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DESIGN EXPERT-SUPPORTED DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR DETERMINATION OF RESVERATROL IN BULK DRUG AND PHARMACEUTICAL FORMULATION

Rudra Pangeni, Javed Ali, Gulam Mustafa, Shrestha Sharma and Sanjula Baboota *

Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, New Delhi-110062, India.

Keywords:

Resveratrol, Box-Behnken design, Optimization, HPLC, Stability indicating method.

Correspondence to Author:
Dr. Sanjula Baboota

Assistant Professor
Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, New Delhi-110062, India.

E-mail: sbaboota@rediffmail.com

ABSTRACT: The aim of the present study was to develop and validate a new, simple, selective and economical stability indicating RP-HPLC method for the quantitative determination of resveratrol in bulk drug and pharmaceutical formulations. A Box-Behnken design supported optimization was carried out to identify the optimum chromatographic conditions. The developed method was validated for linearity, range, accuracy, precision, reproducibility, LOD, LOQ and robustness as per ICH guidelines. Forced degradation studies were carried out in different stress conditions to detect degradation peak using validated method. Optimum chromatographic separation was achieved by mobile phase consisting of methanol, water and acetic acid in 69:30:1 ratio respectively. The flow rate of 1 ml min^{-1} with standard RT of 2.8 min was optimized in the present study. The method was linear in the concentration range of 7.5-60 µg mL^{-1} with a regression coefficient (R^2) of 0.999. The LOD and LOQ was found to be 1.463 and 4.737 µg mL^{-1} respectively. Degradation study showed major decomposition of resveratrol in photolytic stress condition. The stability indicating method was found to be simple, selective and accurate for the quantitative determination of resveratrol and its impurities in drug substance and product.

INTRODUCTION: Resveratrol (3, 4', 5-*trans*-trihydroxy-stilbene or 5-[(E)-2-(4-hydroxy phenyl)-ethyl benzene-1,3-diol;$C_{14}H_{12}O_3$; MW 228.25), is a naturally occurring potent antioxidant and a member of stilbene family of phenolic compounds first isolated from roots of white hellebore (*Veratrum grandiflorum O. Loes*) (**Fig. 1**). Commercially, it is obtained from the roots of the Japanese Knotweed.

It is also reported to be present in different food and food products which include grapes, wine, mulberries, cranberries and peanuts [1]. Resveratrol is extensively used as anti-aging, antioxidant, anti-inflammatory and cardioprotective agent. It improves body metabolism and is also used in chemotherapy. Studies have supported the neuroprotective effects of resveratrol against oxidative stress and cellular death [2].

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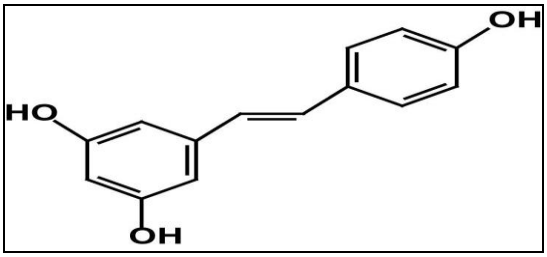


FIG. 1: CHEMICAL STRUCTURE OF RESVERATROL

Parkinson's disease (PD) is the second most common, progressive and age related neurodegenerative disorder of the nervous system mostly affecting the middle aged or elderly people. The basic pathology is the selective and progressive loss of nigral dopaminergic neurons responsible for dopamine production in the substantia nigra pars compacta (SNc) in midbrain and several other factors including free radicals generation, oxidative stress, mitochondrial dysfunction, aging, and over load of calcium [3].

Amongst different causes of dopaminergic neurons loss, oxidative stress is the leading cause of Parkinson's disease because of its ability to metabolize dopamine into hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) [4]. Resveratrol has been widely tested for its neuroprotective activity against Parkinson's disease where it acts by inhibiting Reactive Oxygen Species (ROS) by activating AMP activated kinase (AMPK) [5]. The neuroprotective effects have been confirmed in several animal model studies [6-8]. Resveratrol treatment reduces accumulation of beta-amyloid, a main culprit in Alzheimer's disease [9].

Several analytical techniques including reverse phase high performance liquid chromatography (RP-HPLC) with UV [10-11], electrochemical [12], fluorescence [13] and chemiluminescence detectors [14] have been developed for quantification of resveratrol in wines and grapes. LC-MS method has been developed and used to analyze total resveratrol content in fruit products and wines [15, 1]. HPLC has also been used to determine the concentration of resveratrol in plasma and urine [16-17]. Solid state stability of resveratrol in different stressed conditions was studied [18, 19].

But so far, to our present knowledge, no stability indicating high performance liquid chromatography (HPLC) method has been developed for the determination of resveratrol in the presence of its degradation products using the International Conference on Harmonization (ICH) guidelines for stress-testing. Stability indicating methods are used to discriminate between major active pharmaceutical ingredient and its degradation product(s), process impurities, excipients, or other potential impurities [20]. According to ICH guideline Q1A (R2), the stability testing of drug substances should be carried out under different stress conditions such as hydrolysis, oxidation, photolysis, and thermal degradation to validate the stability indicating supremacy of analytical methods used for the analysis of stability samples.

