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Research Article

A Rapid Reversed-Phase HPLC Method for Analysis of *Trans*-Resveratrol in PLGA Nanoparticulate Formulation

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A rapid reversed-phase high performance liquid chromatography (RP-HPLC) method was developed for the determination of *trans*-resveratrol (*t*-RVT) in PLGA nanoparticle formulation. A new formulation of *t*-RVT loaded PLGA nanoparticles (NPs) with potential stealth properties was prepared by nanoprecipitation method in our laboratory. The desired chromatographic separation was achieved on a Phenomenex C_{18} column under isocratic conditions using UV detection at 306 nm. The optimized mobile phase consisted of a mixture of methanol: 10 mM potassium dihydrogen phosphate buffer (pH 6.8): acetonitrile (63 : 30 : 7, v/v/v) at a flow rate of 1 mL/min. The linear regression analysis for the calibration curves showed a good linear correlation over the concentration range of 0.025–2.0 μg/ml, with determination coefficients, R^2, exceeding 0.9997. The method was shown to be specific, precise at the intraday and interday levels, as reflected by the relative standard deviation (RSD) values, lower than 5.0%, and accurate with bias not exceeding 15% and percentage recovery was found to be in the range between 94.5 and 101.2. The limits of detection and quantification were 0.002 and 0.007 μg/ml, respectively. The method was successfully applied for the determination of *t*-RVT encapsulation efficiency.

1. Introduction

Trans-resveratrol, *trans*-3,4′,5-trihydroxystilbene, (Figure 1) is a natural polyphenolic compound found in a variety of foods, nevertheless essentially in red grapes. It is a potent antioxidant and present mainly in the skin of grapes [1–3]. Small amount of *t*-RVT is also present in the seed and core of the grapes. *t*-RVT is also extracted from *Polygonum cuspidatum*, a plant found in China and Japan [4]. Resveratrol is existing as both *cis*- and *trans*-isomers, however; the *trans*-isomer is the most abundant and biologically active form. *t*-RVT has been suggested to possess cancer chemopreventive [5], antioxidative [6], antiplatelet, antifungal, cardioprotective [7], and HIV/AIDS properties [8]; however, the mechanism(s) of these proposed effects are not fully understood [9].

Results from pharmacokinetic studies indicate that the oral bioavailability of *t*-RVT is almost zero, which casts doubt on the physiological relevance of the high concentrations typically used for *in vitro* experiments [6]. However, its hydrosolubility of 3 mg/100 mL [10–12] makes it "practically insoluble" in water according to the European Pharmacopeia definition, and its log P is 3.1 [13]. Despite this poor water solubility, *t*-RVT exhibits high membrane permeability and can be considered a Class-II compound in the Biopharmaceutical Classification System [14]. *t*-RVT has a very short plasma half-life (~8–14 min). Its oral bioavailability is observed to be very low due to its rapid and extensive presystemic metabolism [15]. Polymeric nanoparticulate system for the encapsulation of *t*-RVT arises as a promising approach in a targeted drug delivery system (TDDS) [16].

In order to fully characterize the *t*-RVT PLGA nanoparticulate formulation, a suitable and validated method is required for a critical assessment of pharmaceutical parameters such as drug content. Literature review reveals that HPLC methods have been reported for the quantitation of *t*-RVT [17] and a few bioanalytical methods [5, 18] are also reported. However, until now, there have been no published reports about the quantitation of *t*-RVT by HPLC in a PLGA nanoparticulate formulation.

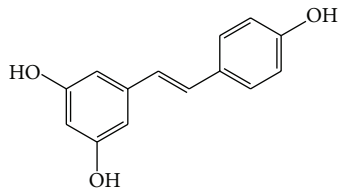

Figure 1: Chemical structures of *trans*-resveratrol.

The purpose of the present study was to develop and validate a simple and time-saving RP-HPLC method with UV detection for the determination of *t*-RVT. The validated method was applied to quantify the content of *t*-RVT incorporated into the PLGA nanoparticulate system after preparation.

