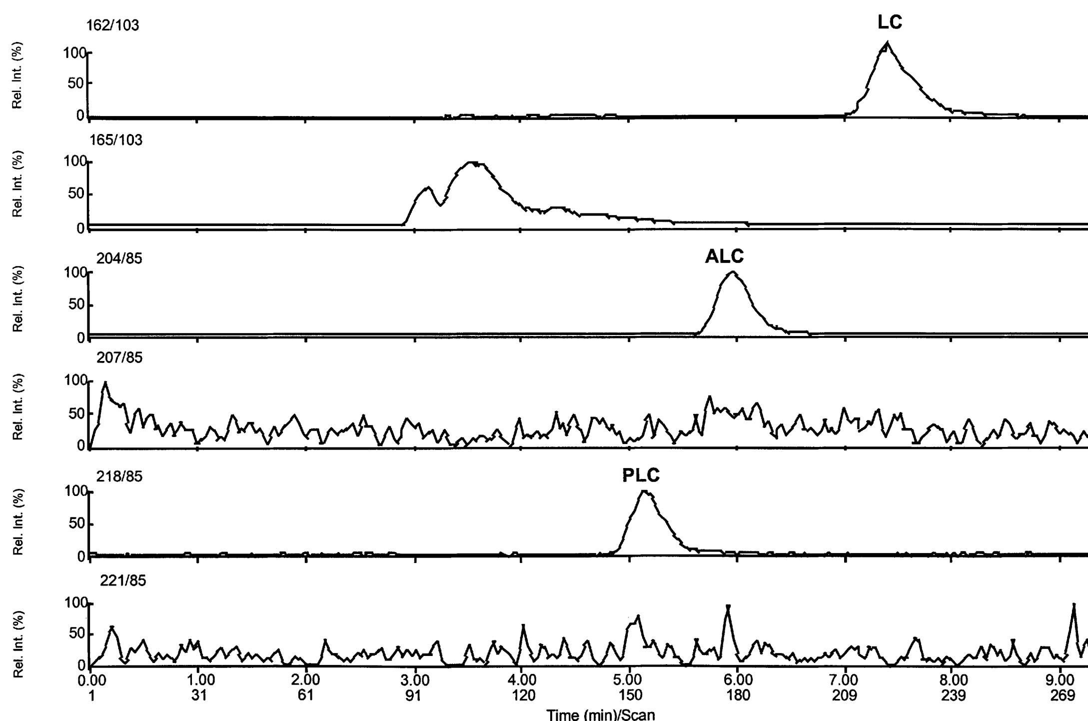

Figure 9. Multiple reaction monitoring chromatograms of a basal plasma sample.

compound. In the first step, a single quadrupole acquisition was run by scanning from *m/z* 100 to 250 with 1 Da steps and 10 ms dwell time. In this way very simple spectra were obtained, containing only the peaks corresponding to the molecular ions, no further fragmentation occurring under these conditions. As expected, the ionization of LC and D_3-LC; of ALC and D_3-ALC; of PLC and D_3-PLC and ST 1085 [I-(−)-4-trimethylammonium-3-chlorobutyric acid (I.S.)] gave rise to the following molecular ions: *m/z* 162, 165, 204, 207, 218, 221 and 180, respectively; the spectra are shown in Figs. 2–4.

The fragmentation spectra of the molecular ions of the above compounds were obtained by operating in MS/MS mode within the range *m/z* 50–200. The spectra are shown in Figs. 5–7. The main fragment arising from LC and D_3-LC at *m/z* 103 was due to loss of the trimethylammonium neutral. The main fragment from ALC, D_3-ALC, PLC, D_3-PLC and I.S., was at *m/z* 85, and was formed through loss of the

