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Sensitive determination of D-carnitine as enantiomeric impurity of levo-carnitine in pharmaceutical formulations by capillary electrophoresis–tandem mass spectrometry

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ABSTRACT

In this work, capillary electrophoresis–electrospray ionization-tandem mass spectrometry was applied to the determination of L- and D-carnitine in pharmaceutical formulations. A simple sample treatment procedure consisting of the use of a dilution or an extraction step with water was employed prior to derivatization with 9-fluorenylmethoxycarbonyl (FMOC). The method was validated in terms of selectivity, linearity, accuracy, precision and sensitivity, with a LOD of 10 ng mL^{-1} for each enantiomer, which was enough to detect enantiomeric impurities up to 0.002% of D-carnitine with respect to the main enantiomer (L-carnitine). Eleven pharmaceutical formulations were analyzed including ampoules, oral solutions, sachets, and tablets. Results showed contents for carnitine comprised between 77 and 101% with respect to the labeled ones in the case of those formulations marketed with the racemate, and from 97 to 102% in those cases where the single enantiomer (L-carnitine) was employed as active ingredient. Percentages for the enantiomeric impurity (D-carnitine) ranging from 0.6 to 1.3% were obtained exceeding the limits established for impurities in drug products. These results corroborate the need of validated analytical methodologies enabling the quality control of pharmaceutical formulations containing carnitine.

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1. Introduction

The impact of chirality in the pharmaceutical field has increased in last years as shown by the development of an important number of drugs using single enantiomers instead of racemic active ingredients. The significance of enantiopurity analysis in chiral drugs is based on the different pharmacokinetic and pharmacodynamic behaviour that D- and L-enantiomers may have. Consequently, the enantiomers of a chiral drug must be carefully considered and investigated in order to understand their role in the drug efficacy.

Levo-carnitine (L-carnitine) is a highly therapeutically effective amino acid which is an essential cofactor of fatty acid metabolism. It is used as a drug since 1960 for the therapy of primary and secondary carnitine deficiency, and in various other diseases such as dislipoproteinemia, anorexia, and dyspepsia. Nevertheless, D-carnitine is considered as a toxic impurity because, as pure substance or in the form of racemic DL-carnitine, it has been found to be not only physiologically inactive but also leading to a functionally relevant depletion of the natural isomer L-carnitine. Thus, serious side effects to human health like muscle weakness and cardiac arrhythmias have been observed with application of racemic DL-carnitine [1–3]. In fact, L-carnitine is the active pharmaceutical ingredient appearing in the European and US Pharmacopoeias for raw material [4,5]. In addition, pharmaceutical regulatory authorities such as the US Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA) require the synthesis and use of drugs as single enantiomers which should provide better therapeutic results and fewer toxic effects. In spite of these facts, some pharmaceutical formulations elaborated and labeled with carnitine racemate are still being legally commercialized while other are marketed with the single enantiomer (L-carnitine). On the other hand, according to the above-mentioned pharmacopoeias, the percentage of any impurity in raw material is limited to only 0.1%. However, only an optical purity control of L-carnitine as raw material is required with a specific optical rotation between −29.0 and −32.0 which lacks of sensitivity.

Several chiral separation methods for carnitine have been reported by using HPLC [6–12] and CE techniques [6,8,13–15]. Among the described methods, only five were applied to pharmaceutical formulations, four out of them employed HPLC with fluorescence or UV detection and the other was based on the use of CE with UV detection. When using HPLC with fluorescence detection, the determination of L- and D-carnitine in two pharmaceutical formulations elaborated with L-carnitine as pharmacological active

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substance was performed in a non-chiral column after previous formation of the diastereomeric carnitine esters using (+)-1-(9-fluorenyl)ethylchloroformate ((+)-FLEC) as chiral reagent for derivatization of carnitine [8]. The content of D-carnitine in the samples was about 0.4% with respect to the total carnitine. A similar methodology was also employed using (+)-FLEC to carry out the determination of D-carnitine in L-carnitine in the range of 0.1–1% [11]. On the other hand, HPLC with a chiral column (ovomucoid-conjugated) and using 9-anthroylnitrile as achiral agent for derivatization of carnitine enabled the determination of carnitine enantiomers with UV detection [9]. In this case, the LOD was 50 $\mu g\, mL^{-1}$ for each carnitine enantiomer, which was too high to detect D-carnitine (only up to 5%) as enantiomeric impurity in L-carnitine pharmaceutical preparations. Finally, the use of another chiral column (Chiralcel D–R) in HPLC with UV detection after derivatization with (α-bromo) methyl phenyl ketone as achiral reagent for carnitine also allowed the enantiomeric separation of D/L-carnitine [12]. The introduction of this lipophilic UV chromophoric group to the carnitine enantiomers improved their retention, enantioresolution and UV detection. The LOD was found to be 0.03% for D-carnitine; however, the content of D-carnitine in three batches of the drug substance (L-carnitine) was found to be below the LOD. With regards to CE, only one article has been published dealing with the quantification of carnitine enantiomers in pharmaceutical formulations [13]. The method was based on the use of FMOC as achiral derivatizing reagent for carnitine, 2,6-dimethyl-β-CD as chiral selector, and UV detection. One pharmaceutical formulation elaborated with L-carnitine was analyzed, and the result (0.42% of D-carnitine) was compared with that obtained by the HPLC method with fluorescence detection developed by the same research group [8], showing good correlation between both methods.