Thus, the aim of the present study was to develop and validate a simple, rapid, precise, economical and accurate isocratic reversed-phase stability indicating HPLC method for the quantitation of resveratrol in bulk drug and pharmaceutical formulation as per ICH guidelines.

MATERIALS AND METHODS:

Chemicals and Reagents:

Resveratrol was obtained as a gift sample from Lactonova, India. HPLC-grade methanol and acetonitrile were purchased from Merck, Mumbai, India. HPLC-grade water was purchased from Millipore, India. All the other chemicals and reagents used were of analytical grade and were purchased from Merck, Mumbai, India.

HPLC Instrumentation and Chromatographic Conditions:

HPLC analysis was performed on a Shimadzu HPLC system comprising of a binary LC-10A VP pump and a UV-visible detector SPD-10AVP. All the chromatographic data collection, acquisition and system control was carried out using Shimadzu class-VP 5.032 HPLC software (Shimadzu, Tokyo, Japan). The chromatographic separation was carried out using a LiChrospher®C18 reversed-phase column (150 mm × 4.6 mm i.d., 5 µm particle size; Merck, Mumbai, India). The mobile phase consisted of a mixture of water, methanol and acetic acid in the concentration of 69:30:1, v/v/v which was filtered through a 0.20 µm pore size nylon membrane, degassed ultrasonically and pumped in isocratic mode at a flow rate of 1.0 mL min^{-1}. Samples (20 µL) were injected by means of a Rheodyne injector and the elute was monitored at 306 nm wavelength.

Optimization of Analytical Method:

Optimization means to improve the performance of any system, process or any product with the aim to obtain best result and benefit from it. Design expert

has been used widely for the optimization of various process parameters. In the present study, Box-Behnken design as response surface methodology was used to optimize flow rate of the mobile phase, injection volume of the analyte and concentration of acetic acid and the effect of these parameters on retention time (RT) and chromatographic area was evaluated *(***Table 1**).

The design was selected as it requires fewer runs compared to Central Composite Design (CCD) which is used in case of three or four variables. A three-factorial three-leveled Box-Behnken design with 17 experimental runs was selected for exploring quadratic response surface and constructing second-order polynomial models with Design Expert® (Version 8.0.0.1, Trial Version).

TABLE 1: VARIABLES WITH THEIR LEVELS SELECTED IN BOX-BEHNKEN DESIGN

Factors	Levels	
	Low (-1)	High (+1)
Flow rate of mobile phase (mL/min)	0.75	1.25
Injection volume	15	25
Concentration of acetic acid (%)	0.50	1.50

Preparation of Sample for Calibration Curve:
A stock solution of resveratrol (1000 µg mL^{-1}) was prepared by dissolving 10 mg of drug in 10 mL of methanol:water (1:1, v/v) mixture. The stock solution was further diluted using methanol:water (1:1 v/v) to obtain working standards in the concentration range of 7.5 – 60 µg mL^{-1}. These dilutions were filtered through 0.20 µm membrane filter before HPLC analysis.

Method Validation:
The optimized analytical method was validated according to the ICH Q2 (R1) guidelines for linearity, range, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness [21].

Linearity and Range:
Standard calibration curve of resveratrol were prepared using ten different concentrations ranging from 7.5 – 60 µg mL^{-1}, obtained after serial dilution of stock solution (1000 µg mL^{-1}). Three replicate injections of each concentration in series were made each day, over three days to determine the linearity of resveratrol over the concentration range. Linear calibration curves of peak area versus drug concentration were plotted using linear least squares regression and evaluated for inter-day linearity and range.

Accuracy as Recovery:
Accuracy was determined by adding 50, 100 and 150% extra resveratrol to standard resveratrol solution (50µg mL^{-1}). The mixtures were analyzed by the proposed method and the experiments were performed in triplicate. The mean, standard deviation and relative standard deviation (RSD) of the peak areas and their corresponding concentrations were calculated for each concentration.

Precision:
Precision (inter- and intra-day) of the method was evaluated by performing replicate analyses (n = 3) in accordance with ICH guidelines. Precision was determined as both repeatability and intermediate precision. Repeatability of sample injection was determined as intra-day variation and intermediate precision was determined by measurement of inter-day variation. For both inter- and intra-day variation, analysis of resveratrol solutions at three different concentrations (20, 40 and 60 µg mL^{-1}) were determined in triplicates.

Reproducibility:
Reproducibility of the analytical method was checked by the different analyst by measuring the precision of the method in another laboratory using a different instrument. Both the intra-day and inter-day precision was determined using three replicates at three different concentrations (20, 40 and 60 µg mL^{-1}).

Limit of Detection and Quantitation:
Detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated under the stated experimental conditions and Quantitation limit is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The limit of detection (LOD) and limit of quantitation (LOQ) was determined using the standard deviation method. The standard deviation

was calculated by blank injection method to determine LOD and LOQ using following formula:

$$LOD = 3.3*\sigma/S \text{ and } LOQ = 10*\sigma/S$$

Where σ is the standard deviation of the blank injection response and S is the slope of the calibration curve.

