Original Article

Development, Optimisation and Validation of RP-HPLC Method for the Quantification of Resveratrol

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ABSTRACT

Background: Trans-resveratrol is a naturally occurring polyphenolic compound extensively marketed as a nutraceutical. It is highly explored molecule in the research community with vast therapeutic potential. Therefore, many inventions were made by pharma-industries for its clinical use and to bring it to the market as a drug. **Objectives:** In this study, a simple, rapid and sensitive Reverse Phase High Performance Liquid chromatographic (RP-HPLC) method for the quantitative determination of resveratrol was optimized and validated. **Materials and Methods:** HPLC system of Shimadzu Corporation, Japan, SPD-M201 model connected to Photo-diode Array was used in this study. Intek chromosol C_{18} column was used to perform the separation. The developed method was validated as per International Conference on Harmonization guidelines. The developed method is further used to estimate the % entrapment efficiency in the bone-targeted resveratrol loaded nanoparticles. **Results:** The optimum chromatographic separation was achieved by the mobile phase consist of acetonitrile and 25 mM ammonium acetate buffer (pH 4.5) in 45:55 ratios respectively. The flow rate of 0.9 mL/min with a standard retention time of 5.8 min at a wavelength 307 nm was optimized. The RP-HPLC method was developed, optimized and validated with the help of Design of Experiment software using 2^3 full factorial design. The desirability value was near to 1, specifies that the method is the ideal and robust. The method is further used to determine the % entrapment efficiency in the bone-targeted resveratrol loaded nanoparticles and found to be 79.27 ± 5.54%. **Conclusion:** The present analytical method can be used for the quantification of resveratrol in pharmaceutical preparations and dietary supplements.

Key words: HPLC, Nanoparticles, Optimization, Resveratrol, Validation.

INTRODUCTION

Trans-resveratrol (RSV) is natural polyphenol categorized chemically as a phytoalexin, have pharmacological potential and marketed as a nutraceutical for supplementing the treatment of diseases. It is approved as dietary supplements under the Federal Food Drug and Cosmetic Act, FDA, USA. Till today, it is not included as a drug for the treatment of any disorder in the United States Pharmacopeia but sold as a dietary supplement.[1] As per the US Food and Drug Administration (USFDA) regulations, RSV is categorized as a nutrient, has been determined to be Generally Recognized as Safe. The increasing number of clinical trials on RSV for cancer, neurological, cardiovascular and metabolic disorders such as diabetes and obesity signifies the growing interest of pharmaceutical industries for its translational use.[2] However, the clinical utility of RSV is limited, mainly due to its instability, inefficient systemic delivery and low bioavailability. To overcome these limitations, several attempts were made to develop an effective nano-formulations as polymeric nanoparticles, cyclodextrins, micelles and liposomes to improve bioavailability and subsequent stability.[3] The literature gives few quantitative HPLC methods for the estimation of RSV while some were associated with the detection system as HPLC-Mass detection (HPLC-MS), HPLC-UV and electrochemical to detect

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RSV in wines or plant extracts. It includes quantification of RSV in wines from Portugal,[4] RP-HPLC coupled with UV and electrochemical detection in wines,[5] HPLC-MS analysis of RSV in Hungarian wines;[6] HPLC using Chromolith columns;[7] microextraction method with Ultra-performance LC (UPLC) for RSV in wines[8] and quantitative analysis of cis- and trans-resveratrol in wines.[7] Vesna Jerkovic *et al.* had assessed RSV from Beer using RP-HPLC-MS/MS method.[9] Other methods for the analysis of RSV from the dietary supplements;[10] for products containing RSV capsules or multi-ingredient formulations along with other phytochemicals,[11] HPLC-MS method[12] and RP-HPLC method to quantify RSV in grape exocarp and seeds extracts.[13]

