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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF RESVERATROL AND CURCUMIN IN HERBAL FORMULATION

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ABSTRACT
A simple, precise, cost effective RP-HPLC method has been developed and validated for the determination of Resveratrol and Curcumin in Herbal formulation. The chromatographic separation was achieved using a Prontosil C18 column (250 x 4.6mm, 5µm) and mobile phase consisting of acetonitrile: 0.02M potassium dihydrogen phosphate buffer (60:40), pH adjusted to 3.0 with Orthophosphoric acid at flow rate 1ml/min was used. Detection wavelength was set at 320 nm for both the drugs. The retention time of Resveratrol and Curcumin were found to be 2.703 and 6.553 minute. The resolution of Resveratrol and Curcumin peak was found to be more than 2. The calibration curves showed good linear correlation coefficients (r^2 > 0.9997) within the tested ranges. The recoveries were found to be between 99.2-101 % for Resveratrol and 98.9-100.8 % for Curcumin. The %RSD for precision and accuracy of the method was found to be less than 2%. The developed method was successfully used for the quantitative estimation of these two markers in a marketed herbal formulation.

KEYWORDS: Resveratrol, Curcumin, simultaneous determination, RP-HPLC, validation, ICH guidelines.

INTRODUCTION
Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities, higher safety margins and lesser costs.[1-2]

Resveratrol (3,4',5-trihydroxystilbene) belong to class of polyphenolic compounds called stillbenes found in many plant species including grapes, peanut, cranberries, Japanese giant knotweed and other herbal drugs.[3-6] Resveratrol acts as antioxidant and protect from damage caused by bacteria, fungi and ultraviolet radiation. Resveratrol exerts anti-aging effects and numerous *in-vitro* animal studies have shown resveratrol has potent antioxidant and anti-inflammatory effect.[3-4] It also promotes vascular endothelial function, enhances lipid metabolism and has anti-cancer activity.[3-4] Resveratrol delays the development of cardiovascular and neurodegenerative diseases, improve glycemic control in type 2 diabetes, and extend lifespan by activating SIRT1 gene which protects the body against the effects of obesity and the diseases of aging.[5-7]

Turmeric (*Curcuma longa*), has long been used in both Ayurvedic and Chinese medicine as an anti-inflammatory, to treat digestive disorders and liver problems, and for the treatment of skin diseases and wound healing.[8-9] The active ingredient in turmeric is curcumin, which has property to stimulate the production of bile and to facilitate the emptying of the gallbladder. It has also demonstrated in animals a protective effect on the liver, anti-tumor action, and ability to reduce inflammation and fight certain infections.[8-9] Curcumin chemically diarylheptanoid used as a traditional medicine for the purpose of healing and in cosmetic preparations. It purifies the blood and is antiseptic by nature. It is comprises of antioxidants and therefore its regular usage protects from the damage caused by free radicals.[8-9] Curcumin gives strength to the immune system and prevents from, cancers, tumors, etc. Curcumin is also known as one of the best anti-ageing supplement.[8-9] Curcumin phytopolylphenol pigment blocks the formation of reactive-oxygen species, possesses anti-inflammatory properties as a result of inhibition of cyclooxygenases (COX) and other enzymes involved in inflammation; and disrupts cell signal transduction by various mechanisms including inhibition of protein kinase C.[10] These effects may play a role in the agent's observed antineoplastic properties, which include inhibition of tumor cell proliferation and suppression of chemically induced carcinogenesis and tumor growth in animal models of cancer.[10]

Although Resveratrol and Curcumin are been used together in many plant products for treatment of various ailments,[11-12] there is no known method for simultaneous quantification of these potent marker constituents. In view of the interesting biological

activities associated with these constituents, it was of interest to develop a suitable, simple, rapid and sensitive novel analytical procedure for simultaneous quantitation of Resveratrol and Curcumin. Such a study would not only facilitate standardization of herbal formulations containing Resveratrol and Curcumin but can also be used for scientific as well as commercial applications. The structure of Resveratrol and Curcumin are shown below.

