RESEARCH PAPER

Ultraviolet-visible Spectrophotometric Method for Estimation of Resveratrol in Presence of Excipients as Impurities.

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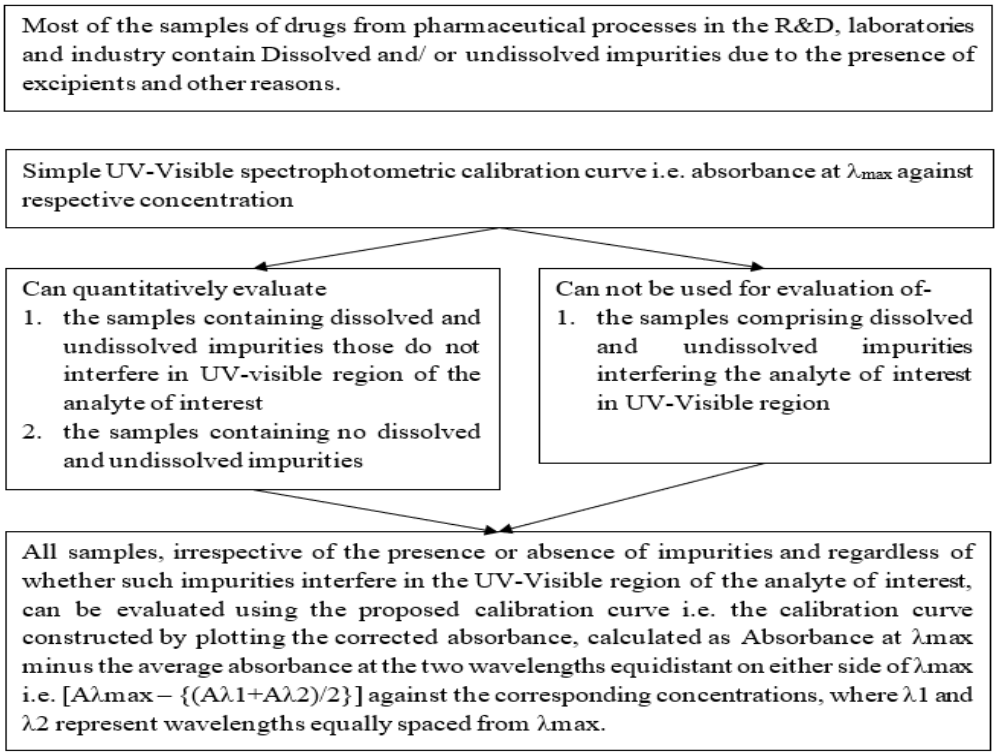
ABSTRACT
In order to prevent inaccurate measurement caused by the presence of soluble or insoluble impurities, a straightforward and sensitive UV spectrophotometric approach for quantitative determination of a model API Resveratrol in the presence of excipients is provided. UV detection was carried out at 306 nm, 296 nm, and 316 nm. The calibration curve was plotted between the resultant of absorbance at these three wavelengths as per the equation [A306 nm - (A296 nm + A316 nm)/2] and the concentration of resveratrol. The calibration curve was determined linear over the tested concentration range i.e. 03-09 µg/ml having limit of detection (LOD) 0.05 µg/ml and limit of quantification (LOQ) 0.16 µg/ml. Percent relative standard deviations, indicating precision, for pure as well as impure drug solutions were confined to the reasonable limits i.e. constantly less than 1.99 for both pure and impure solutions. The accuracy of the devised approach is demonstrated by the mean percent recovery within the range of 98.81%±1.54%-103.00%±1.26% and 99.33% ±1.54-102.22%±2.11% for pure and impure solution correspondingly. In conclusion, the established technique can be used to estimate resveratrol in both pure and impure solutions precisely and accurately using the same calibration curve.