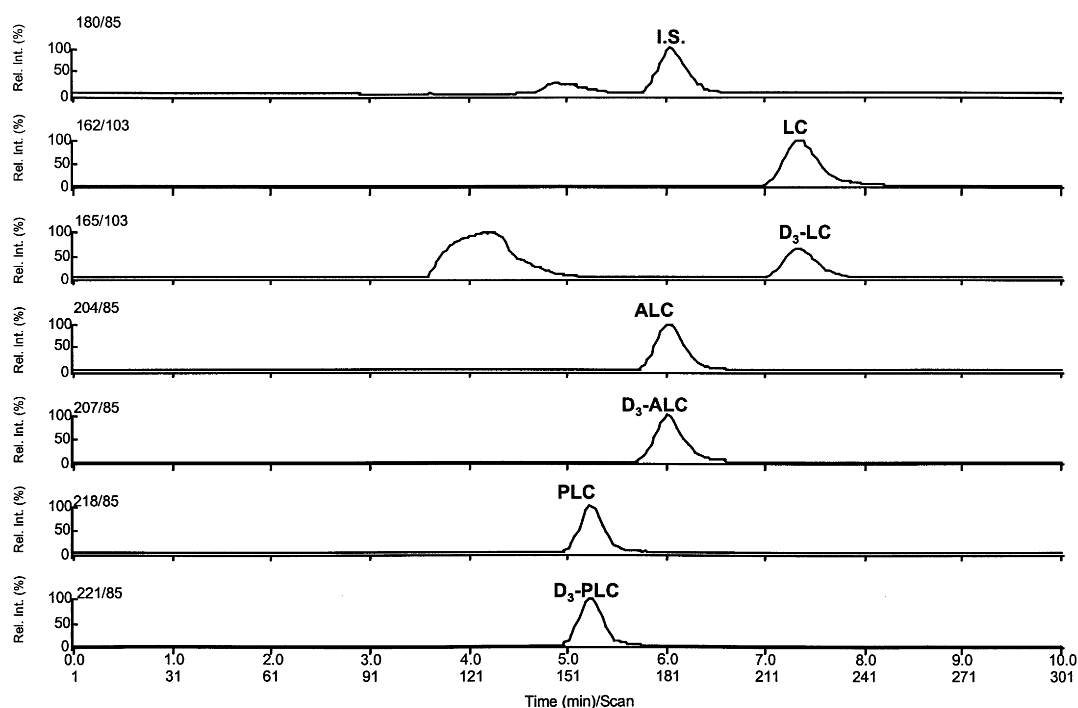

Figure 10. Multiple reaction monitoring chromatograms of a dialysed plasma sample spiked with 160 nmol/mL of LC and D_3-LC, 32 nmol/mL of ALC and D_3-ALC, 8 nmol/mL of PLC and D_3-PLC and 50 nmol/mL of I.S.

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Table 2. Reproducibility of the calibration parameters for LC, D_3-LC, ALC, D_3-ALC, PLC and D_3-PLC

Parameter	LC	D_3-LC	ALC	D_3-ALC	PLC	D_3-PLC
Slope	0.018185	0.018596	0.176451	0.179405	0.240802	0.21007
	0.01757	0.017879	0.179165	0.18599	0.253571	0.219244
	0.016874	0.017477	0.141238	0.137853	0.170882	0.147865
	0.021725	0.021558	0.180636	0.177899	0.213429	0.18976
	0.020229	0.020369	0.175566	0.172996	0.218102	0.187003
	0.018264	0.018446	0.16202	0.162748	0.204172	0.177799
Mean	0.018808	0.019054	0.169179	0.169482	0.216826	0.188624
S.D.	0.0018158	0.0015777	0.0152093	0.0173200	0.0290144	0.0252187
C.V. (%)	9.65	8.28	8.99	10.22	13.38	13.37
Intercept	0.015269	0.012817	0.026194	0.015546	0.000353	0.001745
	0.013512	0.013028	0.014144	0.015026	0.001889	0.002286
	0.010124	0.013861	0.010893	0.008039	−0.000207	0.000967
	0.013934	0.02054	−0.006334	−0.013137	−0.001139	−0.005082
	0.010678	0.011881	0.006959	0.013468	0.002599	0.000688
	0.004054	0.007115	−0.003323	0.001435	0.00308	−0.000096
Mean	0.011262	0.013207	0.008089	0.006730	0.001096	0.000085
S.D.	0.0040460	0.0043187	0.0119319	0.0111031	0.0016775	0.0026635
R^2	0.9988	0.9986	0.9996	0.9997	0.9995	0.9996
	0.9990	0.9990	0.9994	0.9994	0.9995	0.9994
	0.9995	0.9990	0.9991	0.9991	0.9996	0.9994
	0.9997	0.9992	0.9997	0.9998	0.9997	0.9995
	0.9995	0.9995	0.9996	0.9997	0.9998	0.9998
	0.9995	0.9995	0.9997	0.9996	0.9993	0.9991
Mean	0.9993	0.9991	0.9995	0.9995	0.9996	0.9995
S.D.	0.00038	0.00037	0.00023	0.00023	0.00018	0.00024