Mass spectrometry (MS) was never employed for the chiral determination of carnitine in pharmaceutical formulations in spite of its advantages including high sensitivity and selectivity. In fact, CE–MS is a powerful combination for performing rapid, efficient, and sensitive analysis of chiral compounds in order to reach the required low level of quantification for enantiomeric impurities. Our research team has recently developed two analytical methodologies by CE–ESI-MS^2 enabling the determination of carnitine enantiomers in infant formulas [16] and dietary food supplements [17]. A first methodology was developed using a relatively high concentration (2%) of Succ-γ-CD (3 succinyl groups per cyclodextrin ring) making necessary the use of the partial filling technique [16]. This technique is gaining importance in chiral separations by CE–MS because the chiral selector is introduced into the capillary as a plug, shorter than the effective length of the capillary, to avoid its entrance in the MS detection system. Thus, the non-volatile chiral selector does not reach the mass spectrometer avoiding its ionization disturbing effects. Nevertheless, a direct coupling of chiral CE–MS without employing the partial filling of the capillary is very challenging because it increases the separation window available and avoids band-broadening effects in the chiral selector/buffer boundary. Moreover, the selectivity is also more predictable since the same separation principle is active throughout the capillary [18,19]. In the case of carnitine, the use of the above-mentioned chiral selector (Succ-γ-CD) but possessing a different degree of substitution (4 succinyl groups per cyclodextrin ring) led to a considerable improvement of the chiral resolution enabling the use of very low concentrations of CD (0.2% w/v) that were directly introduced in the MS system without a significant contamination [17]. Due to the high interest of the determination of carnitine enantiomers in drugs, the aim of this work was to validate and apply this second analytical methodology to the determination of L- and D-carnitine in pharmaceutical formulations in order to: (i) compare the determined amount of carnitine in the pharmaceutical formulations with the labeled ones for quality control purposes, and (ii) determine D-carnitine as enantiomeric impurity of L-carnitine in those pharmaceutical formulations marketed using L-carnitine as single enantiomer.

2. Experimental

2.1. Reagents and samples

All reagents employed were of analytical grade. Isopropanol was from Scharlau Chemie (Barcelona, Spain). Acetone, 25% ammonium hydroxide solution, sodium hydroxide and hydrochloric acid were from Merck (Darmstadt, Germany). Acetic and formic acids were from Riedel-de Hägen (Seelze, Alemania). Sodium hydrogen carbonate and phosphoric acid were from Panreac Química S.A. (Barcelona, Spain). 9-Fluorenylmethoxycarbonyl (FMOC) was purchased from Fluka (Buchs, Switzerland). Succinyl-γ-cyclodextrin (Succ-γ-CD) with a degree of substitution of 4 was supplied from Cyclolab (Budapest, Hungary). DL-Carnitine and D-carnitine were from Sigma (St. Louis, MO, USA).

Eleven pharmaceutical formulations (ampoules, oral solutions, sachets, and tablets) were analyzed. They were acquired in a pharmacy of Madrid (Spain). As shown in Table 1 four of them were marketed using the single enantiomer (L-carnitine) while the other seven were elaborated with the racemate and labeled as carnitine or DL-carnitine. According to their complexity, different sample treatments were used: (i) oral solutions and ampoules were homogenized and diluted in water up to a estimated concentration of 0.5 $mg\, mL^{-1}$ of carnitine before taking the aliquot for derivatization; (ii) in the case of sachets, four units were homogenized, weighing the necessary quantity to obtain a final concentration of 0.5 $mg\, mL^{-1}$ of carnitine in water; (iii) for tablets ten units were grinded and homogenized, weighing the necessary quantity to obtain a final concentration of 0.5 $mg\, mL^{-1}$ of carnitine after two consecutive water extractions. Each extraction was followed by centrifugation (5000 × *g* for 15 min at 25 °C) and all the supernatants were collected in an appropriate volumetric flask that was completed with water. Finally, derivatization with FMOC was achieved in all cases.

