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High-performance liquid chromatography and capillary electrophoresis of L- and D-carnitine by precolumn diastereomeric derivatization

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Abstract

A new method for the simultaneous assay of D- and L-enantiomers of carnitine is described. The method is based on precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate [(+)FLEC] producing a diastereomeric derivative which can be detected both by UV absorbance and fluorescence detection. Also acyl esters of carnitine can be processed with this method, after alkaline hydrolysis. The D-enantiomer of carnitine and acylcarnitine can be detected at a concentration as low as 0.2% in the raw material and in pharmaceuticals. Assays can be carried out using an autoinjector either by HPLC or capillary electrophoresis (CE) because the derivative proved to be very stable. Its application is proposed for the routine assay of the enantiomeric excess of L-carnitine and their acyl esters in pharmaceutical products.

1. Introduction

L-Carnitine was found to play an important role in the transport of fatty acids across the mitochondrial membrane by the transferase and translocase enzyme system [1,2]. Therapeutic treatment with L-carnitine is needed in primary and secondary L-carnitine deficiency [3]. Recently, some short-chain esters of L-carnitine, namely acetyl-, propionyl-, and isovaleryl-L-carnitine, proved to possess interesting pharmacological properties in central nervous system (CNS) deterioration syndromes, in periferal circulatory disorders and in liver insufficiency, respectively [4–6].

In the past, patients have been treated with the racemate D,L-carnitine. D-Carnitine, however, has been shown to inhibit the carnitine acetyltransferase, leading to a depletion of body L-carnitine storage [7]. According to the current trends and to guidelines on the use of chiral medicinal products, the use of the L-enantiomer of carnitine and its esters is now mandatory. Thus an enantioselective assay for the L- and D-enantiomers of carnitine in pharmaceutical products is required. Previously, this problem

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was resolved by using NMR with chiral shift reagents [8,9] and, *e.g.* in pharmacokinetic studies, enzyme-mediated assays were widely used [10].

In the present paper we describe a chromatographic method with pre-column derivatization leading to a diastereomeric derivative, for the simultaneous determination of the D- and L-enantiomers of carnitine and acylcarnitine. Both HPLC and capillary electrophoresis (CE) can be used with UV absorbance and fluorescence detection. As the derivatization attacks the -OH group of carnitine, acyl esters require a previous hydrolysis step and thus the method is useful only for pharmaceuticals and not for pharmacokinetic studies.

This paper describes the method, its validation and applications to pharmaceutical products.

2. Experimental

2.1. Chemicals

(+)-1-(9-Fluorenyl)ethyl chloroformate [(+) FLEC] (0.5% w/v solution in acetone) was purchased from Janssen Chimica (Geel, Belgium). Carnitine and acylcarnitine derivatives were supplied by Sigma-Tau (Pomezia, Italy). Acetonitrile, acetone, potassium dihydrogen phosphate were from Merck (Bracco, Milan, Italy). Potassium hydroxide (1 *M*) and phosphoric acid (85%, RPE grade) were from Carlo Erba (Milan, Italy). Tetrabutylammonium hydroxide (TBA^+OH^-) (55% aqueous solution, $d_{20}^{20} = 0.97$) was from Nuova Chimica (Milan, Italy).

2.2. Apparatus

The HPLC system consisted of two pumps Model 510 from Waters (Millipore, Milford, MA, USA), a UV detector Model 490 from Waters and a fluorescence detector Model LS-5 from Perkin-Elmer (Norwalk, CT, USA). The separation was carried out on a Nova-Pak C_{18} column from Waters (150 × 3.9 mm I.D.) with 4 μm average particle size. The chromatographic data were processed with a Digital PC-380 equipped with Waters 840 software version 6.2. The derivatizing equipment used was the Reactitherm stirring module Pierce system (Rockford, IL, USA). The CE system was a Quanta 4000 instrument from Waters.