RSV is available in the commercial form with >99.0%. Purity. In this research work, the direct injection of a pure form of the RSV was used without prior purification or extraction method. The pure form of the RSV is used for the development of nano-formulations and in dietary supplements. There is an increasing number of RSV nano-formulations to enhance bioavailability, efficacy and achieve targetability. Therefore, a simple, sensitive analytical HPLC method is necessary to explore RSV nano-formulation research at the laboratory scale. RSV containing dietary supplements gives a greater amount of RSV than present in wines. Pure RSV and RSV containing multi-ingredient formulations are extensively used by the customers as a nutraceutical for health-promoting effects. According to the NIH- Dietary Supplement Label Database, there were 207 RSV containing dietary supplements are available.[14] Most of these dietary supplements are not compliance with the Good Manufacturing Practices (GMP) European laws requirements (95–105% content). A study conducted by Rossi *et al.* 2012, only five out of 14 brands of RSV formulations were passed the GMP criteria.[12] Therefore, an analytical method for RSV containing products needs to be validated and provide a reliable solution to ensure the correct identity, strength, quality, purity and potency. Thus, it was decided to develop a rapid and economic RP-HPLC method which was based on PDA detection for the quantification in the nano-formulation. Systematic validation of the method is essential to achieve the reproducibility and robustness to quantify RSV.

In the present work, the HPLC method has been validated with the help of DOE (Design of Experiment) software to achieve the optimum solution according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines ICH Q2(R1) guidelines.[15]

MATERIALS AND METHODS

Instrumentation and Chemicals

HPLC system of Shimadzu Corporation, Japan, SPD-M201 model connected to PDA (Photo-diode Array), column-oven and auto-sampler was used in this study. Chromatograms were recorded by a computer-aided LC solution 5.57 software. Intek chromosol C_{18} column (250 mm x 4.6 mm id) was used to perform the separation along with guard column. The pH of the buffer was measured using systronics micro-pH system 361, with a glass electrode. Milli-Q from the direct-Q_3 water purification system milli-pore corporation, USA was used. The Design of Experiments (DOE) run was performed by Design-Expert® software, 9.0.4.1 version (Stat-Ease, Inc. Minneapolis). Trans-resveratrol (>99.0%) was purchased from TCI Chemicals, India. The HPLC-grade solvents were procured from Merck Limited, India.

Mobile phase preparation

The mobile phase used for the study is consist of acetonitrile and 25 mM ammonium acetate buffer pH 4.5 in the ration of 45:55. The pH adjusted with acetic acid / ammonium hydroxide solution.

Preparation of calibration standards

A standard stock solution of RSV (1000 µg/mL) was prepared by dissolving in methanol. Further working stocks solutions ranging from 0.1-10 µg/mL were prepared by diluting with methanol.

Development of HPLC method and optimization

The RSV is a strong acid and has three acidic dissociation constants (pKa1= 8.8; pKa2 = 9.8; pKa3 = 11.4).[16] Three different ammonium acetate solution of pH 4.3, 4.5 and 4.7 were considered for DOE runs. The best resolution of the acidic drug was found at the pH 4.5 in a reversed-phase system. Pilot studies were carried out to get separation by using different mobile phase compositions. These buffers included methanol: phosphate buffer pH 6.8; methanol: ammonium acetate buffer pH 4.5; acetonitrile: phosphate buffer, pH 6.8. After several attempts, the best separation of RSV was obtained by isocratic elution with the mixture of acetonitrile and 25 mM ammonium acetate buffer (pH adjusted to 4.5±0.05 with acetic acid/ ammonium hydroxide solution) in the ratio of 45: 55 v/v as mobile phase and Intek Chromasol C_{18} column (250 mm x 4.6 mm id) column as a stationary phase. The flow rate of 0.9 mL/min, injection volume 20 µL with a run time of 10 min was set. All the chromatograms were extracted at λ_{max} for RSV, 307 nm. The method was selected by considering the factors as short run time; the asymmetry factor