Key words: Pure, impure, excipients, UV spectrophotometric method, resveratrol



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INTRODUCTION

The samples from various pharmaceutical processes like dissolution studies of dosage forms, solubility studies and others alike contain dissolved or undissolved matters that might interact with quantitative assessment of the analyte of curiosity until separated (Nounou et.al., 2006)[1]. While the undissolved impurities can be separated by filtration (Nounou et. al., 2006)[1] and/ or centrifugation (Ramprasad et.al., 2002)[2], the dissolved impurities can be separated by HPLC or other chromatographic studies (Mumper et al., 2000)[3] All these separation processes need specific techniques, expertise and equipment resulting in several times increases in the evaluation costs and time duration. While during filtration, the analyte drug might get adsorbed onto the filter medium (Adhikari et al. 2012[4]; Gumieniczek et al. 2011)[5], the HPLC methods use organic solvents and combinations thereof to separate the incompatible analytes. Furthermore, energy and electricity are consumed by all these methods and are laborious, costly and time-consuming. Also, the costly columns might become choked during the process. Although they are all complicated, derivative spect ophotometry, bichromatic techniques, and difference spectrophotometry can also be used to remove background light absorption (Shaodong et.al., 2010)[6]. Additionally, derivative spectrophotometry consequences in more complicated spectrum (Rathod et.al., 2012)[7].

Currently reported resveratrol assay methods involve high performance liquid chromatography with UV detection (HPLC-UV) (Sato et al. 1997[8]; Zhu et al. 1999[9]), fluorometric (Pezet et. al., 1994) [10] and electrochemical (Andlauer et al. 2000[11]; McMurtrey et al. 1994[12]), capillary electrophoresis (Gu et al. 2000[13]; Lin et al. 2001[14]), gas chromatography-mass spectrometry (GC-MS) (Gamoh et.al., 1999)[15] and liquid chromatography-mass spectrometry (LC-MS) (Careri et.al., 2004)[16].

Even if these approaches are helpful, they remain pricey and time-wasting. These also have laborious extraction steps and lengthy retention time.

Moreover, these methods are not suitable for assessing the dissolution samples having both dissolved and undissolved impurities which interact in UV-visible region unless the impurities (dissolved and undissolved) are first separated. The separation of such dissolved as well as undissolved impurities is necessary before estimation by these methods as their interaction in the UV-visible region during detection result in inaccurate quantification of the analyte.

In addition, dissolved impurities are typically removed using chromatographic columns that are labor-intensive, expensive, and onerous. Conversely, undissolved impurities are eliminated through tiresome, expensive, and time-consuming processes i.e. filtration or centrifugation. Moreover, there is a risk of adsorption of the analyte of interest onto the filter medium during filtration. In some cases, separation requires the use of a centrifuge or a high-speed refrigerated ultracentrifuge, which further increases energy consumption.

A simple and easy-to-calculate ultraviolet spectrophotometric method is presented to eradicate the interference caused by dissolved and/ or undissolved interfering substances. The established approach can be effectually rational for the assay of the analyte in the absence of interfering substances i.e. impurities (in pure solutions) as well as in the presence (impure solutions) of interfering substances/ impurities whether dissolved or undissolved.

MATERIALS AND METHODS

All reagent grade chemicals have been employed in the study. Resveratrol was procured from Nutrija Life Sciences, India. Acetonitrile (ACN), potassium dihydrogen orthophosphate, and sodium hydroxide were got from Loba Chemie Pvt. Ltd., India. A Shimadzu PharmSpec UV-1800 UV–Visible spectrophotometer was utilized for the analysis.

Preparation of calibration curve

A primary stock solution (Solution A) with a strength of 1000 µg/ml. was prepared by accurately weighing 50 mg of resveratrol and dissolving it in 15 ml of ethanol in a 50 ml capacity volumetric flask followed by adjusting the volume was to the calibration mark using distilled water.

A secondary working solution (Solution B) having a concentration of 50 µg/ml was obtained by transferring 12.5 ml out of Solution A into a volumetric flask of 250 ml capacity and diluting to volume with distilled water.

An impure working stock solution (Solution C) with a concentration of 50 µg/ml was formulated by adding commonly used formulation ingredients like lactose (diluent), starch (binder), glycerol (humectant), FDA-approved dyes and lakes (colorants), vanilla (flavoring agent), mannitol (sweetener), lecithin (emulsifier), methylparaben (preservative) and standard nanosuspension excipients in their conventional formulation concentrations into the previously prepared working stock solution B (50 µg/ml). The mixture was prepared in a 100 ml volumetric flask, sonicated for 10 minutes to ensure proper dispersion, and finally diluted to the mark using stock solution B.

