International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 16, Issue 3, 2024

Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CURCUMIN AND RESVERATROL IN NANO-MICELLE: DUAL DRUG DUAL FORM SIMULTANEOUS ESTIMATION

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Received: 31 Dec 2023, Revised and Accepted: 13 Feb 2024

ABSTRACT

Objective: To develop a reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous estimation of conjugated form of Curcumin (CCMN) and free form of Resveratrol (RSV) in nano-micelle.

Methods: The conjugation of lipophilic CCMN and hydrophilic Chitosan (CHT) through succinyl linker produce amphipathic molecule that can self-assemble into RSV solution to form micelle. Here RSV exists in micelle core as free form and CCMN with micelle backbone as conjugated form. So it required to estimate conjugated drug and free drug simultaneously from nano-micelle. We developed a RP-HPLC method, utilized C18 column, follow flow rate of mobile phase 1.0 ml/min, which consist of acetonitrile with water (0.5% *Ortho* Phosphoric acid, pH 4.6) in the ratio of 1:1 for 20 min. Injection volume was 10µl and column temperature 25 °CIsosbestic detection of both drugs was at 254 nm.

Results: The retention time of RSV and CCMN were at 8.15 min and 11.41 min respectively, completely distinguished sharp peak of CCMN and RSV developed with resolution 7.360±0.117, wide range of linearity with correlation coefficient value (R^2) of CCMN and RSV were 0.99987 and 0.99992 respectively and recovery value of CCMN and RSV were 100.041±0.22 % and 100.041±0.21 % respectively. The RSD (relative standard deviation) for accuracy, precision and robustness of the method was found to be less than 2%.

Conclusion: The develop method for simultaneous estimation of conjugated CCMN and free form of RSV in the nano-micelle formulation was consider to be accurate, precise, robust and sensitive.

Keywords: Nano-micelle, Amphipathic, RP-HPLC, Estimation, Validation

DOI: https://dx.doi.org/10.22159/ijap.2024v16i3.50276 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Polyphenolic phytocompounds are gaining enormous demand in the treatment of different diseases and ailments of human beings in different countries because of wide safety margin, easy availability and low costs [1-3]. The polyphenolic phytoconstitutent CCMN chemically known as 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione occurs in *Curcuma longa* Linn having diverse therapeutic activity like anti-viral, anti-inflammatory, anti-cancer, antioxidant, wound healing and anti-diabetic [4-6], proved by *in vitro* and *in vivo* studies [7].

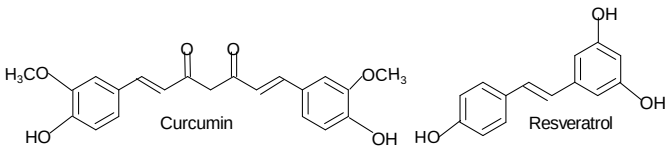

Fig. 1: Structure of curcumin and resveratrol

The polyphenolic phytoconstitutent RSV, also known as phytoalexin, chemically known as 3,5,4'-trihydroxy-trans-stilbene found in red grapes skin, giant knotweed, blueberries, mulberries, eucalyptus and many more having anticancer, anti-inflammatory, antioxidant and type-2 anti-diabetic activity. It diminishes the progress of neurodegenerative disorders and enhances the lifespan of the SIRT1 gene [8-10].

Both drugs have many common therapeutic activities against different diseases. Hence, simultaneous administration of both drugs can produce their synergistic effect against inflammation, oxidative stress and diabetes. Unfortunately, both drugs have very little aqueous solubility and bioavailability. The very slow absorption and fast metabolism of CCMN impose minimal use despite its enormous therapeutic potential. RSV has less bioavailability, faster metabolism and rapid elimination, which arouse delivery problems.

To overcome those problems and simultaneously achieve synergistic therapeutic effects against diabetes, we have developed nano-micelles loaded with two drugs (CCMN and RSV). To construct an amphipathic molecule, here hydrophilic polymer CHT is conjugated with lipophilic polyphenolic compound CCMN via succinyl linker. This amphipathic molecule self-assembled to form micelle in the RSV solution; after completion of micelle formation by dialysis process entire mixture was passesed through 0.45µm filter paper to get nano-size micelle. RSV exists in the nano-micelle core while the amphipathic molecule structure consists of CCMN.

There are very few HPLC methods available for the simultaneous estimation of CCMN and RSV but no HPLC method available for simultaneous estimation of both drugs of two different forms from nano-micelle. Hence, an accurate, precise, rapid and robust RP-HPLC method is required for simultaneous estimation of both drugs from nano-micelles. Our studies focus on the development and validation of the RP-HPLC method for simultaneous estimation of CCMN and RSV from nano-micelles. The common wavelength where both drugs

show maximum absorbance, known as the isosbestic point [11] was found to be 254 nm.

MATERIALS AND METHODS

Chemicals and reagents

Resveratrol (purity>99 %) and rat plasma were procured from Sigma Aldrich chemicals company (St. Louis, Missouri, United States). Curcumin (purity>99 %), Chitosan (low molecular weight), succinic anhydride and dimethyl sulfoxide (DMSO) were purchase from TCI Chemical (India) Pvt. Ltd. HPLC grade acetonitrile (ACN), water, glacial acetic acid, methanol, ammonium acetate and analytical grade *Ortho* Phosphoric were purchased from Merck Specialities Pvt. Ltd., Mumbai, India. Sample solution passes through a 0.45µm pore size Millex syringe.

Preparation and characterization of nano-micelle

Initially amphipathic molecule was synthesized by conjugation of CCMN and CHT through succinyl linker. For the preparation of nano-micelle, 1 mg/ml solution of amphipathic molecule were prepared with 1 mg/ml RSV containing DMSO solution by proper mixing. This mixture was taken into a dialysis bag (Mol. Wt12 kDa), placed into double distilled water for 24 h, and replaced the water in 4 h intervals. Amphipathic molecules were self-assembled in the dialysis bag. During dialysis process unbound-free drugs and other substance were eliminated. After completion of dialysis, it was passed through a filter of 0.45 µm pore size to avoid the large particles and then lyophilized the product. The final nano-micelles were stored in a cool and dry place for further use [12, 13]. The yield of nano-micelles was approximately 88%.

