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Development and Validation of a RP-HPLC Method for Simultaneous Quantitation of Resveratrol and Curcumin: Application to Nanolipid Gel Formulation

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Abstract
A new RP-HPLC method for quantitation of resveratrol and curcumin was developed and validated as per ICH Q2 (R1) guideline. Resveratrol and Curcumin shows synergistic effect. Both the drugs either stand alone and in combination have been utilized in the development of many novel formulations. However, no report is available for their identification and quantitation in combined dosage form. This new method was developed for simultaneous quantitation of resveratrol and Curcumin in a Nanolipid gel formulation.The chromatographic conditions were optimized using Hiber C-18 column, (4.6 mm × 250 mm, 5 µm) with acetonitrile: 0.01 M phosphate buffer pH adjusted to 4.0 ± 0.05 using 1 % v/v *o*-phosphoric acid (60:40, v/v) as mobile phase. The flow rate was 1 ml/min and detection was performed at 345 nm. The retention times of resveratrol and curcumin were found to be 3.13 min and 6.59 min, respectively. Linearity for both the drugs was established in the range of 10-60 µg/ml. The percentage recoveries of resveratrol and curcumin were found to be in the range of 99.5-101.75 and 99.01-101.05, respectively. The % RSD values for intraday and interday precision were found to be 0.10 and 0.8 for resveratrol and 0.30 and 0.58 for curcumin. LOD and LOQ for resveratrol and curcumin were found to be 0.11 & 0.29 µg/ml and 0.34 & 0.90 µg/ml, respectively. Mean % assay ± S.D. for resveratrol and curcumin in Nanolipid gel formulation using the proposed method were found to be 101.67 ± 0.02 and 98.66

Keywords: Resveratrol, Curcumin, Nanolipid gel, RP-HPLC, Validation

1. Introduction
Resveratrol is a phenolic component obtained from skin of grapes, blueberries, raspberries and mulberries. It is an antioxidant and is freely soluble in acetonitrile, methanol and chloroform (Figure 1). It is well known for its anti-inflammatory, analgesic, anti-aging and anticancer activities [Singh G. et.al, 2012]. It enhances the internal functions of the cell,

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particularly mitochondria, which is the energy source for the cell. Mitochondria convert food energy in to energy that the cell can use. Decreased mitochondrial function has been linked to insulin resistance, metabolic syndrome, and cardiovascular disease. Resveratrol has been found to be linked to decreased incidence of many chronic diseases such as neurodegenerative, skin, cancer and heart. [Resveratrol, 2003]

Curcumin is the active component obtained by solvent extraction of turmeric *i.e.*, the ground rhizomes of Curcuma longa L [Priyadarshini KI, 2014] and purification of the extract by crystallization. It is a polyphenolic compound, structurally 1, 7 bis (4-hydroxy-3-methoxyphenyl)-1-6-heptadiene-3, 5-dione (Figure 2). [Gavhad G et al, 2005, Curcumin 2004] It is practically insoluble in water but soluble in acetonitrile and chloroform. Curcumin has antioxidant, anti-inflammatory, antiseptic, anti-diabetic, anti-arthritic, antiatherosclerotic properties and is a natural colorant. The principal colouring components of curcumin exhibits keto-enol tautomerism and antioxidative properties. [Gugulothu DB et al 2012]

Many researchers have explored the potential of resveratrol [Chauhan AS, 2017, Caddeoc, 2013, Basavaraj, 2014, Pando D 2015, Ahirrao M et al, 2017, Shrotriya S N et al, 2017] and curcumin [Onoue S, 2010, Mukerjee A 2009, Zhang F 2011] as individual and in combination with each other for development of various formulations as witnessed from many research publications. Literature survey revealed UV-spectrophotometric [Vidhate B et al, 2015], RP-HPLC [Nikolic VD et al 2015, Singh G et al 2012], and mass spectrometric

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[Buiarelli F et al 2006,] methods for estimation of Resveratrol. Similarly, UV-Spectrophotometric [Sharma K, 2012, Kadam PV, 2013], LC-MS-MS [Liu A, 2006], HPTLC [Ansari M J, 2005] and RP-HPLC [Dandekar PP, 2009, Li J, 2009] methods have been reported for quantitation of curcumin. As resveratrol and curcumin shows synergistic effect [Gonzales AM, 2008, Majumdar AP 2009] it was considered worthwhile to develop a in-house gel formulation containing both the drugs and to quantitate both the drug by using liquid chromatographic technique. The proposed method is simple, accurate, and reproducible for simultaneous determination of resveratrol and curcumin in combined Nanolipid gel formulation. This paper describes development of a RP-HPLC method which is suitable for routine analysis. The method was validated in compliance with ICH Q2 (R1) guideline [30].

