DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF TRANS-RESVERATROL IN ETHOSOMES

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ABSTRACT

The present work describes a simple, accurate and validated UV spectrophotometric method for determination of trans-resveratrol loaded in ethosomal formulation. The method was validated for different parameters like linearity, precision, specificity, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness as per ICH guidelines. The wavelength maximum of resveratrol in phosphate buffer pH 7.4 was found to be at 307 nm. The method was found to be linear in the range of 8 to 38 µg mL^{-1} with a correlation coefficient (r^2) of 0.9957. The accuracy of the method was studied by recovery study and % recovery was found to be in the range of 99.24 to 100.14 %. The method is simple, accurate and requires relatively inexpensive instruments. The proposed method can be successfully used for determination of trans-resveratrol loaded into ethosomes.

INTRODUCTION

Resveratrol is a natural polyphenol compound which has received much interest over the past few years because of its extensive physiological properties. It is an antioxidant compound that occurs naturally on the skins of red grapes and blueberries, among other fruits. It is also found in peanuts and red wine. It can also be referred to as a phytoalexin that is produced naturally by various plants when they are attacked by pathogens like fungi or bacteria[1].

Resveratrol has been shown to exhibit anti-tumor, anti-inflammatory, anti-oxidant and anti-platelet aggregation properties, and these properties have led to it being considered as a potential therapeutic agent for humans. It protects the brain from oxidized fats and free radicals, which are known to kill the cells of the brain, leading to a mental dysfunction always associated with Alzheimer's[2]. Resveratrol has the capability to influence cell growth and turn the genes responsible either on or off, hence the backbone of resveratrol's ability to fight various types of cancer including cancer of the esophageal, breast, colon, skin, ovarian cancer, prostate cancer, liver, pancreas, stomach, oral and cervical, among many others[2, 3].

Resveratrol (3, 5, 4'-trihydroxystilbene) is a stilbenoid, a derivative of stilbene (Fig. 1). Trans-resveratrol is a selective inhibitor of cyclooxygenase-1 (COX-1). It inhibits COX and peroxidase activities of COX-1 with ED50 values of 15 and 3.7 uM, respectively; with essentially no inhibition of the COX activity of COX-2[3]. Resveratrol occurs in both trans and cis isoforms, which may have different biological effects. Due to its stability, trans-resveratrol is
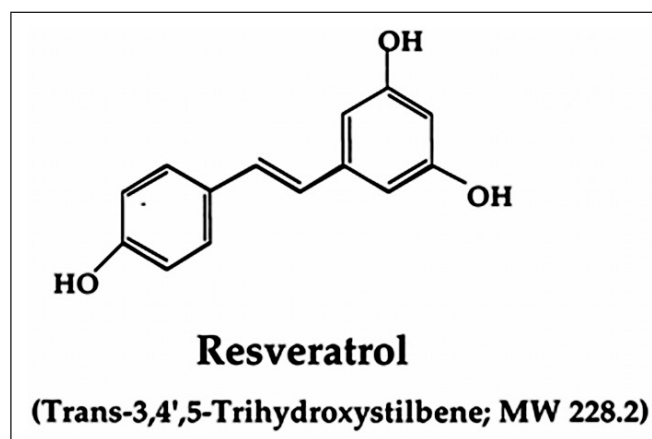

Fig. 1: Chemical structure of trans-resveratrol

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the most commonly used isoform. Cis-resveratrol has been reported to be unstable and is therefore not available commercially[2].

Because of the significant pharmacological activities exhibited by the resveratrol, several researchers have focused on the development of various analytical methods to determine resveratrol in different matrices such as plant extracts, wine and serum[4]. The commonly used methods are high performance liquid chromatography (HPLC), HPTLC and fluorometric and electrochemical detection has also been described[2, 4]. As an alternative to HPLC assays, capillary electrophoresis, gas chromatography–mass spectrometry (MS) or liquid chromatography–MS have also been proposed. Even though these methods are effective, they remain expensive and time-consuming.

In this study, a UV spectrophotometric method was developed for the determination of trans-resveratrol from the ethosomal formulation and the method was validated against the trans-resveratrol loaded ethosomes by evaluating its entrapment efficiency.

MATERIALS AND METHODS

Equipment

UV- VIS double beam spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Japan).