2. Experimental

2.1. Materials and Reagents. *t*-RVT was provided exgratis by M/s Sami Labs (Bangalore, Karnataka, India). The purities of *t*-RVT were >99.5%. HPLC-grade methanol was purchased from M/s SD fine-chem limited (Mumbai, India). Poly(lactic-co-glycolic) acid in a 50 : 50 molar ratio (M.W 14, 500 Da) and an inherent viscosity of 0.53 dL/g (Resomer RG 504 H) received as gift sample from M/s Boehringer Ingelheim Pharma GmbH & Co. KG (Binger Str, Ingelheim, Germany). Tetradecyl trimethyl ammonium bromide was supplied exgratis by M/s SD Fine-Chem Limited, Mumbai. Acetic acid used was of analytical grade and was purchased from M/s Merck (Mumbai, India). Deionized water used in all the experiments was passed through a Milli-Q water purification system (18.2 MΩ/cm) Millipore (Bangalore, Karnataka, India).

2.2. Instrumentation and Chromatographic Conditions. The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with two LC-10 ATVP pumps, SPD-10AVP UV-vis detector, Rheodyne injector with a 50 μL loop. The chromatographic separation was performed on a Phenomenex C_{18} (250 mm × 4.6 mm I.D., 5 μm) column at 30°C. The results were acquired and processed using Shimadzu LC-solution version 6.42 software for data acquisition and processing. The mobile phase consisted of a mixture of methanol: 10 mM potassium dihydrogen phosphate buffer (pH 6.8): acetonitrile (63 : 30 : 7, v/v/v) at a constant flow rate of 1 mL/min. Chromatographic analysis was conducted in isocratic mode. The detection was carried out at 306 nm. An injection volume of 10 μL was used for all standards and samples.

2.3. Special Precautions. To avoid photochemical isomerization from *trans* to *cis* form, all analysis were carried out in dim light and were stored at room temperature and protected from light.

2.4. Preparation of Stock Solutions, Calibration Curve (CC), and Quality Control Samples (QC). Concentrated stock solution of *t*-RVT was prepared by dissolving 100 mg in 100 mL of mobile phase to give 1000 μg/mL stock solution. Two working standard solutions containing *t*-RVT at concentrations of 100 and 10 μg/mL were prepared by further dilution of each stock solution with the mobile phase. Eight standard solutions (0.025, 0.050, 0.075, 0.1, 0.5, 1.0, 1.5, and 2.0 μg/mL) were obtained by appropriate dilution of the working standard solutions with the mobile phase in the concentration range of 0.025–2.0 μg/mL. As quality control (QC), six replicates of 0.025, 0.50, 1.0, and 2.0 μg/mL standards containing the *t*-RVT were considered. For the determination of the limit of detection (LD) and limit of quantitation (LQ) of the method, six standard solutions, between 0.002 and 0.020 μg/mL, were obtained from the 10 μg/mL working solution. All stock solutions were stored at −20°C and working solutions were freshly prepared each day.

2.5. Method Validation. The HPLC method was validated according to the US Food and Drug Administration [19] regulations. The parameters studied for the validation included selectivity, linearity, precision and accuracy, limits of detection and quantitation, stability studies, and recovery.

2.6. System Suitability. Before sample analysis, the chromatographic system employed must pass the system suitability limits in command to assist the precision and accuracy of the developed HPLC method. Other chromatographic parameters, such as injection repeatability (RSD), capacity factor (k'), tailing factor (T), theoretical plates (N), and resolution (R), were also analyzed. The capacity factor is a measure of where the peak of interest is located with respect to the void volume, that is, it corresponds to the elution time of the nonretained components. The system suitability parameters were determined by injecting six times the standard solution containing *t*-RVT at a concentration of 1 μg/mL.

2.7. Limits of Detection and Quantification. The limits of detection and quantitation were determined based on a specific calibration curve obtained from four standard solutions (0.002, 0.010, 0.015, and 0.020 μg/mL) containing the *t*-RVT at concentrations in the closeness of these limits values. LOD and LOQ were calculated according to LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.8. Linearity. Calibration curves were constructed with eight standard solutions of *t*-RVT ranging from 0.025 to 2.0 μg/mL. Linearity was determined through the calculation of a regression line by the method of least squares, representing the peak area as a function of the standard concentration. Data gathered were scrutinized using the Microsoft Excel with linear regression by the least squares method.