2. Experimental

2.1 Equipment and Materials

Resveratrol and Curcumin were procured from Yucca enterprises, Mumbai, India. Acetonitrile was purchased from Merck India Pvt Ltd and HPLC grade water was obtained from ELGA Lab water (UHQ- II), Bucks, England. The liquid chromatographic system was Shimadzu (LC-2010 HT, Japan) equipped with a UV-visible detector (SPA-20A), pump LC-20AD with a SIL-20 AC HT auto-sampler. The chromatographic analysis was performed using LC solution software on a Hiber C-18 column (4.6 × 250 mm, 5 µm particle size). In addition, an electronic balance (Shimadzu, ATY 224), a pH meter (Equip-tronics, EQ-610) and a sonicator (Spectra Lab, UCB 40) were used in this study.

ISSN: 2519-9889
Impact Factor: 3.426

2.2 Preparation of Mobile Phase and Stock Solutions:

Six hundred millilitres of acetonitrile and 400 ml of 0.01 M phosphate buffer pH adjusted to 4.0 ± 0.05 with 1 % v/v *o*-phosphoric acid were mixed. Mobile phase was pumped in ratio of (60:40, v/v).

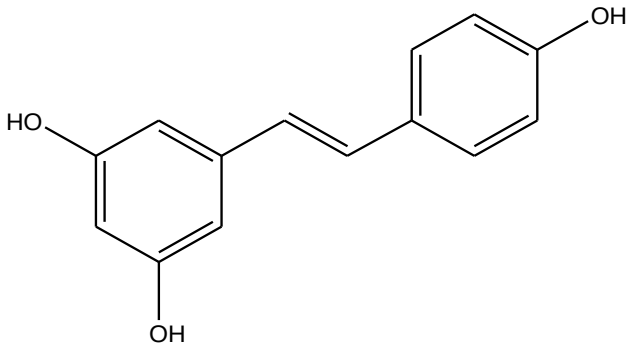

Figure 1: Structure of Resveratrol

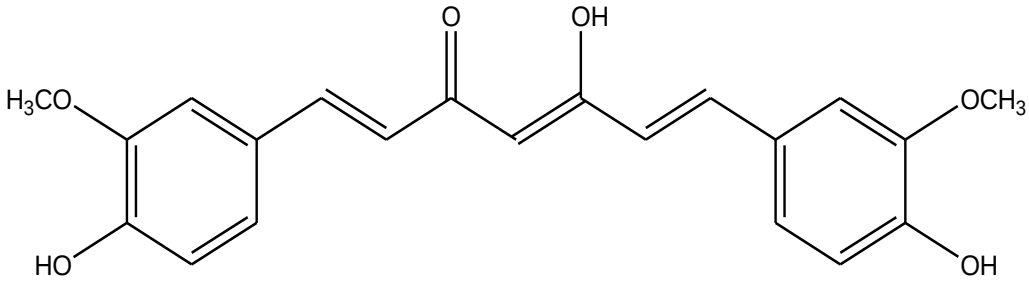

Figure 2: Structure of Curcumin

It was sonicated for 10 min and filtered through 0.22 µm nylon membrane filter (Pall, India) before use. Stock solutions were prepared by dissolving 10 mg each of resveratrol and curcumin in 10 ml of diluent to obtain solutions containing 1000 µg/ml of each drug. HPLC analysis was performed on reversed-phase high-performance liquid chromatographic system

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with isocratic elution mode using a mobile phase of acetonitrile: 0.01 M phosphate buffer of pH 4.0 ± 0.05 adjusted with 1 % v/v *o*-phosphoric acid (60:40, v/v), on Hiber C-18 column (4.6 × 250 mm, 5 µm particle size). The flow rate was 1 ml/min and detection λ was 345 nm.

2.3 Calibration curves for Resveratrol and Curcumin

Appropriate aliquots of resveratrol and curcumin stock solutions were transferred in different 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentrations of 10-60 µg/ml of each drug. Ten µl from these solutions were injected into column under optimized chromatographic conditions. The chromatograms were recorded and peak areas were noted. Calibration curves were constructed by plotting peak areas versus concentrations and regression equations were computed for both the drugs (Table 1).

