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Development &Validation of RP-HPLC Method for Estimation of L-Carnitine and Caffeine as a Energy Boosting Agents in Their Synthetic Mixture

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ABSTRACT:

Over- the- counter energy boosting agents tablet formulation consist of L-carnitine and caffeine and garcinia cumbogia one of the common used. In this research paper we have proposed RP-HPLC method for L-carnitine and Caffeine in synthetic mixture combination, as both the drugs shows synergistic effect as a energy boost which additionally used to reduce triglycerides level with no harmful effect on lipid profile. The HPLC method employed at thermo scientific, hypersil C18column (250mm× 4.6mm, 5µm) with an isocratic mixture of acetonitrile and water (20:80 v/v) as the mobile phase. The column temperature was kept at 30°C.The flow rate was1.0 mL/min and detection was carried out by means of a UV detector at wavelength of 252 nm. Both the components were successfully eluted with mean retention times of 3.2 min &4.5 min for caffeine &L-carnitine respectively. The method was found to be linear (R^2> 0.99), precise (RSD < 0.7 %), accurate (recoveries 97.9–100.8 %), specific, simple, sensitive, rapid and robust. The validated method can be used in routine quality control analysis without any interference by excipients.

Keywords: L-carnitine, Caffeine, RP-HPLC method, Quality control analysis, Energy boosting agents

1. Introduction

Chemically **Caffeine (CA)** is 1, 3, 7-Trimethylpurine-2, 6-dione. (Fig1) The molecular formula of CA is $C_8H_{10}N_4O_2$ and the molecular weight is 194.19 g/ mol.[1] **L.Carnitine (CR)**, having IUPAC name is(3R)-3-hydroxy-4-(trimethylazaniumyl)butanoate. The molecular formula of CR is $C_7H_{15}NO_3$[2] and the molecular weight is 161.201 g/ mol. CA act as a CNS stimulant, it is used in inhibition of adenosine receptors, nucleotide phosphodiesterase enzymes, involvement in adenosine receptor antagonism. Through their effects on the second messengers cAMP and cGMP phosphodiesterase enzymes control cell activity.[3] CR is used as dietary supplements, exports acyl groups from cells and subcellular organelles to urine before they build up to dangerous amounts. it also works as carrier molecule in the transport of long chain fatty acid. The chemical structure of drugs is shown in (Figure 1)

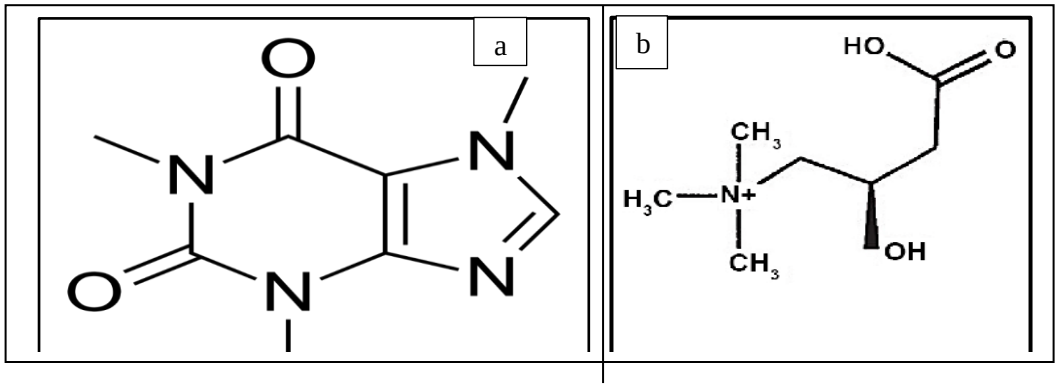

Figure 1: a) Structure of Caffeine b) Structure of Carnitine

Combining caffeine and L-carnitine in a dosage form, such as a supplement or medication, may serve several purposes due to the individual properties of these compounds.