Appropriate aliquot part of solutions B and C were pipetted into separate 10 ml volumetric flasks and further diluted to the final volume to obtain a series of alternating pure and impure standard solutions from 3-9 µg/ml i.e. 3, 4, 5, 6, 7, 8, and 9 µg/ml.

The calibration curve was developed using the approach described by various authors (Arora et al. 2025[17]; Prasad et al. 2013[18]; Prasad et al. 2016[19]; Prasad et al. 2020[20]). Measurements were carried out at three selected wavelengths: the wavelength of maximum absorbance (λmax) at 306 nm, along with two wavelengths located symmetrically on either side of λmax, namely 296 nm (λmax - 10 nm) and 316 nm (λmax + 10 nm). The corrected absorbance values were calculated according to Equation 1, defined as the absorbance at λmax minus the mean of the absorbances at λ1 and λ2. These corrected absorbance values were then plotted against concentration to construct the calibration curve.

$$\textbf{Corrected Absorbance} = \left[\mathbf{A(\lambda max)} - \left(\frac{\mathbf{A(\lambda 1) + A(\lambda 2)}}{\mathbf{2}}\right)\right] \quad -----\mathbf{1}$$

The curve was prepared in the concentration range of 3-9 µg/ml only because the linearity was followed by the samples in the concentration range of 3-9 µg/ml only and not by the samples having concentration beyond 3-9 µg/ml.

Validation

Specificity

Both pure and impure standard solutions of resveratrol at the levels of 4.8, 6.0, and 7.2 µg/ml were independently prepared using the same solvent system as that used in preparing the calibration curve. Each solution was scanned over the wavelength range of 200-400 nm to evaluate any variation in the corrected absorbance values of the pure and impure samples at the selected analytical wavelengths, namely 296 nm, 306 nm, and 316 nm. Additionally, the absorption spectrum was examined to detect any possible shift in the wavelength corresponding to maximum absorbance (λmax) (Guideline, I. H. T., 2005)[21].

Linearity

The linearity of the constructed calibration curve was assessed by applying linear regression analysis (Guideline, I. H. T., 2005)[21]. Furthermore, the adequacy of the regression model was examined through residual analysis by plotting the residual values against the corresponding predicted concentrations (Jurado et al. 2017)[22].

Precision

Repeatability of the method was evaluated by analyzing drug concentrations of 4.8, 6.0, and 7.2 µg/ml prepared from independent stock solutions, both in the presence and absence of impurity in six replicates (n = 6). Intermediate precision was valued by determining intra-day and inter-day variability. For intra-day precision, three concentration levels corresponding to 80%, 100%, and 120% of the midpoint of the linearity range (with and without impurity) were analysed in six replicates, three times within the same day. Inter-day precision was evaluated by repeating the same procedure on three different days. Precision of the method was expressed in terms of relative standard deviation (RSD) (Guideline, I. H. T., 2005)[21].

Accuracy

Accuracy of the method was evaluated at three concentration levels corresponding to 80%, 100%, and 120% of the midpoint of the established linearity range. Known quantities of the drug were spiked into a pre-analyzed standard solution containing 3 µg/ml (representing 50% of the midpoint concentration of the linear range) and assayed the percentage of the spiked concentration the total drug content using the equation of the prepared calibration curve (Guideline, I. H. T., 2005)[21].

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) for the analyte drug using the proposed analytical method were established based on the calibration curve data as per ICH Guidelines Q2 (R1), 2005 (Guideline, I. H. T., 2005)[21] using the following equations-

$$\text{LOD} = 3.3\left(\frac{\text{SD}_{\text{Intercept}}}{\text{Slope}}\right) \text{----------2}$$

$$\text{LOQ} = 10\left(\frac{\text{SD}_{\text{Intercept}}}{\text{Slope}}\right) \text{----------3}$$

where the "$SD_{Intercept}$" is standard deviation of the intercept of the regression line on Y-Axix and the "Slope" is the slope of the prepared calibration curve.

