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Validated Reverse Phase-high-performance Liquid Chromatography Method for Estimation of the Spice: Curcumin in Self-nano Emulsifying Drug Delivery System

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ABSTRACT

An analytical method was developed by employing ultrafast liquid chromatography and a C-18 reverse phase column for estimating and validation of curcumin (CMN). The mobile phase was made up of acetonitrile and water in a 90 : 10 v/v ratio. The flow rate was set at 1 ml/min. A chromatogram of CMN was detected at a wavelength of 420 nm. The retention time for CMN was found to be 3.14 min. The developed method was found to be linear in the range of 2-10 µg/ml with a regression coefficient of 1. The method developed was validated according to the Q2 (R1) requirements of the International Conference on Harmonization (ICH). The percentage recovery was in the range of 95 to 105%, indicating that the procedure was accurate. The relative standard deviation (RSD) as a percentage was found to be less than 2%, indicating the method's precision. The limit of detection was found to be 77.7 ng/ml and the limit of quantification at 235.6 ng/ml. The method developed was proven to be robust because the response did not change significantly with changes in flow rate or wavelength. The method was used to estimate drug loading and release from a self-nano emulsifying drug delivery system. The percent drug loading of CMN in the produced liquid self nano emulsifying drug delivery system (SNEDDS) formulation was 99.95%. At the end of 60 min, dissolution studies revealed a 78.78% CMN release in water.

Key words: Curcumin, SNEDDS, RP-HPLC, validation, analysis

INTRODUCTION

Flavonoids are a class of plant metabolites that are hypothesized to have numerous good health regulating outcomes via modulating cell signalling pathways and acting as antioxidants (Mathesius, 2018). Curcumin is one of the polyphenolic compounds [CMN; (1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione]. It is a bright yellow compound having a molecular weight of 368.38 g/mol and a log p of 3.2 (Chainoglou and Hadjipavlou-Litina, 2019). It is naturally present in *Curcuma longa* having a melting point of 183°C (Kocaadam and Sanlier, 2017). The reported pharmacological effects of CMN are antioxidant, anti-inflammatory (Bulboaca *et al.*, 2019), anticancer (Valizadeh *et al.*, 2021) and antiviral (Zheng *et al.*, 2021) properties. Besides having these pharmacological activities, CMN possesses some other pharmaceutical challenges (Corrie *et al.*, 2021). CMN has low solubility, low bioavailability and low permeability from the intestine (Zheng *et al.*, 2021). As a result, researchers are developing nanoformulations like liposomes to increase the solubility and bioavailability of CMN (Feng *et al.*, 2017). Researchers have also used various drug carriers such as micelles, nanosuspensions, phytosomes to improve the bioavailability of CMN (Ipar *et al.*, 2019). There is a need to create a simple, sensitive, and repeatable method for quantifying these nanoformulations on multiple characterization techniques such as drug loading, drug dissolution and permeability (Zaghary *et al.*, 2019).

High-performance liquid chromatography (HPLC) methods either in pharmaceutical products or in biological samples are reported in the literature for the determination of CMN alone (Chao *et al.*, 2018). There was a method that elaborated the quantification of CMN in self-nano emulsifying drug delivery system (SNEDDS) but it was in a combination of quercetin and in gradient method having

longer retention time using acetonitrile (ACN) and glacial acetic acid (GAA) (2% v/v) as mobile phase (Khursheed *et al.*, 2021). It is well understood that CMN degrades in presence of methanol and therefore methanol might not be used as a gold standard for HPLC method development and validation (Naksuriya *et al.*, 2016). Abdul and co-workers estimated the amount of CMN in SNEDDS formulation using acetonitrile: 0.2 M phosphate buffer. However, the details about the validation of this procedure are not available (Mohd *et al.*, 2015). Nazari-Vanan and co-workers developed an RP-HPLC method for the estimation of CMN using methanol and water (73 : 27, v/v) containing 3.6% glacial acetic acid at a flow rate of 1 ml/min (Nazari-Vanani *et al.*, 2017). Since CMN degrades in methanol this method might not be reliable. Various methods have also been developed using liquid chromatography-mass spectroscopy (LCMS) for CMN (Kunati *et al.*, 2018). However, experiments using LC-MS/MS are more expensive and are utilized less frequently in routine quality control parameter assessments of formulations.