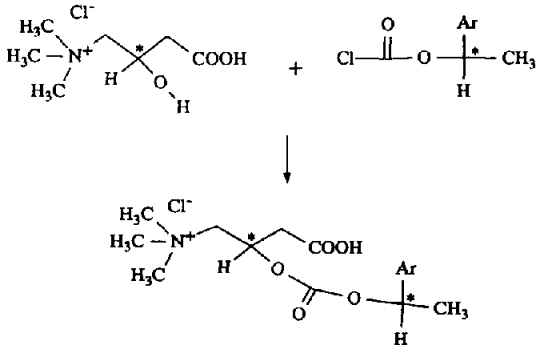
2.3. Derivatization

The derivatization reaction is described in Fig. 1. The FLEC solution was diluted with acetone to a final concentration of 4.5 m*M*. The carnitine used was inner salt or hydrochloride as racemic, pure or scalemic [11] mixture. A reference solution of carnitine was prepared by neutralization of an aqueous solution of 50 m*M* TBA^+OH^- in which 10 mg of carnitine reference standard were dissolved (in a 50-ml volumetric flask) with concentrated H_3PO_4.

A 50-μl aliquot of the carnitine solution and 200 μl of the FLEC solution (4.5 m*M* in acetone) were allowed to react in a sealed 4-ml vial at 80°C for 25 min. After cooling the reaction mixture was diluted with 4 ml of mobile phase A and 5 μl of the resulting solution were analyzed by HPLC.

2.4. HPLC conditions

Gradient elution was performed using the elution program reported in Table 1 at a column temperature of 30°C. The composition of the



Ar = 9-Fluorenyl

Fig. 1. Carnitine derivatization with (+)FLEC.

Table 1
Gradient elution program

	Time (min)	Flow (ml/min)	A%	B%
1	0.00	0.75	100.0	0.0
2	20.00	0.75	100.0	0.0
3	22.00	1.00	0.0	100.0
4	35.00	1.00	0.0	100.0
5	36.00	1.00	100.0	0.0
6	40.00	1.00	100.0	0.0

solvents used was as follows: Solvent A = 25% (v/v) acetonitrile and 75% (v/v) of an aqueous solution of 5 m*M* TBA^+OH^- and 50m*M* KH_2PO_4 neutralized (pH 7.0) with 1 *M* KOH. Solvent B = 75% (v/v) acetonitrile and 25% (v/v) aqueous solution of 5 m*M* KH_2PO_4.

The UV detector was set at 260 nm and the fluorescence detector was set at $\lambda_{ex} = 260$ nm (slitwidth = 10 nm), $\lambda_{em} = 315$ nm (slitwidth = 10 nm.

2.5. Capillary electrophoresis conditions

The column used was a fused-silica capillary (50 μm diameter, 60 cm length) and the mobile phase was an aqueous solution of 50 m*M* KH_2PO_4 adjusted to pH 3.40 with concentrated H_3PO_4. The voltage applied was 14 kV and the UV detector was set at 214 nm. The system was kept at room temperature (*ca.* 25°C).

2.6. Hydrolysis of carnitine acyl esters

The method was tested with acetyl carnitine and propionyl carnitine (inner salts and hydrochloride salts) as racemic, pure or scalemic [11] mixture. Hydrolysis was performed by dissolving 20 mg of acylcarnitine in 20 ml of an aqueous solution of 50 m*M* TBA^+OH^- (pH 12.6) and stirring the solution at room temperature for 15 min. A 1-ml volume of this mixture was diluted with an aqueous solution of 50 m*M* TBA^+OH^- (neutralized to pH 7.0 with concentrated H_3PO_4) in a 10-ml volumetric flask. This resulting solution was derivatized as described above.