Robustness:
Robustness is the measure of method's capability to remain unaffected by small, but deliberate variations in the method parameters [21]. The robustness of the method was evaluated by making small changes in the chromatographic conditions such as percentage of water in mobile phase (±5%), flow rate (±3%) and methanol from different suppliers (Merck and SD Fine Chemicals). The change in the retention time was calculated in triplicates.

Stability:
Stability of resveratrol in solution during analysis was determined by repeated analysis of the samples during the experimentation period of the same day along with the stability of the samples for 48 hours under the laboratory conditions (32 ± 1ºC) and under refrigeration (8 ± 0.5ºC).

Forced Degradation Studies:
Forced degradation studies were carried out by subjecting resveratrol solutions to different conditions such as acidic stress (0.1 M HCL), alkaline stress (0.1 M NaOH), hydrolysis, oxidative stress (3% solution of Hydrogen peroxide), thermal (Dry heat at 100°C), photodegradation stress and neutral degradation. All the degradation studies were followed by the percentage recovery of the drug. Ten milligram of resveratrol was dissolved with 1 mL of methanol in a 10 mL volumetric flask and volume was made up with 0.1 M HCL, 0.1 M NaOH and 3% Hydrogen peroxide solution separately to get the concentration of 1000 µg mL^{-1}. These solutions were refluxed for 8 hours on a water bath at 80°C. The resultant solutions in acidic and basic conditions were neutralized using 0.1 M NaOH and 0.1 M HCL respectively and samples of hydrogen peroxide were further diluted to 50 µg mL^{-1} to develop HPLC chromatograms.

For thermal degradation studies resveratrol (10 mg) powder was heated in an oven at 100°C for 24 hours and the sample was dissolved using 1mL of methanol which was further diluted using HPLC grade water:methanol (1:1) mixture to 50 µg mL^{-1}. The resultant solution was injected to determine the HPLC chromatograms. Similarly for neutral degradation studies resveratrol solution (1000 µg mL^{-1}) was refluxed for 8 hours at 80°C in a water bath and further diluted sample (50 µg mL^{-1}) was studied for HPLC chromatograms.

The photo-degradation study of resveratrol was performed in the presence of UV light (254 nm) for 24 hours and further exposure to daylight for one day. Ten milligrams of light exposed drug was dissolved in a volumetric flask using 1 mL of methanol. The volume was made up using methanol:water (1:1) as a diluent. The resultant solution was used for HPLC chromatograms.

Analysis of Resveratrol in Nanoemulsion and Marketed Dosage Form:
Resveratrol nanoemulsion was prepared by spontaneous emulsification followed by high pressure homogenization technique and consisted of Vitamin E: Sefsol 218(1:1) as an oil phase, Tween 80 as surfactant and Transcutol P as co-surfactant. The obtained nanoemulsion had droplet size in the range of 50 to 120 nm. Nanoemulsion equivalent to 10 milligrams of resveratrol i.e. 2 mL of nanoemulsion was taken in a 50 mL volumetric flask and sonicated in an ultrasonic bath for 5 minutes. The solution was further diluted with the mobile phase. The resultant solution was filtered using 0.2 µm membrane filter and HPLC analysis was carried out.

Similarly resveratrol capsule (marketed dosage form) equivalent to 10 mg resveratrol was accurately weighed and taken in 50 mL volumetric flask. The sample was diluted with mobile phase and sonicated in an ultrasonic bath for 5 minutes. The resultant solution was filtered using 0.2 µm membrane filter and HPLC analysis was carried out.

Statistical Analysis: All the experiments were carried out in triplicate using freshly prepared samples. The results were then expressed as mean.

Standard deviation (SD) and relative standard deviation (RSD) of the peak areas and their corresponding concentrations were then calculated.

RESULTS AND DISCUSSIONS:

Selection of Mobile Phase:

The proper selection of mobile phase was based on the sensitivity of the assay, suitability for stability studies, time period for the analysis, ease of preparation and use of readily available and cost-effective solvents. Different mobile phase using different combination of solvents such as methanol-water, acetonitrile- water, methanol-phosphate buffer (pH 3.5-6.5), acetonitrile-phosphate buffer (pH 3.5-6.5) in different ratios were selected for the analysis of resveratrol. Mobile phase consisting of acetonitrile-water, methanol-phosphate buffer (pH 3.5-6.5) and acetonitrile-phosphate buffer (pH 3.5-6.5) did not give well defined sharp peaks and the retention time was also very high (**Fig. 2A- C**). With methanol-water (70:30 %, *v/v*) sharp peaks were obtained at a retention time of 2.55 minutes but still tailing was observed. To remove tailing acetic acid in different concentration was added and its concentration was optimized using Box-Behnken method (**Fig. 3**).

