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Analytical Method Development and Validation of Content of D-Carnitine in Levocarnitine Using High-Performance Liquid Chromatography

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Abstract

A novel HPLC method was developed and validated for the determination of D-Carnitine in Levocarnitine in accordance with the ICH Q2R1 guidelines. The method demonstrated system precision, specificity, precision at the limit of quantitation (LOQ), accuracy, solution stability, linearity, ruggedness and robustness. The HPLC conditions consisted of a mobile phase composed of pH 2.60 buffer, acetonitrile, and tetrahydrofuran (THF) in a ratio of 850:90:60 (v/v/v), with a flow rate of 1.0 mL/min and UV detection at 244 nm. The entire chromatography run time was set to 65 minutes. Linearity was established over a concentration range of LOQ-160% with a correlation coefficient of 0.996. Accuracy was confirmed within the range between LOQ and 160%. This validated HPLC method is suitable for the precise quantification of D-Carnitine in Levocarnitine Tablets, making it an effective tool for quality control and assurance in pharmaceutical manufacturing.

Keywords

Carnitine, D-Carnitine, Levocarnitine, HPLC

1. Introduction

Carnitine (β-hydroxy-γ-trimethylammonium butyrate) is a vital nutrient predominantly found in meat and dairy products, playing a crucial role in the body's conversion of fat to energy. It exists in two stereoisomers (Figure 1), the biologically active levorotatory form (L-Carnitine) and the non-biologically active dextrorotatory form (D-Carnitine), with a combination of both (DL-Carnitine)

widely available. L-Carnitine, also known as vitamin B6 is the naturally occurring form with a positive impact on growth rate and lipid metabolism [1]. It facilitates fatty acid transport across the mitochondrial membrane [2], making it a popular weight management supplement. Globally, L-Carnitine is utilized for various nutritional and medicinal purposes due to its high therapeutic efficacy. In humans, L-Carnitine promotes fatty acid oxidation, non-oxidative glucose elimination, and acyl transfer, functioning at the intersection of fatty acid and carbohydrate metabolism [3].

D-Carnitine can hinder the absorption and transportation of L-Carnitine [4], impede the β-oxidation of fatty acids, and cause liver damage. However, research on the roles of L- and D-Carnitine in aquatic species has yielded inconsistent results to date. The significant adverse effects of D-Carnitine (D-C) necessitate precise quantification of its content in pharmaceutical and nutritional formulations [5]. Currently, the European Pharmacopoeia [6] and the United States Pharmacopoeia [7] set the limit for the content of D-C to approximately 4% based on optical rotation measurements. However, this approach lacks selectivity and sensitivity. Although modern production methods can yield purer L-C, this analytical limitation precludes the establishment of more stringent limits. Therefore, a novel approach with a sufficient sensitivity range and robustness is extremely desirable.

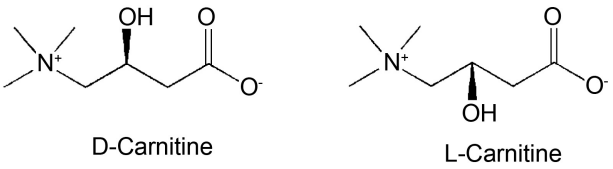

Figure 1. Structure of D and L-Carnitine.

Currently, there are several methods for Enantioselective separation of D-Carnitine from L-Carnitine (**Figure 2**).

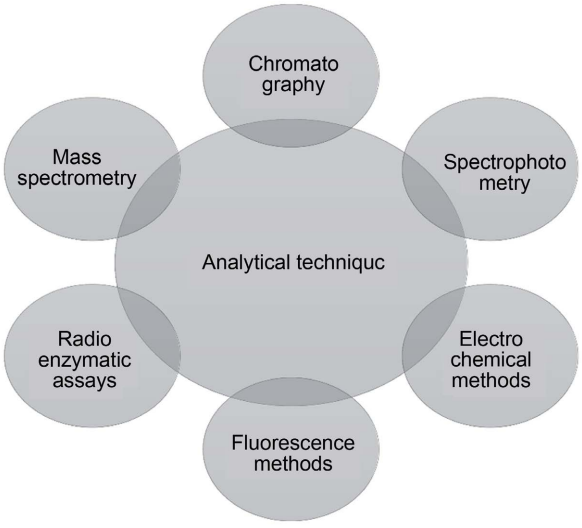

Figure 2. Types of the analytical techniques.

In general, there are two methods for determining L-Carnitine enantiomeric purity. The first method directly estimates the quantity of L-Carnitine without any prior separations by measuring optical rotation and integrating signal reagents. This results from the two enantiomers being separated by NMR or enzyme-mediated tests with chiral shifting reagents. None of the current approaches meets the sensitivity and precision requirements for determining D-Carnitine in L-Carnitine < 1% [8] [9]. The second technique relies on HPLC or CZE separation. Since CZE is more difficult and produces less reproducible findings in analytical labs, HPLC is the recommended approach. Therefore, it is of the utmost importance to detect both L-Carnitine and its Dextrorotatory analogue, D-Carnitine. Researchers have developed new pre-treatment techniques, such as derivatization, to detect carnitine enantiomers and separate their chiral enantiomers.

This paper proposes specificity, Precision, Precision at LOQ, Accuracy, solution stability, linearity, and a rugged and robust method for determining D-Carnitine content in L-Carnitine in the range of 0.1% - 1% by using HPLC. The method was validated in accordance with the ICH Q2R1 principles, and research was conducted to establish its applicability.

