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Determination of L-carnitine in food supplement formulations using ion-pair chromatography with indirect conductimetric detection

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Abstract

A novel method for the determination of L-carnitine in food supplement formulations was developed and validated, using ion-pair chromatography with indirect conductimetric detection. The chromatographic method was based on a non-polar (C_{18}) column and an aqueous octanesulfonate (0.64 mM) eluent, acidified with trifluoroacetic acid (5.2 mM). The retention time was 5.4 min and the asymmetry factor 0.65. A linear calibration curve from 10 to 1000 μg/ml ($r = 0.99998$), with a detection limit of 2.7 μg/ml (25 μl injection volume), a repeatability %RSD of 0.8 (40 μg/ml, $n = 5$) and reproducibility %RSD of 2.6 were achieved. The proposed method was applied for the determination of carnitine in oral solutions and capsules. No interference from excipients was found and the only pretreatment step required was the appropriate dilution with the mobile phase. Recovery from spiked samples was ranged from 97.7 to 99.7% with a precision (%RSD, $n = 3$) of 0.01–2.1%.

Keywords: L-Carnitine; Levocarnitine; Ion-pair chromatography; Food supplement; Indirect conductimetric detection

1. Introduction

L-Carnitine (Levocarnitine, (3R)-3-hydroxy-4-(trimethylammonio)butanoate, inner salt, Fig. 1), is a naturally occurring substance, essential for fatty acid oxidation and energy production in the human body [1]. It is synthesized in the liver (20 g of total L-carnitine is contained in human body) and is mainly present in the skeletal and cardiac muscles of all mammals, where it participates in the transport of long chain fatty acids into mitochondria [2–5]. Deficiency of L-carnitine results in major energy loss and toxic accumulations of free fatty acids. In the last years, L-carnitine has been included in most Pharmacopoeias and used for the treatment of carnitine deficiency or as a dietary supplement for various chronic diseases [6–8]. Several oral formulations (tablets, capsules, solutions) are commercially available from various manufacturers.

Carnitine has been investigated in many biochemical, pharmacokinetic, metabolic and toxicokinetic studies and thus many analytical methods have been developed and published, including enzymic and radioenzymic methods, HPLC methods with UV or fluorimetric detectors, after pre-column derivatization, and tandem mass spectrometry [9,10]. Due to its high polarity and lack of chromophore, quantitative analysis of L-carnitine appears several difficulties, but is challenging. Only a few methods are available for the determination of carnitine in pharmaceutical formulations.

The United States Pharmacopeia [11] proposes HPLC methods suitable for the quantitative determination of L-carnitine in oral solution and tablet formulations. The method for oral solution involves a C_{18} column, heptanesulfonate ion-pairing mobile phase (phosphate buffer pH 2.5–methanol), detection at 225 nm and a preliminary purification using disposable C_{18} microcolumn. The method for tablets involves an aminopropylsilane-bonded silica gel column, acetonitrile–phosphate buffer (pH 4.7) mobile phase, and detection at 205 nm. The later method requires a prolonged equilibration of the column (6 h) and is not suitable for the analysis of formulations containing a high concentration of sugars, since reducing sugars can rapidly degrade the column. In addition, the method is very time

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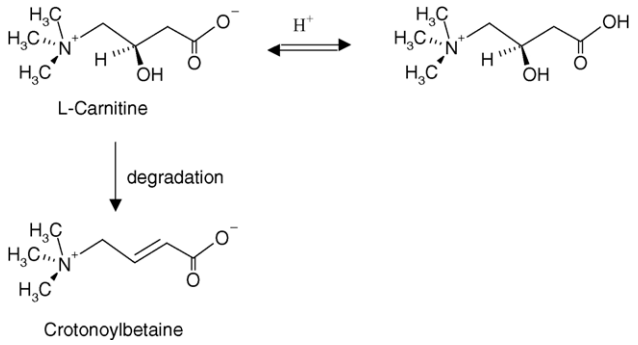

Fig. 1. Structures of L-carnitine and crotonoylbetaine and protonation equilibrium.