2.9. Accuracy and Precision. Precision designates the closeness of agreement, that is, the degree of scatter between a series of measurements got from multiple sampling of the similar homogeneous sample and it was determined by repeatability (intraday) and intermediate precision (interday) for three consecutive days. Four standard solutions

(quality controls), 0.025, 0.50, 1.0, and 2.0 μg/mL, respectively, were prepared six times each and analyzed according to the proposed method (intraday precision) for three consecutive days (interday precision). The relative standard deviation (RSD) determined at each concentration level should not exceed 15%, except for the lower limit of quantitation, where it should not exceed 20% [20]. The accuracy of the method expresses the closeness of agreement between the true value and the value found. It was determined by measuring six replicates of the four quality controls and by calculating the percentage of bias for each compound according to the equation % accuracy = (observed concentration / nominal concentration) × 100. The mean value should be within 15% of the actual value, except at the LOQ, where it should not deviate by more than 20% [21–24].

2.10. Specificity. The specificity of a method may be defined as the ability to accurately measure the analyte in the presence of all potential sample components. In this method, the response of *t*-RVT in a nanoparticle formulation was used as a measure of its specificity.

2.11. Stability. The stability of *t*-RVT quality controls was assessed after a short-term storage at room temperature (~25°C) for 24 h, after a long-term storage for 10 days at −20°C, in order to simulate sample handling. The effect of three freeze–thaw cycles on the stability of the analytes was also investigated. QC samples were stored at −20°C for 24 h and thawed unassisted at room temperature and, when completely thawed, the samples were refrozen for 24 h under the same conditions until completion of the three cycles.

2.12. Recovery. The recovery of *t*-RVT from the nanoparticle supernatant was determined by comparing the respective concentrations with those of standard solutions in the mobile phase at three concentration levels 0.5, 1.0, and 2.0 μg/mL by repeated analysis ($n = 6$).

2.13. Method Applicability

2.13.1. Preparation of t-RVT NPs. The *t*-RVT NPs were prepared by nanoprecipitation technique using Ultra Turrax IKA T25 digital high shear homogenizer, followed by freeze drying. PLGA and drug (100 mg) was dissolved at predetermined ratio in acetone added dropwise to an aqueous solution rotating at 16,000 ×g containing Tetradecyl trimethyl ammonium bromide (TTAB) (200 mg) as emulgent. The resulting dispersion of NPs was vacuum evaporated to eliminate the organic solvent. The resulting NPs was centrifuged at 12,000 ×g for 1 h and washed with deionized water for three times and freeze-dried. The yield of the polymeric NPs was 90.56% with this protocol [25, 26].

2.13.2. Encapsulation Efficiency and Drug Loading. The drug encapsulation efficiency (EE) of *t*-RVT NPs was calculated by determining the amount of free drug using a filtration technique. The EE was determined by the separation of drug-loaded NPs from the aqueous medium containing nonassociated *t*-RVT by ultracentrifugation (REMI high speed, cooling centrifuge, REMI Corporation, India) at 12,000 ×g for 30 min, at 4°C. The unencapsulated *t*-RVT was determined using HPLC. The total drug content in the *t*-RVT NPs was determined by dissolving the *t*-RVT NPs in methanol to release trapped *t*-RVT. The resulting solution was analyzed using HPLC. The drug loading content was the ratio of incorporated drug to polymer (w/w).

EE and % drug loading, each determined in triplicate, were calculated as follows:

$$\text{EE (\%)} = \frac{\text{Amount of } t\text{-RVT in NPs}}{\text{Initial amount of } t\text{-RVT}} \times 100,$$
$$\text{\% Drug loading} = \frac{\text{Weight of drug in NPs}}{\text{Weight of NPs recovered}} \times 100. \quad (1)$$

Table 1: System suitability parameters.