1. **Energy Boost**: Caffeine is a well-known stimulant that can enhance alertness and reduce fatigue by blocking the action of adenosine, a neurotransmitter that promotes sleep. When combined with L-carnitine, which plays a role in energy metabolism by transporting fatty acids into the mitochondria where they can be burned for energy, the combination may provide a synergistic effect in boosting energy levels.[4-6]
2. **Enhanced Exercise Performance**: Both caffeine and L-carnitine have been studied for their potential to improve exercise performance. Caffeine can increase endurance and reduce the perception of effort during physical activity. L-carnitine, on the other hand, may help improve the utilization of fat as an energy source during exercise. Combining the two could theoretically enhance the benefits on exercise performance, particularly for endurance activities.[7-9]
3. **Fat Loss**: There is some evidence to suggest that both caffeine and L-carnitine may aid in fat loss. Caffeine can increase metabolism and promote fat oxidation, while L-carnitine plays a role in the transportation of fatty acids into the mitochondria for energy production. Therefore, combining the two may support weight loss efforts by increasing fat burning and energy expenditure.[10-12]
4. **Cognitive Function**: Caffeine is known to improve cognitive function, including alertness, attention, and concentration. L-carnitine has been investigated for its potential cognitive benefits as well, though the evidence is less robust. Combining the two may provide a comprehensive approach to supporting cognitive function and mental clarity.[13-14]

Shirali et.al (2016)[15] reported the synergistic effect of caffeine-carnitine combination in decreasing body fat and body weight. literature survey revealed various analytical method for estimation of CA alone[16-21] and another drug[22]. Several analytical papers are available on CR alone[23-27] and other drug[28-29]. So not a single analytical paper is reported for the estimation of CA and CR in combined dosage form. In market CA and CR combination is not available in dosage form . “Muscle Blaze

Tablet" is available which contains CA, CR , garcinia combogia & green coffee bean extract. In this tablet CA and CR is the active constituent in this formulation, by referring this tablet formulation. we have prepared synthetic mixture of this combination and then studied the HPLC parameters.

Materials & Methods

Chemical and Reagents-

Pure Drugs CA and CR was procured from Yarrow chem products, Ghatkopar, Mumbai. All the reagents used were of HPLC grade, purchased from MERCK, India. AR-grade Starch, magnesium stearate & talc were used for synthetic mixture.

Instruments-

The HPLC of Shimadzu chromatographic system (Japan) equipped with software Lab solutions and Thermo scientific Hypersil, gold, C18 (250 x 4.6 mm, 5 µm) column with UV detector, Rheodyne 20µl loop capacity manual injector was used throughout the analysis. Digital Analytical Balance– Contech CA223, UV visible spectrophotometer- Jasco V-530 used for experimental work.

Chromatographic Conditions

Before choosing the chromatographic condition, a number of trials were carried out with different ratios of solvent, flow rate, and temperatures to check the retention time (RT),peak shape ,tailing factor(peak symmetry),and theoretical plates of the analytes. A separation was carried out on Thermo scientific, Hypersil gold, C18 column (250 x 4.6 mm, 5 µm pore size) The mobile phase consists of isocratic elution Acetonitrile: Water (20:80 %v/v) with a flow rate of 1.0ml/min and the injection volume of 20µL. All solutions were degassed and filtered through 0.2µm pore size filter. and the UV detector was set at 252nm.

Preparation of Diluent

Homogeneous mixture of acetonitrile and water (20:80 v/v) used as diluent

Preparation of Standard Solution

Accurately weighed quantity of CA(5mg) and CR (10 mg) were transferred in two different 10.0 ml volumetric flask. Then small amount of diluent was added and ultrasonicated for 5 min and diluted up to the mark with diluent. (Concentration:500µg/ml for CAF and 1000µg/ml for CR).

Preparation of mobile phase

The mobile phase was prepared by mixing acetonitrile and water (20:80 v/v). The mobile phase was filtered through 0.45µm and degassed before use.