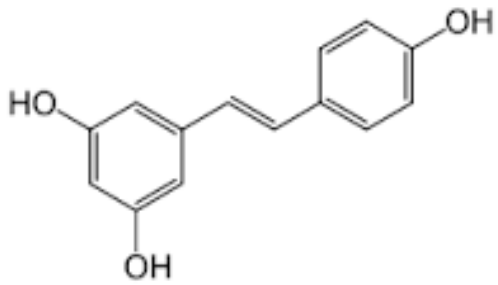

Fig 1: Chemical structure of Resveratrol

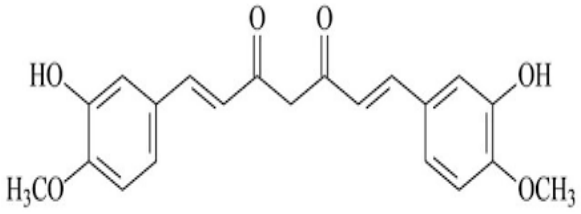

Fig 2: Chemical structure of Curcumin

EXPERIMENTAL

Materials and reagents

Resveratrol and Curcumin were purchased from Yucca Enterprises, Mumbai India with purity of 99% for both by HPLC. A commercial preparation (Kal-Turmeric Resveratrol tablets) used for analysis was procured from local market. HPLC grade acetonitrile (Thomas Baker) and methanol (Thomas Baker) were used. Water was double distilled. Potassium dihydrogen phosphate (Lobachem), Orthophosphoric Acid (Thomas Baker) and Sodium dihydrogen phosphate (Thomas Baker) were used.

Instrumentation

RP-HPLC was performed using Shimadzu HPLC system consisting of a pump LC-20AD, rheodyne sample injection port with 20 microlitre loop, SPD-20A UV-Detector, Spinchrom software, column used was Prontosil C18 (250 x 4.6mm, 5µm), Weighing was done on Contech CA-123 balance and pH was adjusted using PCI analytics Digital pH meter 111. Solvents were filtered through a 0.45 µm filter (Millipore Bedford, MA, USA) and degassed in an ultrasonic bath (Remi Instruments, Mumbai, India).

Principle

Reversed phase liquid chromatography isocratic elution with SPD-20A UV detection.

Optimized Chromatographic Conditions

Column : Prontosil C_{18} (250 x 4.6 mm, 5 µm)
Mobile Phase : Acetonitrile: 0.02 M Potassium dihydrogen phosphate buffer (60:40), adjusted to pH 3.0 with Orthophosphoric acid
Flow Rate : 1.0 mL/min
Wavelength : 320 nm
Injection Volume : 20 µL
Runtime : 11 minutes
Elution : Isocratic

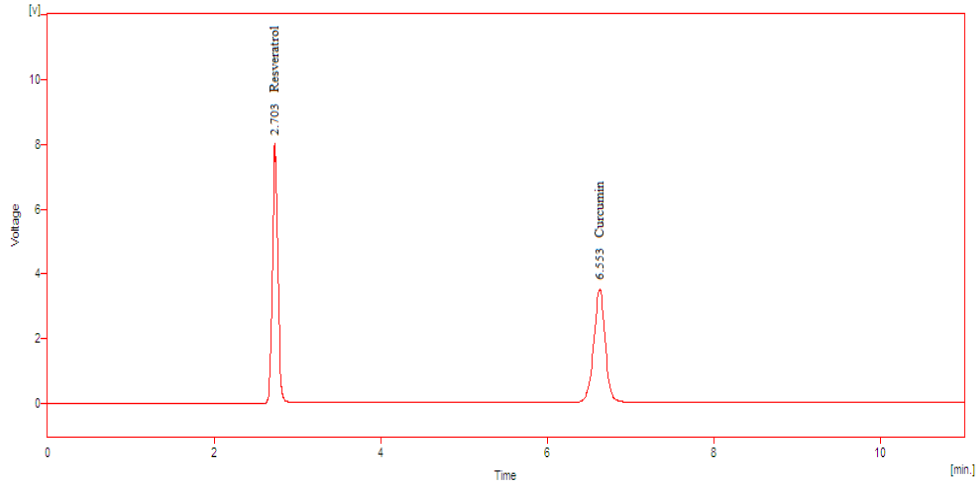

Fig. 3: Chromatogram of Standard solution

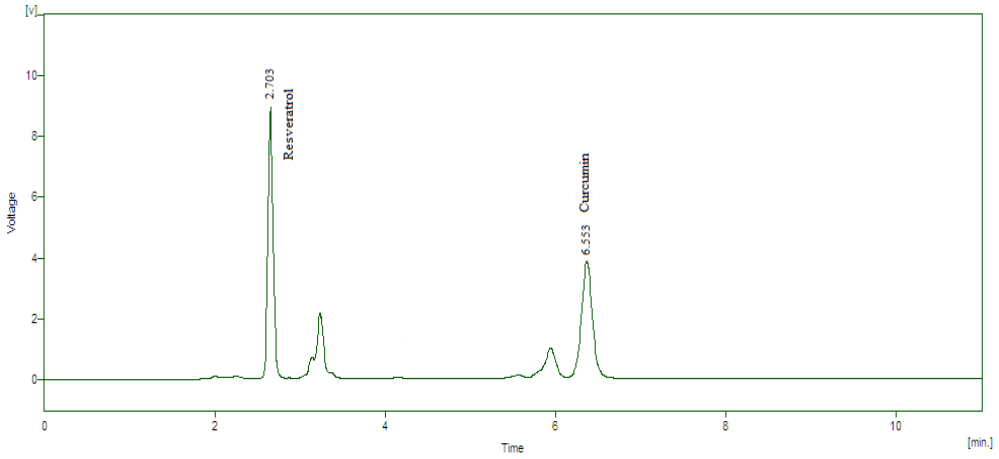

Fig. 4: Chromatogram of Test solution

Preparation of 0.02 M Potassium dihydrogen orthophosphate (pH 3.0)