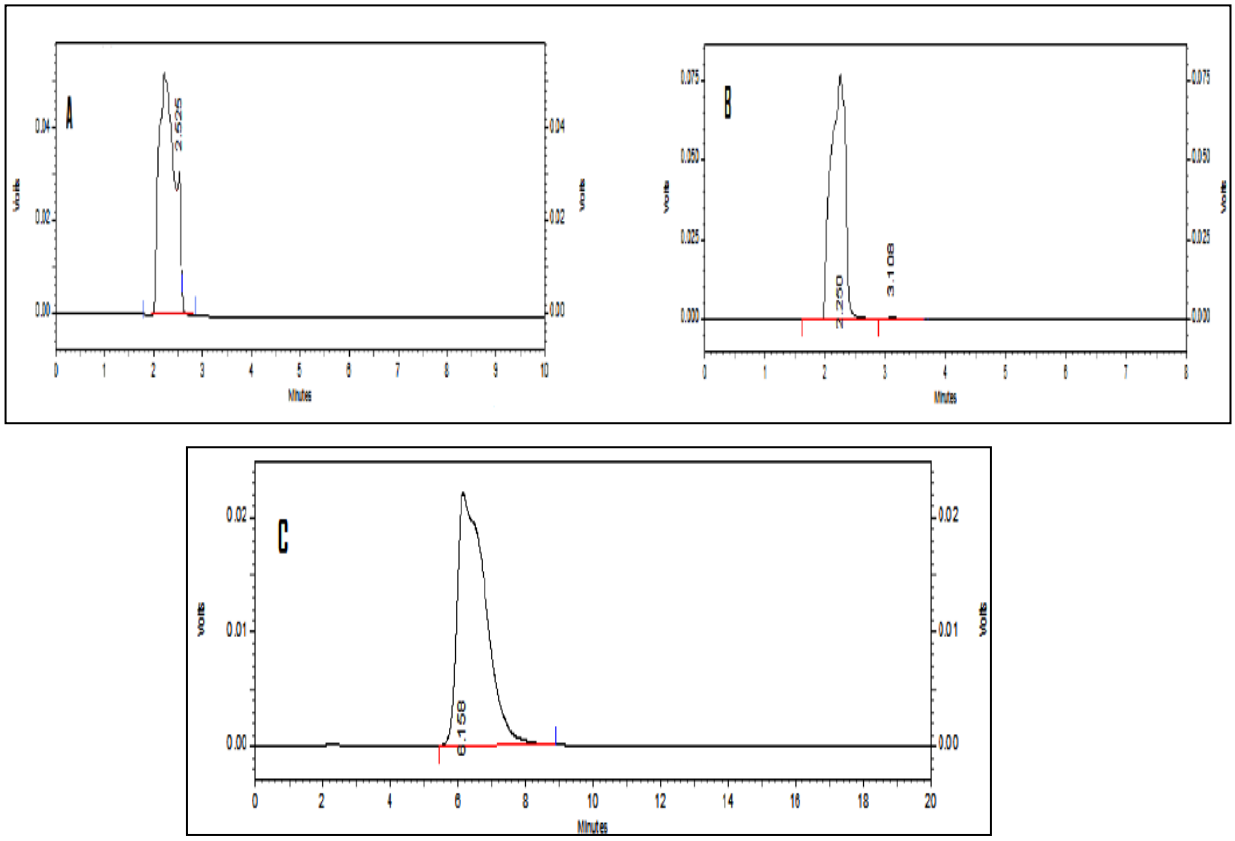


FIG. 2: HPLC CHROMATOGRAMS OF RESVERATROL IN DIFFERENT MOBILE PHASE. (A) ACETONITRILE-WATER (B) METHANOL-PHOSPHATE BUFFER (PH 3.5-6.5) AND (C) ACETONITRILE –PHOSPHATE BUFFER (pH 3.5-6.5)

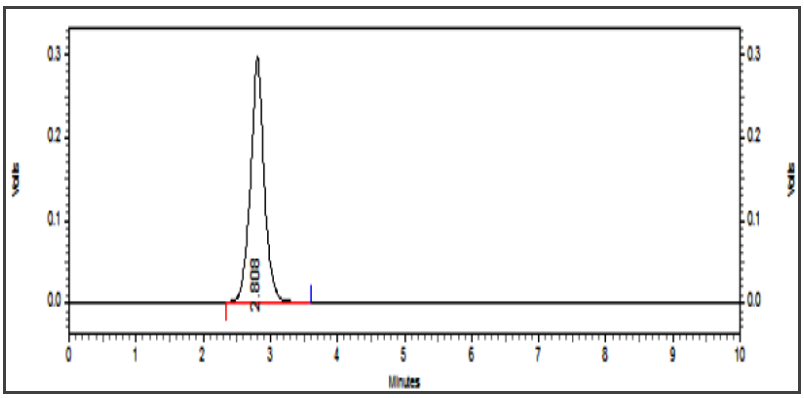


FIG. 3: TYPICAL HPLC CHROMATOGRAM OF RESVERATROL IN SELECTED MOBILE PHASE

Experimental Design Expert:

A Box-Behnken experimental design with three independent factors was performed using 17 standard runs. The selected independent and dependent variables for all the 17 optimized trial runs are given in **Table 2**. Comparison of different proposed models from experimental trials for both the responses favoured quadratic model as best fitted model. The fit summary data exhibited statistically significant *p-values* for all the responses in the model. It was observed that flow rate of mobile phase, injection volume and concentration of acetic acid significantly affected the retention time and chromatographic area *($p<0.0001$)*.