Materials

Trans-resveratrol was procured as a gift sample from Evolva Biotech Pvt Ltd., Chennai, India. Phospholipon® 90H as the matrix material was obtained as a gift sample from Lipoid (Cologne, Germany). Ethanol 96 % and propylene glycol were purchased from KUC (India) and Merck (Mumbai, India), respectively. Sodium chloride, potassium dihydrogen orthophosphate, disodium hydrogen phosphate were purchased from Loba Chemie (Mumbai, India). All other reagents used were of analytical or equivalent grade.

Selection of wavelength maximum (λ_{max})

In order to ascertain the wavelength of maximum absorption (λ_{max}) of the drug, a spectrum of 10 µg mL^{-1} was recorded using UV Visible spectrophotometer by scanning in the range of 200 nm to 400 nm against phosphate buffer pH 7.4 as blank. The λ_{max} of the drug was noted (Fig. 2). The absorption curve showed characteristic absorption maxima at 307 nm for resveratrol.

Preparation of stock solution

Accurately weighed quantity (10 mg) of resveratrol was transferred to 100 mL amber colored volumetric flask. Small quantity of phosphate buffer pH 7.4 was added to ensure complete solubilization of drug and finally volume was made up to the mark with phosphate buffer pH 7.4 solvent mixture to produce 100 µg mL^{-1} solution.

Preparation of working standard solutions

With the help of pipette, 0.1 mL aliquot was withdrawn from stock solution and transferred to 10 mL amber colored volumetric flask. It was then diluted up to 10 mL with phosphate buffer pH 7.4 to produce 1 µg mL^{-1} solution. Similarly solutions of concentration 8, 14, 20, 26, 32 and 38 µg mL^{-1} were prepared, which were used for the construction of the calibration curve (Table I).

Preparation of trans-resveratrol loaded ethosomes[5]

The formulation was prepared by cold method. The trans-resveratrol was dissolved in water and Phospholipon® 90H was dissolved in ethanol in a covered vessel at room temperature by vigorous stirring at 700 rpm on magnetic stirrer. Propylene glycol was then added during stirring in organic medium. Both the media were heated at 30 °C at 700 rpm on magnetic stirrer and the aqueous medium was added to organic medium slowly drop wise. The continuous stirring was applied for another 5 mins and the resultant vesicle suspension was cooled to room temperature. The desired vesicle size of ethosomal formulation was obtained by using bath sonication (JP-4820, Citizen, India). Finally, the formulation was stored under refrigeration conditions.

Determination of % entrapment efficiency (%EE)

% Entrapment Efficiency[5]

The prepared ethosomal formulations were put into a cooling centrifuge (Remi Instruments, India) and were centrifuged at 10,000 rpm for 90 mins at 4-6 °C. The supernant was collected to determine the free drug concentration (C_{free}) at 266 nm and dilution was performed. The total drug concentration (C_{total}) is the total amount of drug to hydration volume. The results were obtained in triplicate. From C_{free} and $C_{total,}$ the % EE was calculated as:

$$\% \ EE = \frac{C_{(total)} - C_{(free)}}{C_{(total)}} \times 100 \qquad \text{Equation (1)}$$

Preparation of ethosomes test solution

Ethosomes dispersion equivalent to 10 mg trans-resveratrol was weighed and transferred to 100 mL amber
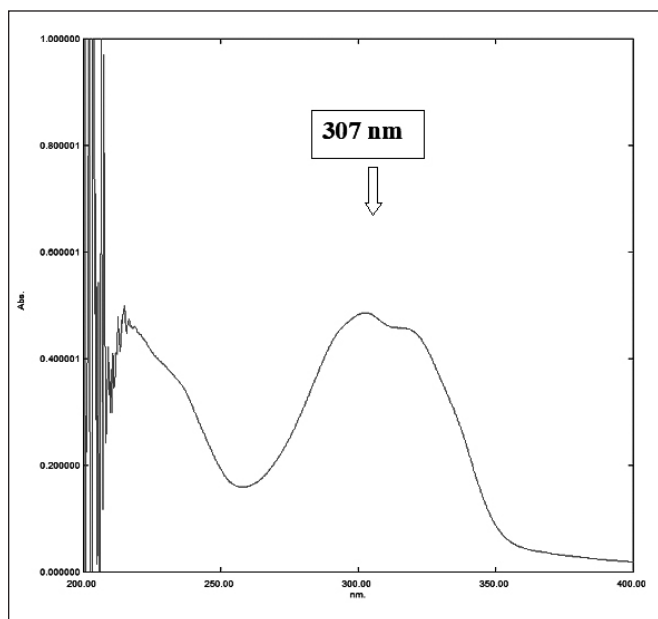
Fig. 2: UV Spectrum of 10 µg mL^{-1} trans-resveratrol in phosphate buffer pH 7.4

colored volumetric flask separately and the volume was made up to 100 mL with phosphate buffer pH 7.4 to produce 100 µg mL^{-1}. Then, 0.8 mL of test solution was diluted up to 10 mL with phosphate buffer pH 7.4 and the absorbance of test solution (8 µg mL^{-1}) was recorded against phosphate buffer pH 7.4 as a blank at 307 nm.