As a result, a simple HPLC-based method for CMN analysis that can be utilized for regular quality control of CMN-containing formulations must be developed and validated (Manoharan *et al.*, 2020). This study illustrated the creation of a cost-effective, accurate, precise and repeatable reverse phase-HPLC (RP-HPLC) method for estimating CMN, as well as its successful use to assess the presence of CMN in SNEDDS (Fatemi Abhari *et al.*, 2020).

MATERIALS AND METHODS

CMN was obtained from M/S Himedia, Mumbai, India. Tween 80 was purchased from Lobachemie Pvt. Ltd, Mumbai, India. Labrafil M 1994 CS (LMCS), Transcutol P (TP) were obtained as a gift sample from M/S Gattefosse, Mumbai India. Acetonitrile was purchased from Rankem, Gujarat, India. The water used was of HPLC grade.

The analytical method was developed using an ultrafast liquid chromatography system that included a binary pump (LC-20 AD; Prominence, Shimadzu, Japan), a degasser unit (DGU-20A5), photodiode array detector (SPDM20A; Shimadzu, Japan), Rheodyne injector with 20 ml sample injector loop, and Nucleodur C18 column with dimensions of 250 × 4.6 mm i.d., 5 µm. The chromatograms were recorded using LC solution software. During the study, the researchers employed a cyclone mixer (CM-101; REMI India), an analytical balance (AX 200; Shimadzu Analytical Pvt. Ltd., India), and a dissolution apparatus (DS 8000; Lab India, Mumbai, India).

Varied solvents ratios (80 : 20, 90 : 10) of acetonitrile and water were analyzed along with their system suitability during the selection of chromatographic conditions for method development, and the peak of CMN was analyzed at 420 nm. Based on the findings, a mobile phase with a certain ratio as well as retention time and flow rate was developed.

A homogeneous mixture of oil, surfactant, cosurfactant and drug made up SNEDDS. CMN was combined with LMCS, Tween 80 and TP in an isotropic mixture. The ternary phase diagram was used to vary the composition of these components to determine their range of potential nanoemulsion. Finally, utilizing StatEase, software and the findings of ternary phase analysis, Box Behnken design was used to create 17 SNEDDS prototypes. The drug loading, mean droplet size, and zeta potential of these 17 prototypes were all evaluated. Analysis of variance was used to analyze the data, and a response surface approach was used to create polynomial equations. The optimized SNEDDS formulation was composed of CMN (25 mg), LMCS (10% w/w), Tween 80 (45% w/w) and TP (45% w/w). The optimized formulation had a droplet size PDI, and zetapotential of 87.24 nm, 0.14 and -22.60 mV. This formulation was assessed for drug loading and dissolution.

CMN (5 mg) was dissolved in 50 ml of acetonitrile in a standard volumetric flask to get the concentration of 1000 µg/ml (Solution A). An aliquot 1 ml/solution A was withdrawn and further diluted up to 10 ml to get a concentration of 100 µg/ml (Solution B). Further, an aliquot of 1 ml of solution B was withdrawn and further diluted up to 10 ml to get a concentration of 10 µg/ml (Solution C). Sample solution was prepared by diluting liquid SNEDDS of CMN to 500 ml, which provided the concentration of 50 µg/ml (Solution D).

Working standard solutions were developed to develop the calibration curve. Suitable aliquots of 2, 4, 6, 8 and 10 ml were collected from solution C and volume was made up to

10 ml using acetonitrile in five different 10 ml volumetric flasks. The samples' final resulting concentrations were 2, 4, 6, 8 and 10 µg/ml. These samples were then utilized to create an HPLC calibration curve. The concentration and mean peak area were plotted on a calibration curve. The mean peak area was calculated by injecting each sample five times.