Table 1: Independent Factors and levels used for the 2^3 Full Factorial design

Independent Factors	Levels	1
Acetonitrile content (%)	42	48
pH of Ammonium acetate buffer solution	4.3	4.7
Flow rate of mobile phase (mL/min)	0.8	1

closes to 1.0. The system suitability parameters such as percentage Relative Standard Deviation (%RSD), theoretical plate count and tailing factor at 10% peak width ($Tf_{10\%}$) were calculated using chromatographic data software. The acetonitrile content (%), pH of ammonium acetate buffer solution and flow rate of mobile phase (mL/min) plays an essential role in the development of the method. Hence, these three factors were considered and optimized with the DOE software as indicated in Table 1.

Robustness studies express to provide consistent results within the changes in the method parameters. The variables were acetonitrile content (45±3%), pH of ammonium acetate buffer solution (4.5±0.2) and flow rate of mobile phase (0.9±0.2 mL/min) used in a multivariate approach (Table 1). The 2^3 full factorial design with two levels as -1 and +1 to yield a total of 08 runs to detect any changes in method responses like retention time, peak area, $Tf_{10\%}$ and a total number of theoretical plates were analyzed by DOE software.

Validation of the optimized method

The method was validated as per the requirements ICH Q2(R1) guidelines by determining parameters as System Suitability, Specificity, Linearity, Accuracy, Precision, Limit of Detection (LOD) and Limit of Quantification (LOQ).

System Suitability

To assess the suitability of instrument, analysts and columns, RSV analyte concentration of 1 µg /mL solution (n=6) was used and system suitability parameters were evaluated.

System Specificity

The interference from the solvent with the mobile phase was assessed by injecting methanol as a blank (n=3).

Linearity

The RSV working stocks solutions ranging from 0.1–10 µg/mL were injected thrice into HPLC System. The coefficient of determination (R^2) was determined from the calibration plot of concentration against the peak area.

Accuracy and Precision

The accuracy was established by determining recovery (%) of RSV at three different concentrations as 0.1, 1 and 10 µg/mL (n=3), whereas precision studies were based on the robustness of the study with inter-day (within a day) and intra-day (two different days) sampling. Precision studies were carried at RSV concentration 10 µg/mL (n=6).

Limit of detection and limit of quantification (LOD and LOQ)

The Limit of Detection (LOD) and the Limit of Quantification (LOQ) was calculated as LOD = 3.3r/SP and LOQ = 10r/SP, where r is the least standard deviation value in response and SP is the slope of the response.

Applicability of the method in PLGA-nanoparticles

In this work, RSV was loaded into Polylactic-co-glycolic Acid (PLGA) nanoparticles to target bone using Alendronate (ALN) as a targeting ligand. The PLGA-Alendronate conjugate was synthesized as per the reported method.[17] The amount of RSV to the conjugate ratio was optimized to get optimum particles size, polydispersity index and zeta potential. The nano-formulation was centrifuged at 13,000 rpm for 20 min. The amount of RSV present in the supernatant was calculated by the standard RSV calibration curve. The %Entrapment Efficiency (%EE) was calculated by the indirect method using the formula $\%EE = [(C_i - C_f)/ C_i] \times 100$, Where C_i = Initial amount of the RSV added, C_f = free non-entrapped RSV content in the supernatant.

RESULTS

Development and optimization of HPLC method

In this study, as per 2^3 full factorial design, a total of 08 runs were performed. The effects of independent factors as acetonitrile content (A), pH of ammonium acetate buffer solution (B) and flow rate of mobile phase (mL/min) (C) on the responses as retention time, area, $Tf_{10\%}$ and number of theoretical plates were as shown in Table 2.

