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Achiral liquid chromatography with circular dichroism detection for the determination of carnitine enantiomers in dietary supplements and pharmaceutical formulations

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ABSTRACT

A simple and enantioselective method for the separation and determination of carnitine enantiomers in dietary supplements and pharmaceutical formulation samples is proposed. This method is based on achiral liquid chromatographic separation of carnitine enantiomers from interferences and direct circular dichroism (CD) detection. The calibration curve of the anisotropy factor (*g*) versus the enantiomeric excess was linear, with a correlation coefficient (R^2) of 0.996. The precision evaluated by UV peak area and CD peak area was suitable (RSD <5% in all cases). The usefulness of the proposed method was demonstrated by analysing natural dietary supplements and pharmaceutical formulation samples. This method has the advantages of being rapid and precise, without using an expensive chiral column. The method was suitable for the simultaneous determination of both enantiomers and for assessing the chemical purity of carnitine.

1. Introduction

Carnitine, 3-carboxy-2-hydroxy-*N*,*N*,*N*-trimethyl-1-propanaminium hydroxide, inner salt, is an endogenous essential chiral substance of mammalian tissues, mainly found in the skeletal and cardiac muscle. Carnitine plays an important role in the metabolism of fatty acids as acyltransferase cofactor and in energy production processes, such as interconversion in the mechanisms of regulation of cetogenese and termogenese, and it is also used in the therapy of primary and secondary deficiency, and in other diseases such as dislipoproteinemia [1–5]. Different oral solutions, tablets and capsules are disposable from different manufacturers. The enantiomers of carnitine show different biological activities. L-Carnitine, the naturally occurring enantiomer, is the therapeutically effective and, therefore, very useful for nutritional and pharmaceutical applications. On the other hand, D-carnitine displays an important toxic influence on biochemical processes [6]. Additionally, interconversion has not yet been observed. Nowadays, 80% of the carnitine is synthesized by chemical processes that generate the racemic mixture D,L-carnitine. Consequently, an elevate content of D-carnitine as subproduct is generated. Hence, the content of D-carnitine must be correctly determined and limited in pharmaceutical and nutritional formulations. In fact, the content of D-carnitine is limited by both, the European Pharmacopoeia and the United States Pharmacopoeia to 4%, determined by optical rotation, a not selective and sensitive enough method [7–8]. Therefore, simple, selective and robust new methods are needed. So far, several analytical methods have been developed to determine the enantiomer purity and to monitor the L-carnitine content in different samples such as foods [9], biological fluids [10], tissues [11], pharmaceuticals and dietary supplements [12]. The most commonly used methods include liquid chromatography using UV diode array (HPLC-DAD) [6,13–15], fluorescence (HPLC-FD) [16,17] or mass spectrometric detection (HPLC–MS) [13], capillary electrophoresis [1,6,13,18,19], radioenzimatic assays [20] and biosensors [21]. Most of HPLC methods require derivatization of carnitine to detect trace amounts of L-carnitine in food and biological samples [22–24]. However, from a practical point of view, the presence of crotonoylbetaine in samples represents an additional problem. In fact, crotonoylbetaine is the major impurity and degradation product (upper limit 0.5%) from carnitine, whose UV absorptivity is 80-fold higher than those of carnitine at 225 nm. Therefore, its content has to be monitored to avoid significant errors in the analytical results. Only few HPLC methods have been proposed for the determination of L-carnitine in pharmaceutical formulations [25,26], although these methods do not consider the separation of crotonoylbetaine from carnitine. In this sense, only few improved HPLC methods, based on ion-pairing conditions, separated crotonoylbetaine and L-carnitine in solution and

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tablet pharmaceutical formulations [27,28]. Due to its high polarity and low absorbance, because of the lack of chromophores, quantitative and enantiomeric analysis of carnitine using achiral high performance liquid chromatography and circular dichroism detection is challenging. An alternative approach involves the use of an achiral stationary phase coupled with a circular dichroism detector, which is specific for optically active compounds. The principle is based on the difference of absorbance between left and right circularly polarized light for two enantiomers [29,30]. The CD detector records simultaneously both the dichroism signal ($\Delta\varepsilon$) and absorbance (ε), and also measures the ratio of these two signals in order to calculate the anisotropy factor (g factor = $\Delta\varepsilon/\varepsilon$). The g factor was found to be proportional to the enantiomeric excess but is independent of concentration [31]. Many authors [32–36] have already demonstrated that this detector, coupled with an achiral stationary phase, was suitable to determine 99.8–98% enantiomeric excess values (0.1–2% enantiomeric purity). Nevertheless, any method has been applied to dietary supplements and pharmaceutical formulations so far.