2.2. Separation electrolytes and standard solutions

Separation electrolytes were prepared by dissolving Succ-γ-CD at a 0.2% (m/v) in the buffer solution. The buffer solution was prepared by diluting the appropriate volume of formic acid with Milli-Q water and adjusting the pH to 2.5 with 25% ammonium hydroxide solution before completing the volume with water to get a 0.5 M buffer concentration. All these solutions were filtered prior use through 0.45 μm pore size disposable nylon filters from

Table 1
Abbreviated name assigned, labeled name of the active pharmaceutical ingredient, labeled amount of carnitine, and price per gram of carnitine for each one of the eleven pharmaceutical formulations analyzed in this work.

Type of pharmaceutical formulation	Labeled name	Labeled amount	Prices (€/g)
Ampoules#1	L-Carnitine	100 $mg\, mL^{-1}$	0.75
Ampoules#2	Carnitine	100 $mg\, mL^{-1}$	0.75
Oral solution#1	Carnitine	50 $mg\, mL^{-1}$	0.73
Oral solution#2	Carnitine	62.5 $mg\, mL^{-1}$	0.40
Oral solution#3	Carnitine	50 $mg\, mL^{-1}$	1.59
Oral solution#4	DL-Carnitine	75 $mg\, mL^{-1}$	0.37
Oral solution#5	DL-Carnitine	50 $mg\, mL^{-1}$	1.80
Sachets#1	DL-Carnitine	40 $mg\, g^{-1}$	2.93
Sachets#2	L-Carnitine	180 $mg\, g^{-1}$	1.42
Tablets#1	L-Carnitine	120 $mg\, g^{-1}$	2.50
Tablets#2	L-Carnitine	300 $mg\, g^{-1}$	2.15

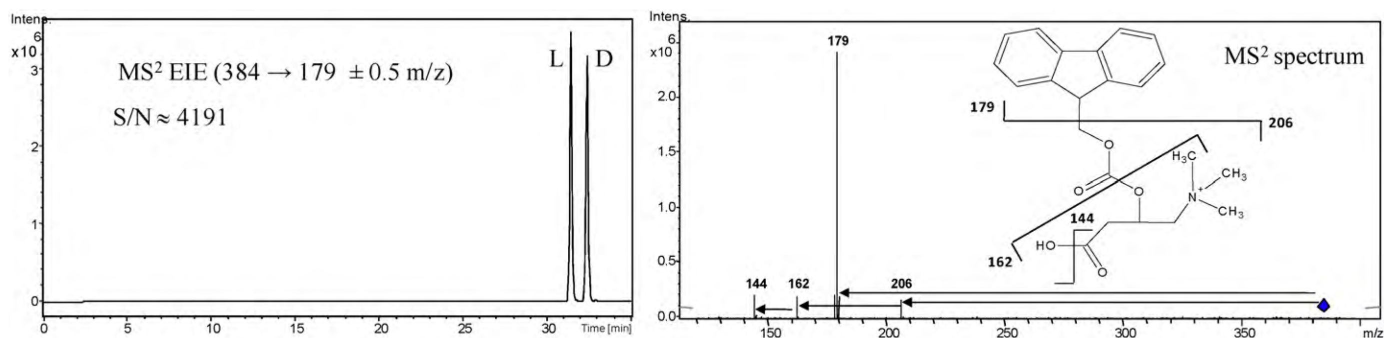

Fig. 1. MS^2 electropherogram, fragmentation pattern and spectrum for a 0.5 mg mL^{-1} DL-carnitine. CE conditions: BGE, 0.5 M ammonium formate buffer (pH 2.5) with 0.2% (m/v) Succ-γ-CD; uncoated fused-silica capillary, 50 μm ID × 100 cm; injection by pressure at 50 mbar × 12 s; applied voltage, 25 kV; temperature, 25 °C. ESI conditions: positive ion mode; spray voltage, 4.5 kV; sheath liquid, isopropanol/water (50/50 v/v) with 0.1% formic acid at 3.3 μL min^{-1}; drying gas flow, 5 L min^{-1}; drying temperature, 300 °C; nebulizer pressure, 2 psi; compound stability, 50%. MS^2 transitions, 384 → 179 *m*/*z*; width, 4 *m*/*z*; fragmentation amplitude, 1.20 V. Scheme of the fragmentation pattern according to [16].

Titan (Eatontown, NJ, USA). Stock standard solutions of DL-carnitine and D-carnitine were prepared from a 1.5 mg mL^{-1} and 1.0 mg mL^{-1} solution respectively and diluting them with Milli-Q water up to the desired concentration. These solutions were stored at 4 °C and warmed at room temperature before use.