The developed method was validated according to the Q2 (R1) requirements of the International Conference on Harmonization (ICH). The system's suitability characteristics, such as peak purity index, theoretical plate, height equivalent to a theoretical plate (HETP), tailing factor, LOD and LOQ were also measured to assess the system's performance. The calibration curve was plotted between the concentration and its mean peak area. The regression equation and coefficient were calculated from the curve.

The accuracy research was conducted based on absolute recovery at three levels : 80, 100 and 120% of the calibration curve's mid concentration (i.e. 6 µg/ml); lower quantified concentration (LQC), medium quantified concentration (MQC) and high quantified concentration (HQC). To achieve these concentrations, aliquots of 4, 8, 6 and 7.2 ml from solution C were added to a 10 ml volumetric flask, followed by 1.2 ml of solution D. The study was repeated six times, with mean and standard deviation values recorded each time. The following equation 1 was used to obtain the percentage absolute recovery.

Absolute recovery = (Actual concentration of analyte/Theoretical concentration of analyte) × 100 ... (Eq. 1)

The degree to which results agree with one another is referred to as precision. The developed method's precision was assessed in terms of repeatability and intermediate precision. On the same day, hexaplicate samples of LQC, MQC and HQC were injected under the identical experimental circumstances to determine repeatability. Similarly, for intermediate precision, LQC, MQC and HQC samples were determined six times on each of three distinct days (inter-day) and by three different analysts (inter-analyst) under the identical experimental settings. The % relative standard deviation (% RSD) was determined using the mean data (n=6).

Per cent relative standard deviation = (Standard deviation of peak area/Average peak area) × 100 ...(Eq. 2)

Robustness is a measure of a method's reliability; it is the ability of a method to stay unaffected by small, deliberate adjustments in method parameters. Variations in flow rate (0.8, 1.0 and 1.2 ml/min) and wavelength (415, 420 and 425 nm) were used to test the analytical method's robustness.

The purpose of the specificity study was to rule out any potential drug-excipient interactions. Each excipient to be employed in the formulation of CMN SNEDDS was diluted in either ethanol or hexane (100 µl) before being injected on HPLC (Zheng *et al.*, 2020).

The stability of CMN standard stock solution (6 µg/ml) was evaluated by placing the sample at normal room temperature (25±2°C). Samples were taken at different time points and analyzed by HPLC at 420 nm.

Peak purity index, HETP, theoretical plate and tailing factor were all used to assess the system suitability of the developed method. The standard deviation of response (sigma) and slope of the calibration curve (S) were used to determine LOD and LOQ (Equations 3 and 4). The standard deviation was calculated using the standard deviation of the Y intercepts of the regression line.

$$LOD = 3.3 \times (\sigma/s) \quad \text{...(Eq. 3)}$$

$$LOQ = 10 \times (\sigma/s) \quad \text{...(Eq. 4)}$$

To 1 ml solution D, 1 ml mobile phase was mixed. It was then subjected to HPLC analysis. To quantify the drug loading, the calibration curve equation was utilized to process the observed area. Drug loading was calculated as:

Drug loading = (Actual amount of drug present/Total amount of drug added) × 100 ...(Eq. 5)

In vitro dissolution tests were performed on SNEDDS containing 5 mg of CMN. A USP type II dissolution apparatus with 900 ml of distilled water was used to evaluate the release of CMN from the capsules. The medium was regulated at 37±0.5°C, and the paddle was rotated at 50

rpm. The formulation was placed in size zero hard gelatin capsules and maintained in an 11 mm diameter stainless steel sinker in the dissolution device. The experiment was conducted for 60 min. At a predetermined time, samples (5 ml) were withdrawn and analyzed in an HPLC at 420 nm.