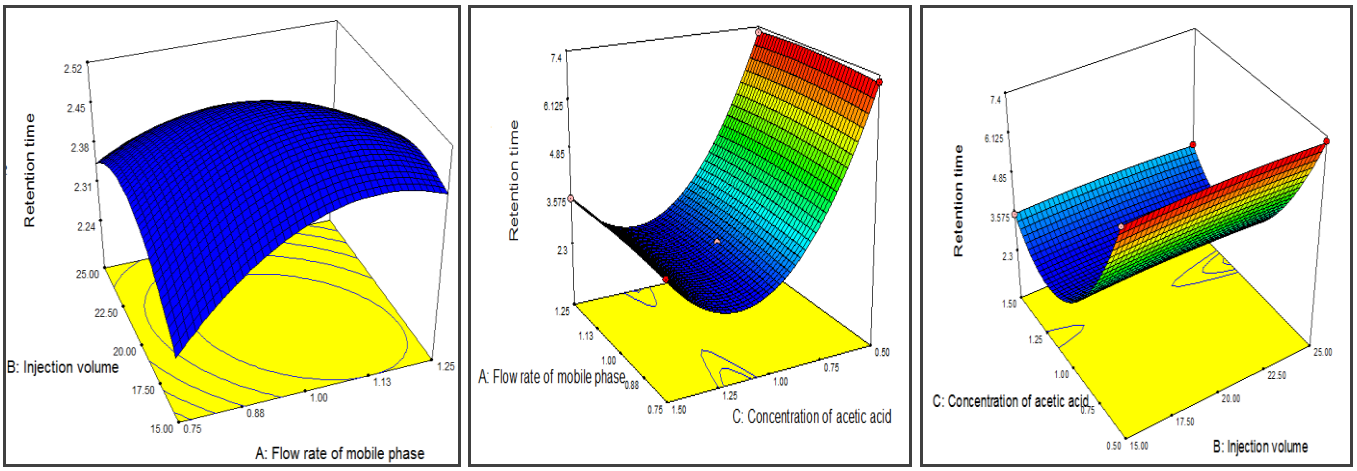
Analysis of variance (ANOVA) for both the retention time and chromatographic area confirmed that the model was statistically significant and fitted well as depicted by the value of *Prob>F* less than 0.05. Polynomial equation generated by the design expert for retention time showed a positive effect of flow rate of mobile phase and negative effect of injection volume and concentration of acetic acid on the retention time whereas polynomial equation for chromatographic area showed positive effect of all the independent variables. A statistically insignificant lack of fit *(Prob>F= 0.0588)* for the chromatographic area was due to noise. The reasonable agreement of predicted R^2 (0.8563) with the adjusted R^2 (0.9754) also showed that the quadratic model was the best fit model. By applying multivariate regression analysis, computer generated quadratic model were obtained for the retention time (Y_1) and chromatographic area (Y_2), given by the following equations:

$$Y_1 = +a_0+a_1X_1-a_2X_2-a_3X_3-a_4X_1X_2-a_5X_1X_3-a_6X_2X_3-a_7X_1^2-a_8X_2^2+a_9X_3^2$$

$$Y_2 = +a_0+a_1X_1+a_2X_2+a_3X_3-a_4X_1X_2-a_5X_1X_3-a_6X_2X_3-a_7X_1^2+a_8X_2^2-a_9X_3^2$$

Where Y_1 and Y_2 are the response of the independent variables, a_0 is the arithmetic mean response, $a_1 - a_9$ are regression coeffecients of the factors X_1, X_2 and X_3.

Response surface, contour and cubic plots were also generated and analyzed to visualize the effect of the parameters and their interactions on the responses. Response surface plots were prepared by keeping one factor constant for each diagram as shown in **Fig. 4**. Surface plot for retention time showed that as the flow rate and injection volumes were increased, the retention time increased. Similarly increase in flow rate also lead to an increase in the chromatographic area. The optimum chromatographic conditions were calculated using numerical optimization. A composite desirability was applied to obtain an optimum set of conditions based on the specified goals and boundaries for the each response. The desirability function showed full desirable response of d=1 which gave the optimal flow rate of 1.0 mL min^{-1}, injection volume of 20 µL and concentration of acetic acid to be 1%.