trimethylammonium group followed by loss of the ester group or chlorine atom for the I.S.; the proposed structures of these fragments are shown in Fig. 8.

Therefore, for the quantitative assay of these compounds, the following transitions were chosen for the multiple reaction monitoring (MRM) experiments: LC: 162/103; D_3-LC: 165/103; ALC: 204/85; D_3-ALC: 207/85; PLC: 218/85; D_3-PLC: 221/85; I.S.: 180/85.

Preparation of the plasma samples

To 50 µL of plasma, 15 µL of a 160 nmol/mL solution of I.S. were added. The deproteinization was carried out by adding 500 µL of a mixture of acetone–methanol (3:1), and centrifuging samples at 20 000 g at 4°C for 10 min. The supernatant phase was transferred into glass conical tubes and dried under vacuum. To the dry residues, 50 µL of the mobile phase were added and 20 µL were injected into the HPLC/MS/MS system. To prepare standard curves dialysed plasma was used, to which known amounts of both undeuterated and deuterated analytes were added.

RESULTS

The validation of the analytical procedure was carried out calculating selectivity, extraction recovery, linearity and intra-assay and inter-assay precision and accuracy.

Specificity

Being endogenous substances, the non-deuterated forms of LC, ALC and PLC are always present in chromatographic profiles of human and animal plasma. Although the specificity of the method was assured by the chromatographic separation and by the MS/MS selectivity, specificity of the assay was also assessed by analysing a set of plasma samples using the procedure here described and by HPLC with fluorimetric detection (HPLC-FL).[7] The results obtained in this experiment are reported in Table 1. There were no significant differences between the two procedures, thus excluding any significant effects of interferences.

More directly, the purity of the HPLC peaks of deuterated carnitines could be demonstrated by the lack of any interfering MS peaks at their retention times in the LC/MS profile of basal plasma. A typical chromatogram of a basal plasma sample is shown in Fig. 9; in Fig. 10 is shown a dialysed plasma sample, to which all seven analytes were added.

Table 3. Intraday precision and accuracy of the method. Values are the means of six replications

Analyte	Actual conc. (nmol/mL)	Mean (nmol/mL)	S.D.	C.V. (%)	Accuracy (%)
LC	5	4.87	0.136	2.80	2.69
	20	20.00	0.320	1.60	0.01
	160	158.99	7.026	4.42	0.63
D_3-LC	5	4.95	0.105	2.12	0.98
	20	20.01	0.585	2.92	0.05
	160	160.13	8.800	5.50	0.08
ALC	1	0.96	0.018	1.87	3.92
	4	4.12	0.091	2.21	2.88
	32	31.60	0.331	1.05	1.24
D_3-ALC	1	0.97	0.050	5.14	2.52
	4	4.09	0.093	2.28	2.37
	32	31.67	0.471	1.49	1.03
PLC	0.25	0.25	0.005	2.04	0.31
	1	1.00	0.049	4.87	0.48
	8	7.97	0.190	2.38	0.40
D_3-PLC	0.25	0.26	0.008	3.07	2.53
	1	1.00	0.014	1.40	0.26
	8	8.00	0.217	2.71	0.02