consuming in case the formulation contains an organic acid, due to the long retention time of the acid under the specified HPLC conditions. Both USP methods deal with high carnitine concentrations (2000–3000 μg/ml). In an improved similar HPLC method (column C_8, mobile phase: phosphate buffer (pH 2.0)–acetonitrile containing octanesulfonate, detection at 215 nm), a working curve of 400–4000 μg/ml was obtained. This method was able to provide a high resolution between L-carnitine and crotonoylbetaine, a major impurity and degradation product (upper limit 0.5%), and was applied in the assay control of pharmaceutical formulations (solution, tablets, capsules) [12]. Crotonoylbetaine (Fig. 1) has two conjugated double bonds, which result in 200 times stronger UV absorptivity than the parent compound (for example, crotonoylbetaine at 0.01%, would falsely increase the results for carnitine by 2%). Therefore, in the HPLC/UV methods the accuracy is strongly influenced by the concentration of crotonoylbetaine, in case of inadequate resolution.

Other analytical methods for the determination of carnitine in formulations involved: HPLC with fluorimetric detection after precolumn derivatization with 9-anthryldiazomethane [13] or pyrene-1-carbonyl cyanide [14] or 1-(9-fluorenyl)ethyl chloroformate [15]; enzymic methods using L-carnitine acetyltransferase [16,17], which was automated using flow injection technique [18], or D-carnitine dehydrogenase [19] in the form of a biosensor [20] and L-carnitine dehydrogenase in a flow-injection system [21]; and capillary electrophoresis (able to separate D/L-carnitine enantiomers) [22].

The majority of the above methods are rather time consuming and most of HPLC methods require derivatization, otherwise they have high detection limits. Thus, there is a need for the development of a fast, accurate (not suffering from the high interference of crotonoylbetaine) and with sufficient detection limit method for the determination of L-carnitine in pharmaceutical and food supplement formulations. In this paper, the ability of carnitine to form ion-pairs with lipophilic counter-anions (due to positively charged trimethyl amino group) and to be protonated (due to carboxylate group), has been exploited to develop an ion-pair chromatographic method with indirect conductimetric detection [23]. To the best of our knowledge this is the first method of this type for carnitine and was found suitable for its accurate quantitation in formulations.

2. Experimental

2.1. Instrumentation

Dionex DX-100 ion chromatographic system consisting of: DX-100 high pressure one piston pump, a sample injector equipped with a 25 μl loop, a Waters Nova Pak® C-18 (3.9 mm × 150 mm, 4 μm spherical silica-based packing material) analytical column and a conductimetric detector (dead volume 1.25 μl) equipped with a thermistor for compensation of temperature variations. The chromatographic peaks were electronically integrated and recorded using an HP 3395 integrator or Class VP 4 data processing software (Shimadzu, Japan) in conjunction with a ss-420× A/D converter board.

2.2. Reagents and standards

All solutions were prepared in HPLC-grade water (specific resistance >17.8 MΩ cm) obtained by Milli-Q water purification system (Millipore) and all chemicals were of analytical reagent grade, unless otherwise stated.

Pure solid L-carnitine (inner salt) was provided by local manufacturer, its exact purity was determined using the European Pharmacopoeia procedure (non-aqueous titration) [24] and was stored in an airtight container. A 10,000 μg/ml standard stock solution was prepared in water and stored in the refrigerator. Working standard carnitine solutions in the range of 10–1000 μg/ml were daily prepared in mobile phase. The analyzed carnitine formulations (oral solutions (100 mg/ml) and capsules (250 and 333 mg)) were purchased from local commercial sources.

Trifluoroacetic acid (TFA) was purchased from Sigma (>99%, spectrophotometric grade), and sodium 1-hexanesulfonate, sodium 1-heptanesulfonate monohydrate and sodium 1-octanesulfonate hydrate were purchased from Acros Organics (HPLC grade). The optimized mobile phase for routine work (aqueous solution of 0.64 mM octanesulfonate and 5.2 mM TFA) was prepared by dissolving 0.15 g of sodium octanesulfonate and 0.40 ml of TFA in 1 l of water.