Method validation[6,7]

Validation can be defined as establishing documented evidence which provides a high degree of assurance that a particular method will consistently produce a product meeting its predetermined specifications. The method was validated for different parameters like linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and robustness as per the ICH guidelines.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. For linearity study, six solutions of different concentration (8, 14, 20, 26, 32 and 38 µg mL^{-1}) were prepared from the stock solution by withdrawing aliquots with the help of pipette and transferring to separate 10 mL amber coloured volumetric flasks and making volume up to the mark with phosphate buffer pH 7.4. The absorbance of the solutions was measured at 307 nm. A graph of concentration versus absorbance was plotted and correlation coefficient (r^2) was calculated.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision of the method was determined in terms of repeatability and intra-day and inter-day precision. Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability of the method was determined by analysing six samples of same concentration of drug. For intra-day and inter-day precision studies six solutions of different concentrations (8, 14, 20, 26, 32 and 38 µg mL^{-1}) were prepared and analysed three times a day and the same procedure was followed for next two days. The results were reported in terms of % relative standard deviation, % RSD (% relative standard deviation).

Specificity

The specificity of an analytical method represents its ability to assess unequivocally the analyte in presence of components which are expected to be present. It was checked by comparing the spectra of standard drug solution and ethosomes test solution.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Recovery study was performed by standard addition method at three levels i.e. 80 %, 100 % and 120 %. At each level, the determination was done in triplicate and the amount of drug recovered was calculated.

Table I: Linearity of resveratrol (8-38 µg mL^{-1}) (λ_{max}: 307 nm)

Concentration µg mL^{-1}	Mean Response± SD (n=6)	%RSD
8	0.114 ±0.0017	1.5193
14	0.333±0.0063	1.8936
20	0.456±0.0090	1.9817
26	0.615±0.0125	2.0377
32	0.788±0.0138	1.7553
38	0.976±0.0119	1.2284
	Linearity Equation	**y = 0.0278x - 0.0919**
	Correlation Coefficient	**0.9957**
	Slope	**0.0278**
	Intercept	**0.0919**

			Table II: Intraday precision		
Conc. (µg mL⁻¹)	Absorbance	Concentration (µg mL⁻¹)	Mean absorbance ± SD (n=6)	Mean concentration ± SD	%RSD
14	0.301	14.1330			
	0.302	14.1690	0.303 ± 0.0032	14.217 ± 0.1156	0.8133
	0.307	14.3489			
26	0.634	26.1115			
	0.632	26.0395	0.632 ± 0.002	26.039± 0.0719	0.2762
	0.630	25.9676			
32	0.810	32.4424			
	0.805	32.2625	0.804 ± 0.0060	32.238 ± 0.2168	0.6725
	0.798	32.0107			

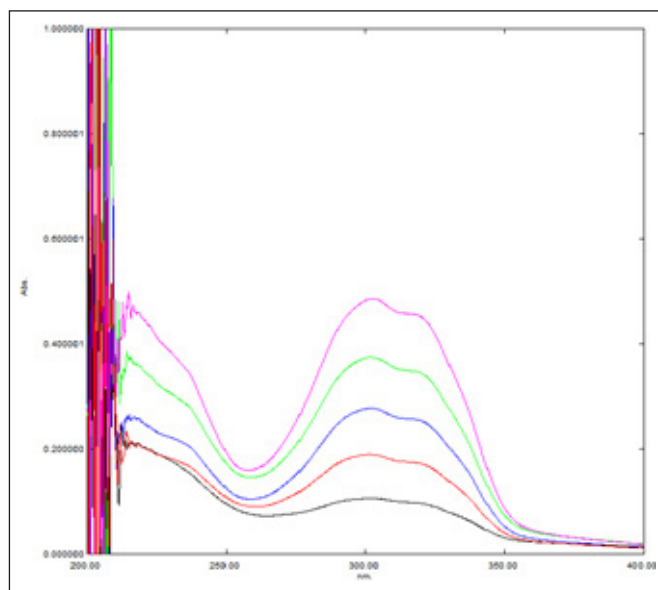|			Table III: Interday precision		
Conc. (µg mL⁻¹)	Absorbance	Conc. (µg mL⁻¹)	Mean absorbance ± SD	Mean concentration ± SD	%RSD
14	0.308	14.3848			
	0.311	14.4928	0.309 ± 0.0015	14.44 ± 0.0549	0.3803
	0.31	14.4568			
26	0.639	26.2913			
	0.636	26.1834	0.638 ± 0.0025	26.27 ± 0.0905	0.3444
	0.641	26.3633			
32	0.808	32.3705			
	0.798	32.0107	0.803 ± 0.0051	32.21 ± 0.1845	0.573
	0.805	32.2625			