2.3. Derivatization procedure

Derivation of carnitine with FMOC was achieved when 50 μL of the standard or samples solutions were mixed with 50 μL of 50 mM carbonate buffer (pH 10.4) and 130 μL of 30 mM FMOC solution (prepared in acetone) and they were allowed to react for 60 min at 45 °C [16,17]. Then, the derivatization reaction was stopped by adding 150 μL of 50 mM acetate buffer (pH 4.2) to the mixture. The derivatization was daily carried out before sample injection in the CE system.

2.4. Capillary electrophoresis conditions

The analyses were carried out in a HP^{3D}CE instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with an on-column DAD working at 254 nm with a bandwidth of 5 nm. The instrument was controlled by a PC running the 3D-CE ChemStation from Agilent Technologies. Uncoated fused-silica capillaries of 50 μm ID and 100 cm length were used. Before first use, a new capillary was conditioned by rinsing at 1 bar with 1 M NaOH for 30 min, then with water for 5 min, followed by 0.1 M HCl for 3 min, and buffer for 60 min. After each run, the capillary was rinsed at 1 bar for 2 min with 0.1 M phosphoric acid, followed by Milli-Q water for 2 min and buffer for 2 min, to maintain an adequate repeatability between injections. Then, 0.2% (m/v) Succ-γ-CD in 0.5 M ammonium formate buffer (pH 2.5) was filled into the capillary at 1 bar for 4 min. Injections were made at the anodic end using a N_2 pressure of 50 mbar for 12 s (≈1% of the total capillary volume, ≈12 nL). The electrophoretic separation was achieved at 25 kV (normal polarity mode) and 25 °C [17].

2.5. Mass spectrometry conditions

Identification and quantification of the target analytes were performed on a 3D Ion Trap mass spectrometer (model 1100 from Agilent Technologies, Palo Alto, CA, USA) coupled to the CE apparatus through an orthogonal electrospray interface (ESI, model G1607A from Agilent Technologies, Palo Alto, CA, USA). LC/MSD Trap Software 5.2 was used for MS control and data analysis. CE–MS conditions were as in a previous work of our research team [17]. Briefly, a sheath liquid based on isopropanol:water (50:50 v/v) with 0.1% formic acid and delivered at a flow rate of 3.3 μL min^{-1} by a syringe pump (model 100, Holliston, USA) was used. The nebulizer and drying gas conditions were 2 psi N_2 and 5 L min^{-1} N_2 at 300 °C. The mass spectrometer operated with the ESI source in the positive ion mode (4.5 kV) and the *m*/*z* range scanned was from 120 to 400. The trap parameters were programmed in ion charge control mode to accumulate 50,000 ions, for a maximum accumulation time of 300 ms, and three scans were averaged per experiment. Other parameters were 86.7 V for capillary exit offset, target mass of 384 *m*/*z*, 50% for compound stability, and 38 for trap drive. The fragmentation was carried out by collision-induced dissociation with the helium present in the trap for 40 ms with 1.20 V for fragmentation amplitude, an isolation width of 4.0 *m*/*z* and a cut off of 106 *m*/*z* to obtain MS^2 experiments in manual mode. Finally, in order to avoid the entrance of air bubbles during the injection step due to the Ventury effect caused by the nebulizer gas, the sample injection was carried out with the nebulizer pressure and the ESI voltage set at zero, and a delay of 2 min was used after sample injection before setting the optimum spray gas and ESI voltage.

3. Results and discussion

The CE–MS^2 method recently developed by our research team [17] was slightly modified in order to analyze pharmaceutical formulations. Thus, the nominal value used in the analysis (maximum concentration of the majority enantiomer that can be injected without loss in resolution or solubility problems) was optimized and increased more than 10 times (from 0.04 mg mL^{-1} to 0.5 mg mL^{-1} carnitine) in order to decrease the relative limit of detection (RLOD) corresponding to D-carnitine. The RLOD is a measure of the minimum enantiomeric impurity that can be detected in mixtures containing high enantiomeric excesses. As a 0.5 M ammonium formate buffer (pH 2.5) in positive polarity and full scan positive ESI ionization was employed [16,17], carnitine (pK_a = 3.8) migrated as positively charged compound towards the cathode end of the capillary coupled to the ion-trap spectrometer via a coaxial sheath liquid interface. Fig. 1 shows the electropherogram, fragmentation pattern, and spectrum for FMOC–carnitine (0.5 mg mL^{-1}) in the MS^2 mode. As reported in our previous work [16], the ion transitions were *m*/*z* 384 → 179, 384 → 206, 384 → 162 and 384 → 144. This CE–MS^2 method was validated for the determination of carnitine enantiomers in pharmaceutical formulations in terms of selectivity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) according to the International Conference on Harmonization (ICH) Guidelines Q2 (R1) [20].

Table 2
Regression equations for calibration straight lines obtained using the CE–ESI-MS^2 method validated for the determination of L- and D-carnitine in pharmaceutical formulations.[a].