In this work, we report for the first time the development of a non-chiral HPLC method for the determination of carnitine enantiomers, based on ion pairing chromatographic conditions, coupled to a circular dichroism detector for the simultaneous monitoring of both ellipticity and UV absorbance signal. This method has been fully validated determining the linearity, limits of detection and quantification, precision and accuracy. Ellipticity and UV absorbance signals allow the evaluation of the g anisotropy factor [37], which is independent of the concentration but proportional to the enantiomeric composition, to determine and calculate an enantiomeric excess of D-carnitine in pharmaceutical formulations and dietary supplements containing L-carnitine as well as L-carnitine tartrate.

2. Experimental

2.1. Instrumentation

Chromatographic analysis was carried out using a Waters 600E pump (Mildford, MA) equipped with a Rheodyne injection valve (model 7725i) coupled to a Jasco CD-2095 Plus (Tokyo, Japan) with an UV absorbance and circular dichroism detector. The CD detector is composed of an Hg-Xe lamp and a 44 μL flow cell with a 25 mm path-length. Data were acquired and analysed using BORWIN software version 1.5 (JBMS Développements, Le Fontanil, France). UV and CD spectra of the carnitine enantiomers were acquired in the 220–420 nm range. Five scans were recorded at a resolution of 2 nm, averaged and blank-subtracted to obtain both spectra. UV and CD detections were performed at 232 nm on the Jasco CD-2095 detector.

2.2. Materials

L-Carnitine (purity: 98%) and D-carnitine (purity: >98%) were supplied from Sigma–Aldrich (Saint Louis, MO). Crotonoylbetaine hydrochloride (2-propen-1-aminium, 3-carboxy-*N*,*N*,*N*-trimethyl-, chloride) was acquired from the US Pharmacopoeia (Rockville, MD). Deionized water purified through a water purification system (Millipore Milli-Q, Millipore, Bedford, MA) was used. Methanol, phosphoric acid (85%) and sodium hydroxide employed to prepare buffers were provided in analytical grade by Panreac (Barcelona, Spain). Sodium octane-1-sulfonate monohydrate was purchased from Fluka (Saint Louis, MO). Capsules of dietary supplements with nominal values of L-carnitine ranging from 100 to 400 mg each capsule were purchased from Korott (Alicante, Spain), Salud Mediterranea (Madrid, Spain) and Naturtierra (Leganés, Spain). Oral solutions of L-carnitine were purchased from Kiluva Diet (Barcelona, Spain) with a nominal value of 1 g each vial of 15 mL. Pharmaceutical formulations of carnitine with nominal values of 300 mg each tablet were acquired from Arkopharma (Carros, France).

2.3. Chromatographic conditions

In this method, the results were obtained using a C_8 column (Ascentis Express, 150 mm × 4.6 mm ID, 2.7 μm particle size) purchased from Supelco (Bellefonte, PA, USA) that was eluted at a flow rate of 1.0 mL min^{-1} with methanol-sodium 1-octanesulfonate (2.5 mM)–sodium phosphate buffer (pH 3; 50 mM) (2.5:97.5, v/v). The injection volume was 20 μL for both standards and samples. A wavelength of 232 nm was used for detection at ambient temperature.

2.4. Sample preparation

2.4.1. Standards and working solutions

Stock standard solutions of L-carnitine and D-carnitine were both weighed and dissolved in distilled water at a concentration of 10 μg mL^{-1}. These solutions were stable for 15 days at least. Crotonoylbetaine standard solution was also prepared as an aqueous solution at a concentration of 50 μg mL^{-1}. All standard solutions were maintained at 4 °C in darkness. Working solutions were prepared daily by diluting the standard solutions with water to obtain the appropriate dilution.