Preparation of working sample solution

Synthetic mixture was prepared containing 50 mg CA,250 mg CR, 0.06 gm of starch,0.03 gm of magnesium stearate and 0.10 gm of talc per tablet. From the prepared mixture, amount equivalent to 5 mg of CA and 25 mg of CR was added into 10 ml volumetric flask & 5 ml diluent added into it, then sonicated for 10 minutes and make the volume to 10 ml with diluent.. Then it was sonicated for 15 min. and filtered through whatman filter paper. From this solution ,1 ml was transferred in 10 ml volumetric flask and add diluent up to the mark to make final concentration of CA and CR, 50 µg/ml and 250 µg/ml respectively. It was then filtered through 0.45µm syringe filter and then used for assay.

Validation of proposed method [30-34]

System Suitability: It was demonstrated by evaluating %RSD (not more than 2%) for the peak area, theoretical plates (not less than 2000) and tailing factor (not more than 2) of CA and CR from five replicates of standard injections.

Linearity: The linearity of the method is it's ability to show that the absorbance is directly proportional to the concentration of analyte in the sample. It is demonstrated by plotting peak area and concentration in the range of 2–10µg/ml for CA and 10-30 µg/ml for CR. respectively and established the regression line with method of best it. Correlation coefficient, slope and intercept of the regression line were calculated.

Precision

The repeatability(intrday) and intermediate precision of the procedure of were used to establish its precision for each six samples. The intraday precision was conducted on (the same day with the sample at certain time. The same sample solution was used on one consecutive day to accomplish the interday precision For the determination 4 µg/ml, 6 µg/ml, and 8 µg/ml concentration of CA and 15µg/ml, 20µg/ml, and 25 µg/ml concentration of CR was tested.

Limit of Detection(LOD) and Limit of Quantification (LOQ)

Sensitivity of the proposed method was estimated in terms of LOD and LOQ .The LOD and LOQ for CA and CR were determined at a signal to noise ratio of 3:1 and 10:1 respectively, by injecting series of dilute solutions with known concentrations.

Accuracy: Accuracy was done by recovery study using standard addition method. Three different concentration levels of known amounts of CA and CR samples were put to a pure drug with consistent weight of 5 µg/ml, and 25 µg/ml (50%,100% and 150% of the label claim) in three triplicates. The recovery was calculated using the peak area.

Robustness

Robustness analysis was used to examine the impact of small deliberate adjustments on the ideal

chromatographic setting created for CA and CR in terms of variations in the column temp (30 ±2 ^{0}C) and flow rate (1 ±0.1 milliliter/min).Three samples of CA (6 µg/ml) and CR (20 µg/ml)) were evaluated using it in all the aforementioned circumstances.

Results and discussion

Method development and optimization

Stationary phase selection-

The polarities of the analytes of interest were taken into consideration when choosing the stationary phase. As the drug molecules are polar or moderately polar, reversed phase stationary phases were tried. A thermo scientific, hypersil gold, C18 column was chosen in order to reduce the time of interaction between the stationary phase and the analytes. This helped to reduce analysis time as there is reduced affinity of the analytes for the stationary phase, and increased interaction of the analytes with the mobile phase.

Mobile phase selection

Preliminary studies with several solvent systems were performed to select the most effective solvent system for the separation of the two APIs. The selection of these solvents as possible mobile phase(s) depended on factors such as cost of solvent(s), polarities of solvent(s) and that of the analyte(s) of interest and the solubility of the analyte(s).. Optimization details of method development trials are given in table 1.

Table 1: Optimization details of Mobile phase

	Trial I	**Trial II**	**Trial III**	**Trial IV**
Mobile Phase	Methanol:Water (50:50%v/v)	Acetonitrile: Water (50:50%v/v)	Acetonitrile: Water (70:30%v/v)	Acetonitrile: Water (20:80%v/v)
Detected Wavelength	252 nm	227 nm	252 nm	252 nm
Conclusion	Extra peaks was shown in trial 1. (Fig2)	L-carnitine has a very low absorbance at this wavelength. (Fig3)	peak does not meet the required criteria for resolution or shape (Fig4)	The peak of both drugs was good, extra peak was not seen. all values are within the acceptable range. (Fig5)