GraphPad Prism version 8 was used to process all of the experimental data, which were expressed as mean±standard deviation (GraphPad Software, Inc., CA).

RESULTS AND DISCUSSION

The effects of different ratios of the mobile phase compositions were investigated and studied. In all of the mobile phase compositions, the CMN peak occurred at varied retention times. In Fig. 2a, it was observed that the peak of acetonitrile : water (80; 20, v/v) had a tailing factor above 2 and theoretical plate less than 2000 with a retention time of 3.4 min. Hence, this method was rejected and the mobile concentration of acetonitrile was increased and used as acetonitrile : water (90 : 10, v/v) which showed a sharp peak with tailing factor above 2, theoretical plate above 200 at 3.152 min (Fig. 1). Although, not statistically significant but this method had a faster retention time. As a result, this chromatographic condition was finalized for further calibration curve construction and validation of the method.

With a correlation coefficient (r^2) of 1, the calibration curve was determined to have excellent linearity in the range of 2-10 µg/ml.

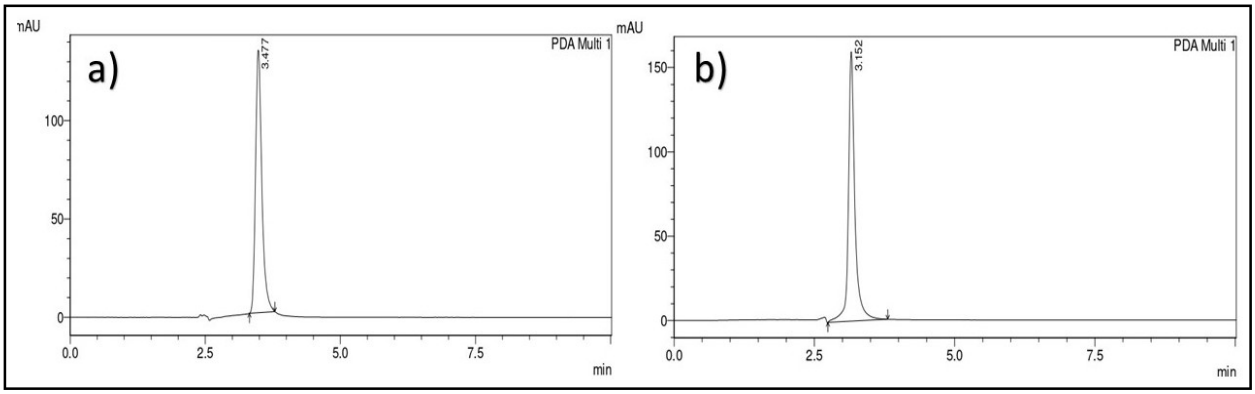

Fig. 1. Chromatograms of CMN in different ratios of acetonitrile and water (a) acetonitrile : water (80 : 20 v/v) and (b) acetonitrile : water (90 : 10, v/v).