2.4.2. Capsule dietary supplements

Ten Korott 250 mg capsules containing 100 mg of carnitine, pollen, pineapple, carbohydrates and proteins were opened and the content was poured out into a 100 mL flask. Water was added to the volume mark and the mixture was homogenized by stirring and then sonicated for 15 min to disintegrate all the solid particles. The resulting solution was filtered through a 45 μm filter and injected into the HPLC system. Additionally, ten Salud Mediterranea 650 mg capsules containing 400 mg of L-carnitine tartrate, dicalcium phosphate, cellulose, magnesium silicone, corn starch and magnesium stearate were dissolved in 400 mL of distilled water. The resulting mixture was stirred and sonicated for 15 min, filtered through a 45 μm filter and injected into the HPLC system. Finally, ten Naturtierra 645 mg capsules with a nominal value of 400 mg of L-carnitine tartrate each capsule, cellulose, dicalcium phosphate, magnesium silicone, magnesium stearate, silicon dioxide and corn starch were poured out into 400 mL of water and stirred. The sample solution was sonicated for 15 min, filtered through a 45 μm filter and injected into the HPLC system.

2.4.3. Dietary supplement solution

Fifteen milliliters of Kiluva Diet solution containing 1 g of carnitine, water, fructose, concentrate of lemon, citric acid, lemon flavour and Fucus algae were transferred to a 1000 mL flask, water was added to the volume mark and mixed vigorously. The mixture was stirred and then solid particles were disintegrated by 15 min of sonication. The resulting solution was filtered through a 45 μm filter and injected into the HPLC system.

2.4.4. Pharmaceutical formulation

Ten Arkopharma tablets with a nominal value of 300 mg of carnitine each tablet, sucrose, corn starch, magnesium stearate and natural orange flavour were ground and placed into a flask where water was added to obtain a 300 mL solution. The mixture was homogenized by stirring and then sonicated for 15 min. The final sample solution was filtered through a 45 μm filter and injected into the HPLC system.

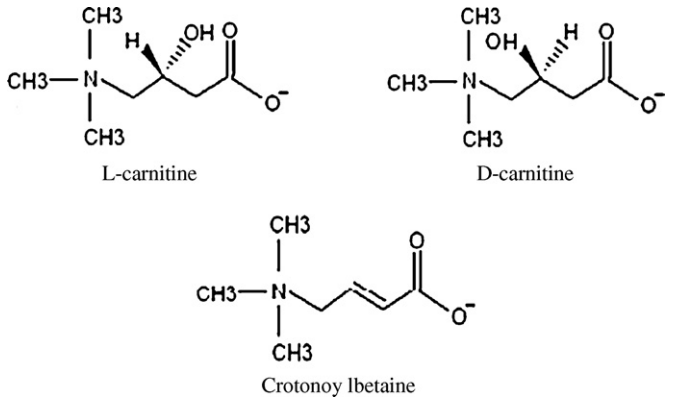

Fig. 1. Chemical structures of L-carnitine, D-carnitine and crotonoylbetaine.

3. Results and discussion

The structures of the analytes, L-carnitine, D-carnitine and crotonoylbetaine used for the proposed method are shown in Fig. 1. The corresponding CD and UV spectra of the enantiomers of carnitine show that the UV spectra are identical for both enantiomers. On the other side, CD spectra have the same intensity of the signal but with opposite sign. A wavelength of 232 nm was indicated as an appropriate one to monitor L-carnitine and D-carnitine (Fig. 2). The ellipticity signal was employed for the direct determination of L-carnitine instead of the UV signal due to its selectivity. Thus, the CD signal directly obtained will correspond only to carnitine and the separation between carnitine and crotonoylbetaine will be used for the correct determination of the enantiomeric excess.