	L-Carnitine high level	D-Carnitine high level	D-Carnitine low level
External standard calibration method[b]			
Correlation coefficient	0.9997	0.9998	0.9997
Slope	$3.62 (\pm 0.09) \times 10^6$	$3.48 (\pm 0.54) \times 10^6$	$3.42 (\pm 0.14) \times 10^6$
Intercept	$-11.6 (\pm 36.0) \times 10^3$	$-2.70 (\pm 21.0) \times 10^3$	$1.50 (\pm 1.67) \times 10^3$
Standard additions calibration method[c]			
Correlation coefficient	–	0.998	0.996
Slope	–	$3.61 (\pm 0.21) \times 10^6$	$3.54 (\pm 0.90) \times 10^6$
Intercept	–	$49.5 (\pm 9.75) \times 10^3$	$20.4 (\pm 9.01) \times 10^3$

[a] Experimental conditions as in Fig. 1.
[b] Five standard solutions for high concentration levels, ranging from 0.1 mg mL^{-1} to 0.6 mg mL^{-1} of each carnitine enantiomer, and five standard solutions for low concentration levels, ranging from 0.1 μg mL^{-1} to 25 μg mL^{-1} of each carnitine enantiomer, were employed. Average values of triplicate measurements were considered. Values in parentheses are confidence intervals at 95%: $\pm t \times s_{slope}$ or $\pm t \times s_{intercept}$.
[c] Four solutions with three high spiked levels (0.1 mg mL^{-1}, 0.25 mg mL^{-1}, and 0.5 mg mL^{-1}) for Sachet#1 and three low spiked levels (2 μg mL^{-1}, 10 μg mL^{-1} and 20 μg mL^{-1}) for Tablet#1. Average values of triplicate measurements were considered. Values in parentheses are confidence intervals at 95%: $\pm t \times s_{slope}$ or $\pm t \times s_{intercept}$.

3.1. Selectivity

The precursor → product ion transitions for the target compound (384 *m*/*z* of FMOC–carnitine molecular ion) was used to verify the selectivity. Thus, as it can be seen in Fig. 1 an adequate selectivity is obtained since carnitine can be determined without any interferent peak. Moreover, an enantiomeric resolution of about 3 is obtained and the unequivocal identification of carnitine was possible from the MS/MS spectrum. In fact, since more than two precursor/product transitions were obtained, the unambiguous identification of carnitine by CE–MS was performed [21].

3.2. Linearity

Calibration plots using the external standard calibration method were obtained by plotting corrected peak areas (peak area to migration time ratio) from the EIE of the MS^2 experiment at *m*/*z* 179 as a function of standard concentrations in mg mL^{-1}. The nominal concentration for the carnitine analysis was set to 0.5 mg mL^{-1} for each carnitine enantiomer. Five standard solutions at high concentration levels (0.1 mg mL^{-1}, 0.2 mg mL^{-1}, 0.3 mg mL^{-1}, 0.5 mg mL^{-1}, and 0.6 mg mL^{-1}, ranging from 20 to 120% relative to 0.5 mg mL^{-1}) for each carnitine enantiomer, and five standard solutions at low levels (0.1 μg mL^{-1}, 0.25 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, and 25 μg mL^{-1}, ranging from 0.02 to 5% relative to 0.5 mg mL^{-1}) for D-carnitine were used. As shown in Table 2, in all cases, regression lines with correlation coefficients (r) above 0.999 and with the intercepts including the zero value for a 95% confidence level, were obtained. A comparison between confidence intervals for the slopes of the calibration curves obtained for each enantiomer showed that there were not statistically significant differences between them (confidence level of 95%). Thus, since the relative response factor (RRF) between both enantiomers was about 1.04 (obtained by dividing their slopes at high levels) and 1.06 (obtained by dividing their slopes at high levels for L-carnitine and low levels for D-carnitine), the response for D-carnitine could be considered equivalent to L-carnitine because the RRF was in the range between 0.8 and 1.2 [4]. As a consequence, the percentage of D-carnitine could be determined from the ratio between the areas of the L- and D-enantiomers. On the other hand, the effect of matrix interferences was tested for two types of formulations (Tablets#1 and Sachets#1) selected by its higher complexity and diverse composition. A comparison between confidence intervals for the slopes obtained by the external standard and the standard additions calibration methods showed that no significant differences were found at a confidence level of 95% (see Table 2), which indicated the absence of matrix interferences at high and low concentration levels of D-carnitine. For the liquid samples analyzed (oral solutions and ampoules), which have a similar or even less complex composition than drinks studied in a previous work [17], the absence of matrix interferences was assumed. As a consequence, the simpler calibration method using an external standard was used for the quantitation of carnitine enantiomers in the pharmaceutical formulations analyzed in this work.