TABLE 1: LINEAR REGRESSION DATA FOR CALIBRATION CURVES

Parameters (Units)	Resveratrol	Curcumin
Linearity range (µg/ml)	10-60	10-60
r^2 ± S.D.	0.997 ± 0.000153	0.9974 ± 0.000321
Mean Slope ± S.D.	22953.3 ± 17.89	14770 ± 50.56
Mean Intercept ± S.D.	2477.5 ± 783.17	3512.33 ± 1335.70

S. D is standard deviation where n= 3 observations.

2.4 Formulation of Nanolipid Gel of Resveratrol and Curcumin

Solid lipid nanoparticles (SLN) of resveratrol [12] and curcumin were prepared by using ultraprobe sonication method and were incorporated in gel base to form Nanolipid gel. Carbopol 940 was used as a gelling agent along with preservatives like methyl and propyl

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paraben. Triethanolamine was added in the dispersion of Carbopol 940 till it got gel under magnetic stirring.

2.5 Analysis of Nanolipid gel Formulation

10 gm of Nanolipid gel (containing 5 mg each of resveratrol and curcumin) was accurately weighed and transferred into a 100 ml volumetric flask containing 40 ml of acetonitrile and 10 ml of chloroform. It was heated on water bath for 10 mins until gel got dissolved and later diluted upto mark using acetonitrile to obtain final concentration of 50 µg/ml of each drug. The above solution was filtered using 0.45 µm nylon filter paper and first few ml of filtrate was discarded. Appropriate aliquot from stock was transferred to a 10 ml volumetric flask and the volume was made upto the mark with mobile phase to obtain a solution containing 30 µg/ml resveratrol and curcumin. A 10 µl volume of above sample solution was injected into HPLC in triplicate under optimized chromatographic conditions and peak areas were measured. Contents of both the drugs were found using regression equations.

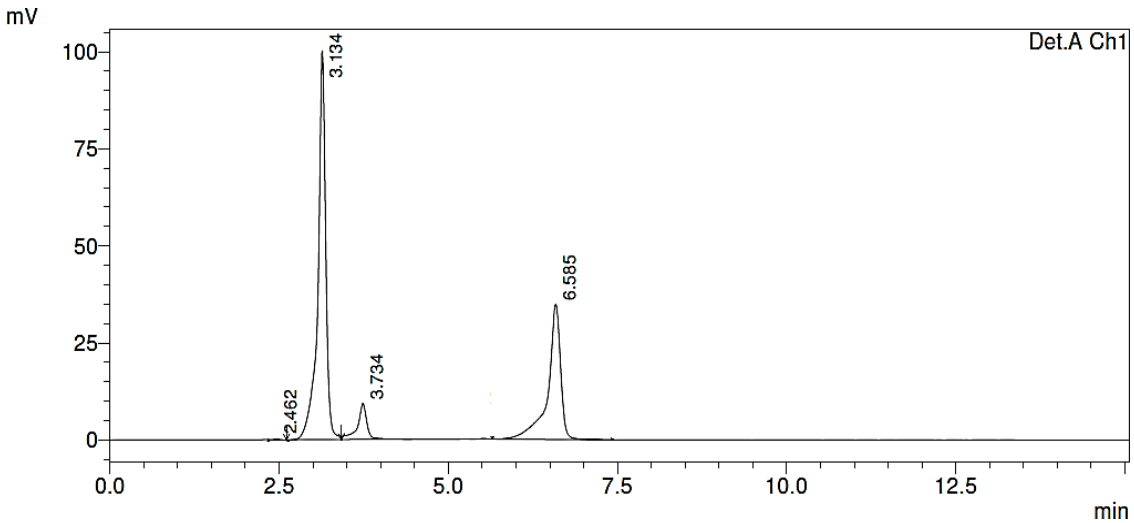

Figure 3: Chromatograph of mixture of Resveratrol and Curcumin, Resveratrol with retention time of 3.13 min and Curcumin with retention time of 6.59 min.

ISSN: 2519-9889
Impact Factor: 3.426

2.6 Validation

The method of analysis was validated as per ICHQ2 (R1) guideline [30] for the parameters like precision, accuracy, robustnessand detection limit, quantitation limit. The precision studies (Intraday and Interday) was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days for the concentration of 20, 40 and 60 µg/ml respectively. The precision is expressed as the % RSD of Peak areas and it should not be more than 2 %. System suitability tests are an integral part of any chromatographic analysis method which are used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting each drug solution at 50 µg/ml concentration. The results are shown in Table 2. The accuracy of the method was determined by calculating percentage recovery of resveratrol and curcumin from combined gel formulation. Recovery studies were carried out by applying the method to gel sample containing resveratrol and curcumin at 75, 100 and 125% levels.