Instrumentation and chromatographic conditions

The HPLC system used was Agilent 1260-infinity (Minneapolis, MN), solvent delivery pump equipped with 20 µl, loop and agilent sample injector (G1329B), and photodiode array (PDA) detector (G13150) using Lab Solution software (Version 1.5). Chromatographic separation was accomplished using Phenomenex Gemini (250 mm X 4.6 mm i.d., 5 µm particle, C18 reversed-phase) Column. The constituents of mobile phase and their ratio were optimized after many trials. The optimized condition of analysis was that the flow rate of mobile phase 1.0 ml/min for run time 20 min, which consist of acetonitrile with water (0.5% *Ortho* Phosphoric acid, pH 4.6) in the ratio of 1:1. Sample injection volume was 10µl and column temperature was 25 ℃ Before utilization of both mobile phase and test sample those were sonicated for 20 min and filtered through 0.45µm membrane filter. The isosbestic wavelength, which is the common peak absorbance of both drugs, was determined by superimposition of individual peak absorption spectra of CCMN and RSV of the range of 200 nm to 400 nm (UV–vis spectrophotometer, Shimadzu, Japan) and it was found that both absorption spectra curve cross at 254 nm. The isosbestic detection wavelength was fixed at 254 nm.

Preparation of calibration standards and sample solution

Preparation of RSV standard

Quantitatively, 10 mg of RSV was transferred quantitatively into a 100 ml volumetric flask after being accurately weighted. 80 ml of ethanol was added dissolved well, and the volume was made up with ethanol to obtain a solution of 100 µg/ml. The working standard solution with a concentration range of 0.1-32µg/ml was prepared by serial dilution with a mobile phase. Before use all those solution were kept at 4 ℃

Preparation of CCMN standard

Accurately weighted 10 mg CCMN was transferred quantitatively into a 100 ml volumetric flask. 80 ml of methanol was added, shaken well to dissolve and the volume was made up with methanol to get a concentration 100 µg/ml. In a similar way like RSV the working standard solution in the concentration range of 0.1-64µg/ml was prepared by dilution with mobile phase. Before use all those solutions were kept at 4 ℃

Preparation of sample solution for investigation of drugs loading and loading efficacy

A concentration of 2 mg/ml of nano-micelle was prepared with 2N HCl, incubated for 1.5 h at 50 ℃for acid hydrolysis of ester bonds exist in between CHT and CCMN. The sample solution was diluted with methanol to produce 1000 ppm. Before filtration through 0.22µ membrane, it was sonicated for 10 min. Filtrate was evaporated to produce dry powder than re-constituted with mobile phase solution centrifuge the solution for 15 min at 5000rpm and then 1.0 ml supernatant was diluted to 10 ml. A known concentration of CCMN and RSV (1:1 ratio) was prepared and treated as above (to know percent degradation of CCMN and RSV in 2N HCl). All the samples were subjected to RP-HPLC analysis within 2 h to investigate drug loading efficacy (DEE) and drug loading (DL) [14-17].

Method validation

For the validation of simultaneous estimation of CCMN and RSV in nano-micelle, we follow the guideline of International Conference on Harmonisation (ICH) stated in Q2(R1). The guideline includes selectivity, system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and robustness [18].

Selectivity

The objective of this study is to clearly separate peaks of drugs and eliminate other substance involve in the nano-micelle. Through HPLC systems separately analyzed standard drugs, standard drugs mixture, nano-micelle sample solution, and nano-micelle free sample solution. For the method selectivity confirmation, Rs (resolution) should be>1.5 [19].

System suitability

To evaluate system suitability, a mixture (1:1 ratio) of CCMN and RSV at a concentration of 01 µg/ml in methanol was prepared. It was analyzed six times through HPLC with different time interval and noted down area under the curve or peak area (AUC), numbers of theoretical plate (N), retention time (t_R), tailing factor (T_f) and resolution (R_s). The data generated was calculated to obtain statistical values such as standard deviation (SD) relative standard deviation (RSD). The acceptance limit for system suitability RSD value should be less than 2%.

CCMN and RSV calibration solutions preparation for linearity assessment

Separately 01 mg/ml stock solution of CCMN and RSV in methanol were prepared and diluted by mobile phase to produce calibration standard solution of strength 02µg/ml, 04µg/ml, 06µg/ml, 08µg/ml, 16µg/ml and 32µg/ml for CCMN and 01µg/ml, 02µg/ml, 04µg/ml, 08µg/ml, 12µg/ml and 16µg/ml for RSV. Before injection to HPLC system, all were passed through 0.22µ filter. The obtained peak areas were graphically plotted against the corresponding strength of CCMN and RSV to obtain the regression correlation coefficient (R^2) [20, 21] for the linearity assessment of both drugs.

Sensitivity

LOD and LOQ were calculated to know the sensitivity of test. LOD signify how much less quantity our developed HPLC analysis can sense and LOQ signify how much less quantity our developed HPLC analysis can quantify. Standard deviation (σ) and slop (S) of linear regression for CCMN and RSV were obtained from data generated in calibration curve by statistical calculation [19]. Based on the equation below, LOD and LOQ were calculated.

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \quad \ldots\ldots\ldots \mathrm{Eq1}$$

$$\mathrm{LOQ} = \frac{10\sigma}{S} \quad \ldots\ldots\ldots \mathrm{Eq2}$$

Where σ = the standard error of the response, S = the slope of the calibration curve.