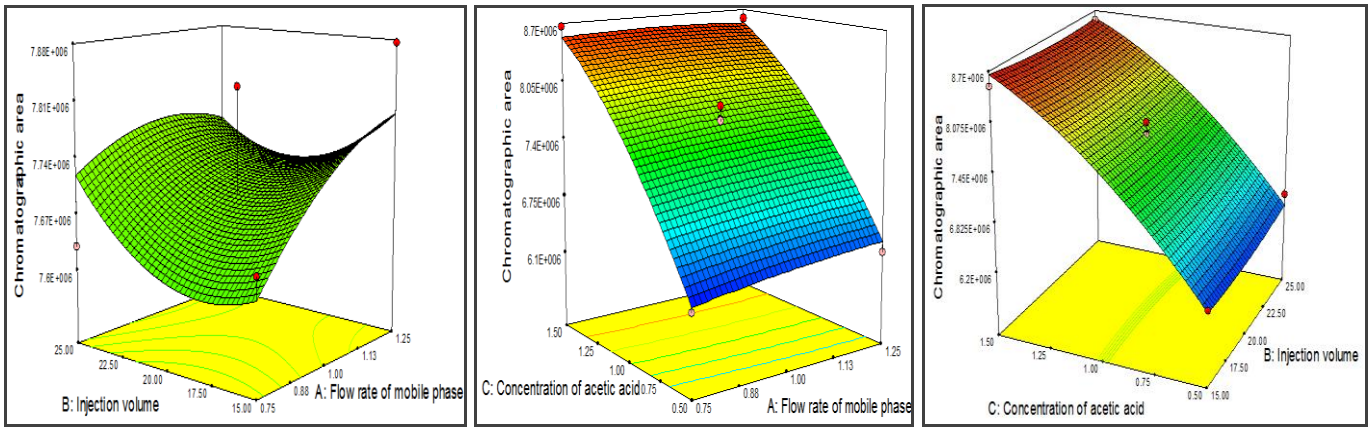


FIG.4: RESPONSE SURFACE PLOT SHOWING EFFECTS OF DIFFERENT INDEPENDENT VARIABLES ON RETENTION TIME AND CHROMATOGRAPHIC AREA

TABLE 2: OBSERVED RESPONSES IN BOX–BEHNKEN DESIGN FOR 17 ANALYTICAL RUNS

Run	Independent variables			Dependent Variables	
	Flow rate of mobile phase (mL/min)	Injection volume (µL)	Concentration of acetic acid (%)	Retention time (min)	Chromatographic Area
1.	1.00	25.00	1.50	3.525	8567480
2.	1.00	20.00	1.00	2.808	7824800
3.	1.25	15.00	1.00	2.806	7876453
4.	1.00	15.00	1.50	3.525	8517850
5.	0.75	25.00	1.00	2.588	7629470
6.	0.75	15.00	1.00	2.565	7646730
7.	1.00	20.00	1.00	2.808	7669837
8.	1.00	20.00	1.00	2.804	7662987
9.	1.00	20.00	1.00	2.806	7659845
10.	1.25	25.00	1.00	2.552	7724640
11.	0.75	20.00	0.50	7.225	6117584
12.	1.00	15.00	0.50	7.242	6328754
13.	1.25	20.00	0.50	7.234	6268743
14.	1.25	20.00	1.50	3.522	8592530
15.	1.00	25.00	0.50	7.246	6574839
16.	0.75	20.00	1.50	3.550	8664980
17.	1.00	20.00	1.00	2.806	7658739

Method Validation:

After optimization of flow rate of mobile phase, injection volume and concentration of acetic acid, the developed method was subjected to method validation as per ICH guidelines. The method was validated using standard procedure to demonstrate its suitability for analysis.

Linearity and calibration:

The standard calibration plot between peak area and drug concentration showed good linearity (n=3) in the concentration range of 7.5-60 µg mL^{-1}. The RSD of the slopes was < 0.5% which indicated that the method was precise. The linear regression data for the calibration plot also indicated a good linear relationship between peak area and concentration over a wide range. The linear regression equation was y = 15361x–46959 with the regression coefficient of 0.999. The regression data are given in **Table 3**.

TABLE 3: LINEAR REGRESSION DATA FOR THE CALIBRATION PLOT

Linearity range (µg/mL)	7.5-60
Regression equation	y = 15361x−46959
Correlation coefficient	0.999
Slope ± SD	15360.33 ± 73.14
Intercept ± SD	46958.66 ± 2326.40
% RSD of slope	0.47
% RSD of intercept	4.95
Limit of Detection	1.46 µg/mL
Limit of Quantitation	4.73 µg/mL

Accuracy as Recovery:

The accuracy of the method as recovery was examined by analyzing resveratrol standard

solution (50 µg mL^{-1}) at three concentration levels of 50, 100 and 150 % (n=3) which showed the recovery of 99.23 – 100.36 % of the spiked samples. The value of % recovery and % RSD is listed in **Table 4**. As shown in the table, the % RSD obtained was 0.428, 0.857 and 1.46% which indicated that the method was accurate.

TABLE 4: ACCURACY OF THE METHOD

Amount (%) of the drug added to analyte	Theoretical content (µg/mL)	Concentration found (µg/mL)±SD	Recovery %	%RSD
0	50	50.12 ± 0.23	99.22	0.32
50	75	75.20 ± 0.32	100.26	0.42
100	100	100.33 ± 0.86	100.33	0.85
150	125	124.22 ± 1.81	99.38	1.46

Precision: The intra- and inter- day precision of the analytical method was examined by analysis of three different concentrations of resveratrol i.e. 20, 40 and 60 µg mL^{-1} in triplicate on the same day and consecutive three days. The analytical results obtained from the investigation are summarized in **Table 5**. The obtained % RSD for both the intra- and inter- day precision was below 2 % indicating that the method was precise.