About 2.7218 g of Potassium dihydrogen orthophosphate was accurately weighed and dissolved in 1000ml of water and adjusted the pH with o-phosphoric acid to 3.0±0.05. The solution was then filtered using 0.45µ membrane filter.

Preparation of Mobile Phase

The pH of (0.02 M) Potassium dihydrogen orthophosphate was adjusted to 3.0 with Orthophosphoric acid, and mixed with Acetonitrile in the ratio 40:60 and was sonicated.

Preparation of standard solution

100mg of Curcumin and 100mg of Resveratrol were accurately weighed and transferred into 100ml volumetric flask respectively. About 70ml of mobile phase was added, sonicated and diluted to 100ml using mobile phase. Accurately 1ml of the above solutions was transferred into 10ml volumetric flask and diluted up to the volume with mobile phase. Then, 9.9ml of Curcumin and 1 ml of Resveratrol solution were diluted to 10ml respectively with the mobile phase. Final concentration of Curcumin and Resveratrol were made to 99µg/ml and and 10µg/ml respectively by suitable dilutions.

Preparation of sample solution

20 tablets were weighed and powdered. The quantity of powder equivalent to 495 mg of Curcumin and 50 mg of Resveratrol were transferred into a 1000 ml volumetric flask. About 700 ml mobile was added and solution was sonicated for 30 mins with intermittent shaking. The volume was made up using the mobile phase, mixed and filtered through 0.45µ PVDF filter. Accurately 2 ml of the above solution was transferred into 10 ml volumetric flask and diluted up to the volume with mobile phase. Final concentration of Curcumin and Resveratrol were made to 99 µg/ml and and 10 µg/ml respectively by suitable dilutions.

RESULT AND DISCUSSION

The proposed RP-HPLC method was validated as per ICH guidelines

Analysis of tablet formulation (% Assay)

$$\% \text{ Assay of Drug} = \frac{A_T \times \text{Dilution of standard preparation} \times P \times \text{Avg wt (mg)} \times 100}{A_s \times \text{Dilution of test preparation} \times 100 \times L.C}$$

Where,

A_T= Area of peak obtained from the chromatogram of sample preparation

A_s = Area of peak obtained from the chromatogram of standard preparation

P = % Potency of drug.

LC= Label claim of drug in mg/tablet. **Results are shown in Table 1**

Selectivity and Specificity

To assess the selectivity of the developed method, solutions of both the drugs were injected into the system. Two sharp peaks of Resveratrol and Curcumin were obtained at retention time of 2.703 min and 6.553 min respectively in reference to standard solution. Specificity was determined by comparison of the chromatogram of mixed standards and sample solutions. As the retention time of standard drugs and the retention time of the drugs in sample solutions were same, so the method was specific. The parameters like resolution (Rs) and asymmetric factor were calculated. Good correlation was found between the results of mixed standards and sample solutions. Results are shown in the **Table 2.**

Linearity

The linearity of an analytical method is its ability to obtain results, which are directly proportional to the concentration of analyte in the sample. It was carried out by preparing the sample solutions containing 6-16 µg/ml

for Resveratrol and 77-132 µg/ml for Curcumin respectively. A calibration curve was drawn by plotting concentration on X-axis Vs. area on Y-axis and regression equation, correlation coefficient, y-intercept, slope of the equation was calculated. Result are shown in the **Table 3 and Figure 6, 7.**

Accuracy

The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 75%, 100% and 125%. The recovery studies were carried out by adding known amounts of standard Resveratrol and Curcumin were added to pre-analyzed samples and they were subjected to proposed HPLC method. The recoveries results of Resveratrol and Curcumin in Herbal formulation are shown in the **Table 4.**

Precision

Precision study was performed to find out intraday and interday variations. The intraday and interday precision study of Resveratrol and Curcumin was carried out by estimating the corresponding response 3 times on the same day and on 3 different days for 3 different concentrations of Resveratrol and Curcumin and the results are reported in terms of % relative standard deviation (%RSD) however, all results fall within acceptance limits (RSD < 2), as shown in **Table 5.**

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD is ability of analytical method able to detect the lowest concentration of the analyte. LOQ is lowest concentration of the analyte which can be quantitatively analyzed with acceptable precision and accuracy. It was calculated based on the slope and blank response from the calibration curve as per ICH guidelines. LOD and LOQ were calculated based on the standard deviation of the response and slope. Result are shown in **Table 5.**

Table 1: Assay Determination of Resveratrol and Curcumin.