Accuracy and precision

As per the guide line of ICH Q2(R1) accuracy is the closeness of obtained results to the actual value and precision is the closeness of obtained results among each other or it may be define as

repeatability of obtained results. For the accuracy and precision assessment three concentration of CCMN (02µg/ml, 04µg/ml and 08µg/ml) and RSV (02µg/ml, 04µg/ml and 08µg/ml) were prepared from standard solution. All those were analyzed through HPLC system in triplicate within same day (Intra-day) and in three consecutive days (inter-day), follow the same experimental condition for all. The measured peak responses of chromatogram of intra-day and inter-day were statistically calculated for accuracy and precision measurement. Statistically, we have calculated mean, SD, and % RSD [22-26].

Robustness

The develop method consider to be a robust method when minor intentional change of chromatographic condition like flow rate, pH, and composition unaffected the reproducibility of results or no significant change occur in expected results. The chromatographic analysis data obtained after small changes in parameters were analyzed to calculate RSD, which should remain less than 2% for the chromatographic method to be robust.

***In vitro* release study of CCMN and RSV from nano-micelle**

The cumulative releases of CCMN and RSV from nano-micelle were studied in PBS buffer pH 5.0 and 7.4. Separately a fixed quantity of nano-micelle (10 mg) was dispersed in 10 ml of PBS buffer pH 5.0 and 7.4 solutions. All those solutions were taken into two separate dialysis bags and transferred it into 90 ml respective buffers solution. Those were incubated at 37 °C for 9 d with moderate shaking. During the incubation period, with an increasing time interval, 2 ml of the sample was withdrawn from each beaker and replaced with the respective buffer [27]. The samples were investigated to investigate percent cumulative drug release [14-17] using the developed RP-HPLC method.

Data analysis

All the data were statistically analyzed as required to obtain the least square (R^2), standard deviation, mean, relative standard deviation and equation of the calibration curve by Excel Microsoft INC USA.

RESULTS AND DISCUSSION

Finding of common maximum absorbance wavelength

The spectral analyses of CCMN and RSV at concentration 05µg/ml and 10µg/ml solutions were done respectively in methanol. The absorption spectra in the range of 200 nm to 400 nm of both drugs were recorded and plotted. Here the superimposition of two different absorption spectra crosses to each other at wavelength 254 nm, which is the common maximum absorbance wavelength (isosbestic wavelength) of both drugs shown in fig. 2.

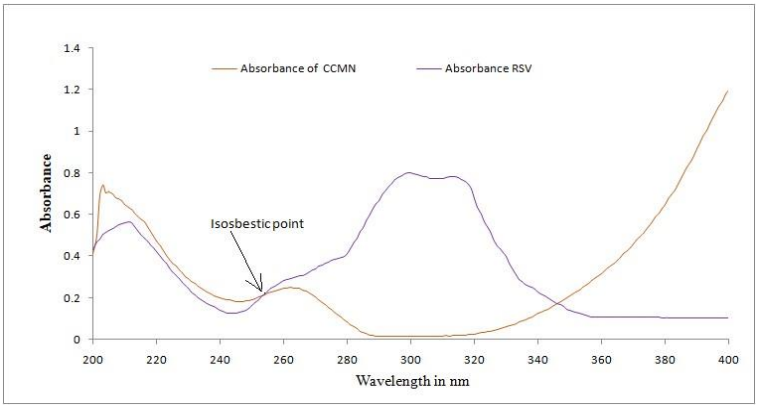

Fig. 2: Superimposition UV spectrum of CCMN and RSV

Optimization of chromatographic parameters

Extensive analyses were done to optimize various parameters like the flow rate of the mobile phase, pH of the mobile phase, column temperature, ratios of the mobile phase components like acetonitrile, *Ortho* Phosphoric acid and water. After many trials, it was optimized that the flow rate of the mobile phase was 1.0 ml/min, pH of the mobile phase was 4.6, Column temperature was 25 ℃ ratio of water and 0.5% *Ortho* Phosphoric acid (pH 4.6) containing acetonitrile was 1:1. Before utilization of above composition as mobile phase, we utilize water and methanol at a different ratio as a mobile phase but the results were not satisfactory. For the simultaneous estimation of CCMN and RSV, the isosbestic wavelength 254 nm was fixed.

Under optimized chromatographic conditions, the chromatogram of the mixture of both standard drugs showed distinctly separated acute sharp peaks with retention times of 8.15 min and 11.41 min for RSV and CCMN, respectively in simultaneous analysis fig. 3. Both drugs were analyzed separately under the same chromatographic condition also; chromatograms were shown in fig. 4 and fig. 5. The chromatographic results execute a higher number of theoretical plate, lesser height of equivalent theoretical plate and lesser tailing factor; those signify the acceptance criteria of HPLC analysis.