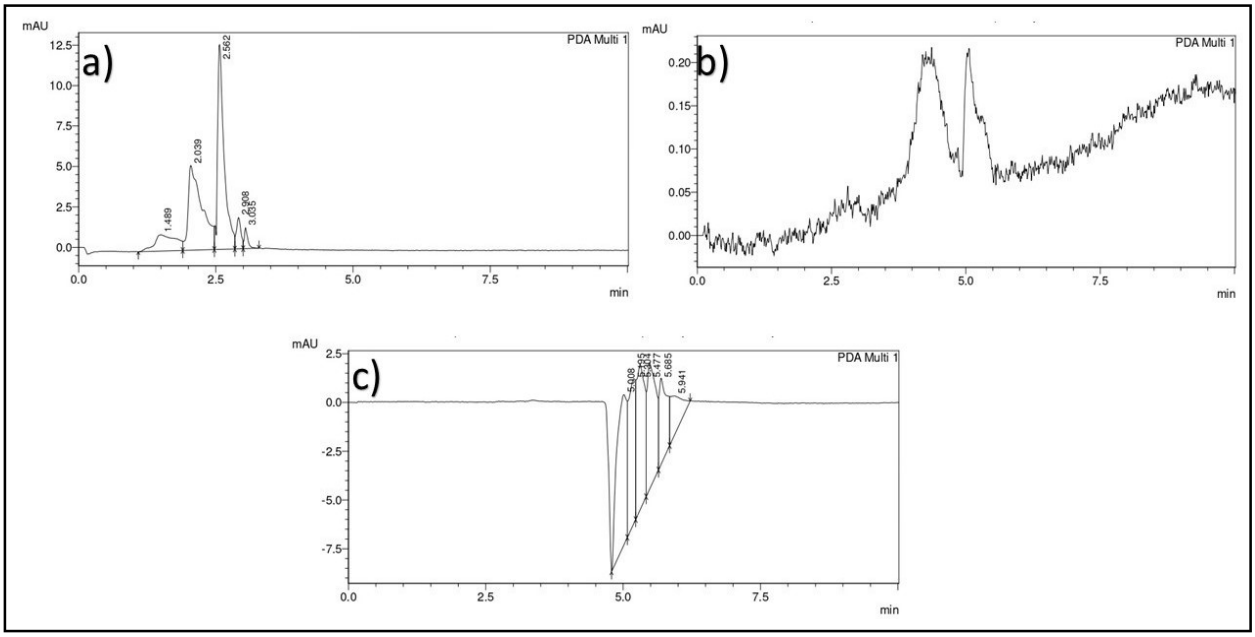

Fig. 2. Specificity studies of CMN SNEDDS (a) LMCS, (b) TP and (c) Tween 80.

Calculating the mean percentage recovery of the drug from the LQC, MQC and HQC, samples were used to assess the accuracy of the method developed. The data demonstrated that the mean per cent recovery for all three levels i.e. LQC, MQC and HQC was within the prescribed ranges i.e. 95-105%. This indicated that the developed method was accurate (Table 1).

The precision of the developed method was assessed by calculating the % RSD for intraday, interday and interanalyst measurements of the LQC, MQC and HQC solution under the same experimental settings. The percentage relative variation was found to be less than 2%, indicating that the developed procedure was precise. The results are tabulated in Table 2.

The results of % RSD after making small deliberate changes in the flow rate, wavelength revealed that it was less than 2% indicating excellent robustness of the method. With a change in flow rate, the retention time varied, which was not significant and the % RSD of the altered was still less than 2% (Table 3).

No peak was seen at the retention time of CMN in the blank samples of all the excipients. Therefore, the developed method was found to be specific.

The samples were determined to be stable at room temperature up to one week, with a percentage RSD of 2%.

Before the use of a chromatographic system, it is necessary to perform a system suitability test to determine its suitability and efficacy (Table 5). Any chromatographic system's performance might fluctuate with time, affecting the reliability of the analytical data. The method developed was sensitive enough to detect CMN in formulations, based on the LOD and LOQ values. A theoretical plate value of >2,000, lower HETP readings and a decreased

Table 1. Results of accuracy studies

Levels	Theoretical concentration of standard solution (µg/ml)	Conc. of sample solution (µg/ml)	Actual concentration (µg/ml)	Actual concentration recovered from mobile phase (µg/ml) (mean± S. D.) (N=6)	Recovery (%) (mean±S. D.)
LQC	4.8	6	10.8	10.64±1.17	98.51±0.95
MQC	6.0	6	12.0	12.00±1.94	100.00±1.32
HQC	7.2	6	13.2	13.16±1.42	98.93±0.81