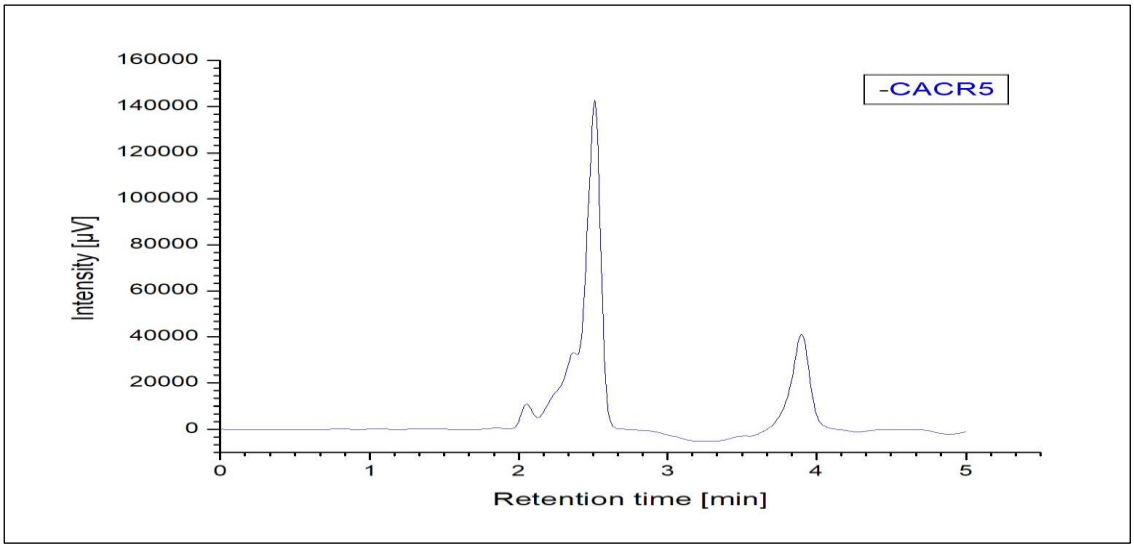

Figure 2 - Trial I Chromatogram of combination of CA & CR

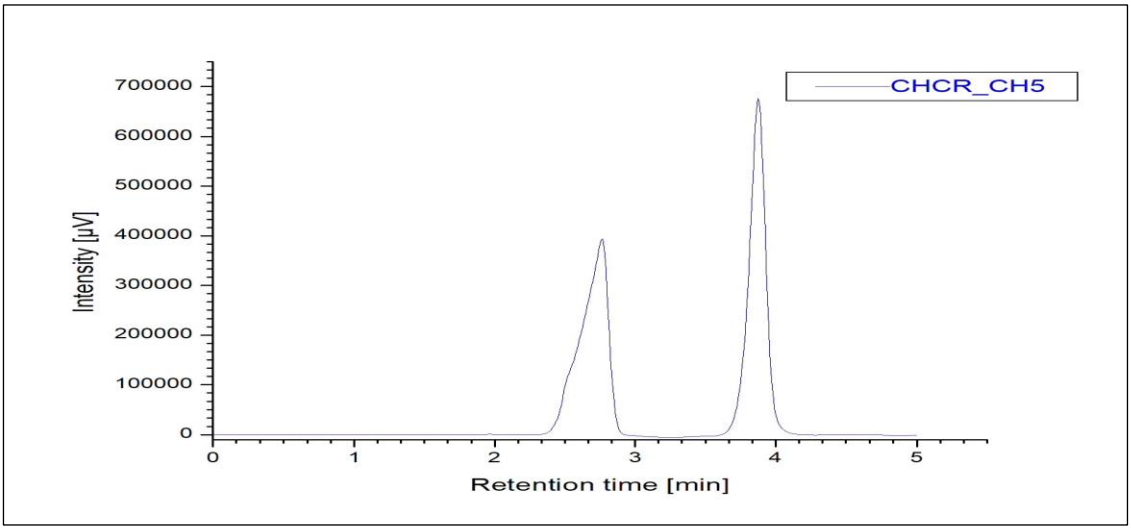

Figure 3- Trial II Chromatogram of combination of CA & CR

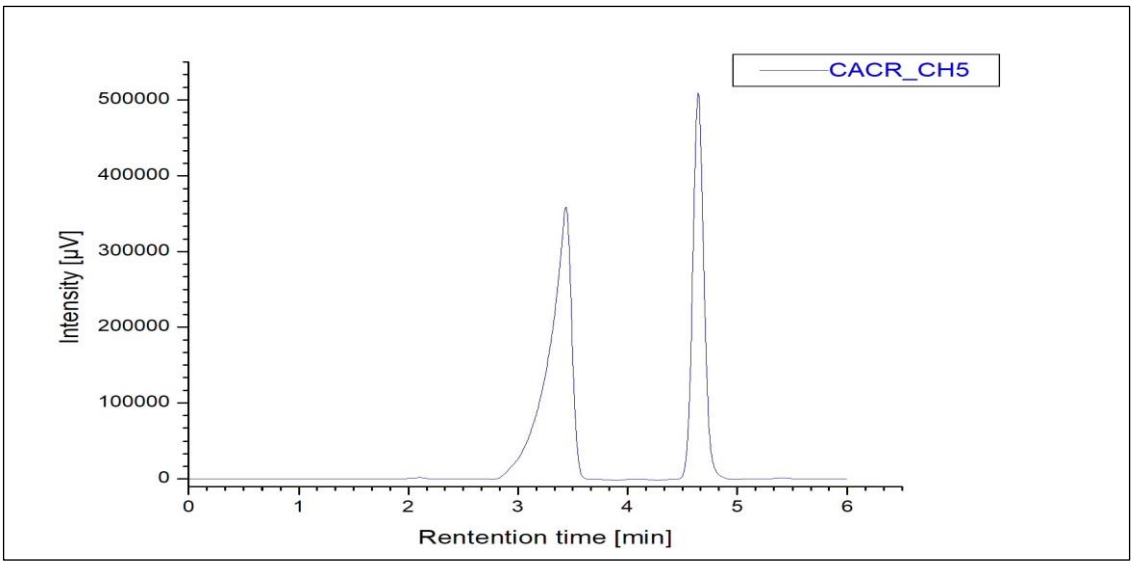

Figure:4 Trial III Chromatogram of combination of CA & CR

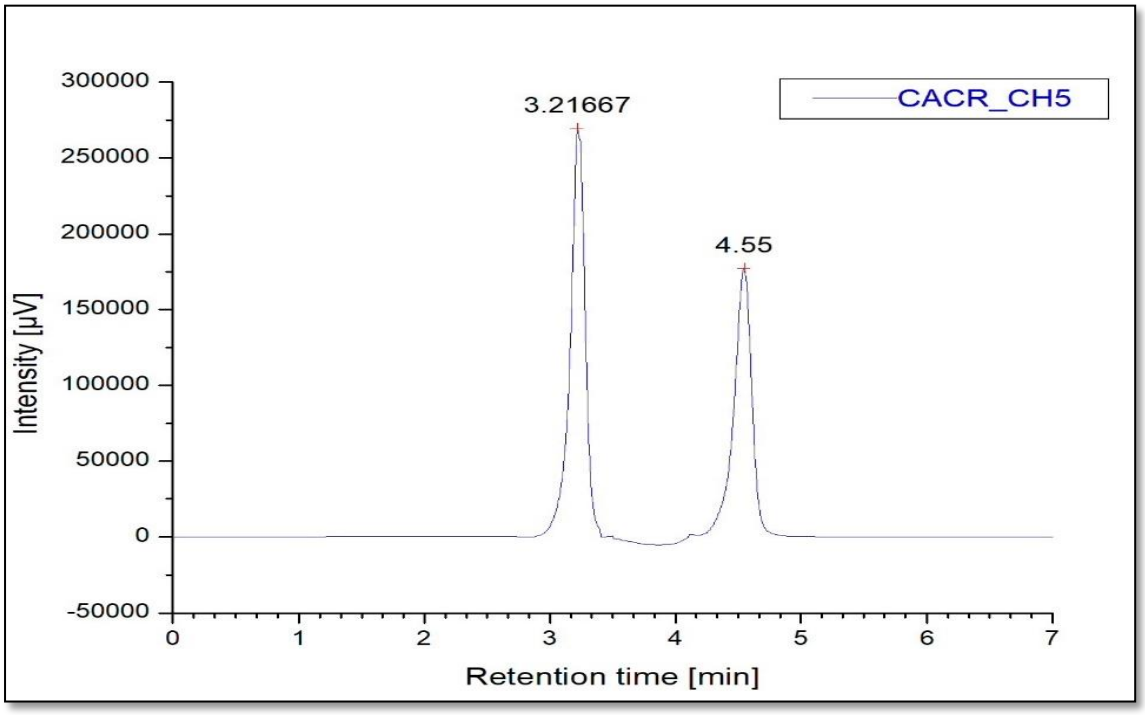

Figure 5- Chromatogram of Optimized condition of combination of CA & CR

Method Validation

System suitability

All the system suitability parameters including peak area, theoretical plates tailing factor, retention time and resolution met the acceptance limits as per ICH guidelines. Results are summarized in Table

Table no 2: System suitability parameters