2.3. Sample preparation

Oral solutions were simply diluted with water and further diluted with mobile phase to obtain working sample solutions in the range 100–700 μg/ml. For capsules, the content of 10 units was accurately weighed and dispersed in water in a 500 ml volumetric flask with the help of shaking. After filtration through a 0.45 μm filter the solution was diluted with mobile phase to obtain a concentration within the range of 100–700 μg/ml.

2.4. Procedure

The chromatographic elution was performed at room controlled temperature (25 °C) in isocratic mode at 1.2 ml/min flow rate. Eluent solutions were filtered through a 0.45 μm membrane filter before usage. The flow path was rinsed for about 15 min, until stabilization of baseline. Other instrumental parameters involved were: air pressure at 10 p.s.i. and conductivity range 1000 μS (microsiemens).

3. Results and discussion

3.1. Selection and optimization of chromatographic system

In order to develop an ion-pair chromatographic retention mechanism for L-carnitine, an aqueous mobile phase containing a lipophilic anionic ion-pairing reagent (long chain alkanesulfonate) is required in conjunction with a stationary phase of low polarity, such as the chemically bonded silica phase of octadecyl (C_{18}) type.

Since L-carnitine is an inner salt, the net electric charge of the molecules is zero, except in strong acidic environment, and therefore, it cannot be determined with suppressed direct conductimetric detection. However, indirect unsuppressed detection of L-carnitine can be achieved with protonation of carboxylate group. In this case, proton cations (with high conductance) are consumed to produce cations of L-carnitine (with lower conductance) and therefore the analytical signal is negative. Also, acidification of mobile phase establishes a net positive electric charge to carnitine molecule (Fig. 1) and thus enhances ion-pair formation with alkanesulfonate, as well as improves the symmetry of the chromatographic peaks due to inhibition of cation-exchange interactions between the analyte and free deprotonated silanolic groups. TFA, among other acids, was selected for two reasons: (a) it is a strong acid and therefore, it can fully protonate carboxylate groups (enhanced sensitivity) and (b) the counter anions (trifluoroacetate) appear lower conductivity than inorganic anions and therefore smaller background signal and noise.

Selection of mobile phase was performed in three steps: (a) selection of length of the aliphatic chain of alkanesulfonate ion-pairing reagent, (b) optimization of alkanesulfonate ion-pairing concentration and (c) optimization of TFA concentration.

In order to select the length of the aliphatic chain of alkanesulfonate anion, the three most commonly used alkanesulfonate ion-pairing reagents (hexane-, heptane- and octane-sulfonate as sodium salts) were examined. Calibration curves of L-carnitine in the range of 100–1000 μg/ml (0.62–6.2 mM) were obtained using as mobile phase aqueous solutions of 0.64 mM alkanesulfonate and 3.9 mM TFA (pH 3.2). Table 1 shows the chromatographic and calibration characteristics obtained with the three different alkanesulfonate reagents. It is obvious that the retention time, controlled by the lipophilicity, increases drastically with the chain length of the alkanesulfonate. Therefore, in order to achieve an acceptable retention time of L-carnitine, mobile phase with lower concentration of octanesulfonate is required comparing to other alkanesulfonate reagents. Since background noise is directly related to mobile phase salt concentration, the lower salt concentration is generally preferable. Furthermore, the background conductivity of the examined mobile phases (with equivalent molecular alkanesulfonate concentration) was lower for octanesulfonate salt due to its higher molecular size and smaller mobility (Table 1). The sensitivity of the method (slope of calibration curve) was higher (and approximately equal) using heptane- and octane-sulfonate. Octanesulfonate was superior in respect of the asymmetry factor of L-carnitine peak (0.6), and the detection limit (32 μg/ml). Therefore, octanesulfonate was chosen for further optimization. Resolution from system peak ($R > 3.8$) and linearity of calibration curve ($r > 0.999$) were excellent in all cases.