3.1.1. Optimisation of chromatographic conditions

Prior to the application of the proposed method, optimal values of the parameters causing variability of the measurements were selected. Thus, pH, ionic strength of the buffer solution, concentration of sodium 1-octanosulfonate, selection and content of the most appropriate organic modifier were optimised. Methanol-sodium 1-octanesulfonate (2.5 mM)–sodium phosphate buffer (pH 3; 50 mM)

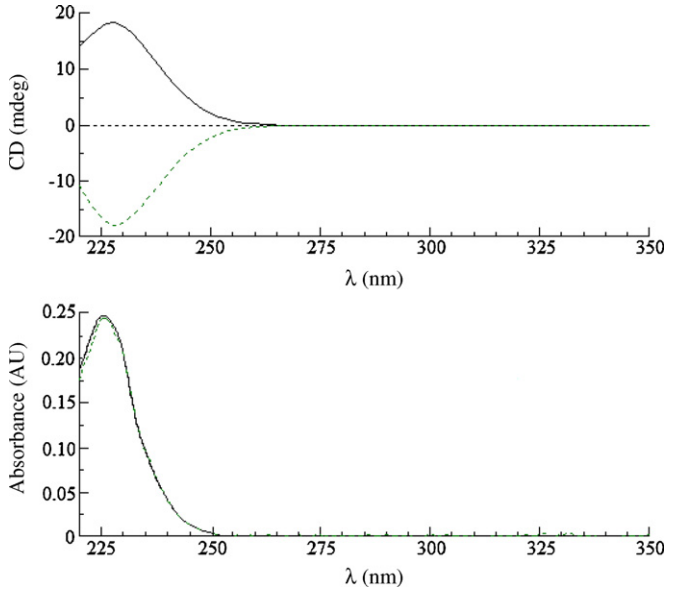

Fig. 2. CD and UV spectra of L-carnitine (dash line —) and D-carnitine (continuous line —) at a concentration of 10^3 μg mL^{-1}.

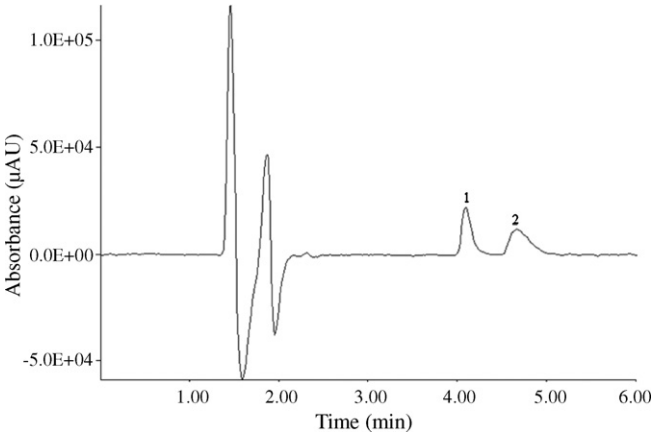

Fig. 3. HPLC chromatogram obtained after injection of crotonoylbetaine (1) and L-carnitine (2). Column (Ascentis Express, 150 mm × 4.6 mm ID, 2.7 μm particle size); mobile phase, sodium phosphate buffer/MeOH (pH 3, ionic strength: 50 mM), 98.5:2.5 (v/v); detector wavelength, 232 nm; flow rate, 1 mL min^{-1}; injection volume, 20 μL; ambient temperature, carnitine concentration, 10^3 μg mL^{-1}; crotonoylbetaine concentration, 10 μg mL^{-1}. Peaks between 1.00 and 2.00 min correspond to dead time.