Parameter	CA	CR
% RSD of Retention time [a]	0.8384	0.5673
%RSD of peak area [a]	0.6342	0.9562
Tailing factor	1.021	1.217
Resolution [b]	12.47	-
Theoretical plate	5541	4954

[a] mean of six determination

[b] resolution with respect to former peak

Linearity, Precision, LOD and LOQ

Linearity of the standards was examined on 2–10µg/ml for CA and 10-30 µg/ml for CR. According to ICH guidelines, the correlation coefficient (R^2) for a calibration curve must be greater than 0.995. The correlation coefficient was found to be more than 0.995 for both the drug indicating good linearity of calibration curve. Overlay graph of linearity determinations as shown in fig.6. The linearity curves were shown in figure 7 &8. Table 3 represent the result of precision study. Table 4 observations showed that the proposed method provides acceptable intra-inter day variations for CA and CR in

their simultaneous determination, The % RSD for each of the drug was found to be less than 2, indicating a high degree of precision in the developed HPLC method. The calculation of the limit of detection (LOD) and limit of quantification (LOQ) for the drug involved using the standard deviation of the intercept and the slope of the calibration curve. LOD and LOQ values for Caffeine, and L-carnitine are also summarized in table 4.

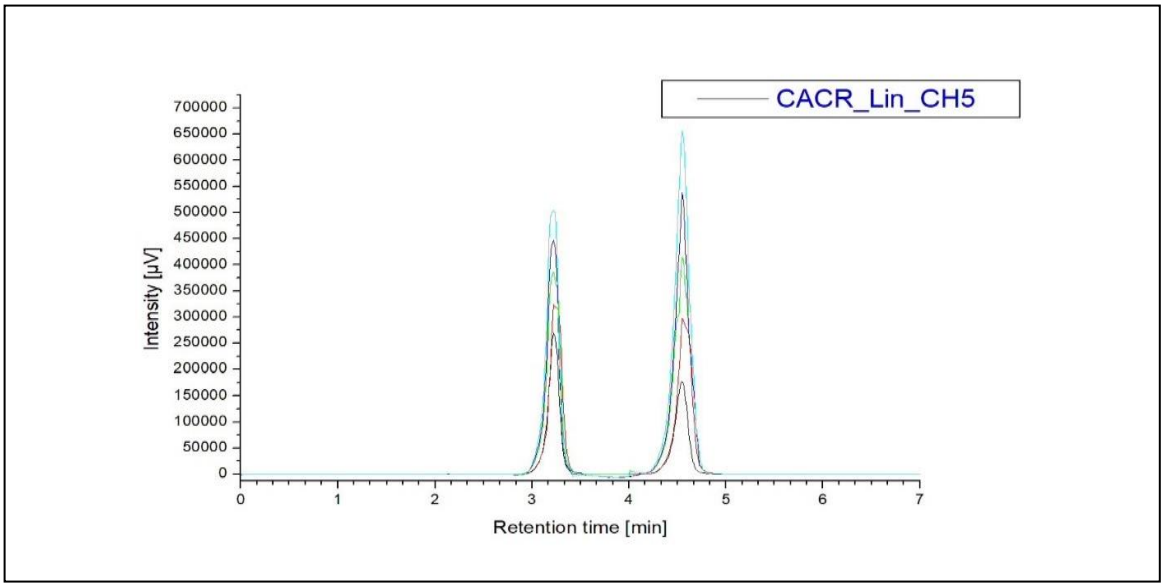

Figure 6: Overlay graph of Linearity determination (CA +CR)