2. Literature Review

2.1. Research and Use of Carnitine in Therapeutics

Carnitine (3-hydroxy-4-*N*-trimethylammoniobutanoate) has been researched extensively since its development 100 years ago. Research to date has expanded the knowledge of carnitine's function in metabolism, and an increase in research interest in the use of carnitine in therapeutics has been noticed. This is partly due to the discovery of mechanisms for both primary and secondary carnitine deficit, as well as carnitine's use as a therapeutics and supplement. Furthermore, characterizing the biological processes of carnitine production has resurrected this characteristic of carnitine homeostasis (Almannai *et al*., 2019).

Human carnitine status fluctuates depending on body composition, gender, and food. Carnitine consumption in the diet is favorably correlated with plasma carnitine levels. The approach used to determine carnitine content of the food is outdated and ineffective. Nonetheless, carnitine in the diet is vital. The carnitine biosynthesis enzymes' molecular biology has been completed. Carnitine synthesis is a very effective system that requires pathways in several organs. The abundance of trimethyllysine from tissue proteins determines overall biosynthesis. There has yet to be a case of carnitine depletion caused by a biosynthetic error (Flanagan *et al*., 2010; Ringseis *et al*., 2018; Steiber *et al*., 2004).

Carnitine is produced endogenously from two essential amino acids, methionine and lysine, when it is not received from the diet. This can occur in the brain, liver, and kidneys (Cave *et al*., 2008). Because skeletal and cardiac muscles have the greatest quantities, they are unable to synthesis carnitine and must obtain it from plasma. Microbes in the intestinal tract mostly destroy unabsorbed LC (Rebouche, 2004). Carnitine is almost entirely intracellular (99%) (Cave *et al*., 2008).

Carbohydrate metabolism is influenced by carnitine. Carnitine regulatory abnormalities have been attributed to diabetic complications, trauma, hemodialysis, starvation, obesity, cardiomyopathy, fasting, endocrine imbalances, medication interactions, and other conditions (Guerra *et al*., 2021).

2.2. Role of Carnitine in Disease

In this work, [1] provided a description of carnitine and its relevance to human health as well as its possible involvement in different diseases. Carnitine plays the part of the shuttle of the long-chain fatty acid to the mitochondria, where they undergo β-oxidation for energy production. The authors further note that while there is an abundance of literature on L-Carnitine as a therapeutic agent, there are relatively few publications on the effects of D-Carnitine and that it may, in fact, be toxic due to the inhibition of L-Carnitine. Different metabolic and mitochondrial diseases are associated with carnitine deficiency, thus urging precise control of the carnitine level in practice.

2.3. Pharmacokinetics of L-Carnitine

Regarding the pharmacokinetics of L-Carnitine, [2] published an extensive analysis of the absorption, distribution, metabolism, and excretion of the compound. They pointed out that L-Carnitine is actively transported in the small intestine and is found in considerable concentration in skeletal muscles and cardiac tissues. The pharmacokinetics of L-Carnitine are non-linear, and thus, the above supplementation can affect the endogenous levels. Notably, they observed that D-Carnitine, one of the isomers of L-Carnitine, could affect carnitine's metabolism and exhibit toxicities.

2.4. Determination of D-Carnitine in L-Carnitine Using HPLC

Another approach for the analysis of L- and D-Carnitine is chiral HPLC, which is [3] after derivatization of L-Carnitine with (+) FLEC. This method is important in the synthesis of L-Carnitine supplements, especially because of the negative impact linked to D-Carnitine. The authors pointed out that if even small amounts of D-Carnitine posed a threat to the normal functioning of the body's metabolism, then arguably accurate analytical methods would be critical in the pharmaceutical and clinical use of the product.

2.5. Obesity, Inflammation, and Pharmaconutrition

Some of the important studies that have been conducted on the topic include the work of [10] on obesity, inflammation, and pharmaconutrition: Evidence for a therapeutic role for L-Carnitine. As observed earlier by other researchers, carnitine's pro- and anti-inflammatory effects, particularly in the context of overweight and obese patients with MS, point to its potential to prevent obesity and its comorbidities. The authors continued with the argument that supplementation with L-Carnitine could assist in the attainment of metabolic homeostasis, with

some words of caution being sounded over the levels of D-Carnitine in certain supplements.

2.6. Metabolism of D-Carnitine by *Escherichia coli*

It was found that *E. coli* can metabolize D-Carnitine through an enzymatic process that unleashes the formation of substances other than those generated by L-Carnitine. These findings shed some light on the metabolic differences between L and D-Carnitine and pointed to the biological individuality of both enantiomers. The work is also relevant to industrial biotechnology and knowledge of bacterial metabolism in the gut.

2.7. Functional Differences between L- and D-Carnitine in Metabolic Regulation

In a low-carnitine, Nile tilapia [5] investigated the functional differentiation of L-Carnitine and D-Carnitine in metabolism. Their findings also showed that L-Carnitine is directly involved in lipid metabolism and helps energy production, whereas D-Carnitine has no positive effects and may interfere with the metabolism process. The current literature also depicts the therapeutic use of L-Carnitine as the effective one and D-Carnitine as having almost negligible or no beneficial actions in the body's metabolism.

2.8. Pharmacopoeia Standards for Carnitine

According to European Pharmacopoeia (Ph.Eur) and United States Pharmacopoeia (USP) L-Carnitine is used in pharmaceutical preparations to be of high purity and composition. Such a standard also requires that the use of D-Carnitine should be monitored well to eliminate the possibility of impurity effects. Both pharmacopoeias emphasize the need for enantiomeric purity of L-Carnitine product and the significance of those aspects for patient protection and safety of the therapy [6] [7].

2.9. Enzymes in Stereoselective Pharmacokinetics of Endogenous Substances

Explicated the part played by enzymes in the stereoselective pharmacokinetics of endogenous substances, carnitine included. Their work also showed that, first, stereoisomers are selective substrates preferentially targeted with the enzymes of the body; second, depending on the type of stereoisomers, the D-form is often a biological inaccuracy or toxicological equivalent of the L-form, in this particular case, L-Carnitine. The stereoselectivity of this enzyme has an important bearing on the process of drug design and the progress of Carnitine-based therapeutics.