The effect of octanesulfonate concentration in the mobile phase was studied in the range of 0.10–0.64 mM (TFA 3.9 mM) and calibration curves in the range 100–1000 μg/ml were constructed. As it is shown in Table 2 (and Fig. 2), the increase of octanesulfonate concentration resulted in increase of retention time and sensitivity of the method. Furthermore, asymmetry factor and precision was better using 0.64 mM and therefore, the 0.64 mM concentration was

Table 1
Chromatographic and analytical characteristics of L-carnitine using aqueous mobile phase of 3.9 mM TFA and various alkanesulfonate ion-pairing reagents

Characteristic	Alkanesulfonate in mobile phase, 0.65 mM		
	Octanesulfonate	Heptanesulfonate	Hexanesulfonate
Retention time (t_R) (min)	7.1	4.3	2.3
Asymmetry factor	0.60	0.50	0.55
Theoretical plates (N)	2.4×10^3	2.1×10^3	1.4×10^3
Mobile phase (background) conductivity (μS)	628	652	671
Resolution[a]	7.9	5.4	3.8
Slope of calibration curve (μV s ml μg^{-1})	$(10.0 \pm 0.17) \times 10^2$	$(9.33 \pm 0.21) \times 10^2$	$(6.97 \pm 0.12) \times 10^2$
Correlation coefficient (r), 100–1000 μg/ml	0.9997	0.9994	0.9998
%RSD (at 100 μg/ml, $n = 3$)	1.7	2.0	1.5
Detection limit[b] (μg/ml)	32	43	36

[a] From negative system peak.
[b] Injection volume, 25 μl.

Table 2
Effect of octanesulfonate concentration in mobile phase on the chromatographic and analytical characteristics of L-carnitine (TFA 3.9 mM)

Characteristic	Octanesulfonate concentration (mM)		
	0.64	0.30	0.10
Retention time (t_R) (min)	7.1	5.7	3.5
Asymmetry factor	0.60	0.58	0.57
Theoretical plates (N)	2.4×10^3	2.3×10^3	1.8×10^3
Mobile phase (background) conductivity (μS)	628	574	563
Resolution[a]	7.2	6.2	3.4
Slope of calibration curve (μV s ml μg^{-1})	$(10.0 \pm 0.17) \times 10^2$	$(7.94 \pm 0.11) \times 10^2$	$(7.01 \pm 0.15) \times 10^2$
Correlation coefficient (r), 100–1000 μg/ml	0.9997	0.99990	0.9997
%RSD (at 100 μg/ml, $n = 3$)	0.6	2.1	1.7
Detection limit[b] (μg/ml)	32	29	47

[a] From negative system peak.
[b] Injection volume, 25 μl.

chosen as optimum. Mobile phase of higher octanesulfonate concentration resulted in a further increase of retention time, which is undesirable for routine analysis.

In the final step, the effect of TFA concentration in the range of 1.3–5.2 mM was studied using the optimun octanesulfonate concentration (0.64 mM). As it is shown in Table 3 (Fig. 2A and Fig. 3), increase of TFA concentration resulted in decrease of retention time and improvement of asymmetry factor due to protonation of stationary phase free silanolic groups and inhibition of cation-exchange interactions with positively charged L-carnitine. Also, increased theoretical plates, resolution (from system peak), linearity, detectabilitty and precision was observed. Therefore, the optimum TFA concentration was chosen to be 5.2 mM. Higher TFA concentration was useless to be examined, since retention time of L-carnitine would had been close to void time and the detector signal would had been saturated (background conductivity close to or higher than 1000 μS).

3.2. Calibration curve—analytical characteristics

Fig. 3A shows a typical chromatogram of L-carnitine (500 μg/ml, 3.1 mM) using the optimum mobile phase (0.64 mM octanesulfonate, 5.2 mM TFA) at a flow-rate 1.2 ml/min. The negative peak at 2.7 min is the so called system peak due to mobile phase pH disturbances produced by sample injection [25].