(2.5:97.5, v/v) at a flow rate of 1 mL min^{-1} were selected due to the good chromatographic performances obtained (peak efficiencies of 7760 ± 325 theoretical plates for crotonoylbetaine and 2030 ± 90 theoretical plates for carnitine) and a short run time of 6 min. Retention times (expressed as mean ± SD) under these conditions were 4.11 ± 0.01 min for crotonoylbetaine and 4.67 ± 0.01 min for carnitine (Fig. 3). Moreover, a high resolution of 3.16 between carnitine and crotonoylbetaine was obtained and no impurities coeluted with these two analytes when samples were injected into the HPLC system. The readings of absorbance, circular dichroism and anisotropy factor were taken at the retention time of carnitine for both enantiomers of carnitine (Fig. 4). The shape of anisotropy factor peak obtained is caused by the scarce absorptivity of carnitine that generates a higher noise level and consequently less sensitiveness in the detection. Additionally, *g* factor peaks observed by other authors (for instance, reference [38]) were not absolutely a constant signal throughout the retention time of the analytes. This fact does not imply that *g* factor would depend on the concentration of the analyte, only on the ratio of the enantiomers. In this sense, the anisotropy factor was calculated at the retention time of carnitine.

3.2. Calibration and figures of merit

To analyse the linearity of the UV-CD detector, calibration curves were plotted as peak area versus concentration of each analyte. The tested concentrations were 1, 2.5, 5, 10, 20, 30, 40, 46.8 and 50 mg mL^{-1} in the calibration of crotonoylbetaine. In the analysis of linearity for L-carnitine and D-carnitine 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2500, 3000, 4000 and 5000 μg mL^{-1} concentrations were tested. Linearity of the calibration curve of enantiomeric excess (e.e.) was analysed by plotting the *g* factor versus the enantiomeric excess in the −100 to +100% range. Concentrations of L-carnitine and D-carnitine were adjusted to keep the carnitine concentration constant at 800 mg L^{-1}. The linearity was analysed with a series of 13 e.e. levels: −100, −98, −90, −80, −50, −20, 0, +20, +50, +80, +90, +98 and +100% and the correlation coefficient (R^2) was calculated to prove the linearity of the calibration curves. The LOD and LOQ were considered to be the concentrations that produced a signal-to-noise ratio of 3 and 10 respectively and were calculated as $3S_{y/x}/s$ and $10S_{y/x}/s$, respectively, where $S_{y/x}$ is the standard deviation of residuals, and *s* the slopes of the standard calibration curves. The precision and accu-