3.3. Accuracy

The recovery was evaluated for each carnitine enantiomer in two representative formulations, selected due to their diverse complexity and composition (Sachets#1 and Tablets#1). Tablets#1 (elaborated with L-carnitine) was spiked with D-carnitine at low levels (2 μg mL^{-1}, 10 μg mL^{-1}, and 20 μg mL^{-1}) and at high levels (0.1 mg mL^{-1}, 0.25 mg mL^{-1}, and 0.5 mg mL^{-1}). However,

Table 3
Precision obtained for the CE–ESI-MS^2 method.

Precision	L-Carnitine high level	D-Carnitine high level	D-Carnitine low level
Instrumental repeatability (n = 6)[a]			
A_c, RSD (%)			
Standard solution	3.0	5.0	3.9
Ampoules	5.3	–	5.5
Oral solutions	–	–	–
Sachets	5.0	–	3.6
Tablets	3.4	–	3.1
t, RSD (%)			
Standard solution	1.2	1.2	1.8
Ampoules	1.3	–	1.9
Oral solutions	–	–	–
Sachets	1.1	–	1.3
Tablets	0.9	–	0.8
Intermediate precision (n = 4 or 3)[b]			
A_c, RSD (%)			
Standard solution	7.7	8.0	8.6
Ampoules	2.8	1.0	9.4
Oral solutions	6.5	6.8	–
Sachets	5.9	4.0	8.9
Tablets	7.4	–	9.6
t, RSD (%)			
Standard solution	2.5	2.5	1.6
Ampoules	2.2	3.4	1.1
Oral solutions	2.6	3.1	–
Sachets	3.1	3.1	2.7
Tablets	3.8	–	2.3

[a] Six consecutive injections in the same day of a 0.5 mg mL^{-1} carnitine solution.
[b] One solution prepared daily for carnitine standard (0.5 mg mL^{-1} for high concentration level and 10 μg mL^{-1} for low concentration level) injected by triplicate in four consecutive days or one solution prepared daily for each sample injected by triplicate in three consecutive days. Average values of triplicate measurements were considered.

Table 4
Determined amounts of L- and D-carnitine, percentages of D-carnitine referred to total carnitine, and percentages of L-carnitine with respect to the stated content in the eleven pharmaceutical formulations analyzed ($n = 3$).

Samples	L-Carnitine	D-Carnitine		
	Determined amount	Determined amount	% D-Carnitine	% Stated content
Ampoules#1	102 ± 2[a]	0.6 ± 0.2[a]	0.9 ± 0.3	102 ± 3
Ampoules#2	50 ± 2[a]	50.8 ± 0.3[a]	50.0 ± 0.7	101 ± 2
Oral solution#1	19.7 ± 0.8[a]	19 ± 1[a]	49.1 ± 0.9	77 ± 2
Oral solution#2	26 ± 2[a]	26 ± 3[a]	50.0 ± 0.6	83 ± 4
Oral solution#3	20.5 ± 0.7[a]	20.0 ± 0.7[a]	49.2 ± 0.4	81 ± 1
Oral solution#4	37.6 ± 0.3[a]	36.9 ± 0.4[a]	49.1 ± 0.1	99.3 ± 0.4
Oral solution#5	20.5 ± 0.4[a]	20.2 ± 0.8[a]	49.2 ± 0.1	81 ± 1
Sachets#1	20.0 ± 0.1[b]	19.6 ± 0.2[b]	49.6 ± 0.6	99.0 ± 0.1
Sachets#2	174 ± 1[b]	2 ± 2[b]	1.3 ± 0.9	97 ± 1
Tablets#1	120 ± 2[b]	1.2 ± 0.4[b]	1.2 ± 0.4	100 ± 1
Tablets#2	299 ± 5[b]	1.5 ± 0.9[b]	0.6 ± 0.1	100 ± 2

[a] $mg\,mL^{-1}$.
[b] $mg\,g^{-1}$.

Sachets#1 due to its racemic composition, only was spiked with 0.5 $mg\,mL^{-1}$ of DL-carnitine (0.25 $mg\,mL^{-1}$ of each carnitine enantiomer). Each one of these solutions was injected in triplicate. Recovery values close to 100% were obtained for both samples. Thus, recoveries of 106 ± 11% were obtained for the sample Tablets#1 at high levels of D-carnitine and 99.0 ± 1.4% at low levels of D-carnitine. For the sample Sachets#1 recoveries of 100.2 ± 3.2% for L-carnitine and 109 ± 12% for D-carnitine were obtained.