3. Results and discussion

3.1. Reaction time and temperature

The derivatization kinetics were determined at different temperatures, *i.e.* 50, 60, 70, 80, 90 and 100°C, for both L-carnitine and D-carnitine. Samples were taken at 5-min intervals for 30 min. The optimum proved to be 25 min at 80°C (Fig. 2). Under these conditions the areas of the chromatographic peaks did not increase appreciably with longer reaction times. Moreover, a check with HPLC confirmed the absence of non-derivatized carnitine. The column used was a SGE-SCX (5 μm) (250 × 4 mm I.D.) kept at 30°C, and the mobile phase was a 4:6 (v/v) mixture of acetonitrile and an aqueous solution of 50 m*M* $NH_4H_2PO_4$. The flow-rate was 0.75 ml/min. The UV detector was set at 200 nm.

At higher temperatures and very long reaction times formation of crotonyl betaine chloride (2-propen-1-aminium, 3-carboxy-N,N,N-trimethyl chloride) could be observed. However, secondary reactions of this type do not interfere with the determination of the enantiomeric excess.

3.2. Influence of pH

Derivative formation proved to depend markedly on the pH of the buffer. The UV absorbance of the two diastereoisomers increased with increasing pH reaching a maximum at pH 7, followed by a rapid decrease of the peak area, due to the instability of the derivatives (Fig. 3).

3.3. FLEC/carnitine ratio

When the amount of FLEC (4.5 m*M* in acetone) was gradually increased from 50 to 250 μl, an increase in peak area of the diastereoisomers was detected. Thus, 200 μl of FLEC solution and 50 μl of 3 m*M* carnitine were taken as the optimum amounts (Fig. 4).

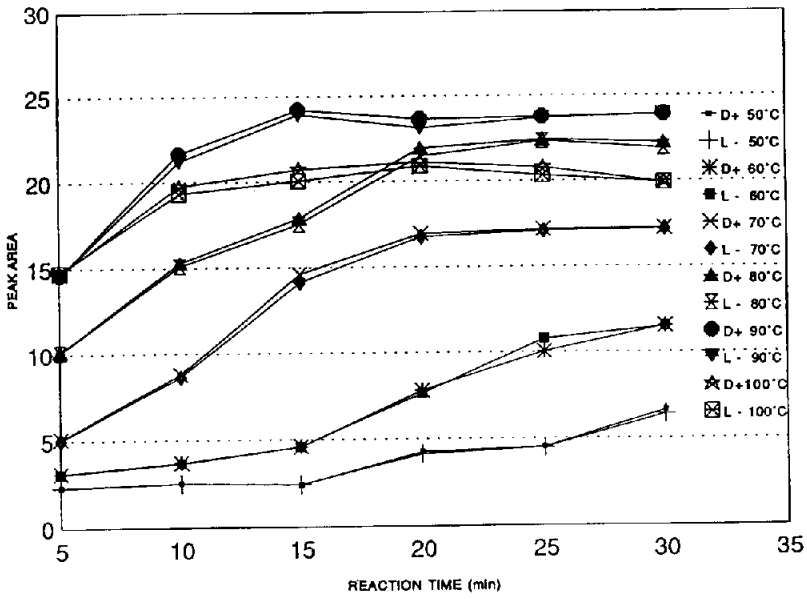


Fig. 2. Absorbance behaviour of both L- and D-carnitine-(+)FLEC derivatives *versus* reaction time at 50°C, 60°C, 70°C, 80°C, 90°C and 100°C in the optimization of the derivatization procedure.

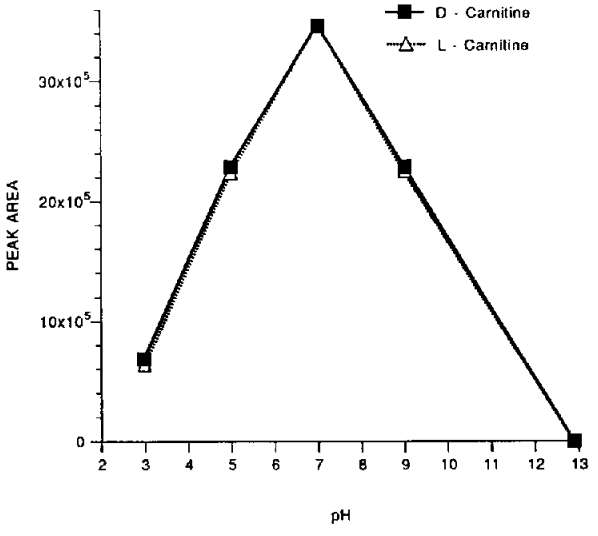


Fig. 3. Influence of pH on the derivatization kinetics of both L- and D-carnitine-(+)FLEC.