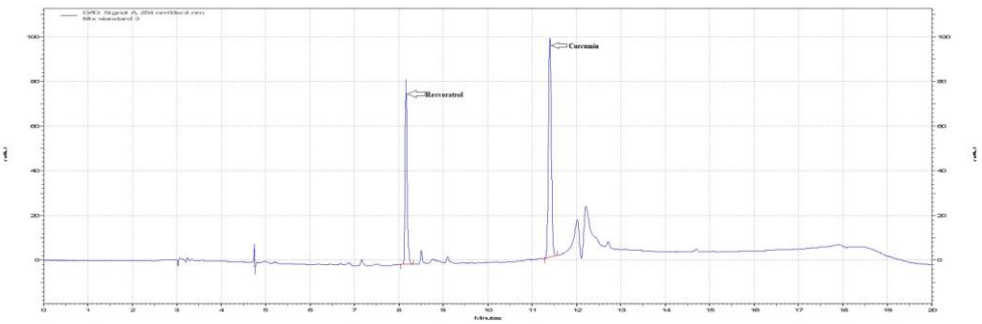

Fig. 3: RP-HPLC chromatogram of resveratrol and curcumin standard

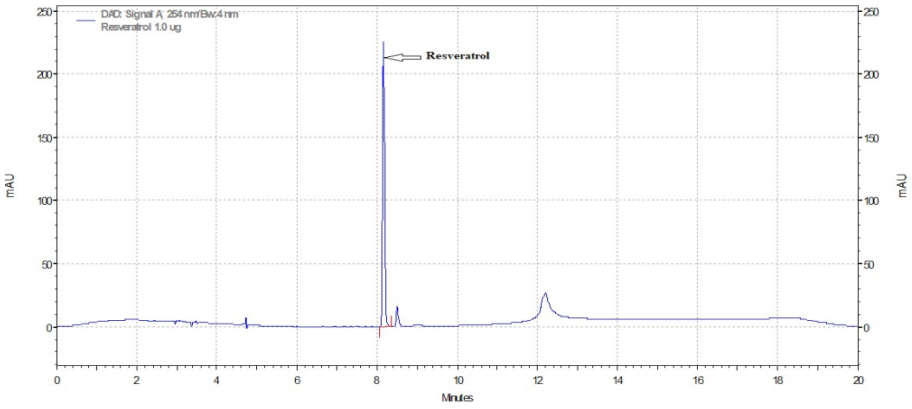

Fig. 4: RP-HPLC chromatogram of resveratrol standard (t_R-8.15 min)

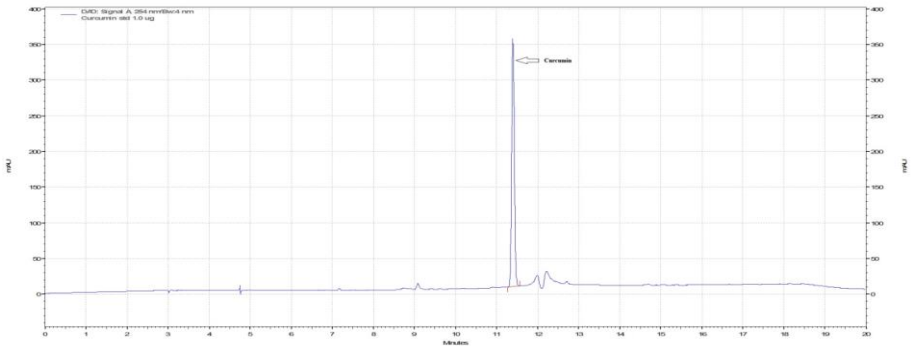

Fig. 5: RP-HPLC chromatogram of curcumin standard (t_R-11.41 min)

Method validation

For the RP-HPLC method validation we follow the guideline of ICH stated in Q2(R1).

System suitability test

The mixture of CCMN and RSV solution was injected in the HPLC column six times to analyze the system suitability of the developed method. The analyzing concentration of CCMN and RSV in the mixture was 01 µg/ml each. To analyze system suitability, different parameters like retention time, number of theoretical plates, area under the curve, tailing factor and resolution were determined (table 1). The average resolution of both drugs was 7.360±0.117 which signifies the complete separation of peak of the drugs and the RSD value of all parameters was less than 2% which signifies that our developed method fulfils the requirements of ICH guideline as well as USP guideline. So our developed RP-HPLC method is very suitable and highly effective [28].

Table 1: System suitability of the developed method

Sample	t_R		N		AUC		T_f		Rs
	RSV	CCMN	RSV	CCMN	RSV	CCMN	RSV	CCMN	
1	8.109	11.381	8085	8270	109456	174443	1.108	1.158	7.479
2	8.105	11.317	7987	8207	109237	173806	1.126	1.211	7.342
3	8.281	11.404	7894	8181	109975	174378	1.142	1.201	7.138
4	8.169	11.408	7913	8102	109981	173800	1.139	1.184	7.403
5	8.167	11.405	7992	8064	109635	174049	1.131	1.214	7.401
6	8.165	11.401	7882	8188	109579	174202	1.148	1.198	7.397
Average	8.166	11.386	7958.833	8168.667	109644	174113	1.132	1.194	7.360
SD	0.064	0.035	77.484	74.382	293	277	0.014	0.021	0.117
RSD (%)	0.778	0.309	0.974	0.911	0.267	0.159	1.260	1.736	1.591

n=6; t_R: Retention time, N: Number of theoretical plate, AUC: Area under the curve, T_f: Tailing factor, Rs: Resolution

Linearity, range and sensitivity

Six different concentration of CCMN (02µg/ml, 04µg/ml, 06µg/ml, 08µg/ml, 16µg/ml and 32µg/ml) and RSV (01µg/ml, 02µg/ml, 04µg/ml, 08µg/ml, 12µg/ml and 16µg/ml) were analyzed separately and AUC of different concentration of both compound were plotted against their respective concentration in MS-Excel fig. 6a and 6b. It was found that the linearity range of CCMN was 2-32 µg/ml and RSV was 1-16 µg/ml, equation of calibration carve were Y= 171178.89X+19619.91 for CCMN and Y = 108179.53X+6862.02 for RSV, the correlation coefficient value (R^2) of CCMN and RSV were 0.99987 and 0.99992 respectively, very near to 1, that signify the criteria of linearity. To know the sensitivity of the test LOD and LOQ were calculated. LOD and LOQ of CCMN were 0.284939359 µg/ml and 0.863452604µg/ml, respectively. LOD and LOQ of RSV were 0.12463075µg/ml and 0.37766894µg/ml, respectively. From LOD and LOQ results it was concluded that the developed method

is very sensitive, <01 µg/ml solution of CCMN and<0.37766894µg/ml solution of RSV can be quantified. All the above data is summarized in table 2.