Table 2. Results of precision study

Parameters	Levels	Conc. (µg/ml)	Area (mean±S.D.) (N=6) (cm^2)	RSD (%)
Repeatability (intraday precision)	LQC	4.8	470130.7±183.42	0.59
	MQC	6.0	602590.4±195.81	0.46
	HQC	7.2	722232.3±224.32	0.40
Interanalyst	LQC	4.8 (A1, A2, A3)	471108.6±81.43	0.43
	MQC	6 (A1, A2, A3)	603469.2±265.12	0.27
	HQC	7.2 (A1, A2, A3)	720771.7±305.50	0.63
		Intermediate precision (interday)		
Day 1, Day 2, Day 3	LQC	4.8	471537.2±106.03	0.34
Day 1, Day 2, Day 3	MQC	6.0	607788.5±176.46	0.78
Day 1, Day 2, Day 3	HQC	7.2	722101.7±129.47	0.44

Table 3. Results of robustness study

Variables	Value	Conc. (µg/ml)	Mean area±S. D. (N=6) (cm^2)	RSD (%)	Mean R_t (min) (mean ±S. D.) (N=5)	RSD (%)
Flow rate (ml/min)	0.8	6	673353.6±328.33	0.29	3.89±0.07	0.24
	1.0	6	602134.7±209.47	0.17	3.13±0.15	0.36
	1.2	6	466367.9±155.69	0.74	2.61±0.03	0.22
Wavelength (nm)	415	6	590825.8±240.29	0.49	3.13±0.02	0.36
	420	6	605706.3±232.14	0.62	3.13±0.02	0.36
	425	6	595651.4±194.40	0.45	3.13±0.02	0.36

Table 4. Results of stability study of standard stock solution (6 µg/ml)

Sample days							Standard deviation	RSD (%)
1	2	3	5	6	7	Mean (cm^2)		
604227.4	603134.7	602922.3	604337.2	606123.9	608291.1	604839.4	2038.2	0.33
614311.3	608244.3	612456.2	614387.3	606123.9	606289.4	610302.1	3878.05	0.63
604426.5	608341.2	609452.3	604817.4	607423.6	601798.2	606043.2	2861.62	0.47