3. Materials and Methods

3.1. Drugs and Chemicals

L-Carnitine reference standard was bought from Sigma Aldrich, L-Carnitine API

was obtained from Chengda Pharmaceutical Co. Ltd-China and D-Carnitine was purchased from Toronto Research Chemicals (TRC, Canada).

3.2. Instrumentation

The HPLC system (Waters Alliance 2695) programmed by Waters Empower-3, Synergi 4µ Hydro-RP80 Å, 4.6 × 250 mm; P# 00G-4375-E0 column was used. Digilab sonicator has been used to degass the diluent and mobile phase. To measure the weight of material, used an electronic balance from Sartorius. A Metrohm pH meter was used for all of the pH adjustments. (Figure 3)

Figure 3. Waters alliance 2695 HPLC system.

3.3. Mobile Phase Preparation

- **Buffer Preparation**: Accurately weighed and transferred about 5.78 g Potassium dihydrogen in a suitable flask containing 850 mL of water and allowed to dissolve after the pH was adjusted to 2.60 ± 0.05 with diluted Ortho-phosphoric acid. Filtered through 0.45 µm membrane filter.
- **Mobile phase A Preparation**: Transferred 850 mL of Buffer solution pH 2.60, 90 mL and HPLC grade tetrahydrofuran and 60 mL in a suitable container; contents were mixed well and sonicated to degas.
- **Mobile phase B Preparation**: Transferred 900 mL of acetonitrile and 100 mL of water in a suitable container, mixed well and sonicated to degas.

3.4. Diluent Preparation

Ethyl alcohol, which has good solvent properties and compatibility with the concentration of compounds in this procedure, was used as a diluent. That capability was important for the analysis in ensuring that the analytes were fully dissolved in the solution without any precipitation and for maintaining the stability of the solution.

3.5. Solution Preparation

- **Preparation of Solution-1:** Transferred about 0.2 mL of Ethyl chloroformate

into a 100 mL volumetric flask. Diluted to volume with Chloroform and mixed well.

- **Preparation of Solution-2:** Transferred about 0.7 mL of Triethylamine into a 100 mL volumetric flask. Diluted to volume with Chloroform and mixed well.
- **Preparation of Solution-3:** Weighed and transferred about 225 mg of L-Alanine-β-naphthylamide into a 50 mL volumetric flask. Sonicated to dissolve and dilute to volume with diluent and mixed well.
- **Preparation of Solution-4:** Weighed and transferred about 420 mg of Sodium bicarbonate into a 200 mL volumetric flask. Sonicated to dissolve and diluted to volume with water and mixed well.

3.6. Preparation of Standard Solution

- **Preparation of Standard Stock Solution:** Weighed accurately and transferred about 50 mg of the D-Carnitine WRS to a 10-mL volumetric flask. Added about 5.0 mL of diluent into the flask and sonicated to dissolve. Poured the diluent to the volume and shake well.
- **Derivatization Procedure for Standard:** Taken 25 mL in a dry volumetric flask with a magnetic bar, transferred 1.0 mL of Standard stock solution and allowed to start agitation at 990 rpm. Added 2.0 mL of solution-1, then pipetted out 1.0 mL of solution-2 and 2.0 mL of solution-3 into the flask sequentially. Allowed to stir the solution for 10 minutes at 990 rpm. Immediately, added 5.0 mL of solution-4 and shaken the solution vigorously for a minute. Allowed to stand for 20 minutes at room temperature. After 20 minutes, collected the upper aqueous layer. Transferred 1.0 mL of aqueous solution into a 50 mL volumetric flask, diluted to volume with water, and mixed well. Further, diluted 5.0 mL of the solution to 25 mL with water and mixed well.

3.7. Preparation of Resolution Solution

- **Preparation of Resolution Stock Solution:** Taken a weighing balance and weighed carefully about 50 mg of D, L-Carnitine and transferred it to a 10 mL volumetric flask. To dissolve, added about 3/4th of the volume of diluent to the flask and allowed for sonication. Taken diluent and dissolved it in one litre of the above preparation, then stirred it vigorously in order to enhance its homogeneity, to a concentration of about 5000 µg/mL of D-Carnitine.
- **Derivatization Procedure for Resolution Solution:** Taken a 25 mL in a dry volumetric flask with a magnetic bar, transferred 1.0 mL of Resolution Stock solution and allowed to start agitation at 990 rpm. Added 2.0 mL of solution-1, then pipetted out 1.0 mL of solution-2 and 2.0 mL of solution-3 into the flask sequentially. Allowed to stir the solution for 10 minutes at 990 rpm. Immediately, added 5.0 mL of solution-4 and shaken the solution vigorously for a minute. Allowed to stand for 20 minutes at room temperature. After 20 minutes, the upper aqueous layer is collected. Transferred 1.0 mL of aqueous solution into a 50 mL volumetric flask, diluted to volume with water, and mixed well.

3.8. Preparation of Sample Solution

1) Preparation of Sample Stock Solution: Weighed accurately and transferred about 250 mg of L-Carnitine API into a 50-mL volumetric flask. Added diluent about 3/4th of the volume of the flask and sonicated to dissolve. Diluted to volume with diluent and mixed well (Concentration of about 5000 µg/mL of D-Carnitine).

2) Derivatization Procedure for Sample: Taken 25 mL in a dry volumetric flask with a magnetic bar, transferred 1.0 mL of Sample stock solution and allowed to start agitation at 990 rpm. Added 2.0 mL of solution-1, then pipetted out 1.0 mL of solution-2 and 2.0 mL of solution-3 into the flask sequentially. Allowed to stir the solution for 10 minutes at 990 rpm. Immediately, added 5.0 mL of solution-4 and shaken the solution vigorously for a minute. Allowed to stand for 20 minutes at room temperature and collected the upper aqueous layer for injection.