The calibration curve was linear in the range 40–1000 μg/ml, with a regression equation:

$$A = -1.40(\pm 0.83) \times 10^3 + 1.001(\pm 0.002) \times 10^3 C_{\mu g/ml},$$

$$S_{y/x} = 1.28 \times 10^3, \quad r = 0.999996, \quad n = 5.$$

The calibration curve passed through the origin (t-test) allowing the use of the one-standard approach for routine determinations. Detection limit was 2.7 μg/ml and repeatability %RSD ranged from 0.1 to 1.4. The robustness of the method was evaluated by performing six independent series of measurments on a 100 μg/ml fresh standard solution within one year, using three different analysts and two different columns. Reproducibility %RSD of peak area was 2.6.

Two important features of the new method are:

(a) Crotonoylbetaine, unlike HPLC/UV methods with inadequate separation, is not expected to cause significant analytical error. Conductimetric detector in indirect mode

Table 3
Effect of TFA concentration in mobile phase on the chromatographic and analytical characteristics of L-carnitine

Characteristic	TFA concentration (mM)			
	1.3	2.6	3.9	5.2
Retention time (t_R) (min)	10.7	8.6	7.1	5.4
Asymmetry factor	0.54	0.58	0.60	0.65
Theoretical plates (N)	0.5×10^3	2.2×10^3	2.4×10^3	4.6×10^3
pH of mobile phase	3.9	3.5	3.2	2.8
Mobile phase (background) conductivity (μS)	268	362	628	738
Resolution[a]	2.2	4.1	7.2	8.4
Slope of calibration curve (μV s ml μg^{-1})	$(3.81 \pm 0.58) \times 10^3$	$(1.89 \pm 0.09) \times 10^3$	$(1.00 \pm 0.02) \times 10^3$	$(1.001 \pm 0.002) \times 10^3$
Correlation coefficient (r), 100–1000 (μg/ml)	0.98	0.998	0.9997	0.999998[b]
%RSD (at 100 μg/ml, $n = 3$)	6.5	1.4	0.6	0.8[c]
Detection limit[d] (μg/ml)	33	33	32	2.7

[a] From negative system peak.
[b] 10–1000 μg/ml.
[c] 40 μg/ml.
[d] Injection volume, 25 μl.