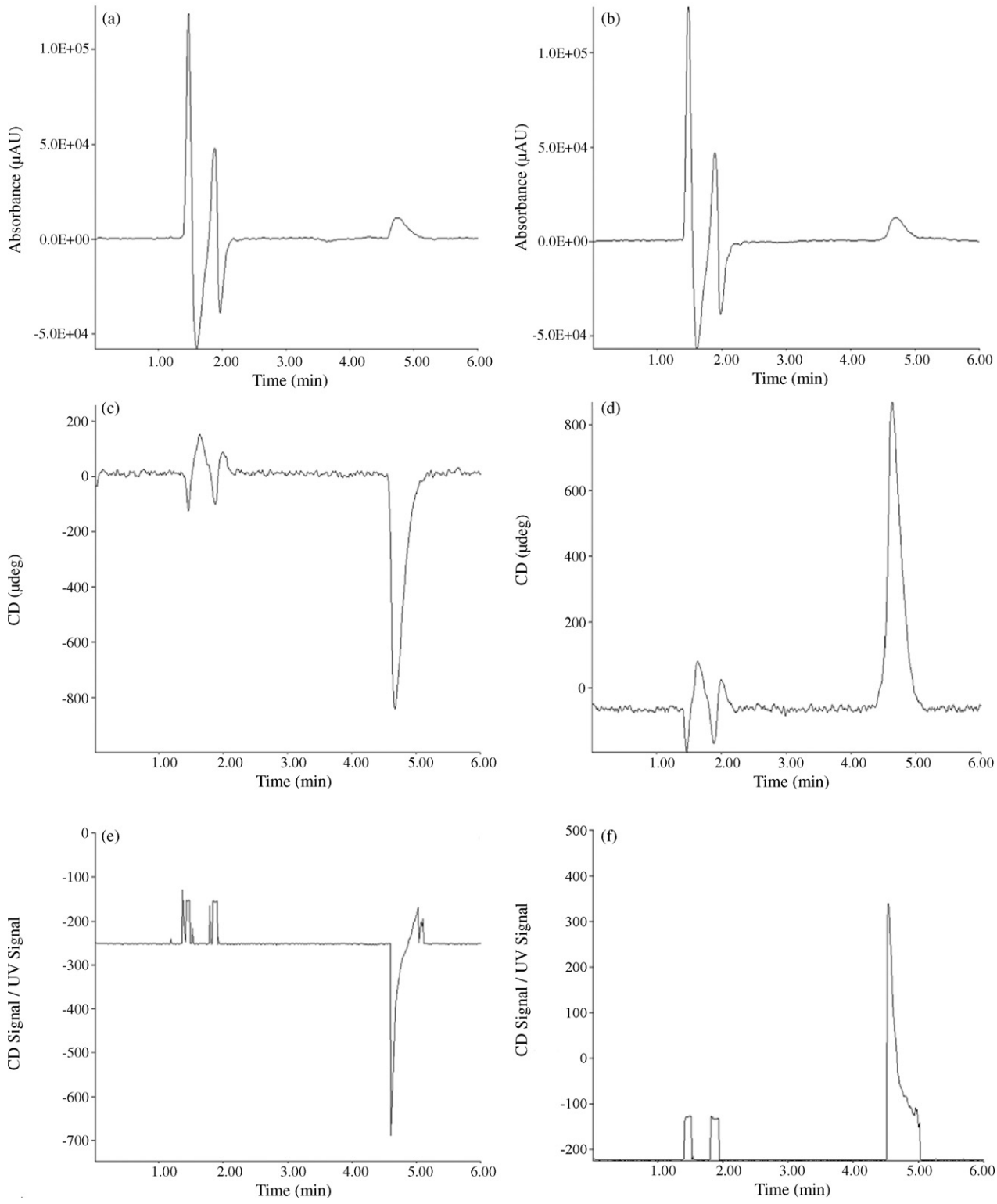

Fig. 4. Chromatographic profiles corresponding to UV (a and b), CD (c and d), and *g* factor (e and f) signals obtained after injection of L-carnitine (a, c and e) and D-carnitine (b, d and f) into the HPLC system. Chromatographic conditions as reported in Fig. 3.

racy were defined as the relative standard deviation (%RSD) and as the error from the theoretical nominal concentration, respectively. Assay concentrations were calculated by comparing the HPLC peak areas of samples to those of L-carnitine standard solutions (10^3 μg mL^{-1}). To evaluate the intra-day precision, ten replicate injections of standard solutions of L-carnitine and crotonoylbetaine at concentrations of 10^3 and 10 μg mL^{-1} respectively were conducted. The same procedure was repeated on 3 consecutive days to determine the inter-day precision.

Under the working conditions, standard calibration curves were obtained for peak areas from absorbance signals versus concentrations of standard solutions. Ranges of concentrations of 100–10^3 μg mL^{-1} for L-carnitine and of 1–50 μg mL^{-1} for crotonoylbetaine were found to be linear ($n = 8$). The straight-line

Table 1
Validation parameters for the HPLC method to determine L-carnitine (ellipticity signal) and crotonoylbetaine (UV signal) contents.

	Linear range ($\mu g\,mL^{-1}$)	$Y=(a\pm S_a)X+(b\pm S_b)$	R^2	$S_{y/x}$	LOD	LOQ	Precision	
							Intra-day	Inter-day
L-Carnitine	100–10^3	$Y=(0.011\pm1.4E^{-4})\,C_L-(0.187\pm0.069)$	0.999	0.108	18.4	61.4	3.68	4.75
Crotonoylbetaine	1–50	$Y=(0.016\pm2E^{-4})\,C_C-(0.009\pm0.005)$	0.999	0.009	0.91	3.03	3.33	3.83

a: slope (mdeg mg L^{-1} for L-carnitine and AU mg L^{-1} for crotonoylbetaine); S_a: standard deviation of the slope; b: intercept (mdeg for L-carnitine and AU for crotonoylbetaine); S_b: standard deviation of the intercept; R^2: regression coefficient; $S_{y/x}$: standard deviation of residuals; LOD: limit of detection ($\mu g\,mL^{-1}$); LOQ: limit of quantification ($\mu g\,mL^{-1}$); precision expressed as relative standard deviation (%, $n=10$).