RESULTS AND DISCUSSION

The UV absorption spectra of both pure and impure standard solutions of resveratrol displayed a distinct light absorption maximum at 306 nm, which was selected as the wavelength of maximum absorbance (λmax) for subsequent analysis. It was observed that the impurities were interacting with the absorbance throughout the scanning range. This was because either the dissolved impurities were absorbing the light in the scanning range or there was detected false absorbance due to the diffraction of light after being striking to the edges of the undissolved particles. So, to nullify this interaction, calibration curve (**Figure 1**) of resveratrol was prepared by plotting the corrected absorbance according to Equation 1 against respective concentration. The concept behind this is that this interaction of absorption of light in the UV-Visible region (i.e. 200 nm - 800 nm) is nullified according to Equation 4, provided concentration of the pure standard of the drug and that of the impure standard of the drug is same.

$$\left[\mathbf{A(\lambda max)^P} - \left(\frac{\mathbf{A(\lambda 1)^P + A(\lambda 2)^P}}{\mathbf{2}}\right)\right] = \left[\mathbf{A(\lambda max)^I} - \left(\frac{\mathbf{A(\lambda 1)^I + A(\lambda 2)^I}}{\mathbf{2}}\right)\right] \quad -----\mathbf{4}$$

where λmax is wavelength of maximum absorbance of resveratrol i.e. 306 nm; λ1 and λ2 are wavelengths of

absorbance of resveratrol equidistant on either side of its λmax i.e. 296 nm (λmax - 10) and 316 nm (λmax + 10) respectively; superscript P indicates pure standard and superscript I indicates impure standard.

Compliance with Beer–Lambert's law was observed over the concentration range of 3–9 µg/ml. The regression analysis yielded the equation Y = 0.004x + 0.0003, where Y and x denote absorbance and concentration respectively, with a correlation coefficient (r) of 0.9995 (**Table 1**).

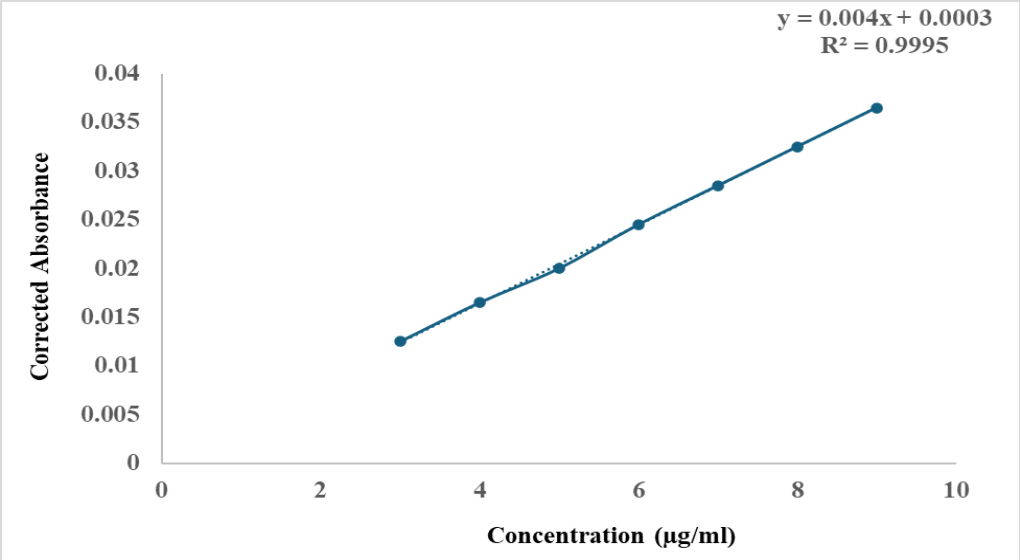

Figure 1: Calibration curve of resveratrol in water

Table 1: Regression parameters for calibration curve of resveratrol

No.	Parameter	Value
1	Analytical wavelengths (nm)	296, 306 and 316
2	Equation for corrected absorbance	$[A_{306} - (A_{296} + A_{316})/2]$
3	Linearity range (µg/ml)	3-9
4	Regression equation (Y = ax+b)[a] Slope (a) Intercept (b)	Y = 0.004x + 0.003 0.004 0.003
5	SD of intercept (n = 6)	6.32×10^{-5}
6	Correlation coefficient	0.9995

[a] Y= Absorbance and x= Concentration

Linearity was further confirmed by performing method of residual analysis, in which residual values were plotted against the predicted concentrations. The presence of a random distribution pattern in the residual plot i.e. residual amount versus concentration predicted supported the linear relationship (**Table 2** and **Figure 2**).