3.9. Preparation of Blank Solution

Derivatization Procedure for Blank: Taken 25 mL in a dry volumetric flask with a magnetic bar, transferred 1.0 mL of diluent and allowed to start agitation at 990 rpm. Added 2.0 mL of solution-1, then pipetted out 1.0 mL of solution-2 and 2.0 mL of solution-3 into the flask sequentially. Allowed to stir the solution for 10 minutes at 990 rpm and immediately, added 5.0 mL of solution-4 and shaken the solution vigorously for a minute. Allowed to stand for 20 minutes at room temperature and after 20 minutes, collected the upper aqueous layer for injection.

3.10. Chromatographic Parameters

For chromatographic separation, a Synergi 4µ Hydro-RP80 Å, 4.6 mm × 250 mm (P# 00G-4375-E0) HPLC column was used. The mobile phase comprises pH 2.60 buffer, acetonitrile, and THF in a ratio of (850%:90%:60%) with a flow rate of 1.0 mL/min, detected by UV at 244 nm. The total chromatography run time is 65 minutes. The column oven temperature is 30˚C, the sampler temperature is maintained at 25˚C, and the injection volume is 25 µL.

The following validation parameters were determined for the developed method: Precision, Accuracy, Linearity, Specificity, Ruggedness (Intermediate Precision) and Robustness, as per the ICH guidelines.

4. Results and Discussion

4.1. System Precision

To ensure a level of system precision, standard and resolution solutions are prepared and injected according to the method. The relative standard deviation for peak area response of D-Carnitine was obtained using data obtained from six (6) replicate injections of the standard solution. Between D-Carnitine and L-Carnitine, the resolution is documented in Table 1. The % RSD of the peak response of D-Carnitine from six (6) replicate injections of the standard solution preparation solution was less than 10 and meets the acceptance criteria for the resolution

between D-Carnitine and Levocarnitine from derivatized resolution solution was more than 1.5, thus making the system suitable.

These responses, which stand slightly above 135,075 to slightly below 135,793, are proportional to the signal elicited by the HPLC system in D-Carnitine for each injection. The overall mean response value obtained for all six injections is computed to be 135,316. Only for assessment of the system precision is the Relative Standard Deviation (RSD) computed and based on it, the value is 0%. This shows that the system had nearly the same response to each of the injections, which is an indication of the high level of precision that exists in the system. This is further evidenced by the low % RSD, which shows that the HPLC method has a small variation between injections and can thus accurately quantify the amount of D-Carnitine in the samples that were analyzed. It is important for the analysis that the method and equipment used are functioning properly, and this validation ensures this.

Table 1. System precision of D-Carnitine.

	Sample Name	Injection No	D-Carnitine Response
1	Standard	1	134,775
2	Standard	2	135,079
3	Standard	3	135,084
4	Standard	4	135,518
5	Standard	5	135,793
6	Standard	6	135,649
Mean			135,316
% RSD			0

4.2. Linearity and Range

To establish the solutions of D-Carnitine at varying concentrations ranging from LOQ to 160% of the specification levels (0.05% of D-Carnitine with respect to sample concentration) were prepared and injected into the HPLC system (**Figure 4**). The correlation coefficient square (r^2) must not be less than 0.99 (**Table 2**).

The linearity data of D-Carnitine contains details of sample concentration and peak response as read by the HPLC instrument. This linearity work in method validation is important in ascertaining if the instrument response is proportion to the analyte concentration within the range of calibration. The table shows the concentration of D-Carnitine studied, including the Limit of Quantitation (LOQ) concentration and a high concentration at 160%; concentrations in μg/mL are also in the table. For each concentration level, the peak area is measured, and it refers to the intensity of the signal that the HPLC detects.

The lowest concentration (1.4250 μg/mL) is at LOQ and the peak response was found to be 9265. When the concentration rises to 80% (1.9000 μg/mL), the peak area increases to 13,258, which is also an influence of the contrast. This trend is

also observed in the maximum peak area, arising to 15,727 at 100% concentration (2.3750 µg/mL), 20,243 at 120% concentration (2.8500 µg/mL and highest at 26,998 at 160% concentration (3.8000 µg/mL). These results denote a direct proportion between the concentration of D-Carnitine and the area of the peak area; this is in agreement with the findings of the same study that the HPLC assay can provide an accurate determination of D-Carnitine from samples with fairly high concentrations as well as those with low concentrations. This is a useful concept in many ways to help avert any deviations in the method by aiding in the precision to deliver accurate and dependable quantitative analyses of relative concentrations.

Table 2. Linearity of D-Carnitine.

S. No.	Level (%)	Concentration (µg/mL)	Peak Area
1	LOQ	1.4250	9265
2	80%	1.9000	13,258
3	100%	2.3750	15,727
4	120%	2.8500	20,243
5	160%	3.8000	26,998

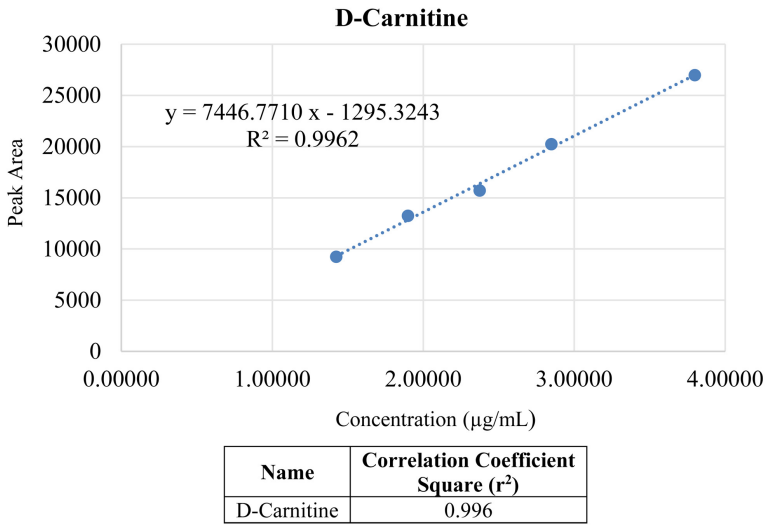

Figure 4. Linearity plot for D-Carnitine.