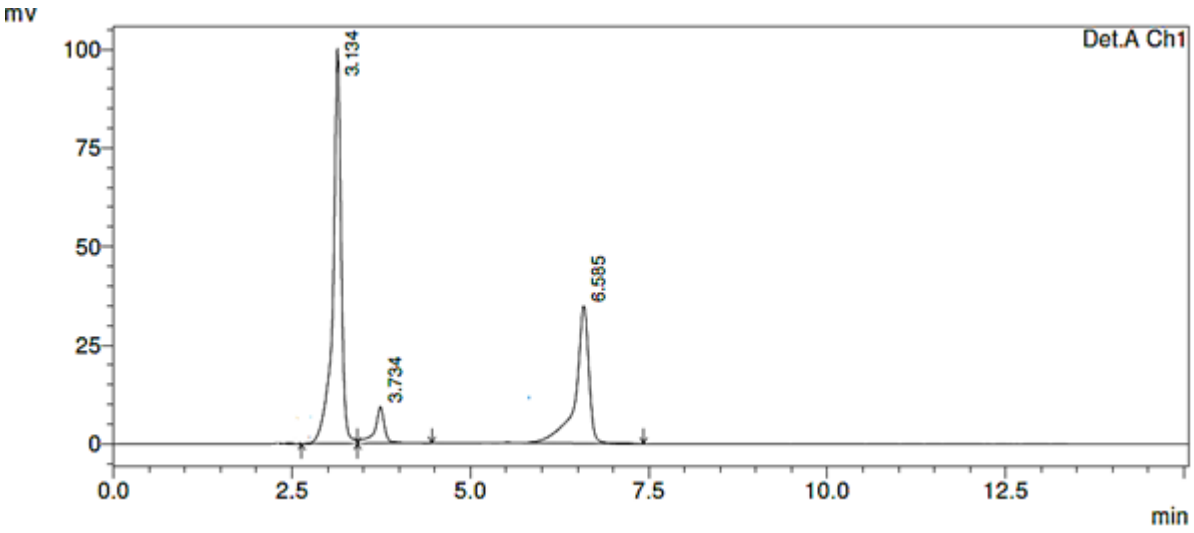

Figure 4: Chromatograph of Nanolipid gel formulation of Resveratrol and Curcumin, Resveratrol with retention time of 3.13 min and Curcumin with retention time of 6.58 min.

At each level three determinations were carried out and the results obtained were compared. Robustness of the method was studied by deliberately changing few parameters like percentage of acetonitrile and phosphate buffer in the mobile phase, flow rate and column oven temperature during analysis. One factor was changed at one time to estimate the effect.

ISSN: 2519-9889
Impact Factor: 3.426

Each factor selected was changed at three levels (-2, 0, +2) with respect to optimized parameters. Robustness of the method was done at the concentration level of 30 µg/ml for both the drugs and the results are shown in (Table 3). The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using following formulae: LOD= 3.3(SD)/S and LOQ= 10 (SD)/S, where SD=standard deviation of response (peak area) and S= average of the slope of calibration curve.

3. Results and Discussion

Various trials were performed for elution of resveratrol and curcumin by changing mobile phase, column and mobile phase ratio. In the present study, columns from two different manufacturers were tested. Using water: methanol (50:50, v/v) resolution between resveratrol and curcumin was poor. Peak for curcumin was splitted in mobile phase comprising of phosphate buffer and acetonitrile in ratio of 60:40, v/v. In phosphate buffer: acetonitrile (70:30, v/v) peak shape of curcumin was not proper and SST parameters were not within acceptable limits.

The mobile phase consisting of acetonitrile and 0.01 M phosphate buffer having pH 4.0 ± 0.05 adjusted with 1 % v/v *o*-phosphoric acid in 60:40 v/v ratio using 1ml/min flow rate gave two sharp, well-resolved peaks with minimum tailing factor for each drug (Figure 3). The retention times for resveratrol and curcumin were 3.13 min and 6.59 min, respectively. UV overlain spectra of both resveratrol and curcumin showed that both drugs absorbed appreciably at 345 nm, so this wavelength was selected as the detection wavelength.

ISSN: 2519-9889
Impact Factor: 3.426

The values of correlation coefficient for both the drugs demonstrated the good relationship between peak area and concentration. Therefore, the developed method was linear in concentration range of 10-60 µg/ml for both the drugs.The data of regression analysis of the calibration curves is shown in Table 1.The results for validation and system suitability test parameters are summarized in Table 2.