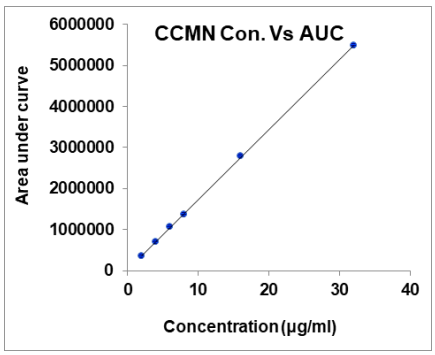

Fig. 6a: Calibration curve of CCMN

Accuracy and precision

Three different concentrations of CCMN (02µg/ml, 04µg/ml and 08µg/ml) and RSV (02µg/ml, 04µg/ml and 08µg/ml) were prepared for accuracy and precision study. Those entire samples were analyzed in intra-day and inter-day as per the guidelines of ICH [18]. The analysis results were summarized in tables 3, 4, and 5. HPLC analysis data obtained were statistically calculated to analyze %RSD and percent recovery. The intra-day and inter-day recovery (percent accuracy) of CCMN were 100.041±0.22 % and 99.75±0.42 %, respectively, in correspondence to RSV were 100.041±0.21 % and 100.14±0.29 %, respectively, it signify the acceptance criteria of accuracy. Similarly, intra-day and intra-day % RSD of CCMN were 0.44±0.28and 0.28±0.02respectively, in correspondence to RSV were 0.24±0.05and 0.32±0.19 respectively, it signifies the acceptance criteria of precision. As per the guideline of acceptance criteria, the percent RSD of accuracy and precision should be below 2% but our method achieves below 0.5 % in this regard, so our developed method has more reliability than other methods [29].

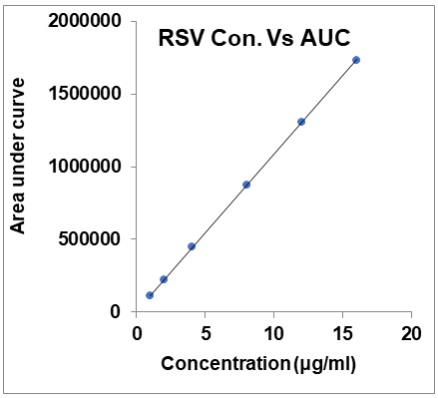

Fig. 6b: Calibration curve of RSV

Table 2: Validation parameters for curcumin and resveratrol

Parameter	CCMN	RSV
Linearity range (µg/ml)	2.00-32.00	1.00-16.00
Linear equation	Y = 171178.89 X+19619.91	Y = 108179.53X+6862.02
Correlation coefficient of (R^2)	0.99987	0.99992
Slope	171178.89	108179.53
Intercept	19619.91	6862.02
Standard error (σ)	14780.48582	4085.60492
LOD (µg/ml)	0.284939359#	0.12463075#
LOQ (µg/ml)	0.863452604$	0.37766894$

All the values are considered as mean±SD, n=6; #Calculated from Eq1, $Calculated from Eq2

Table 3: Summary of intra-day and inter-day precision and accuracy of the method

Type of analysis	Nominal strength in (µg/ml)	Mean strength found, µg/ml		Mean Accuracy %		RSD% (Precision)	
		CCMN	RSV	CCMN	RSV	CCMN	RSV
Intra-day	2	2.003±0.015	1.997±0.006	100.166667	99.83333	0.7625	0.2892
	4	4.007±0.012	4.010±0.010	100.166667	100.25	0.2882	0.2494
	8	7.983±0.021	8.003±0.015	99.7916667	100.0417	0.2608	0.1909
Inter-day	2	1.987±0.006	1.997±0.006	99.3333333	99.83333	0.2906	0.2892
	4	4.007±0.012	4.017±0.021	100.166667	100.4167	0.2882	0.5183
	8	7.980±0.020	8.013±0.012	99.75	100.1667	0.2506	0.1441

Intra-day and inter-day accuracy of CCMN were 100.041±0.22 % and 99.75±0.42 %; Intra-day and inter-day accuracy of RSV were 100.041±0.21 and 100.14±0.29; Intra-day and inter-day % RSD of CCMN were 0.44±0.28 and 0.28±0.02; Intra-day and inter-day % RSD of RSV were 0.24±0.05% and 0.32±0.19 % respectively; All the values are presented as mean±SD, n=3.

Table 4: Intra-day analysis results of CCMN and RSV

Conc.	Intra-day analysis of CCMN				Intra-day analysis of RSV				Intra-day % accuracy	
	Found	Mean	SD	RSD%	Found	Mean	SD	RSD%	CCMN	RSV
2	1.99 2 2.02	2.0033	0.0153	0.7624	1.99 2 2	1.9967	0.0058	0.2892	100.1667	99.3333
4	4 4.02 4	4.0067	0.0115	0.2882	4.01 4.02 4	4.01	0.01	0.2494	100.1667	100.25
8	7.96 7.99 8	7.9833	0.0208	0.2608	7.99 8 8.02	8.0033	0.0153	0.1909	99.7917	100.0417

All the values are considered as mean±SD, n=3

Table 5: Inter-day analysis results of CCMN and RSV

Conc.	Day	Inter-day analysis of CCMN				Inter-day analysis of RSV				Inter-day % Accuracy	
		Found	Mean	SD	RSD%	Found	Mean	SD	RSD%	CMN	RSV
2	Day-1	1.98	1.9867	0.0058	0.2906	1.99	1.99667	0.0058	0.2892	99.3333	99.8333
	Day-2	1.99				2					
	Day-3	1.99				2					
4	Day-1	4	4.0067	0.0115	0.2882	4.04	4.0167	0.0208	0.5183	100.1667	100.4167
	Day-2	4.02				4.01					
	Day-3	4				4					
8	Day-1	8	7.98	0.02	0.2506	8.02	8.0133	0.0115	0.1441	99.75	100.1667
	Day-2	7.96				8					
	Day-3	7.98				8.02					

All the values are considered as mean±SD, n=3

Robustness

Different concentrations of CCMN and RSV were prepared like LQC, MQC and HQC (i.e., Low, Medium and high-quality control) for CCMN those were 01µg/ml, 16µg/ml and 32 µg/ml, respectively, for RSV which were 01µg/ml, 08µg/ml and 16 µg/ml respectively. Minor changes in chromatographic conditions like flow rate (1±0.1 ml/min), pH (4.6±0.2) wavelength (254±2 nm), and composition of mobile phase were done to observe the effect in terms of retention time and assay at different concentration levels. All the results signify the acceptance criteria of ICH as well as USP guidelines table 6. RSD values of retention time and assay were less than 2% which proves that our developed method fulfills the acceptance criteria of robustness.