4.3. Precision at LOQ

Precision at the LOQ Level was determined by preparing and injecting D-Carnitine at the LOQ level.

The % RSD for peak response of D-Carnitine from the six (6) replicate injections of LOQ solution met the acceptance criteria of not more than (NMT) 10. Hence, the method is sensitive and precise at LOQ. (Table 3)

The precision study was carried out at the level of LOQ, the lowest detectable level of D-Carnitine, which can be confidently quantitated. The table has six dif-

ferent "injections" of the D-Carnitine sample, all of which are made in parallel to check the reproducibility of the analysis.

In each injection, the peak response of the D-Carnitine is obtained. The values vary between 8624 and 10,023, and the highest peak area was found during the third injection and the lowest during the sixth one. The mean peak area over the six injections is 9164, thus giving the overall average response at this low concentration level. On the accuracy of these measurements, the percent Relative Standard Deviation (% RSD), giving a value of 5, is determined.

It is noteworthy that a % RSD of 5 implies a moderate variation between the injections, which is acceptable within most methods, especially enantioselective impurity at such low concentrations as the LOQ. From this data, it is possible to infer that the method has an acceptable level of accuracy at the LOQ level and is able to provide a consistent response for the D-Carnitine at this level of concentration. Such a fine distinction is particularly important to establish the reliability and reproducibility of the quantitation in routine uses and for the detection of low levels of the contents.

Table 3. (a) Precision at LOQ Level for D-Carnitine. (b) Concentration and Percentage of D-Carnitine.

(a)			
	Sample Name	Injection No	D-Carnitine Response
1	LOQ Precision	1	9231
2	LOQ Precision	2	9037
3	LOQ Precision	3	10,023
4	LOQ Precision	4	8873
5	LOQ Precision	5	9197
6	LOQ Precision	6	8624
Mean			9164
% RSD			5

(b)		
Name	Concentration (µg/ML)	% Level
D-Carnitine	1.4250	0.03%

4.3.1. Method Precision

Method Precision for the content of D-Carnitine was determined by injecting six (6)-individual sample solutions of Levocarnitine, USP. The samples were prepared using this method (**Figures 5-7** for typical chromatograms). The % RSD for % Content values of D-Carnitine from six (6)-individual sample preparations met the acceptance criteria of less than 10, and hence, the method is precise (**Table 4**).

Method Precision for D-Carnitine lists the findings of an experiment conducted to determine the precision of the analytical technique employed for the determi-

nation of D-Carnitine. Accuracy relates to the preciseness of the method when repeated measurements are made in the same circumstances.

There are six samples marked as "Method Precision" with D-Carnitine in each sample. The values vary from 0.042% to 0.046%. It is, therefore, clear that there is slight variability across the measurements. By dividing the total of D-Carnitine measurements by six, the mean of D-Carnitine for these six measurements is found to be 0.043%.

However, the degree of accuracy of these measurements is again checked with the help of percent Relative Standard Deviation (% RSD), which comes to about 3%. The coefficient of variance, % RSD, is at 3%; this shows that the variation of the measurements is much less; thus, the method used to measure D-Carnitine has very good precision. Such low variability is very important in analytical methods, particularly when determining the concentration of D-Carnitine in various samples where one needs to get reproducible results. This is important in order to maintain and achieve a high degree of confidence in the results of the method, especially in routine analysis.

Table 4. Method precision for D-Carnitine.

	Sample Name	% of D-Carnitine
1	Method Precision_1	0.043
2	Method Precision_2	0.044
3	Method Precision_3	0.046
4	Method Precision_4	0.043
5	Method Precision_5	0.042
6	Method Precision_6	0.042
Mean		0.043
% RSD		3

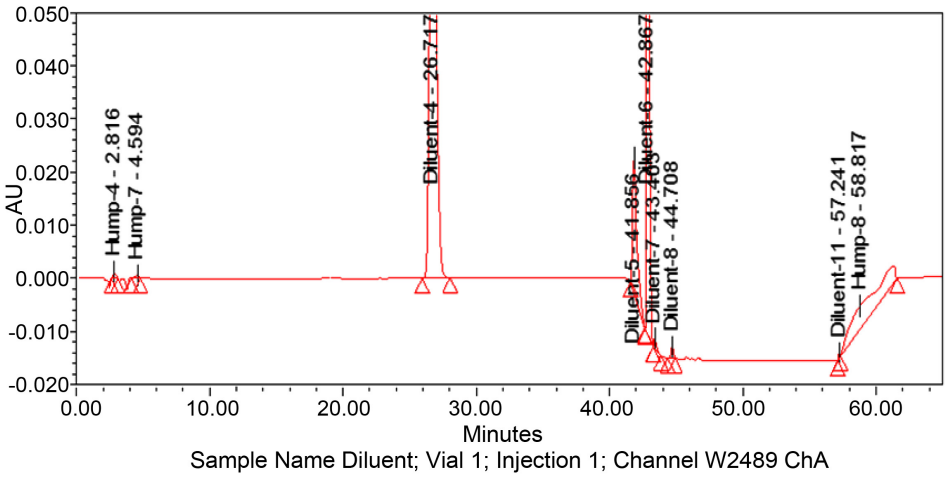

Figure 5. Typical chromatogram of blank.