Table 2: Test for residuals

Predicted Concentration (µg/ml)	Observed Concentration (µg/ml)	Residual Amount (µg/ml)
3	2.98	0.02
4	4.01	-0.01
5	5.26	-0.26
6	5.93	0.07
7	6.99	0.01
8	7.89	0.11
9	9.24	-0.24

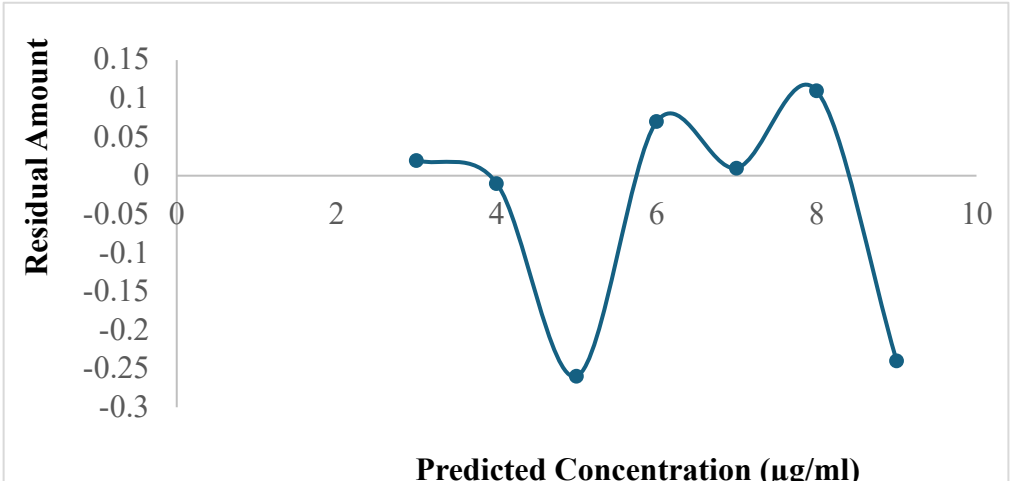

Figure 2: Residual amount v/s variable (concentration predicted) curve.

The specificity of the method was demonstrated by the minimal variation observed in corrected absorbance values between pure and impure resveratrol solutions at concentrations of 4.8, 6.0, and 7.2 µg/ml, with differences consistently below 1.88% (**Table 3**).

Table 3: Specificity studies for the developed analytical method for resveratrol

Concentration taken (µg/ml)	Corrected absorbance* for		Corrected absorbance difference (A_P-A_I)	%Corrected absorbance difference (w. r. t. A_P)
	Pure solution (A_P)	Impure solution# (A_I)		
4.8	0.0190±0.0001	0.0189±0.001	0.0001	0.52
6	0.0244±0.0001	0.0243±0.001	0.0001	0.41
7.2	0.0289±0.000	0.0290±0.001	-0.0001	-0.35

*Mean±SD of 6 replicate determinations, #Solution of resveratrol with added excipients

The method also exhibited a high degree of precision, as evidenced by relative standard deviation (RSD) values always below 1.99% for both pure and impure samples (Tables 4–6).

Table 4: Repeatability studies for the developed analytical method for resveratrol

Solution	Concentration of drug solution (µg/ml)								RSD
	Prepared	Found							
		1	2	3	4	5	6	Mean*	
Pure Solution	6	6.33	6.12	6.33	6.12	6.20	6.10	6.20±0.11	1.72
Impure# Solution	6	6.42	6.33	6.40	6.20	6.33	6.33	6.34±0.08	1.22

*Mean±SD, #Solution of resveratrol with added excipients, RSD- Relative Standard Deviation