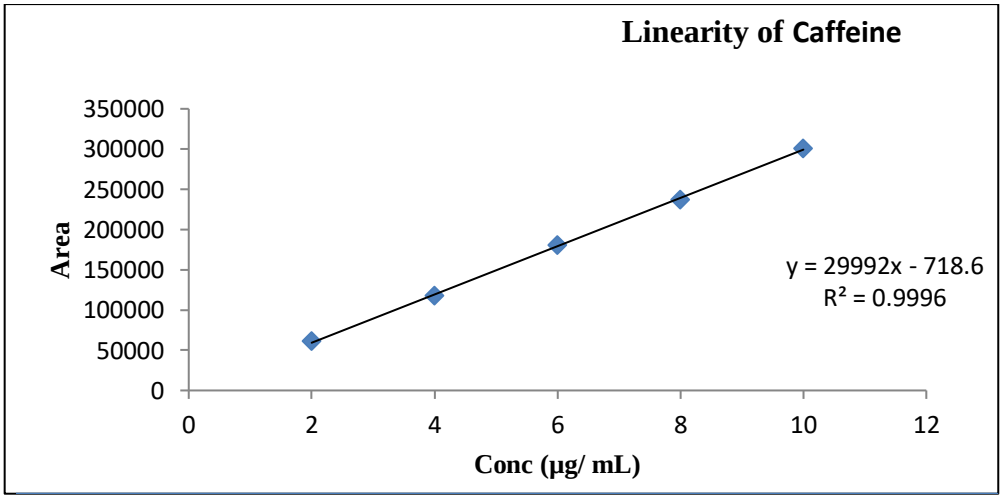

Figure 7- Calibration curve of Caffeine

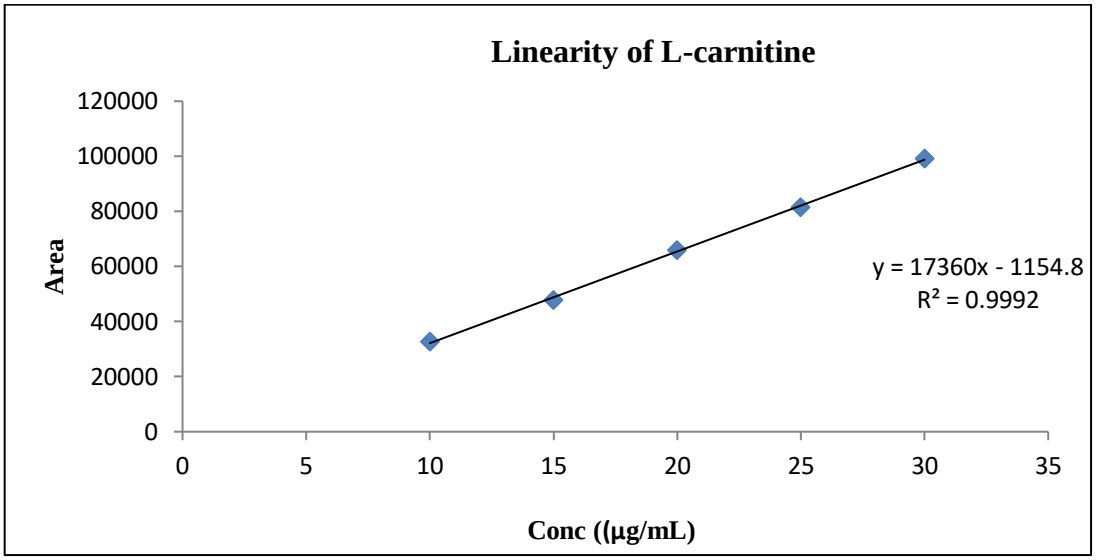

Figure 8- Calibration curve of CR

Table 3: Data of Precision study

Parameters/ Markers	Intra-day				Inter-day		
	Conc. (µg/ mL)	Mean Area (n=3)	SD	%RSD	Mean Area (n=3)	SD	%RSD
Caffeine	4 µg/mL	111564	346.41	0.7161	111342	348.15	0.7412
	6 µg /mL	167149	672.22	0.7821	168113	677.73	0.7427
	8 µg /mL	212767	789.21	0.8384	217698	796.75	0.7407
L-carnitine	15 µg /mL	45136	271.71	0.7687	45776	281.93	0.7551
	20 µg /mL	63522	423.75	0.6311	63548	431.31	0.7660
	25 µg /mL	78757	381.14	0.6424	78142	337.10	0.7414

Table 4: Data of Linearity, Precision, LOD &LOQ

Parameters	Limits	CA	CR
Linear equation	-	29992x-718.6	17360x-1154.8
Correlation coefficient (R^2)	≥0.995	0.9996	0.9992
Linearity range		2–10µg/ml	10-30 µg/ml
Precision (Intra day)	%RSD<2%	0.7788	0.6807
Precision (Inter day)	%RSD<2%	0.7415	0.7541
LOD		0.55	2.65
LOQ		1.68	4.97

Accuracy

The % recovery of Caffeine and L-carnitine were found within a range of 99.92 to 100.10% and 99.87 to 100.02% respectively. All experimental results are in the range of acceptability for accuracy (98-102%). The results are shown in Table 5.

Table 5: Data of Recovery studies

Parameters/ Markers	Amount of Standard Added (µg/ mL)	% Recovery ± SD
CA	50 %	99.96±0.037
	100 %	99.92±0.029
	150 %	100.10±0.065
CR	50 %	99.92±0.034
	100 %	99.87±0.035
	150 %	100.02±0.041

Robustness

The developed HPLC method was found to be robust in terms of variations in the column temp (30 ±2 ^{0}C) and flow rate (1 ±0.1 milliliter/min). The results obtained for robustness studies, and % RSD shown in table 6.