Fig. 3: Overlay of the spectra of trans-resveratrol (8-38 µg mL⁻¹) in phosphate buffer pH 7.4

Limit of detection (LOD)

It is the lowest concentration of analyte in sample that can be detected but not necessarily quantified. It was calculated based on standard deviation of response and slope of the curve using following equation:

LOD = 3.3 σ/s

where σ - standard deviation of the response

s – slope of curve

Limit of quantification (LOQ)

It is the minimum concentration of analyte that can be quantified with suitable precision. It was calculated using following equation:

LOQ = 10 σ

where σ is standard deviation of response.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It was done by measuring absorbance of a 10 μg mL^{-1} solution at detection wavelength 307 ± 2 nm. Each measurement was done in triplicate.

Solution stability

For stability study solution was stored at room temperature for 24 h. The initial absorbance and absorbance at 24 h was measured and the difference was noted. The similarity factor was calculated as follows:

$$\text{Similarity factor} = \frac{\text{Absorbance of initial solution}}{\text{Absorbance of solution after 24 h}}$$

RESULTS AND DISCUSSION

Selection of wavelength maximum (λ_{max})

The UV spectrum of resveratrol in phosphate buffer pH 7.4 has maximum absorption (λ_{max}) at 307 nm as shown in Fig. 2. The absorbance of excipients in ethosomes solution did not interfere with resveratrol. As a result, 307 nm wavelength was selected for quantitative analysis and validation of resveratrol in ethosomes.

Linearity

The drug obeyed Beer–Lambert's law in the concentration range of 8-38 μg mL^{-1} with regression 0.9957 at 307 nm with % RSD <2 %, as shown in Table I. Overlay spectra of resveratrol are shown in Fig. 3 and calibration curve is shown in Fig. 4.

Precision

The developed method was found to be precise as the % RSD values for intraday (Table II) and interday (Table III) precision were found within limit (< 2 %). Repeatability expresses the precision under the same operating conditions over a short interval of time. So, as per Table IV, this method is repeatable.
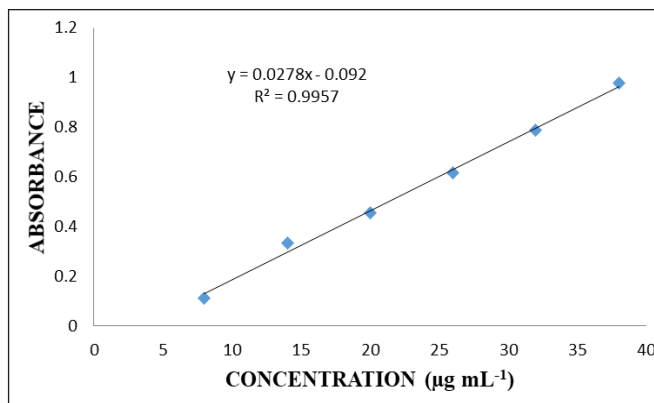

Fig. 4: Linearity curve of trans-resveratrol (8-38 μg mL^{-1})