equations, correlation coefficients, the limits of detection and quantification were calculated. The precision of the method was assessed by analysing the standards at concentration of 10^3 $\mu g\,mL^{-1}$ ten times consecutively. The results showed that the intra- and inter-day precision, expressed as relative standard deviation (%), for L-carnitine and crotonoylbetaine were not over 3% (Table 1). The linearity of the anisotropy factor (g) versus enantiomeric excess was determined at different e.e. levels over the range from −100 up to 100% and the regression was found to be lineal all over this range. The straight-line equation over 13 points is fitted to $y=(0.0008\pm2.0E^{-5})x$ (e.e.) $-(0.0019\pm0.0012)$, where y is g factor and x the enantiomeric excess. A correlation coefficient of 0.996 was obtained. Precision of the method for the enantiomeric excess determination was in the range of 0.1–4.8% (Table 2).

3.3. Determination of L-carnitine in dietary supplements and pharmaceutical formulations

To test the proposed method a quantitative analysis of commercially available L-carnitine formulations (capsules, oral solution and tablets of a pharmaceutical) was conducted. No matrix interferences were detected at none of the analysed samples and this method allowed a high resolution between carnitine and crotonoylbetaine, which was not found in any of the analysed samples (Fig. 5). Time of each analysis is short (6 min) and reproducible results with good recovery values were obtained (Table 3).

3.4. Enantiomeric excess determination in dietary supplements and pharmaceutical formulations

To determine the excess of one enantiomer compared to the other, the following equation was applied:

$$\text{e.e.}_L(\%)=\frac{[L]-[D]}{[L]+[D]}\times 100 \quad (1)$$

where $[L]$ and $[D]$ are the concentrations of L-carnitine and D-carnitine, respectively [39].

Table 2
Precision for enantiomeric excess determination ($n=3$).

Enantiomer excess (%)	RSD on CD (%)	RSD on UV (%)	RSD on g-factor (%)
−100	3.5	3.3	0.3
−98	1.5	1.4	2.6
−90	3.4	1.6	1.9
−80	3.6	0.7	2.7
−50	0.8	1.2	1.5
−20	1.3	0.5	0.6
0	1.0	3.1	0.7
20	2.4	4.8	2.4
50	3.8	4.8	3.7
80	3.3	0.7	1.9
90	1.4	1.8	1.4
98	4.3	0.9	3.6
100	1.8	0.1	1.1

Enantiomeric excess <0 indicate ratios L-carnitine/D-carnitine >1. Enantiomeric excess >0 correspond to ratios L/D < 1. 0 corresponds to ratio L/D = 1.