Parameter	Compound	
	t-RVT	TTAB
Retention time (R)	3.26	5.89
Tailing factor (T)	1.23	1.75
Injection repeatability (RSD)	0.387	0.512
Resolution (R)	—	6.43
Capacity factor (K')	3.81	4.07
Theoretical plates (N)	2967	2519

3. Results and Discussion

3.1. Method Development and Optimization. A high performance liquid chromatography method for the estimation of *t*-RVT in a nanoparticulate dosage form has been developed according to the principles of Good Laboratory Practices. Optimization runs were carried out using a Phenomenex C_{18} column, by testing different proportions of methanol: 10 mM potassium dihydrogen phosphate buffer (pH 6.8): acetonitrile. Since *t*-RVT is poorly water soluble and more soluble in organic solvents, a higher percentage of methanol was considered to diminish the retention time. The optimized mobile phase was 63 : 30 : 7 (v/v/v) of methanol: 10 mM potassium dihydrogen phosphate buffer (pH 6.8): acetonitrile at a flow rate of 1 mL/min. Under these conditions, *t*-RVT eluted at 3.26 min. The method was validated over the range 0.025–2.0 μg/mL.

3.2. Method Validation

3.2.1. System Suitability Tests. To assure the feasibility and adequacy of the proposed method for estimation of *t*-RVT in routine pharmaceutical application and verify the resolution, column efficiency, and chromatographic repeatability system suitability tests were performed (Table 1). The capacity factor (k') was between 1 and 10, indicating good resolution with respect to the void volume. The RSD of peak areas of six consecutive injections was found to be less than 2%, thus showing good injection repeatability and excellent chromatographic and environmental conditions. The tailing factor (T) for the *t*-RVT was found to be close to 1, reflecting

Table 2: Intraday and interday precision and accuracy results for *t*-RVT ($n = 6$).

Nominal concentration (μg/mL)	Intraday ($n = 6$)			Interday ($n = 18$)		
	Measured concentration (μg/mL) Mean ± SD	Precision % RSD	Accuracy % bias	Measured concentration (μg/mL) Mean ± SD	Precision % RSD	Accuracy % bias
0.025	0.024 ± 0.001	4.16	−4	0.024 ± 0.001	4.16	−4
0.5	0.508 ± 0.016	3.14	1.6	0.502 ± 0.023	4.58	0.4
1.5	1.521 ± 0.011	0.72	1.33	1.51 ± 0.017	1.125	0.66
2.0	1.994 ± 0.025	1.25	−1	1.97 ± 0.034	1.72	−1.5

good peak symmetry. The resolution (R) between the peaks was found to be greater than 2, indicating good separation of the *t*-RVT. The values for theoretical plate number (N) demonstrated good column efficiency. Resolution between *t*-RVT and TTAB was 6.43.

3.2.2. Limits of Detection and Quantitation. The estimated LD for *t*-RVT was 0.002 μg/mL. The LQ found for *t*-RVT was 0.007 μg/mL.

3.2.3. Linearity. Linearity was evaluated over the concentration range 0.025–2.0 μg/mL for *t*-RVT, estimating the regression equation and the determination coefficient (R^2) obtained from the least squares method. The coefficients of determination for the calibration curves of the three compounds were higher than 0.9997, which is generally considered as evidence of an acceptable fit of the data to the regression line, indicating a good linearity over the concentration range proposed.

3.2.4. Accuracy and Precision. Accuracy and precision for the quality controls in the intraday and interday run are shown in Table 2. The intra- and interday RSD values did not exceed 5.0%. The intra- and interday bias values were found in the interval 0.4 to −4.0%. These data indicate that the developed method is accurate, reliable, and reproducible, because RSD nor exceeded 15%, which is in agreement with acceptance recommendations.

3.2.5. Specificity. The specificity of the method was analyzed in the presence of *t*-RVT and the supernatant of the NPs, containing polymer and emulgent (Figures 2 and 3, resp.). As shown, neither the nanoparticle content nor the supernatant exhibits peaks interfering with those of the analytes, thus indicating that the method is specific.

3.2.6. Stability. The stability data for *t*-RVT under conditions likely to be found during the analytical process and sample storage included short-term, long-term, and freeze–thaw stability analysis are gathered in Table 3. According to the results, it can be inferred that the analytes are stable under the studied conditions.