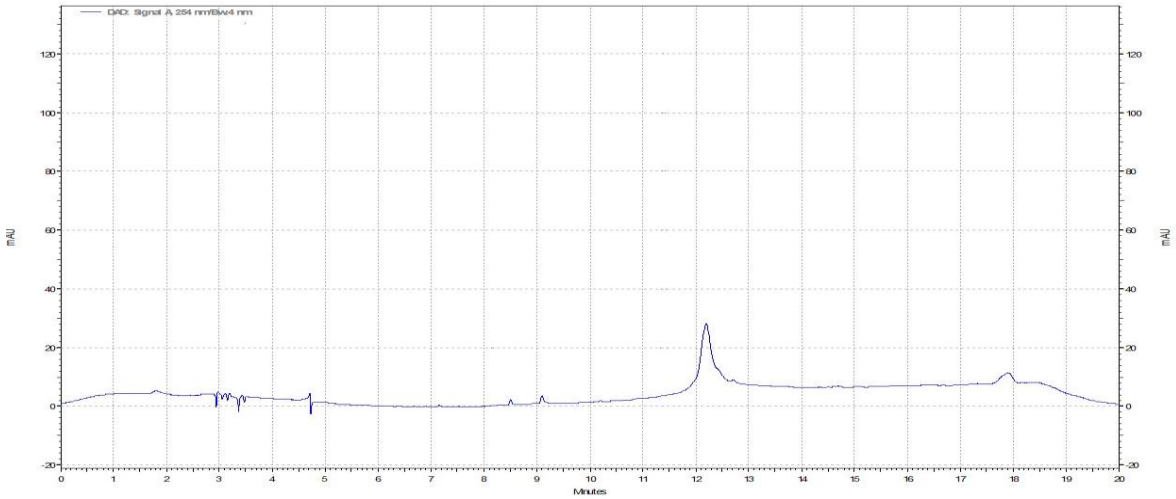

Fig. 7: A blank sample RP-HPLC chromatogram

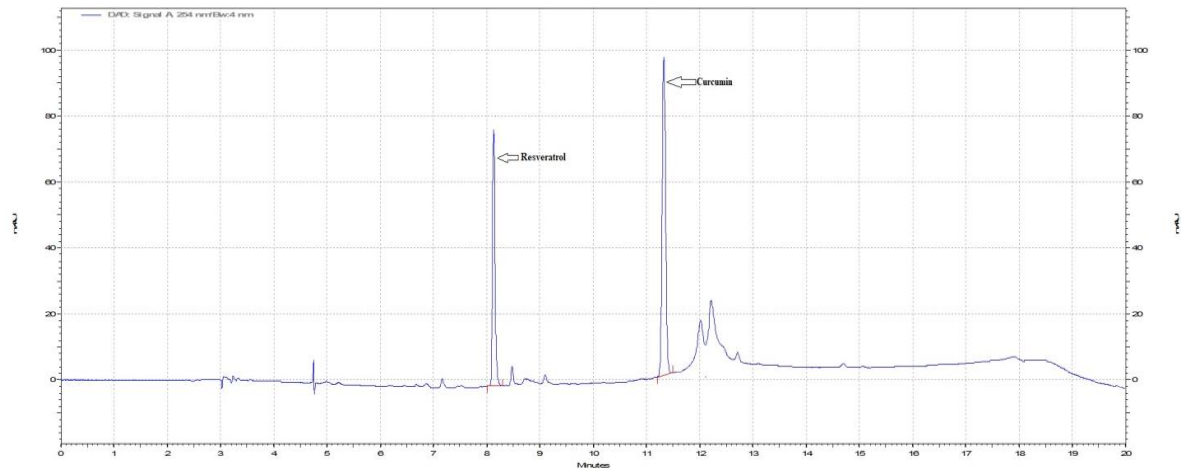

Fig. 8: RP-HPLC chromatogram of CCMN and RSV treated with 2N HCl

Table 6: Summary of robustness of method

Variables	Value	Strength	CCMN								RSV							
			RT	RT (mean)	SD of RT	%RSD of RT	Assay %	Assay % (mean)	SD of assay %	% RSD of assay	RT	RT (mean)	SD of RT	%RSD of RT	Assay %	Assay % (mean)	SD of assay %	%RSD of assay
Flow rate (pH=4.6, Water: CAN= 50:50, Wavelength= 254)	0.9	LQC	11.85	11.847	0.0153	0.1289	100.41	99.797	0.6531	0.6544	8.64	8.573	0.0586	0.6835	100.85	99.840	0.8822	0.8836
		MQC	11.86				99.87				8.53				99.45			
		HQC	11.83				99.11				8.55				99.22			
	1	LQC	11.45	11.413	0.0351	0.3077	99.49	99.950	0.4927	0.4930	8.18	8.153	0.0306	0.3747	99.17	99.863	0.6008	0.6016
		MQC	11.41				99.89				8.12				100.23			
		HQC	11.38				100.47				8.16				100.19			
	1.1	LQC	11.08	11.023	0.0551	0.4996	100.41	99.907	0.7136	0.7143	7.91	7.817	0.0950	1.2159	99.41	100.117	0.6574	0.6566
		MQC	11.02				100.22				7.82				100.71			
		HQC	10.97				99.09				7.72				100.23			
pH (Flow rate= 01 ml/min Water: CAN= 50:50, Wavelength= 254)	4.4	LQC	11.18	11.110	0.0700	0.6301	100.48	100.097	0.7077	0.7070	7.98	7.943	0.0321	0.4047	100.11	99.807	0.2793	0.2799
		MQC	11.04				99.28				7.93				99.56			
		HQC	11.11				100.53				7.92				99.75			
	4.6	LQC	11.45	11.413	0.0351	0.3077	99.49	99.950	0.4927	0.4930	8.18	8.153	0.0306	0.3747	99.17	99.863	0.6008	0.6016
		MQC	11.41				99.89				8.12				100.23			
		HQC	11.38				100.47				8.16				100.19			
	4.8	LQC	11.71	11.607	0.1106	0.9529	99.79	99.787	0.6250	0.6263	8.38	8.307	0.0666	0.8016	99.48	99.580	0.2088	0.2097
		MQC	11.62				99.16				8.25				99.82			
		HQC	11.49				100.41				8.29				99.44			
Composition (Water: ACN) (Flow rate= 01 ml/min, pH=4.6 Wavelength= 254)	50:50	LQC	11.45	11.413	0.0351	0.3077	99.49	99.950	0.4927	0.4930	8.18	8.153	0.0306	0.3747	99.17	99.863	0.6008	0.6016
		MQC	11.41				99.89				8.12				100.23			
		HQC	11.38				100.47				8.16				100.19			
	55:45	LQC	11.27	11.210	0.0600	0.5352	100.75	99.803	0.8259	0.8275	8.11	7.997	0.1266	1.5834	99.74	99.870	0.1253	0.1255
		MQC	11.21				99.23				8.02				99.88			
		HQC	11.15				99.43				7.86				99.99			
	60:40	LQC	10.89	10.817	0.0702	0.6493	99.14	99.563	0.9395	0.9436	7.83	7.867	0.0404	0.5137	100.01	100.287	0.3958	0.3946
		MQC	10.81				100.64				7.91				100.11			
		HQC	10.75				98.91				7.86				100.74			
Detection wavelength (Flow rate= 01 ml/min (pH=4.6, Water: CAN= 50:50)	252	LQC	11.5	11.423	0.0709	0.6211	99.97	99.660	0.5543	0.5562	8.28	8.203	0.0681	0.8298	99.94	99.937	0.1050	0.1051