3.4. Precision

Precision was measured for carnitine standard solutions and for all the samples analyzed in this work. Precision at the high concentration level was measured injecting 0.5 $mg\,mL^{-1}$ carnitine solutions whereas at the low concentration level was calculated for 10 $\mu g\,mL^{-1}$ carnitine solutions. Table 3 shows the values of precision in terms of: (i) *instrumental repeatability*, obtained from six consecutive injections in the same day of the standard and sample solutions; and (ii) *intermediate precision*, assessed from four (for standard solutions) or three (for sample solutions) at each level injected in four (for standard solutions) or three (for sample solutions) consecutive days. Acceptable precision was obtained with RSD values for corrected peak area lower than 10%, which are quite good for a CE–MS hyphenation where usually higher RSD values are reported. With respect to the migration times, all RSD values were lower than 4%. The results showed no statistically significant differences between the precisions achieved with the standards and samples.

3.5. LOD and LOQ

The limits of detection (LOD, S/N = 3) and quantification (LOQ, S/N = 10) were determined by injecting low amounts of carnitine standard. A LOD of 10 $ng\,mL^{-1}$ and a LOQ of 33 $ng\,mL^{-1}$ were calculated for the carnitine solution previously to its derivatization. These values correspond to amounts of carnitine injected of about 0.12 pg and 0.40 pg, respectively. The RLOD obtained allowed to detect enantiomeric impurities of D-carnitine up to 0.002% in pharmaceutical formulations elaborated with L-carnitine.

3.6. Analysis of pharmaceutical formulations

Quantification is a subject of prime importance, particularly for the pharmaceutical industry, where the reliability of analytical data is essential. The CE–ESI-MS^2 method was used to determine the content of L- and D-carnitine, and the percentage of D-carnitine, with respect to the total carnitine, in 11 pharmaceutical formulations containing carnitine. Although the use of internal standards improves the quantitative performance when injection-related sources of error are possible or when using complex sample treatments where analytes may be lost, in this work, water solutions obtained after simple sample treatments (samples were only diluted for liquid solutions, dissolved in water, for sachets, or

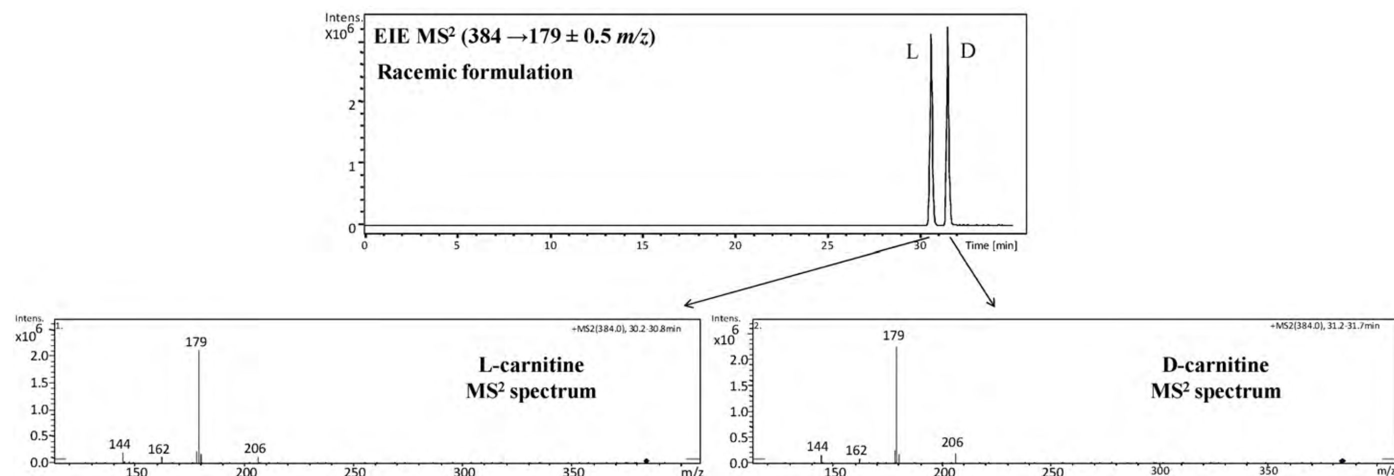

Fig. 2. CE–ESI-MS^2 EIE for the sample Ampoules#2 which contains racemic carnitine at m/z 179 and the corresponding MS^2 spectra for the peaks of L- and D-carnitine. Experimental conditions as in Fig. 1.