Table IV: Repeatability

Sr. No.	Conc. (μg mL^{-1})	Absorbance	Conc.(μg mL^{-1})
1		0.642	26.399
2		0.645	26.5071
3	26	0.638	26.2553
4		0.639	26.2913
5		0.642	26.3992
6		0.641	26.3633
	MEAN	0.641	26.3693
	S.D.	0.0024	0.08932
	R.S.D.	0.3873	0.3387
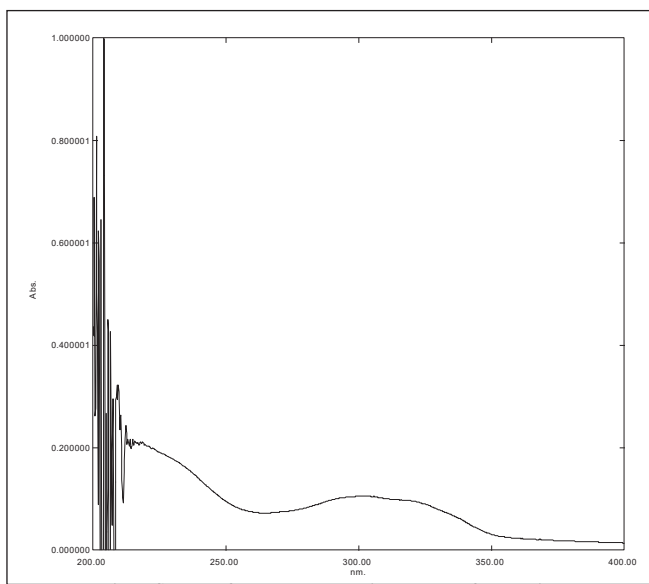
Fig. 5: Spectra of trans-resveratrol in ethosomal formulation

Table V: Accuracy							
Level of recovery	Sample concentration (µg mL⁻¹)	Std. added (µg mL⁻¹)	Total amount (µg mL¹)	Absorbance	Amount recovered	% recovery	Mean % recovery
80	5	4	9	0.156	8.91	99.08	100.14
	5	4	9	0.161	9.09	101.07	
	5	4	9	0.159	9.02	100.27	
100	5	5	10	0.188	10.06	100.68	99.24
	5	5	10	0.184	9.92	99.24	
	5	5	10	0.18	9.78	97.8	
120	5	6	11	0.213	10.9676	99.71	99.92
	5	6	11	0.216	11.0755	100.69	
	5	6	11	0.212	10.9317	99.38	

Specificity

The excipients in ethosomes did not interfere with absorbance of resveratrol which indicates that the method was specific, as shown in Fig. 5.

Accuracy

Recovery study shows that the overall % recovery was found to be 99.76 % as per in Table V. So, this method was found to be accurate.

Limit of detection (LOD)

LOD calculated using the equation as mentioned earlier is 0.63 µg mL⁻¹.

Limit of quantification (LOQ)

LOQ calculated using the equation as mentioned earlier is 1.92 µg mL⁻¹.

Robustness

The method was found to be robust when checked for the effect of change in detection wavelength (Table VI).

Solution stability

The stability of solution was evaluated by determining similarity factor (0.97), which was found within the acceptance criteria of 0.98-1.02. The developed method was found to be precise, specific and accurate

Table VI: Robustness						
Concentration (µg mL⁻¹)	Absorbance		Concentration (µg mL⁻¹)		%Assay	
26	305 nm	309 nm	305 nm	309 nm	305 nm	309 nm
	0.634	0.638	26.11	26.26	100.43	100.98
	0.635	0.636	26.15	26.18	100.57	100.71
	0.635	0.637	26.15	26.22	100.57	100.84
Average	0.635	0.637	26.14	26.22	100.52	100.84
S.D.	0.0006	0.001	0.0208	0.0360	0.0799	0.1384
RSD	0.0910	0.1570	0.0795	0.1372	0.0795	0.1371

Table VII: Solution stability (26 µg mL⁻¹)			
Concentration (µg mL⁻¹)	Absorbance ± SD	Concentration (µg mL⁻¹) ± SD	Assay (%)± SD
Initial	0.629 ± 0.003	25.9 ± 0.124	99.73 ± 0.47
After 24 h	0.646 ± 0.002	26.5 ± 0.09	102.08± 0.36

(Table VII). The overall summary of validation parameters shown in Table VIII.

Table VIII: Summary of validation parameters

Parameter	Trans-resveratrol loaded ethosomes
Linearity	8-38 µg mL^{-1}
Regression equation	y = 0.0278x - 0.0919
R^2	0.9957
LOD	0.63 µg mL^{-1}
LOQ	1.92 µg mL^{-1}
Repeatability (%RSD, N=6)	0.382
Intraday precision (%RSD, N=3)	0.5873
Interday precision (%RSD, N=3)	0.4325
Accuracy	99.24-100.14 %

CONCLUSION

The proposed UV-spectrophotometry method for estimation of *trans*-resveratrol in pharmaceutical dosage form of ethosomes was successfully developed and validated for its intended purpose. The method was shown to be linear, precise, repeatable, specific, accurate, robust and stable. Therefore, it can be used for the determination of trans-resveratrol loaded into ethosomes.

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