		MQC	11.36				99.99				8.15				100.04			
		HQC	11.41				99.02				8.18				99.83			
	254	LQC	11.45	11.413	0.0351	0.3077	99.49	99.950	0.4927	0.4930	8.18	8.153	0.0306	0.3747	99.17	99.863	0.6008	0.6016
		MQC	11.41				99.89				8.12				100.23			
		HQC	11.38				100.47				8.16				100.19			
	256	LQC	11.46	11.413	0.0503	0.4410	100.42	99.793	0.6742	0.6756	8.25	8.163	0.0757	0.9275	99.05	99.650	0.6940	0.6964
		MQC	11.42				99.88				8.11				100.41			
		HQC	11.36				99.08				8.13				99.49			

All the values are considered as mean±SD, n=3

Drug loading and entrapment efficacy

To break the micelle architected we utilized concentrated HCl that helps to acid hydrolyze ester bonds that exist between CHT and CCMN, the amphipathic molecular unit of the micelle disappeared after separation of CHT and CCMN and consequently, micelle core materials were exposed to the solution. Similarly, a known concentration of CCMN and RSV mixture solution and a blank sample were treated in the same way before being subjected to our developed RP-HPLC analysis. From HPLC analysis, the results were found in terms of AUC and it was converted in terms of concentration through standard curve equations. The obtained results of the entire sample (fig. 7, 8 and 9) were calculated and it was found that the percent loading of CCMN in nano-micelle was 26.52±0.67 w/w and entrapment efficacy was 93.72%±1.02w/w, the percent loading of RSV in nano-micelle core was 14.56±0.18 w/w and entrapment efficacy was 98.72%±0.12 w/w. In the nano-micelles, CCMN exists in conjugated form and RSV exists in free form. It is very difficult to estimate conjugated drugs and free drugs simultaneously from nano-micelle by HPLC analysis or by any other method of analysis. A lot of papers [27, 30-34] are available on drug conjugates where therapeutic properties were being evaluated with more emphasis, but very few papers are available which explain the estimation of conjugated drug. There are many research articles are available [29, 35, 36] on drug delivery through micelle, where drugs are entrapped in a micelle core in most cases and it is easy to estimate either by UV-Vis Spectroscopy or by chromatographic method. First time we developed an analytical method to estimate dual drugs of dual molecular form (conjugated and free) simultaneously from nano-micelle. Here we also considered the impact of percent degradation during the estimation of drugs. The conjugated CCMN and free RSV were measured simultaneously through our developed RP-HPLC method.