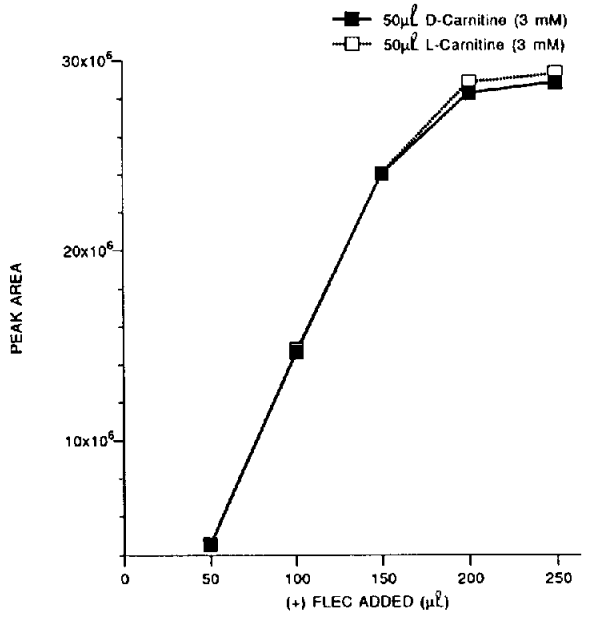


Fig. 4. Carnitine derivatization behaviour *versus* the quantity of (+)FLEC (4.5 m*M* in acetone) added to 50 µl of 3 m*M* L- or D-carnitine.

3.4. TBA^+OH^- concentration

The effect of the TBA^+OH^- concentration on the derivatization procedure was carefully investigated. The hypothesis that formation of micelles would stabilize the diastereoisomers and increase the reaction rate was confirmed. The optimum concentration of TBA^+OH^- was found to be 50 m*M* (Fig. 5).

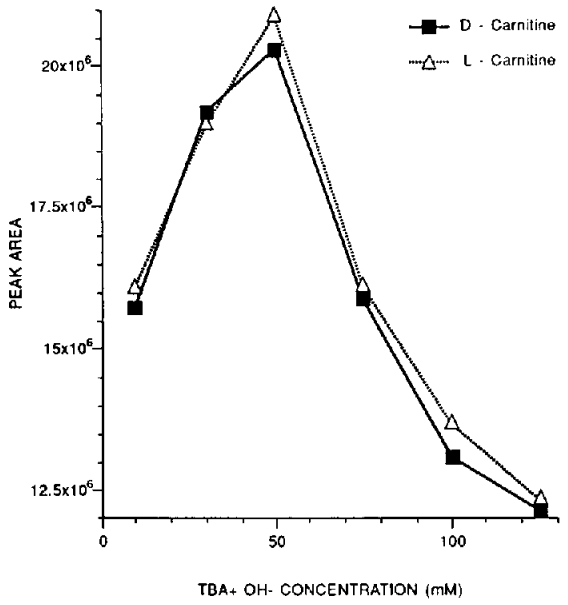


Fig. 5. Influence of the TBA^+OH^- concentration on the rate of L- and D-carnitine derivatization with (+)FLEC.

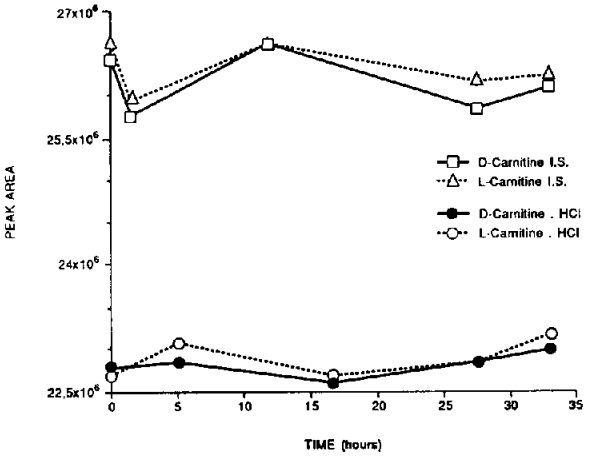


Fig. 6. Stability of L- and D-carnitine-(+)FLEC derivatives at room temperature.