TABLE 5: PRECISION OF THE METHOD

Conc (µg/mL)	Repeatability (Intra-day)		Intermediate precision (Inter-day)	
	Mean Recovery (µg/mL)± SD	RSD %	Mean Recovery (µg/mL)± SD	RSD %
20	20.06±0.20	1.13	20.19±0.15	0.25
40	40.63±0.80	1.79	40.34±0.40	1.07
60	60.70±0.12	1.20	59.48±0.01	0.76

Reproducibility: The reproducibility of the method was checked by different analyst by measuring the precision of the analytical method in different laboratory using different instrument. Both the intra- and inter-day precision was determined. The obtained results showed no significant difference in the % RSD which indicated that the method was reproducible. The obtained reproducibility results are listed in **Table 6**. As shown in table, the % RSD were 1.78, 1.877 and 1.982 % for intra-day precision and 1.272, 1.874 and 1.645% for inter-day precision which showed that the method was reproducible.

TABLE 6: REPRODUCIBILITY OF THE METHOD

Conc (µg/mL)	Repeatability (Intra-day)		Intermediate precision (Inter-day)	
	Mean Recovery (µg/mL)± SD	RSD %	Mean Recovery (µg/mL)± SD	RSD %
20	20.14±0.38	1.78	20.08±0.13	1.27
40	39.63±0.54	1.87	39.20±0.42	1.84
60	59.76±0.82	1.98	59.54±0.52	1.64

Limit of Detection and Quantitation: The LOD and LOQ of the analytical method was 1.463 and 4.737 µg mL^{-1} respectively, determined by standard deviation method which indicated that the method can be used for detection and quantification of resveratrol over a wide range of concentrations.

Robustness: The robustness of the analytical method was determined by deliberately changing certain parameters such as composition of the mobile phase, flow rate and suppliers of methanol. Each parameters was studied at three levels expect for the methanol, which was studied from two different manufacturers. The study did not show any significant changes in the retention time of the resveratrol. The low value of % RSD i.e. below 0.5% for all the parameters (**Table 7**) indicated robustness of the method.

TABLE 7: ROBUSTNESS OF THE METHOD

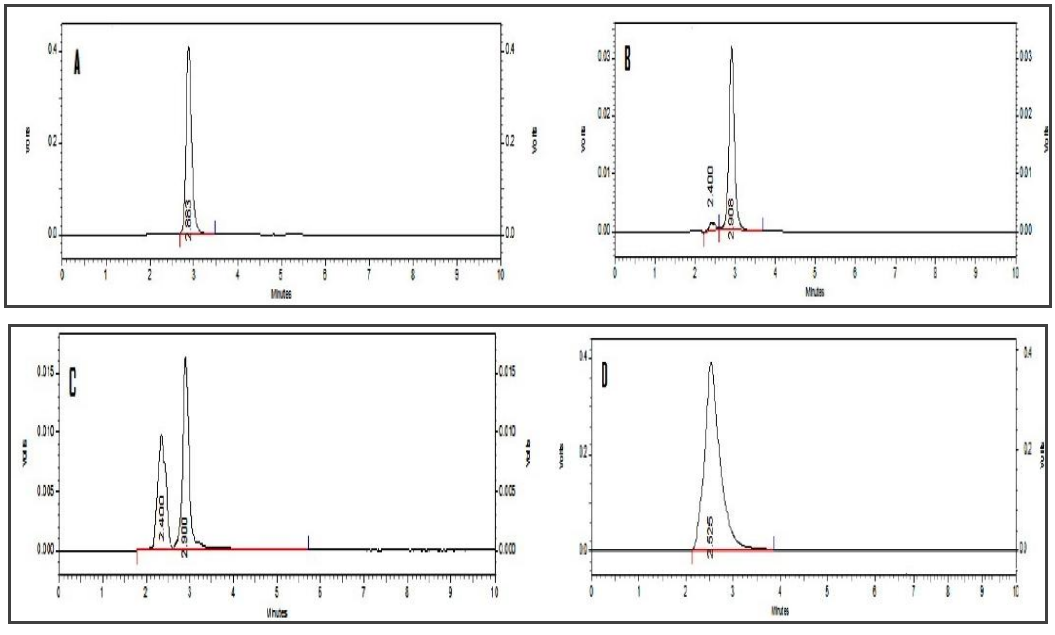
A. Percentage of water in mobile phase		
% of water	**Mean RT(min)±SD**	**%RSD**
25	2.806±0.008	0.30
30	2.808±0.025	0.22
35	2.804±0.016	0.20
B. Flow rate of the mobile phase		
Flow rate (mL/min) (±3%)	**Mean RT(min)±SD**	**%RSD**
0.97	2.812±0.006	0.22
1	2.808±0.012	0.31
1.03	2.808±0.012	0.42
C. Methanol from different companies		
Company	Mean RT(min)±SD	%RSD
Merck, India	2.806±0.006	0.21
SD Fine Chemicals, India	2.809±0.020	0.38

Stability:

The drug solution was stable when stored for 48 hours at laboratory temperature and under refrigeration in methanol.