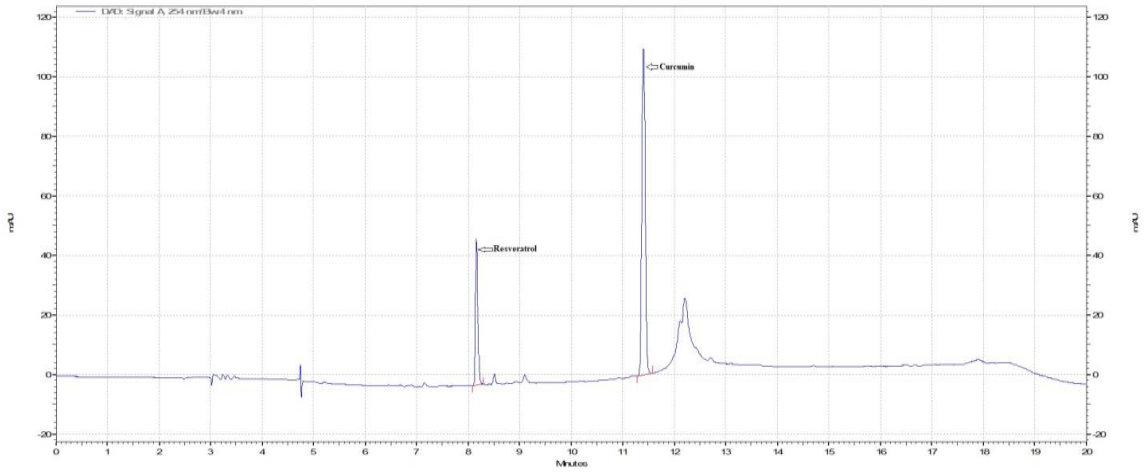

Fig. 9: Nano-micelle sample RP-HPLC chromatogram

Cumulative drug release

The cumulative releases of CCMN and RSV from nano-micelles were observed in phosphate buffer pH 5.0 and pH 7.4 through the dialysis method and consequently, the percent degradation of CCMN and RSV in the same environment at the same time interval was evaluated. In different time intervals, up to 9 d samples were taken from each vessel and subjected to RP-HPLC analysis. It was observed that more than 90% of drugs (CCMN and RSV) were released from the nano-micelle within 7 d at pH 5.0, whereas more than 50% of the drugs remained in the nano-micelles to be released within 7 d at pH 7.4. It was also observed that after 9 d 100% of drugs were released at pH 5.0, whereas more than 45% of the drugs remained in the nano-micelles to be released at pH 7.4. Fig. 10 and table 6. From HPLC analysis the results were found in terms of AUC and it was converted in terms of concentration through standard curve equations. During the cumulative drug release study we also consider the impact of percent degradation of drugs throughout the study. Here we observed and compared the selected drug release pattern simultaneously from nano-micelle, which couldn't be possible in UV-Vis spectroscopy.

Table 7: Cumulative percent release results of CCMN and RSV in pH 5.0 and pH 7.4

Time (h)	CCMN release at pH 5		RSV release at pH 5		CCMN release at pH 7.4		RSV release at pH 7.4	
	AUC	Cumulative % release	AUC	Cumulative % release	AUC	Cumulative % release	AUC	Cumulative % release
0	0	0	0	0	0	0	0	0
1	358414	3.769869498	101224	2.995439719	188414	1.878225477	51224	1.408232817
2	714708	7.772164751	154809	4.726403754	364708	3.858687091	74809	2.171000641
4	1094689	12.07767875	264901	8.268143893	604689	6.567430986	134901	4.100138568
6	1544679	17.20448682	374699	11.83549901	808679	8.902395035	194699	6.039019405
8	2144794	24.0518553	514684	16.39596886	1084794	12.06261549	264684	8.320260136
12	3077681	34.66886027	757771	24.27375985	1597681	17.88819743	377771	11.99195284
24	4498705	50.82132605	1458697	46.76242114	2398705	26.97704189	758697	24.20186223
36	5499347	62.45418959	2099436	67.56300091	2799347	31.6998411	1149436	36.84419852
48	6229891	71.19293147	2389891	77.44751409	3129891	35.68721829	1309881	42.30008701
72	7049983	81.00939236	2686874	87.63145545	3509983	40.2627125	1386874	45.15779557
168	8291286	95.60405613	2997987	98.35821618	4001286	46.1179809	1487987	48.80561351
216	8610021	100.0711363	3020131	100.0106652	4284421	49.71156665	1580131	52.20081573

All the values are considered as mean±SD, n=3

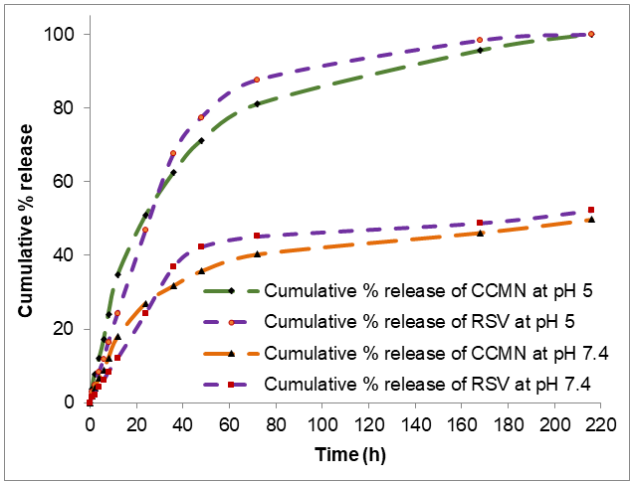

Fig. 10: Cumulative percent release pattern of CCMN and RSV in pH 5.0 and pH 7.4

CONCLUSION

Conjugated curcumin and free form of resveratrol that exist in nano-micelle were evaluated simultaneously by developing and validating the RP-HPLC method. As per the guideline of ICH Q2(R1), the developed RP-HPLC method was validated. It was found that our develop method proved its robustness, accuracy, linearity, system suitability and criteria of precision. From the chromatogram, it was found that the peaks of drugs arouse from nano-micelle extract and peaks of drugs arouse from known concentration drugs mixture were well resolved and completely separated. There were negligible interferences of other substances in the resolution of peaks. In our development method, there was no such compound which took a huge time to eliminate from the column, reduced column lifetime and disturbed the integrity of column.

ACKNOWLEDGMENT

The authors would like to thank Jadavpur University, Departments of Chemistry and Pharmaceutical Technology for technical assistance and expertise and also thank to Emami Limited for technical assistance and expertise.

FUNDING

Self-funding

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICTS OF INTERESTS

The authors have declared no conflict of interest.

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