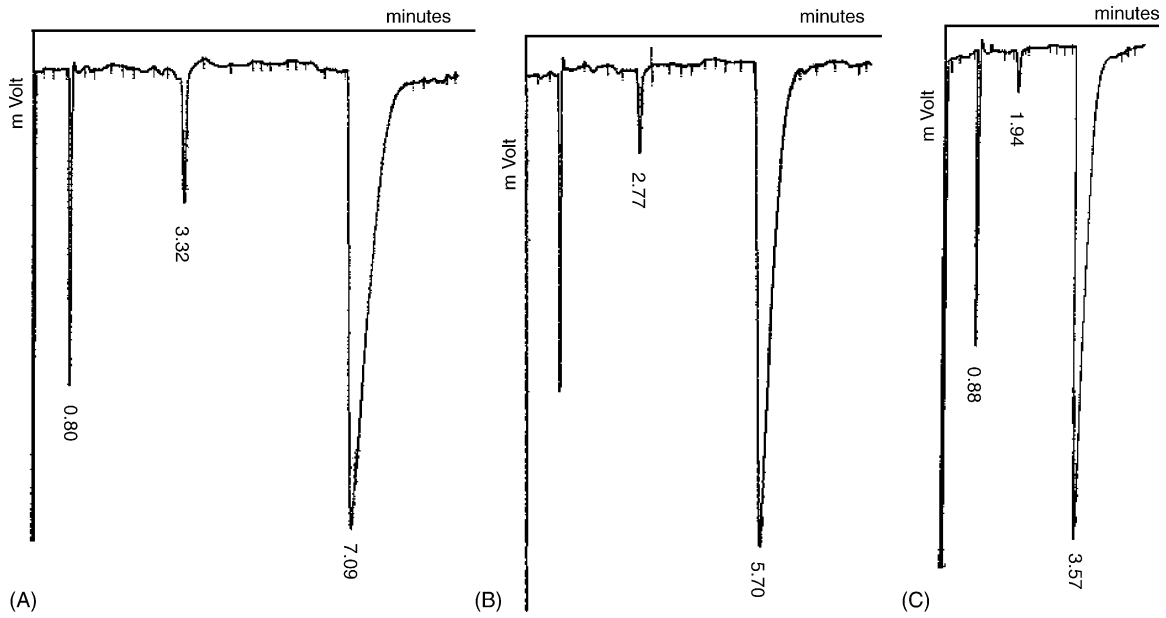

Fig. 2. Effect of octanesulfonate concentration in mobile phase on chromatographic characteristics of L-carnitine (TFA concentration 3.9 mM): (A) 0.64 mM (t_R L-carnitine: 7.1 min, system peak: 3.3 min), (B) 0.3 mM (t_R L-carnitine: 5.7 min, system peak: 2.8 min) and (C) 0.10 mM (t_R L-carnitine: 3.5 min, system peak: 1.9 min).

appears equal response factor for crotonoylbetaine and carnitine, since in both molecules the analytical signal is produced by the protonation of carboxylate group (Fig. 1). Therefore, as long as crotonoylbetaine exists in low concentration (Pharmacopoeia upper limit 0.5%), the analytical relative error is expected to be in the range of the method variation (%RSD $\leq$ 1.4).

(b) Long retention times of organic acids, which limit the applicability of the USP method for tablets, are not observed with the proposed chromatographic method, since it is based on a C_{18} analytical column and strongly acidic mobile phase. In the USP method, long retention times of organic acids are caused by anion-exchange mechanism, which is established by the aminopropyl-analytical column.

3.3. Determination of carnitine in pharmaceutical formulations—recovery experiments

The developed method was used to determine the amount of L-carnitine in commercial formulations (oral solutions and capsules) as described in Sections 2.3 and 2.4. In the sample chromatograms, no matrix component was detected by the conductimetric detector. Only the peak of L-carnitine

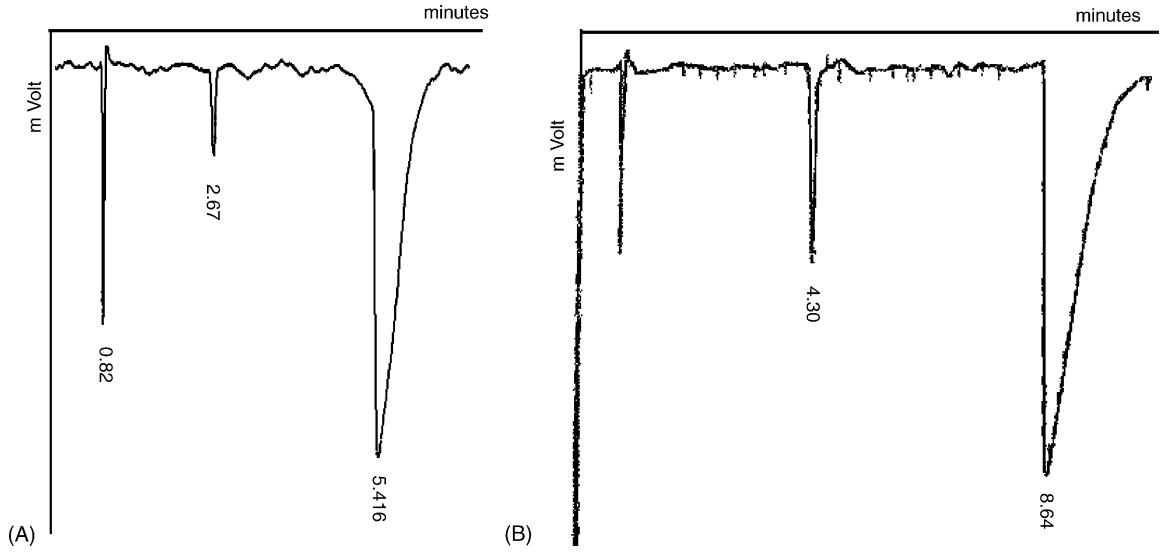

Fig. 3. Effect of TFA concentration in mobile phase on chromatographic characteristics of L-carnitine (octanesulfonate concentration 0.64 mM): (A) 5.2 mM (t_R L-carnitine: 5.4 min, system peak: 2.7 min), (B) 2.6 mM (t_R L-carnitine: 8.6 min, system peak: 4.3 min).