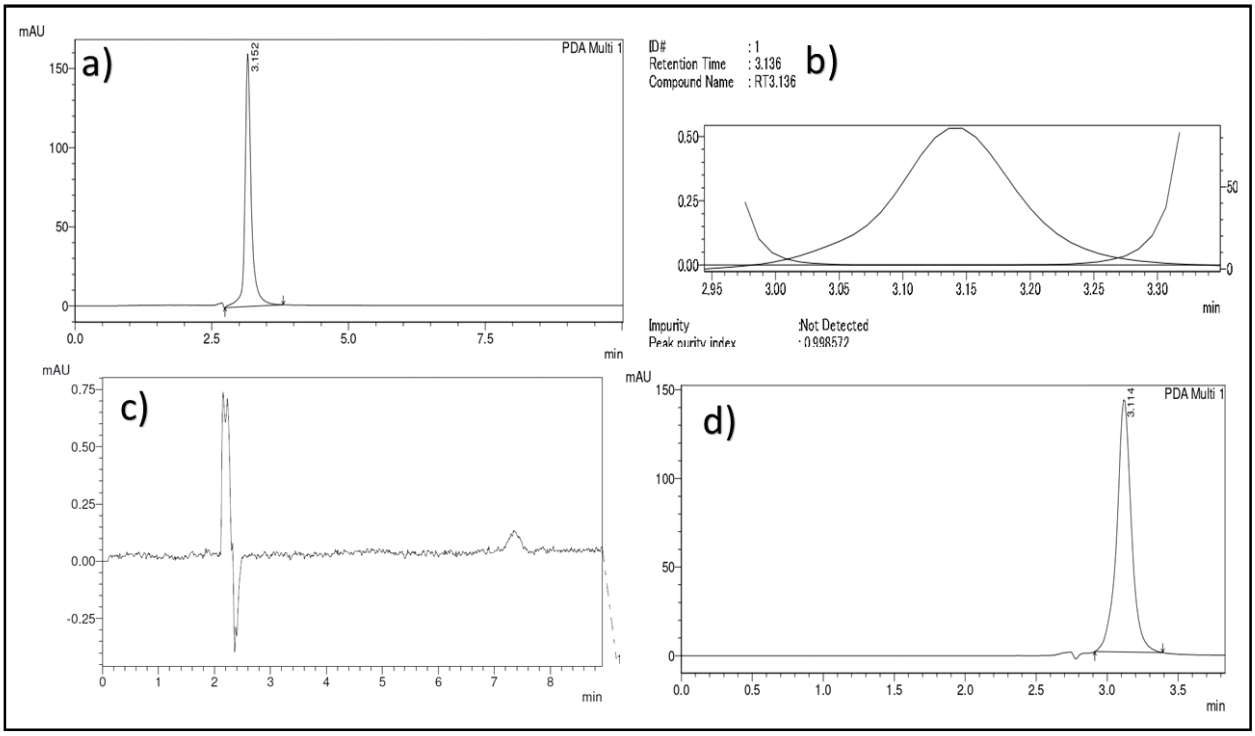

Fig. 3 (a). Chromatogram of unprocessed CMN, (b) Peak purity of CMN, (c) Chromatogram of placebo SNEDDS formulation and (d) Chromatogram of CMN SNEDDS.

Table 5. System suitability parameters

Parameters	Value (mean±S.D.)
HETP	38.45±3.21
Theoretical plate	3900.46±14361.14
Theoretical plate/meter	377512±2146.87
Tailing factor (Symmetry factor)	1.361±0.78
Peak purity index	0.999±0.54
LOD	77.7 ng/ml
LOQ	235.6 ng/ml

Where HETP–Height equivalent theoretical plate, LOD–Limit of detection and LOQ–Limit of quantification.

tailing factor suggested improved column efficiency. The findings of our research revealed that the method developed had excellent system suitability (Fig. 3). The drug loading of CMN in SNEDDS was found to be 99.95%. The results of the dissolution study indicated that 78.78% of CMN was released at the end of 60 min in water (Fig. 4). The results of stability are given in Table 4.

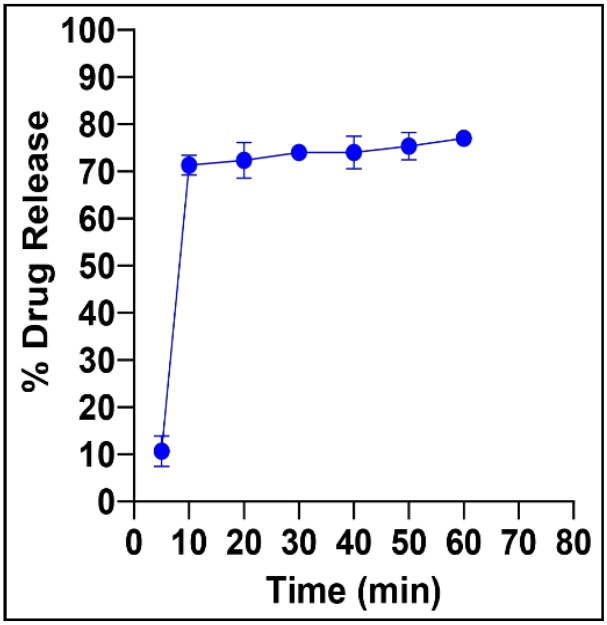

Fig. 4. Dissolution study of CMN SNEDDS in water.

CONCLUSION

An attempt was made in this study to establish an RP-HPLC method for CMN quantification. The technique was created effectively and met all method validation parameters, including linearity, range, precision and accuracy. Furthermore, the method was effectively used to quantify the amount of CMN in SNEDDSs by dissolution and calculating the drug loading, demonstrating the established method's selectivity and sensitivity. The favourable results of this study suggest that the established method can be used to estimate the presence of CMN in biological samples during preclinical or clinical trials.

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