Forced Degradation Studies:

Forced degradation study determined by exposing stock solution of resveratrol to 0.1M HCL, 0.1M NaOH, 3% H_2O_2, dry heat and UV light proved the method as stability indicating and highly specific to resveratrol (**Fig. 5**). There was no degradation of resveratrol in the presence of 0.1M HCL for 24 hour and no significant change in the peak area and retention time was observed. In the presence of 0.1M NaOH, it was observed that the retention time for parent peak was the same but an undistinguishable degradation peak near to the parent peak was observed. Oxidative stress with 3% H_2O_2 resulted in a degradation peak at lower retention time with a significant decrease in peak area compared to parent peak. Thermal degradation study carried out by storing resveratrol powder in oven at 100ºC for 24 hours showed shift of drug peak to 2.525 minutes showing degradation. Similarly neutral degradation carried by refluxing the sample for 8 hours at 80ºC did not show any degradation peak with no significant decrease in retention time. Resveratrol was found to be sensitive to photolytic degradation as it converts into *cis*-form in the presence of UV-Visible light. A degradation peak at 9.367 minutes was found along with substantial decrease in the percentage recovery of the drug after 24 hours. The RT and total percentage drug recovery after each degradation studies are listed in **Table 8**.



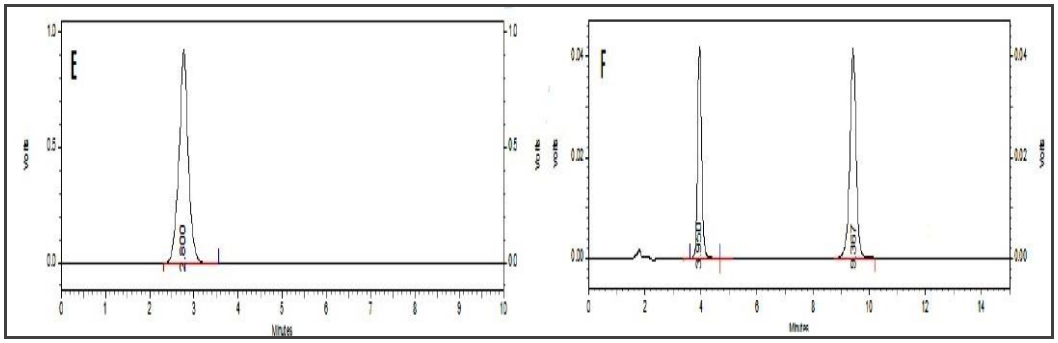


FIG. 5: HPLC CHROMATOGRAMS SHOWING FORCED DEGRADATION STUDIES. (A) ACIDIC DEGRADATION IN 0.1 M HCL (B) ALKALINE DEGRADATION IN 0.1 M NAOH (C) OXIDATIVE DEGRADATION IN 3% H_2O_2 (D) THERMAL DEGRADATION IN OVEN AT 100° C (E) NEUTRAL DEGRADATION IN WATER BATH AT 80° C (F) PHOTOLYTIC DEGRADATION IN UV CHAMBER AND DAYLIGHT.

TABLE 8: FORCED DEGRADATION STUDIES

Sample Exposure Condition	RT of resveratrol after Exposure (min)±SD	RT of degradation products (min)±SD	% RSD	% Recovery after 24 hours
Acidic Degradation	2.86±0.046	–	1.61	95.03± 0.28
Alkaline Degradation	2.93±0.043	2.40±0.032	1.47	63.02±0.53
Neutral Degradation	2.80±0.005	–	0.20	77.82±0.36
Oxidative Degradation	2.89±0.013	2.40±0.018	0.44	45.43±0.98
Thermal Degradation	2.63±0.018	–	0.69	63.65±0.51
Photolytic Degradation	3.94±0.008	9.36±0.012	0.21	38.15±0.94

Analysis of Resveratrol in Nanoemulsion and Marketed Dosage Form:

The validated method was used to estimate the resveratrol content in nanoemulsion formulation formulated by aqueous titration method and marketed resveratrol capsule. Sample analysis was carried out in triplicates after proper extraction. A single peak was observed at the retention time of resveratrol. There was no interaction between resveratrol and excipients present in the nanoemulsion. The resveratrol content was found to be 99.28±0.48% for nanoemulsion and 98.2±0.92% for marketed capsule. The low RSD value indicated the suitability of this method for routine analysis of resveratrol in pharmaceutical dosage forms.

CONCLUSION: A new, selective and simple stability-indicating HPLC method has been developed for the quantitative determination of resveratrol and its impurities in drug substance and product. The optimum experimental conditions were determined by the use of response surface methodology with the use of least number of runs as possible using Box-Behnken design. The developed method is sensitive, rapid and reliable and is validated in accordance with ICH guidelines. Stress degradation study showed well separation of degradation peaks from the resveratrol main peak. The method seems to be robust.

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