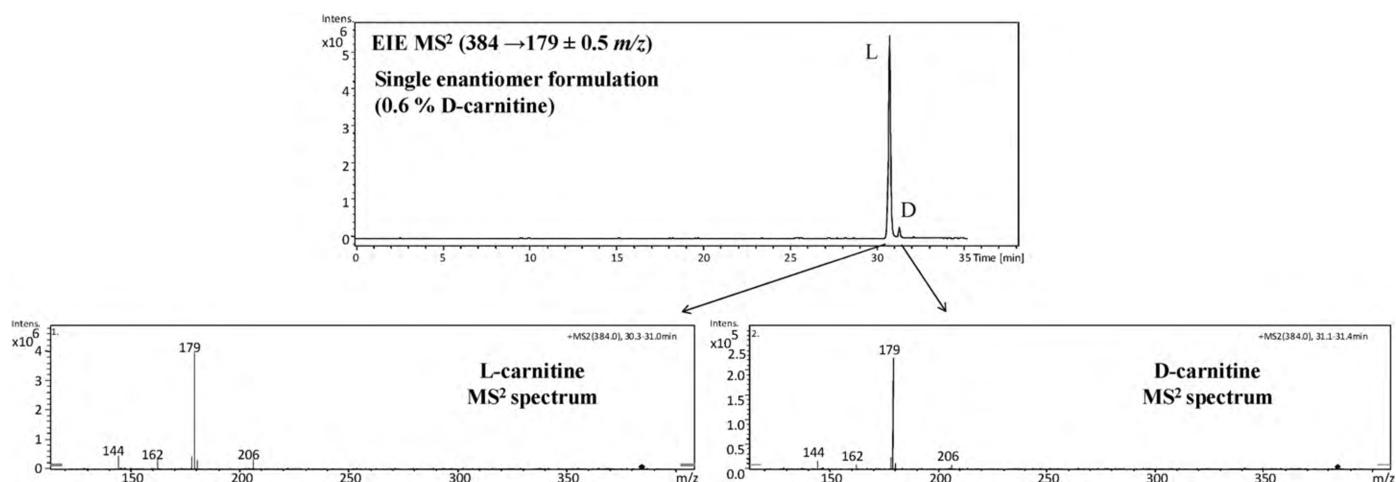

Fig. 3. CE–ESI-MS^2 EIE for the sample Tablets#2 and the corresponding MS^2 spectra for the peaks of L- and D-carnitine. Experimental conditions as in Fig. 1.

extracted with water, for tablets) were injected in CE–MS. As a consequence, the external standard method was employed. The results obtained are grouped in Table 4. The D-form was quantified through a calibration curve at high or low concentration levels depending on the content of this enantiomer in the samples. In all samples analyzed, the amount of D-carnitine was higher than the LOQ. The results obtained confirmed that in the seven samples elaborated with carnitine racemate (one ampoule, one sachet, and the five oral solutions analyzed), both enantiomers exist at a 1:1 ratio. Thus, the % of D-carnitine in these seven samples was around 50%. It is important to take into account that in the case of these racemic samples only half of the content correspond to the active pharmaceutical ingredient (L-carnitine) whereas the other half correspond to D-carnitine. Fig. 2 shows the EIE for a representative sample (Ampoules#2) that confirmed the existence of both enantiomers and their unequivocal identification through their MS^2 spectra. It is remarkable that in four of the eleven samples studied (one ampoule, one sachet, and the two tablets analyzed) elaborated with L-carnitine, percentages up to 1.3% D-carnitine were obtained, which exceed the limits established for impurities in drugs. Fig. 3 shows the EIE for the sample with the lowest percentage of D-carnitine (Tablets#2, with 0.6%). Despite of the low concentration of D-carnitine present in the solution analyzed (2.5 μg mL^{-1}), its unequivocal identification was possible through its MS^2 spectrum. Finally, for all samples, the percentages of active ingredient (carnitine or L-carnitine) with respect to the stated content (indicated in the label) were comprised between 77 and 102%. According to the price per gram included in Table 1 for each pharmaceutical formulation, it can be observed that the use of a single enantiomer does not always correspond with the most expensive pharmaceutical formulations.

4. Conclusions

A specific, sensitive, and reliable CE–ESI-MS^2 method was validated and applied for the first time to the identification and determination of L- and D-carnitine in pharmaceutical formulations. A simple sample treatment procedure consisting of the use of a dilution or an extraction step with water was employed. The LOD of the method (10 ng mL^{-1}) enabled to detect enantiomeric impurities up to 0.002% in drug product, and was considerably lower than the limit established by the current legislation (0.1%). The results obtained showed that when L-carnitine is employed as single enantiomer for the elaboration of the pharmaceutical formulation (four of the eleven samples analyzed) enantiomeric impurities (D-carnitine) up to 1.3% were determined exceeding the legal limits. The use of racemic carnitine in seven of the eleven samples analyzed was corroborated (% D-carnitine between 49.1 and 50.0%). Contents for carnitine comprised from 77 to 101% with respect to the labeled ones were obtained in the case of those formulations marketed with the racemate and from 97 to 102% in those cases where the single enantiomer (L-carnitine) was employed as active ingredient. These results show the high potential of the method proposed to achieve the quality control of pharmaceutical formulations containing carnitine.

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