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Table 4. Interday precision and accuracy of the method. Values are the means of six replications

Analyte	Actual conc. (nmol/mL)	Mean (nmol/mL)	S.D.	C.V. (%)	Accuracy (%)
LC	5	4.91	0.077	1.57	1.85
	10	10.12	0.153	1.51	1.24
	20	20.35	0.437	2.15	1.76
	40	39.96	0.504	1.26	0.11
	80	79.84	1.670	2.09	0.21
	160	160.39	1.162	0.72	0.24
D_3-LC	5	4.90	0.080	1.62	1.92
	10	10.11	0.072	0.72	1.07
	20	20.31	0.182	0.90	1.53
	40	40.13	0.679	1.69	0.32
	80	79.55	1.441	1.81	0.56
	160	160.61	1.309	0.81	0.38
ALC	1	0.98	0.015	1.54	1.74
	2	2.01	0.021	1.06	0.56
	4	4.09	0.079	1.92	2.24
	8	7.97	0.096	1.21	0.38
	16	15.97	0.390	2.44	0.18
	32	32.07	0.305	0.95	0.23
D_3-ALC	1	0.99	0.011	1.14	1.50
	2	2.01	0.035	1.74	0.60
	4	4.07	0.045	1.11	1.74
	8	8.00	0.083	1.04	0.03
	16	15.92	0.362	2.27	0.50
	32	32.10	0.321	1.00	0.31
PLC	0.25	0.25	0.001	0.49	0.35
	0.5	0.50	0.005	0.96	0.52
	1	1.01	0.010	0.97	1.23
	2	2.00	0.013	0.65	0.10
	4	4.00	0.051	1.27	0.05
	8	8.01	0.031	0.38	0.08
D_3-PLC	0.25	0.25	0.003	1.19	0.12
	0.5	0.50	0.009	1.77	0.67
	1	1.01	0.014	1.43	1.10
	2	2.00	0.012	0.59	0.04
	4	3.97	0.057	1.45	0.84
	8	8.04	0.041	0.51	0.48

Sensitivity

The limits of detection of the method were 1 nmol/mL for LC and D_3-LC, and 0.1 nmol/mL for ALC, D_3-ALC, PLC and D_3-PLC.

Recovery

The recovery of the assay for all the analytes ranged between 80–96%.

Linearity

The linearity of the assay was assessed in the concentration ranges 5–160 nmol/mL for LC and D_3-LC, 1–32 nmol/mL for ALC and D_3-ALC, and 0.25–8 nmol/mL for PLC and D_3-PLC. Calibration parameters obtained for six different curves are reported in Table 2. As shown, good reproducibility was obtained.

Precision and accuracy

Intra-assay and inter-assay variability was determined by replicate analyses of the same set of dialysed plasma samples containing known amounts of the six analytes. The analyses were performed on six different days. Results obtained are shown in Tables 3 and 4; precision, expressed as C.V. (%), was in the range 0.38–5.5%; accuracy expressed as % error, was in the range 0.01–3.92% for all six carnitines.

CONCLUSIONS

A new HPLC/MS/MS method for the determination of carnitines and their deuterated analogues in plasma is described in this paper. It is based on a fast step of deproteinization of plasma, followed by HPLC separation and MS/MS detection. The results obtained demonstrate that the performance is suitable for both animal and human pharmacokinetic studies. Once again these results show that the HPLC/MS/MS technique can be used to analyse small molecules in plasma, and to quantify them with an accuracy and precision comparable to those of more conventional techniques; on the other hand, HPLC/MS has the enormous advantage over other techniques of being so specific that tedious and expensive purification steps can be minimized.

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