Table 5: Intraday precision of developed analytical method for resveratrol

Solution	Concentration of drug solutions (µg/ml)					RSD
	Taken	Found				
		t1**	t2**	t3**	Mean*	
Pure Solution	**4.8**	4.76±0.12	4.79±0.13	4.78±0.11	4.78±0.09	1.88
	6.0	6.02±0.17	5.98±0.17	5.99±0.18	6.01±0.12	1.99
	7.2	7.20±0.14	7.17±0.17	7.21±0.17	7.19±0.13	1.81
Impure# Solution	**4.8**	4.81±0.07	4.82±0.07	4.81±0.08	4.81±0.08	1.66
	6.0	6.01±0.11	6.01±0.10	6.01±0.11	6.01±0.11	1.99
	7.2	7.19±0.15	7.20±0.12	7.21±0.11	7.20±0.13	1.67

*Mean±SD of 18 determinations (6 replicate determinations every time for 3 points of time in a day), **Mean±SD of 6 replicate determinations, #Solution of resveratrol with added excipients, RSD- Relative Standard Deviation

Table 6: Interday precision of developed analytical method for resveratrol

Solution	Concentration of drug solutions (µg/ml)					RSD
	Taken	Found				
		Day 1**	Day 2**	Day 3**	Mean*	
Pure Solution	**4.8**	4.76±0.12	4.78±0.12	4.77±0.14	4.77±0.09	1.88
	6.0	6.02±0.17	5.99±0.09	5.99±0.07	6.01±0.11	1.83
	7.2	7.20±0.14	7.18±0.11	7.20±0.13	7.19±0.13	1.81
Impure[#] Solution	**4.8**	4.81±0.07	4.80±0.07	4.80±0.08	4.81±0.07	1.46
	6.0	6.01±0.11	6.03±0.13	6.03±0.13	6.03±0.12	1.99
	7.2	7.19±0.15	7.20±0.13	7.20±0.12	7.20±0.14	1.94

*Mean±SD of 18 determinations (6 replicate determinations every time for 3 different days), **Mean±SD of 6 replicate determinations, [#]Solution of resveratrol with added excipients, RSD- Relative Standard Deviation

Accuracy was evaluated through recovery trials using standard addition method. The percentage recovery of known quantities of drug added to a pre-analysed sample ranged from 98.81% ± 1.54% to 103.00% ± 1.26% for the pure solution and from 99.33% ± 1.54% to 102.22% ± 2.11% for the impure solution (**Table 7**), indicating satisfactory accuracy of the method.

Table 7: Accuracy studies for the developed analytical method for resveratrol

C_s (µg/ml)	C_a (µg/ml)	Pure solution		Impure solution[#]	
		C_t* (µg/ml)	%Recovery*[†]	C_t* (µg/ml)	%Recovery*[†]
3	1.8	4.83±0.15	101.67±1.45	4.84±0.14	102.22±2.11
	3	6.09±0.15	103.00±1.26	5.98±0.15	99.33±1.54
	4.2	7.15±0.13	98.81±1.54	7.19±0.14	99.76±1.75

C_s= Concentration of standard solution, C_a= Concentration of sample solution added and C_t= Total concentration found, [†]%Recovery= $[(C_t-C_s)/C_a]x100$, *Mean±SD of 6 replicate determinations, [#]Solution of resveratrol with added excipients

The limit of detection (LOD) and limit of quantification (LOQ) for resveratrol using the developed method were determined to be 0.05 µg/ml and 0.16 µg/ml, respectively.

CONCLUSION

The developed analytical method was demonstrated to be easy, accurate, rapid, and dependable. It was validated consistent with the parameters defined in the ICH Q2(R1) guidelines (Guideline, I. H. T., 2005)[21], including specificity, linearity, limits of detection and quantification, intra-day and inter-day precision, and accuracy. The results obtained for all validation characteristics were within the acceptable criteria. Accordingly, the method is suitable for the determination of resveratrol in various pharmaceutical dosage forms as well as API, irrespective of the presence or absence of excipient-related impurities.

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ABBREVIATIONS

UV: Ultraviolet; **HPLC:** High performance liquid chromatography; **ACN:** Acetonitrile; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **$SD_{intercept}$:** Standard deviation of intercept of the calibration curve; **ICH:** International conference on harmonization; **SD:** Standard deviation; **RSD:** Relative standard deviation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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