3.2.7. Recovery. The % recovery of *t*-RVT from the nanoparticle supernatant was comprised between 94.5 and 101.2 (Table 4). This indicates that the developed method is adequate to quantify the *t*-RVT.

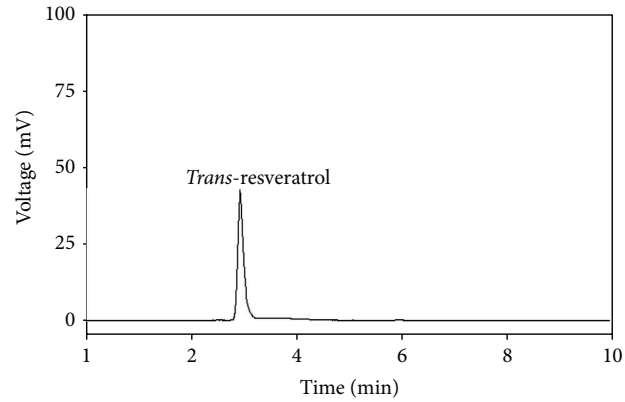

Figure 2: Chromatogram of *trans*-resveratrol.

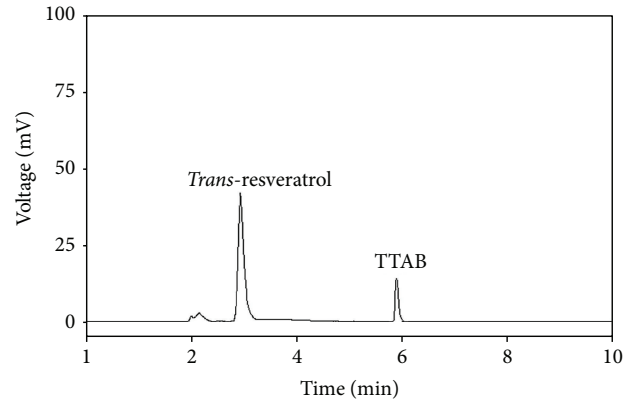

Figure 3: Chromatogram of nanoparticle supernatant.

3.3. Method Applicability. The method developed in this work was used to determine the content of *t*-RVT PLGA NPs with a mean particle size of 181 nm and a zeta potential of −28.41 mV. A total percentage yield of 97.35 for *t*-RVT was obtained from the nanoparticle dispersion. This yielded an EE of 73.62 ± 0.05% for *t*-RVT which corresponded to a drug loading of 91.34%. The results substantiate the suitability of the developed method for determining *t*-RVT concentration in nanoparticulate formulation.

Table 3: Short-term, long-term, and freeze-thaw stability of *t*-RVT quality controls ($n = 6$).

Stability conditions	Nominal concentration (μg/mL)			
	0.025	0.5	1.5	2.0
Short-term stability				
Mean determined concentration (μg/mL)	0.024	0.47	1.474	1.992
Accuracy (%)	96.0	94.6	98.26	99.6
Long-term stability				
Mean determined concentration (μg/mL)	0.023	0.482	1.469	1.967
Accuracy (%)	94.0	96.4	97.93	98.35
Freeze-thaw stability				
Mean determined concentration (μg/mL)	0.024	0.491	1.483	1.986
Accuracy (%)	96.0	98.2	98.87	99.3

Table 4: Percentage of recovery of *t*-RVT from the nanoparticle supernatant ($n = 6$).

	Nominal concentration (μg/mL)		
% Recovery	0.5	1.0	2.0
	101.6	97.1	94.5

4. Conclusion

A specific, linear, accurate, reliable, and reproducible new method for the quantitation of *t*-RVT was developed and fully validated over the range of 0.025–2.0 μg/mL. The method was successfully applied to measure the drug content in nanoparticulate formulation after preparation. The optimized nanoparticulate formulation with a mean particle size of 181 nm and a zeta potential of −28 mV renders encapsulation efficiency in excess of 73% for *t*-RVT, which indicates that these NPs are efficient carriers for encapsulation of poorly soluble drug *t*-RVT.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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