Table 6: Robustness result

Parameters/ Markers	Conc. (ug/ mL)	Column temp. (^{0}C)	Mean Area	%RSD	Flow rate (mL/min)	Mean Area	%RSD
CA	6 µg /mL	28 ^{0}C	119719	1.025	0.9 mL/min	117917	1.167
	6 µg /mL	30 ^{0}C	110942	0.7465	1.0 mL/min	118342	0.7654
	6 µg /mL	32 ^{0}C	111943	1.107	1.1mL/min	118015	1.121
CR	20 µg /mL	28 ^{0}C	63543	1.025	0.9 mL/min	62981	1.215
	20 µg /mL	30 ^{0}C	63981	0.7424	1.mL/min	62971	0.7214
	20 µg /mL	32 ^{0}C	63279	1.112	1.1mL/min	63107	1.167

Analysis of synthetic mixture

20 µl of sample mix solution was injected in HPLC system. The procedure was replicated six times. The mean % purity for CA and CR were found to be respectively. The results of assay suggest that the method can be suitably applicable to formulation mixture too. Table 7 represents concentration obtained and %purity calculated for CA and CR.

Table no :7 Assay of synthetic mixture

	CA	CR
STD (Peak Area)	139671	78957
Synthetic Mixture (Peak Area)	139115	78451
% ASSAY	99.60	99.03

Conclusion-

The estimation of CA and CR can be done in the presence of pharmaceutical additives using the proposed method suggest it applicability to formulations for estimation of CA and CR simultaneously. The results of validation parameters indicate the implementation of method for the routine analysis. The result of analysis of standard and sample mixture by proposed method were found to be substantially reproducible and accurate. Hence the proposed validated method is appropriate for routine analysis in quality control labs.

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