TABLE 2: SST PARAMETERS AND SUMMARY OF VALIDATION STUDIES

Parameter (Units)	Resveratrol	Curcumin
Retention Time (min)	3.1±0.2	6.5±0.2
Theoretical Plates	4821.55	10326.03
Tailing Factor (asymmetry factor)	0.803	0.694
Linearity range (µg/ml)	10-60	10-60
Correlation coefficient	0.997	0.9974
Precision (%RSD)		
Interday (n=3)	0.8	0.58
Intraday (n=3)	0.10	0.30
Recovery (%)	99.5- 101.75	99.01- 101.05
Robustness	Robust	Robust
LOD (µg/ml)	0.11	0.29
LOQ (µg/ml)	0.34	0.90
Mean Assay (%)±S.D.	101.67±0.02	98.66±0.88

SST stands for system suitability test

TABLE 3: ROBUSTNESS STUDIESFOR RESVERATROL AND CURCUMIN

Factor	Level[a]	RESVERATROL[b]	CURCUMIN[b]
A. Percentage of acetonitrile and phosphate buffer in mobile phase (v/v)			
58:42	-2	3.17	6.94
60:40	0	3.11	6.50
62:38	2	3.00	5.75
Mean ± S.D.		3.09 ± 0.08	6.39 ± 0.60

ISSN: 2519-9889
Impact Factor: 3.426

B. Flow rate (ml/min)			
0.8	-2	3.84	7.74
1.0	0	3.11	6.50
1.2	2	2.59	5.52
Mean ± S.D.		3.18 ± 0.62	6.58 ± 1.11
C. Column Temperature			
28˚ C	-2	3.10	6.36
30˚ C	0	3.11	6.50
32˚ C	2	3.08	6.28
Mean ± S.D.		3.09 ± 0.01	6.38 ± 0.11

Robustness studies carried out at concentration level of 30 µg/ml, [a] *Three factors were slightly changed at three different levels (-2, 2, 2) and* [b] *retention time.*

TABLE 4: ACCURACY DATA FOR RESVERATROL AND CURCUMIN

Drug	Amount added (µg/ml)	Amount Found (µg/ml)*	% Recovery*	Mean % Recovery± S.D.	% R.S.D.
Resveratrol	30	30.30	101.00	100.32 ± 0.38	0.37
		30.22	101.75		
		30.36	101.22		
	40	39.85	99.62	99.86 ± 0.22	0.22
		40.02	100.07		
		39.95	99.89		
	50	50.13	100.26	100.01 ± 0.44	0.43
		49.75	99.5		
		50.14	100.28		
Curcumin	30	30.15	100.52	100.83 ± 0.27	0.26
		30.27	100.92		
		30.31	101.05		
	40	39.60	99.01	99.13 ± 0.16	0.16
		39.62	99.07		
		39.72	99.32		
	50	50.39	100.78	100.59 ± 0.19	0.18
		50.19	100.39		
		50.30	100.61		

The %RSD values for intraday and interday precision values were found to be 0.10 and 0.8 for resveratrol and 0.30 and 0.58 for curcumin. The developed method was found to be specific, for quantitation of resveratrol and curcumin as no other peak was observed at the same retention time values of 2 components (Figure 4).

ISSN: 2519-9889
Impact Factor: 3.426

The recovery studies were carried out at 30, 40 and 50 µg/mlconcentrations corresponding to 75, 100 and 125 % level. The mean % recovery ± S.D. values corresponding to 3 levels were found to be 100.32 ± 0.38, 99.86 ± 0.22 and 100.01 ± 0.44for resveratrol and 100.83 ± 0.27, 99.13 ± 0.16 and 99.13 ± 0.16 for Curcumin. The results are shown in Table 4. The proposed method was successfully applied for quantitation of resveratrol and curcumin in their combined Nanolipidgel formulation. The results for the assay were comparable with the corresponding amount added in Nanolipid gel formulation. Results for robustness evaluation for both the drugs are presented in Table 3. Insignificant differences in peak areas and less variability in retention times were observed. The % The LOD for resveratrol and curcumin were found to be 0.11 and 0.29 µg/ml, respectively, while LOQ were 0.34 and 0.90 µg/ml respectively. Statistical analysis proved that the developed method was accurate, precise, and repeatable. Assay results for combined dosage form using proposed method showed 101.67±0.02 of Resveratrol and 98.66±0.88% of Curcumin. Further this method can be extended to study the stress degradation behaviour of both the drugs in combination.

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