3.5. Spectroscopic characteristics

The maximum UV absorbance of the carnitine derivative was detected at 260 nm. The most suitable wavelengths for fluorimetric detection were found to be 260 nm (λ_{ex}) and 315 nm (λ_{em}).

3.6. Stability of diastereoisomeric derivatives

The peak areas observed over a 35-h period after the derivatization at room temperature of D- or L-carnitine inner salt or hydrochloride with (+)FLEC did not show any significant variation with time (Fig. 6). Thus it was concluded that the diastereoisomers are very stable, which enabled the use of an automated sample injector.

3.7. Choice of the optimum HPLC and CE conditions

Different buffer solutions were used as eluents in order to optimize the chromatographic resolution and the retention times. The capacity factor (k'), selectivity (α) and resolution factor (R) of D-carnitine-(+)FLEC and L-carnitine-(+)FLEC eluted with 12.5m*M*, 25m*M* or 50m*M* KH_2PO_4 added to the eluent mixture at various pH values are shown in Table 2. The capacity factor and resolution factor increased with pH, reaching a maximum at pH 5 and 7, respectively. No difference was observed in selectivity under the various conditions tested.

Fig. 7 shows the separation of the (+)FLEC derivatives of D- and L-carnitine by HPLC, while Fig. 8 illustrates the sensitivity of the method. Good results were obtained even by CE; the

Table 2
Capacity factor (k'), selectivity (α) and resolution (R) factor for the HPLC analysis of D- and L-carnitine at different buffer molarity and pH values

KH_2PO_4	pH	k'_D	k'_L	α	R_S
12.5 m*M*	3	2.10	2.49	1.18	1.35
	5	4.96	5.89	1.19	1.68
	7	5.15	6.20	1.20	3.68
25.0 m*M*	3	3.41	4.11	1.20	2.03
	5	4.72	5.66	1.20	1.59
	7	4.52	5.45	1.21	3.60
50.0 m*M*	3	2.79	3.34	1.19	1.93
	5	5.05	6.12	1.21	1.89
	7	4.19	5.09	1.22	3.59