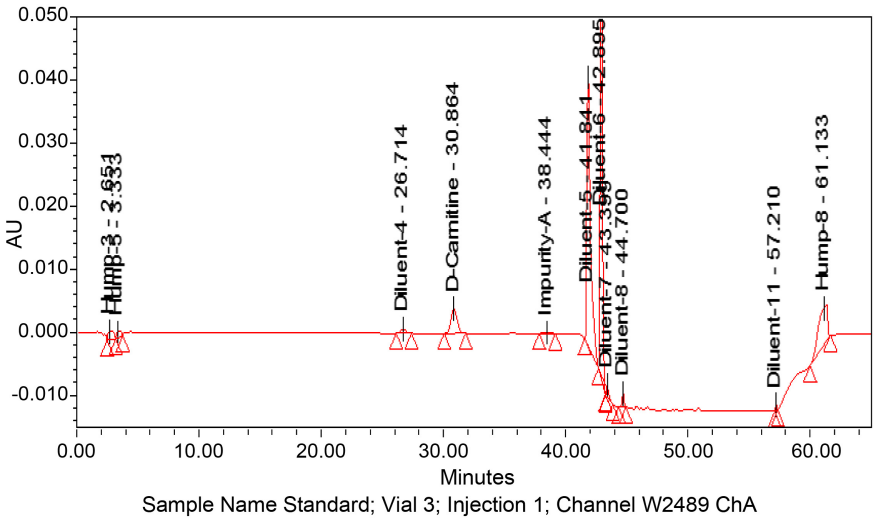

Figure 6. Typical chromatogram of standard.

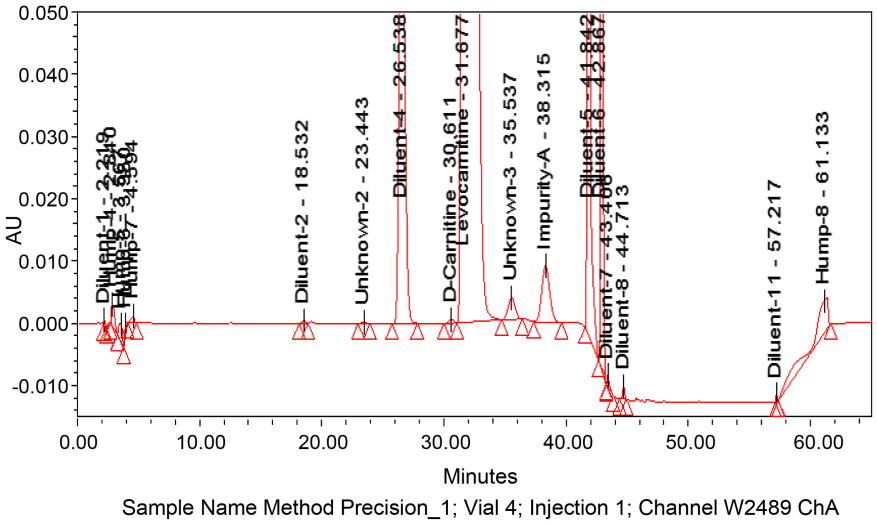

Figure 7. Typical chromatogram of sample.

4.3.2. Ruggedness (Intermediate Precision)

The intermediate precision (reproducibility) of the D-Carnitine content was evaluated by the second analyst preparing the standard and six (6)-sample individuals on different days. Studied samples were prepared according to the method (**Figure 8** for typical chromatogram).

- The % RSD for the content (%) of D-Carnitine from six (6)-individual sample preparations met the acceptance criteria of less than 10, and hence, the method is reproducible (**Table 5**).

Intermediate Precision (Analyst 1) gives data on the analyst's method precision in determining the D-Carnitine in six samples. The following procedure was carried out by Analyst 1 to determine the repeatability of the method at the same set of conditions.

The values of the D-Carnitine for the six samples are presented as a range,

where the values are somewhat different, 0.042% and 0.046%. Taking the mean of such samples into consideration, their average of D-Carnitine is 0.043%, which means that, on average, the method defined here is capable of measuring D-Carnitine at this concentration.

In order to make a more accurate measurement, the % Relative Standard Deviation (% RSD) was determined, which was obtained as 3%. The percent relative standard deviation or % RSD is very low, thus implying that the measurements have a small difference between them, and therefore, the method is precise. In analytical chemistry, such a value of % RSD is acceptable because it means that the method is accurate and will yield similar results if employed by the same operator under the same circumstances. Such high accuracy is needed in the practice of D-Carnitine measurements in any laboratory testing.

Table 5. Method precision (Analyst 1).

	Sample Name	% of D-Carnitine
1	Method Precision_1	0.043
2	Method Precision_2	0.044
3	Method Precision_3	0.046
4	Method Precision_4	0.043
5	Method Precision_5	0.042
6	Method Precision_6	0.042
Mean		0.043
% RSD		3

Intermediate Precision (Analyst 2 on a different day) provides information regarding the precision of D-Carnitine measurement done by the second analyst on the given date. There are six types of samples, and they are joined together in a table where they are labelled as "Method Precision" with numbers that range from 1 to 6. The proportion of D-Carnitine depicted in these samples varies from 0.035% to 0.038%; of the samples analyzed, the concentration ranged as follows, most of the samples falling within the above bracket of 0.037% or 0.038%. The mean of D-Carnitine for all six samples is estimated to be the mean, which is the measure that is close to the average; it is represented by 0.037%. Precision is expressed as the relative standard deviation (%), which shows the distribution of the results in relation to the mean of the RSD, which has been determined to be 3%. Such % RSD estimate points to accurate and repeatable measurement, as it is known that lower % RSD values are associated with higher accuracy of analytical techniques.