Brand name of herbal dosage form		% Amount Found
Kal-Termeric and Resveratrol Tablet (495mg curcumin+50mg resveratrol)	Resveratrol	98.87%
	Curcumin	98.78%

Table 2: System suitability parameters of Resveratrol and Curcumin

System Suitability Parameters	Resveratrol	Curcumin
Retention time (min)	2.703	6.553
Theoretical plates	8426	11321
Asymmetric factor	1.0	1.1

Table 3: Data indicating linearity of the proposed method for Resveratrol and Curcumin

PARAMETERS	Resveratrol	Curcumin
Linearity range	6-16µg/ml	77-132µg/ml
Slope	72.169	6.951
Intercept	2.8341	4.2801
Correlation coefficient	0.9998	0.9998

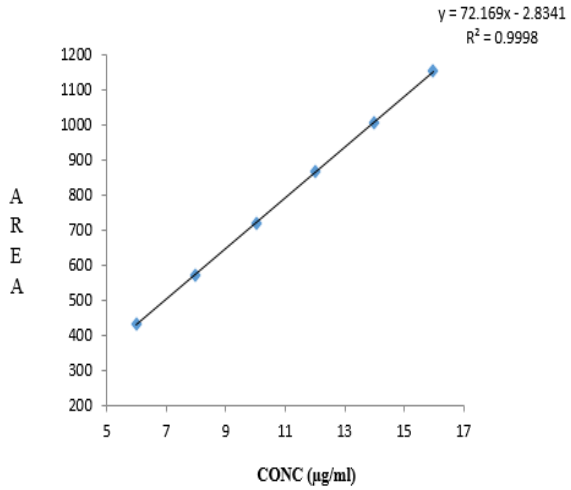

Fig. 5: Calibration curve of Resveratrol

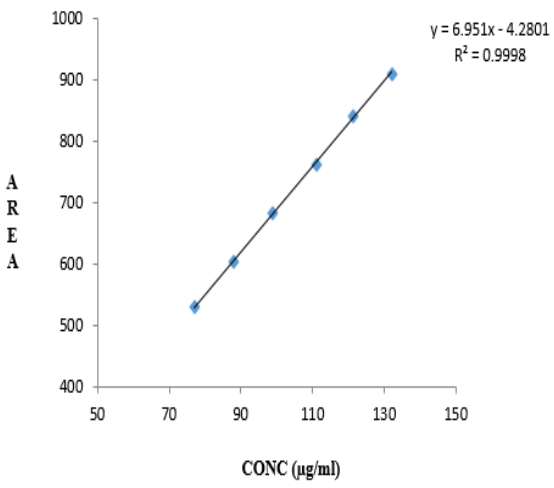

Fig. 6: Calibration curve of Curcumin

Table 4: Results for Accuracy studies of Resveratrol and Curcumin

Pre-analyzed sample solution[µg/ml]	Sample concentration [µg/ml]	Excess drug added [µg/ml]	Amount recovered [µg/ml]	% Recovery
Resveratrol	6.25	3.125	9.375	101.00
	6.25	6.25	12.50	99.20
	6.25	9.375	15.625	100.48
Curcumin	62	31	93	98.92
	62	62	124	100.80
	62	93	155	100.35

Table: 5 Results of precision, LOD and LOQ for Resveratrol and Curcumin.

PARAMETERS	Resveratrol	Curcumin
	Precision (%RSD)	
Intra-day (n=3)	0.203	0.773
Inter-day (n=3)	0.230	0.802
LOD	0.55 µg/ml	2.98 µg/ml
LOQ	0.18 µg/ml	0.95 µg/ml

CONCLUSION

The developed method is the first report for simultaneous estimation of estimation of Resveratrol and Curcumin in Herbal formulation. The developed HPLC method was found to be more accurate, precise and reproducible. The analysis of tablets containing two drugs gave the satisfactory results. The statistical parameter of this method showed good results. The recovery studies revealed excellent accuracy and high precision of the method. The statistical parameters and recovery studies of HPLC method were compared with the developed spectrophotometric method of analysis of the same dosage form. The HPLC method was found to give better results. Therefore the proposed method could be applied for routine analysis in quality control laboratories.

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