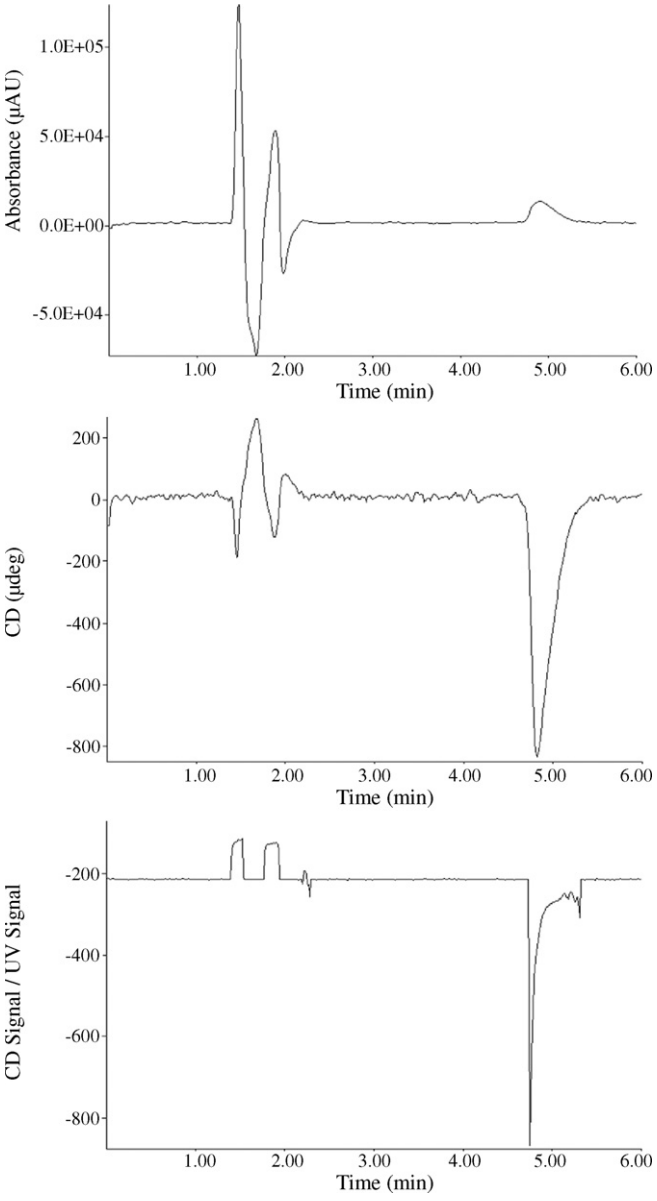

Fig. 5. Chromatographic profile corresponding to UV, CD and g factor signals after the injection of the pharmaceutical formulation sample into HPLC system. Chromatographic conditions as reported in Fig. 3.

Samples of L-carnitine known concentration were spiked with different enantiomeric excess of D-carnitine in the range 0.5–10%. No interferences from excipients were observed. Accuracy has been expressed as % recovery of added D-carnitine, and the relative standard deviation is presented related to precision (Table 4). Good recovery values were obtained with a precision in the range 2.9–4.3%. It can be concluded than the use of a silica C_8 column

Table 3
Quantitative analysis of commercially available L-carnitine products.

Sample	Theoretical content (mg)	Found content (mean ± SD; $n = 3$)	Recovery (%)
L-Carnitine capsule[a]	100	96 ± 1	96
L-Carnitine capsule[b]	273	253 ± 3	93
L-Carnitine capsule[c]	273	271 ± 3	99
L-Carnitine oral formulation	1000	1010 ± 12	101
L-Carnitine pharmaceutical tablets	300	286 ± 3	95

[a] Korott.
[b] Naturtierra.
[c] Salud Mediterránea.

Table 4
Results of the determination of enantiomeric excess of D-carnitine obtained by spiking samples of known L-carnitine concentration ($n = 15$).

D-Carnitine % spiked	Found enantiomeric excess	
	Recovery (%)	RSD (%)
0.5	101	3.7
1	99.5	4.3
2	101	3.3
5	98.5	2.9
10	97.3	4.3

with CD detection was adequate to estimate in a first approach the enantiomeric excess of D-carnitine.

4. Conclusions

This HPLC validated method with an achiral support and circular dichroism detector is suitable for quantitative analysis of L-carnitine in capsules, oral solutions and tablets of commercially available formulations, and the determination of D-carnitine enantiomeric excess, which has been successfully performed thanks to simultaneous UV and CD detections, throughout the *g* factor calculation. This method lacks of derivatization step and of a chiral support to separate the enantiomers which imply a low cost of the analysis. Additionally, a simple sample pre-treatment is required and no matrix interferences were detected. Moreover, a large number of samples can be analysed due to the short analysis time (6 min) and a high resolution between carnitine and its main impurity, crotonoylbetaine remains stable. This method could also be useful to detect carnitine enantiomers or its L-acylated metabolites in biological samples, such as urine or plasma samples, from individuals with metabolic disorders related to the levels of these metabolites in the organism. The results obtained and the reported data on precision, accuracy, linearity and sensitivity show that the developed method is useful for the application purposes. The lower precision obtained respect to the chiral HPLC methods are balanced by the rapidness, accuracy, economical advantages and simplicity of the proposed method, and its usefulness as screening and a first approach for the determination of enantiomeric excess of D-carnitine.

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