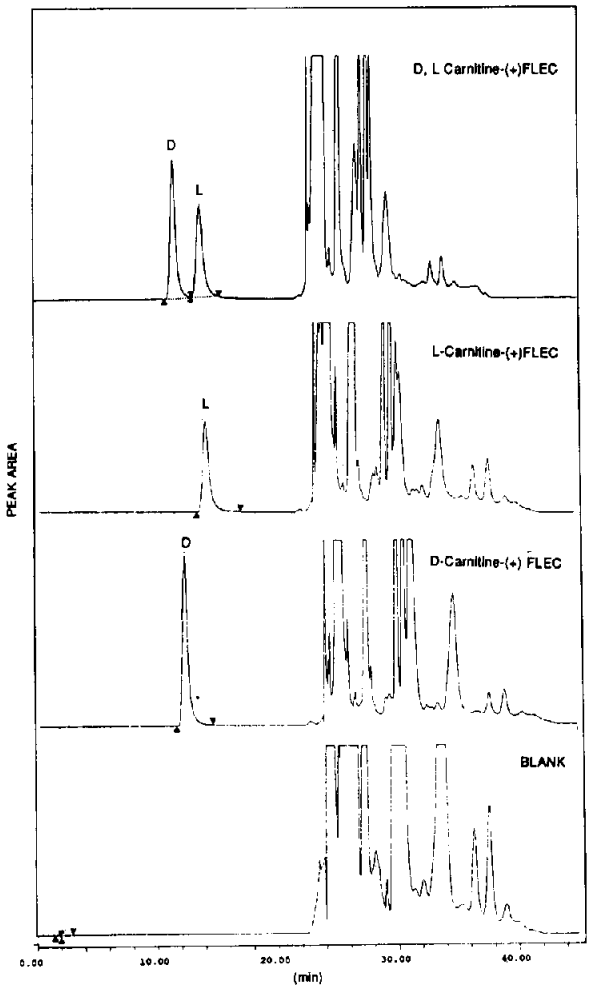


Fig. 7. HPLC separation of (+)FLEC derivatives of L- and D- carnitine.

dependence of the separation on the voltage applied is shown in Fig. 9.

3.8. Linearity of the enantiomeric excess evaluation

Increasing amounts of D-carnitine were added to L-carnitine in order to obtain mixtures ranging from *ca.* 0.2% to *ca.* 10.0% of D-carnitine. Table 3 summarizes the amounts of L- and D-carnitine standards mixed together, their recovery and the enantiomeric excess. The following straight line

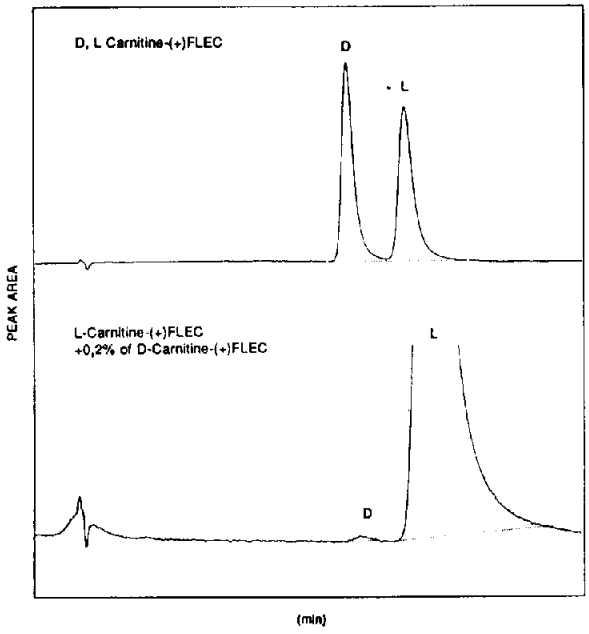


Fig. 8. HPLC separation of (+)FLEC derivatives of L- and D- carnitine: sensitivity of method.

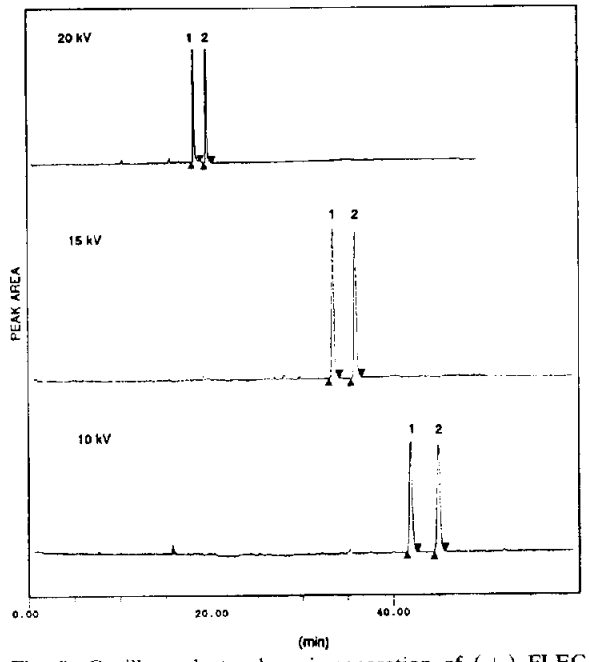


Fig. 9. Capillary electrophoresis separation of (+) FLEC derivatives of D- and L-carnitine: effect of increasing voltage. Conditions: capillary, 50 cm (L_d) × 60 cm (L_t) × 50 μm O.D.; temperature, 25°C; detector, UV 214 nm; buffer 0.05 *M* KH_2PO_4, pH 3.4 with H_3PO_4. Peaks: 1 = L-carnitine-(+)FLEC, 2 = D-carnitine-(+)FLEC.