Table 4
Assay of content and recovery results of carnitine commercial formulations

Formulation, Claimed content	Content found (±SD, $n = 3$)	Recovery range(%)[a] (mean)
Maledrol® oral solution, 100 mg/ml (malic acid, sodium methylparaben, sodium propylparaben, saccharin sodium)	100.4 ± 0.9	98.5–99.7 (99.1)
Ensial® oral solution, 100 mg/ml (methyl hydroxybenzoate, propyl hydroxybenzoate, malic acid, saccharin sodium)	96.0 ± 0.8	100.4–102.5 (101.8)
Merlit® oral solution, 100 mg/ml (malic acid, methylparaben, propylparaben, saccharin sodium, cherry flavor)	97.3 ± 1.2	97.2–101.4 (98.6)
Minoa® oral solution, 100 mg/ml (malic acid, methyl-*p*-hydroxybenzoate sodium, propyl-*p*-hydroxybenzoate sodium, saccharin sodium, cherry flavor)	99.3 ± 0.9	99.9–103.1 (101.9)
Power Burner® oral solution, 100 mg/ml (citric acid, potassium sorbate, sodium cyclamate, acesulfame-K, sodium saccharin, flavours)	98.8 ± 1.4	95.7–98.7 (97.5)
Lamberts® capsules, 250 mg (L-tartrate, gelatin, magnesium stearate, silicon dioxide)	243.7 ± 5.3	97.7–99.2 (98.7)
Ultimate Burner® capsules, 333 mg (Garcinia Cambogia, vitamin B_6, chromium picolinate, gelatin, magnesium stearate)	324.6 ± 1.2	97.8–98.6 (98.1)

[a] From three recovery experiments at different spiking levels, 100, 250 and 500 μg/ml.

and system peak were obtained. The assay results shown in Table 4 revealed good precision and conformance to the Pharmacopoeia requirement for content within the range of 95–105% of the label content.

The accuracy of the proposed method was evaluated by recovery experiments with spiking diluted sample solutions or dispersions. The recoveries presented in Table 4 (95.7–103.1%) reveal sufficient accuracy. Further study of the matrix (excipients) effect on the determination was carried out by dilution experiments (determination of L-carnitine content in commercial formulations using a varying dilution factor D ($V_{initial}/V_{final}$) at three different levels). The correlation curves of the concentration found (in the diluted solution) versus D were very linear ($r > 0.999$) with a slope equal to the content of the formulation and a statistically (proven by *t*-test) zero intercept. Similarly, the correlation curves of content found versus D were very linear with statistically (poven by *t*-test) zero slope. These results reveal the absence of any constant or proportional determinate error from the excipients.

4. Conclusions

L-Carnitine, a substance administered for the treatment of carnitine deficiency and as a dietary supplement for various chronic diseases, can be determined using ion-pair chromatography with indirect conductimetric detection and a non-polar (C_{18}) analytical column along with an aqueous octanesulfonate—TFA eluent. This LC-conductimetric method for L-carnitine has the advantages of no derivatization step (unlike the most LC methods), short analysis time ($t_R = 5$ min), sufficient detection limit (2.7 μg/ml) (much lower than that of the official [11] or other published methods [12] for the assay of pharmaceutical formulations), and very simple sample pretreatment. The lack of derivatization step implies low cost of the analytical procedure. As far as it concerns the safety of the method, special attention is required for the preparation and handling of the mobile phase, since TFA is a strong acid and corrosive reagent.

As concluded from the literature, this the first work dealing with an LC method with conductimetric detection for L-carnitine and can be applied to the assay of other substances with similar (carboxylate inert salt) structure.

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