- The % Difference in % D-Carnitine content between Method Precision and Intermediate Precision met the acceptance criteria of not more than 10, and hence, the method is rugged (**Table 6**).

Table 6. Intermediate precision (Analyst 2).

	Sample Name	% of D-Carnitine
1	Intermediate Precision_1	0.037
2	Intermediate Precision_2	0.038
3	Intermediate Precision_3	0.037
4	Intermediate Precision_4	0.035
5	Intermediate Precision_5	0.037
6	Intermediate Precision_6	0.038
Mean		0.037
% RSD		3

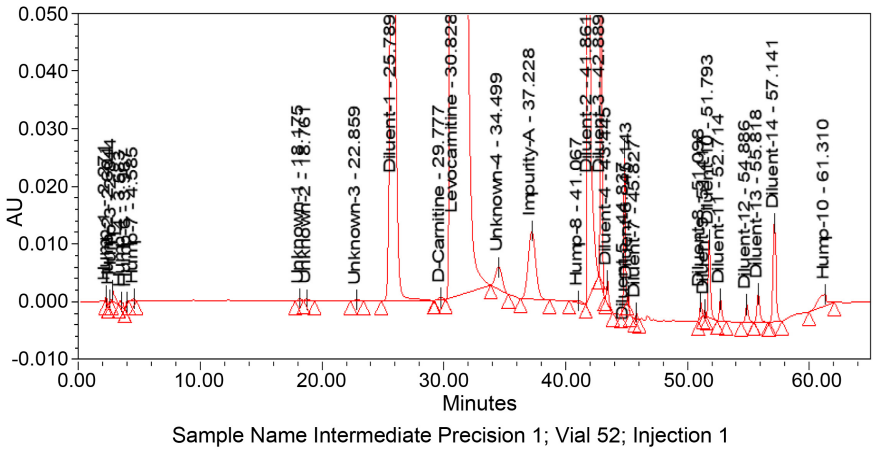

Figure 8. Typical chromatogram of spiked sample.

4.4. Solution Stability

Both reference and exemplar solutions were kept at ambient temperature and were injected at the following time intervals: Initial (0 hours) and 78 hours for Standard, Initial (0 hours) and 68 hours for the sample. The % difference in the area from the initial and time point for standard and the % difference in % D-Carnitine content for sample solution was generated.

- The % difference in response between initial and time points is less than 10% for standard (Table 7).

Standard D-Carnitine solution stability at room temperature in the lab over 78 hours. It presents the first peak area measured for D-Carnitine and the peak response obtained at 78 hours. Actually, it began with a response of 135,316, and after a total of 78 hours, it became 136,091. The difference between these two peak responses is found to be about 1%. This means that there was a progressive increase in the peak area of D-Carnitine from time to time. However, it was less, this could be a result of minor degradation active peak and/or evaporation of the solvents (diluent) in prepared solutions since they were sensitive to changes in stability. However, a variation of about 1% is still within range of tolerance, which means that the concentration of D-Carnitine in standard solution is still fairly sta-

ble when stored at room temperature for up to 78 hours.

Table 7. Solution stability of standard at room temperature.

Standard	Peak Area		% Difference
	Initial	78 hours	
D-Carnitine	135,316	136,091	1

The solution is stable for D-Carnitine sample for a 68-hour period at room temperature. The proportion of D-Carnitine in the sample at the beginning and after 68 hours is noted in Table 8. After 68 hours, the D-Carnitine level increased from 0.043% to 0.044%. A 2% difference is calculated. This rather substantial rise in level suggests that the D-Carnitine in sample solution may be less stable than the reference solution when stored at room temperature. The accumulation of D-Carnitine over time could be a result of minor degradation or evaporation of the solvents (diluent) since they were sensitive to changes in stability. A 2% increase in impurity content highlights how important it is to control storage circumstances in order to preserve the sample.

- The % difference in content (%) of D-Carnitine between the initial and 68-hour time point is less than 10% for the sample solution (Table 8).

Table 8. Solution stability of sample at room temperature.

Sample	% Impurity		Absolute difference	% Difference
	Initial	68 hours		
D-Carnitine	0.043	0.048	0.005%	2

The aforementioned information leads to the conclusion that sample and standard solutions are consistent up to 78 hours and 68 hours, respectively, when stored at room temperature.

4.5. Specificity

Blank, standard, and individual impurity solutions were prepared and injected into the chromatographic system for identification and blank interference with the D-Carnitine and L-Carnitine peaks.

1) No interference was observed at the retention time of D-Carnitine from the diluent.

2) All the peaks are well separated from each other. Hence, the method is specific (Table 9).

Table 9. Specificity of D-Carnitine. L-Carnitine RT details also in the same table (RT in Standard solution: 31.8 minutes and RT in Sample solution: 31.6 minutes).

Sample Name	RT in Standard Solution (minutes)	RT in Sample Solution (minutes)
D-Carnitine	30.8	30.6

4.6. Accuracy

The drug substance was taken, and varying amounts of D-Carnitine of the specification level (0.05%) were added to the flasks. The duplicate spiked samples were prepared and implanted in accordance with the method validation protocol. The Average % Recovery at LOQ, 100%, and 160% meet the acceptance criteria, and hence the method is accurate (Table 10).

Provided an assessment of the accuracy of the method used to quantify D-Carnitine at three different concentration levels. The following parameters were defined: LOQ (Limit of Quantitation), 100%, and 160%. An individual test for each level was performed, and the quantity of D-Carnitine added was compared with the quantity found in the sample. The recovery, which may be used to extend the efficiency of the method in detecting the added D-Carnitine, was determined in the test.