Table 3
Evaluation of the enantiomeric excess of L-carnitine at increasing percentage of D-carnitine in the range 0.26–9.53%

Concentration (μg/ml)		D-Carnitine (%, w/w)		Enantiomeric excess (e.e.)	
L-carnitine	D-carnitine	theorical	found	theorical	found
237	0.63	0.26	0.22	99.47	99.55
237	1.50	0.63	0.56	98.74	98.88
237	1.88	0.78	0.74	98.42	98.51
237	2.49	1.04	0.97	97.92	98.06
237	5.01	2.06	2.06	95.87	95.88
237	6.26	2.56	2.48	94.87	95.04
237	12.52	5.00	4.83	89.99	90.34
237	18.77	7.32	7.29	85.35	85.42
119	12.52	9.53	9.38	80.94	81.24

was obtained with the linear regression method: $y = -1.982x + 100.1$, $R^2 = 0.9998$

4. Conclusions

The precolumn derivatization procedure, previously proposed for carnitine by Engewald *et al.* [12] using HPLC, was modified in the present study in order to assay also the acyl esters of D- and L-carnitine. In this respect this method is the first enantioselective chromatographic assay of carnitine acyl esters reported in the literature. The assay can be carried out by HPLC or CE and allows the detection of the derivative either by UV absorbance or fluorescence detection. As the derivative proved to be stable for more than 35 h, the assay can be carried out with an autoinjector. In addition, only inexpensive reagents and instrumentation are needed.

All the conditions of the assay were carefully verified through optimized validation. This method is adequate for routine assay of the enantiomeric excess of carnitine, acetyl-carnitine and propionyl-carnitine in raw material and in pharmaceuticals.

References

[1] J. Bremer, *J. Biol. Chem.*, 237 (1962) 3628–3632.
[2] S.V. Pande and R. Parvin, *J. Biol. Chem.*, 251 (1976) 6683–6691.
[3] G.N. Breningstall, *Pediat. Neurol.*, 6 (1990) 75–81.
[4] N. Martucci, A. Agnoli and V. Manna, in A. Bes, J. Cahn, R. Cahn, S. Hoyer, J.P. Marc-Vergnes and H.M. Visniewski (Editors), *Senile Dementias: Early Detection*, John Libbey Eurotext, Montrouge, France, 1986, pp. 401–407.
[5] R. Ferrari, E. Pasini, E. Condorelli, A. Boraso, R. Lisciani and A. Marzo, *Cardiov. Drug Ther.*, 5 (1991) 17–24.
[6] F. Di Lisa, R. Menabò, R. Barbato, G. Miotto, R. Venerando and N. Siliprandi, in A.L. Carter (Editor), *Current Concepts in Carnitine Research*, CRC Press, London, 1992, pp. 27–36.
[7] M. De Grandis, C. Mezzina, A. Fiaschi, P. Pinelli, G. Bazzato and M. Morachiello, *J. Neurol. Sci.*, 46 (1980) 365–371.
[8] R. Voeffrey, J.C. Perlberger and L. Tenud, *Helv. Chim. Acta*, 70 (1987) 2058–2064.
[9] A. Marzo, G. Cardace, N. Monti, S. Muck and E. Arrigoni-Martelli, *J. Chromatogr.*, 527 (1990) 247–258.
[10] A. Marzo, G. Cardace and E. Arrigoni-Martelli, *Chirality*, 4 (1992) 247–251.
[11] J.H. Brewster, *Chem. Eng. News*, 3 (1992) 70.
[12] W. Engewald, H. Engelhardt, W. Gotzinger, P. Klosser and H.P. Kleber, *Pharmazie*, 45 (1990) 629–630.