At the LOQ level, the aim was to attain a concentration of 1.4250 µg/mL. The recovery was 74%, 75%, and 78%, and the average recovery was 76%. This means that at low concentrations, the method samples less D-Carnitine than is actually present.

At the 100% level, there was a target concentration of 2.3750 µg/mL. The recovery was 80%, 80% and 80%, respectively, which gave an average recovery of 80%. From this, it can be deduced that the recovery rate has improved and is satisfactory at this concentration level.

Lastly, at the 160% level, the target concentration was set at 3.8000 µg/mL. The method showed the highest accuracy with overall recovery of 83%, 84%, and 84%, respectively, giving an average recovery of 84%. This indicates that the method enhances its accuracy, especially at high concentrations of D-Carnitine.

Table 10. Accuracy for D-Carnitine.

Name	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	Average % recovery
Accuracy-LOQ-1	1.4250	1.0546	74	
Accuracy-LOQ-2	1.4250	1.0626	75	76
Accuracy-LOQ-3	1.4250	1.1141	78	
Accuracy-100%-1	2.3750	1.8896	80	
Accuracy-100%-2	2.3750	1.8883	80	80
Accuracy-100%-3	2.3750	1.9107	80	
Accuracy-160%-1	3.8000	3.1685	83	
Accuracy-160%-2	3.8000	3.1878	84	84
Accuracy-160%-3	3.8000	3.1988	84	

4.7. Robustness

In the robustness study, both the standard solution and sample were prepared on

the basis of the conditions mentioned in the method, and both were injected into the chromatographic system. To remain relevant, the same standard and sample solution was re-inserted, changing one of them at a time while the others remained constant. System suitability data were computed for the standards prepared and injected under different method conditions and compared with those of normal method conditions (Table 11).

Robustness Study-HPLC Parameters contains the findings of the D-Carnitine robustness check in compliance with the HPLC method. The robustness study determines the effect of slight and controlled changes in critical method parameters on the retention time of D-Carnitine and the precision of the method in terms of % RSD.

Under the reference conditions where the HPLC column temperature is at 30˚C and the mobile phase pH is at 2.60, the HPLC method has been used for the determination of the retention time for D-Carnitine, which is 30.8 minutes, and the % RSD is 0, showing high accuracy in the experiment.

When the column temperature is reduced to 25˚C, the retention time becomes 33.9 minutes, indicating that the compound of lower temperatures eluted at a slower rate than at the other temperature. On the other hand, when the column temperature was raised to 35˚C, the peak was shortened to 27.2 minutes, which is indicative of the fact that the elution process is faster at higher temperatures.

The effect of changing the mobile phase pH is also investigated in the present study. When the pH is at 2.40, the time to retain the site is increased to 31.2 minutes. On the other hand, they are increasing the pH to 2.80 causes it to retain samples at 32.7 minutes of the retention time, which also shows that changes in the pH also influence the changes in the retention time of the eluting peaks but to a certain extent in comparison to temperature changes.

In all the tested conditions, the % RSD remains at 0, indicating that the method has high precision regardless of the variation in temperature and pH. This robustness study proves that the HPLC method for the detection of D-Carnitine has a certain degree of stability and can operate with slight changes in analytical method conditions.

HPLC Parameters:

1) Column Operating Temperature (Procedural temperature is 30˚C)

a) Temperature Plus (+5˚C) → 35˚C

b) Temperature Minus (−5˚C) → 25˚C

2) Buffer Mobile Phase (Procedural pH 2.60)

a) pH Minus (−0.2) → pH 2.40

b) pH Plus (+0.2) → pH 2.80

Derivatization Procedure Parameters:

Six (6) derivatized sample solutions were prepared in duplicate by changing the derivatization procedure parameters such as reaction time, reaction temperature, and concentration of Solution-3, and they were poured into the chromatographic apparatus in accordance with the protocol's guidelines. The peak areas obtained

for D-Carnitine in all the derivatized samples were compared with the peak area obtained for D-Carnitine in the derivatization sample prepared under normal conditions as per the method.

1) Reaction Temperature (Procedural temperature is 25˚C)

a) Temperature Plus (+5˚C) → 30˚C

b) Temperature Minus (−5˚C) → 20˚C

2) Reaction Time (Procedural Time is 10 minutes)

a) Time Plus (+5 Minutes) → 15 minutes

b) Time Minus (−5 Minutes) → 5 minutes

3) Volume of Solution-3 added (Procedural 2.0 mL)

a) Volume Plus (+0.5 mL) → 2.5 mL

b) Volume Minus (−0.5 mL) → 1.5 mL

Acceptance Criteria:

- All the system suitability requirements must be met for standard variations in HPLC parameters.
- The results should be comparable to those of Derivatized samples prepared by varying the Derivatization procedure parameters.

Table 11. Robustness study-HPLC parameters.

Parameter		Retention time for D-Carnitine (Minutes)	% RSD
Normal Condition	Column Temp: 30˚C Mobile phase pH: 2.60	30.8	0
Column Temp. Minus 5˚C	25˚C	33.9	0
Column Temp. Plus 5˚C	35˚C	27.2	0
pH Minus 0.2	2.40	31.2	0
pH Plus 0.2	2.80	32.7	0

5. Conclusion

The above studies concluded that the method for determining the content of D-Carnitine in Levocarnitine Drug substance is specific, precise, accurate, rugged, robust, sensitive, and linear over the concentration range. Standard and sample solutions are stable for up to 78 hours and 68 hours, respectively, when stored at room temperature. The developed method is satisfied with the regulatory approach standards outlined in the ICH guidelines. Therefore, it's simple to use this D-Carnitine in Levocarnitine method in routine analysis of production samples and as well as stability samples.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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