Perturbation plot interpretations

The Design Expert version 9.0.3.1 provided perturbation plots interpretations to check the effects of independent factors as acetonitrile content (A), pH of ammonium acetate buffer solution (B) and flow rate of mobile

phase (mL/min) (C) on the responses as retention time (R1), area (R2), $Tf_{10\%}$ (R3) and number of theoretical plate (R4) as indicated in Figure 1.

Effect of factors on retention time

Perturbation chart showed that retention time is significantly influenced by changing the acetonitrile content (A) and the flow rate of mobile phase (C). The retention time is decreasing, with the increase in factors A and C while it is not affected by the changes in the pH of the mobile phase (B) as shown in Figure 1(1).

Effect of factors on peak area

Peak area of RSV is significantly influenced by the change in the flow rate of the mobile phase (C). Peak area reduced with an increase in flow rate, while it remains unaffected by acetonitrile content (A) and pH of the mobile phase (B), as shown in Figure 1(2).

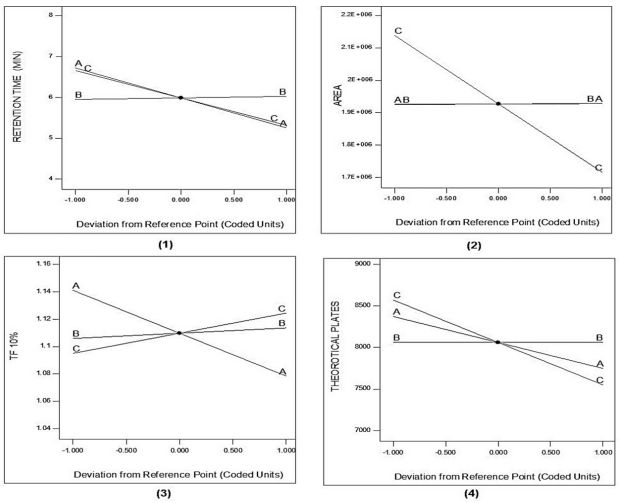

Figure 1: Effect of factors Acetonitrile Content (A), pH of Ammonium Acetate buffer solution (B) and flow rate of mobile phase (mL/min) (C) on (1) Retention Time (2) Area (3) $Tf_{(10\%)}$ and (4) Number of Theoretical Plates.

Effect of factors on Tailing Factor$_{10\%}$

The $Tf_{10\%}$ was significantly influenced by changing the factor A, acetonitrile content (A) and the flow rate of the mobile phase (C). $Tf_{10\%}$ is decreased with an increase in acetonitrile content while increased with the flow rate of the mobile phase. The pH of the mobile phase (B) did not have a significant effect on $Tf_{10\%}$ as mentioned in Figure 1(3).

Effect of factors on Theoretical Plates

Figure1(4) defined the decrease in theoretical plate count with an increase in acetonitrile content (A) and the flow rate of the mobile phase (C) and unaffected with the changes in the pH of the mobile phase (B).

Analysis of Robustness Study

The polynomial equations corresponding to the responses were generated by DOE software and analyzed by ANOVA with significance method. The polynomial equations for each response were given below:

Retention Time (R1) = 5.98687 + -0.733208* A + 0.0377083* B + -0.668708* C + -0.0300417 * AB + 0.0655417 * AC + -0.0322083 * BC

Peak Area (R2) =1.92701e+006 + 840.25 * A + 2052.33 *B + -210661* C + 2382.5* AB + -711.25 * AC + -2555.67 * BC

$Tf_{10\%}$ (R3) = 1.10975 + -0.03125 * A + 0.00383333 * B + 0.0146667 * C + 0.00233333 * AB + 0.00633333 * AC

Theoretical Plate Count (R4) = 8059.98 + -311.999 * A + -510.21 * C + -130.208 * AB + -82.9635 * ABC

Where A, B and C are acetonitrile Content, pH of buffer solution and flow rate of mobile phase (mL/min). The correlation Coefficient (R^2), level of significance (ρ value) and relative errors were calculated by DOE software. (Table 3). The robustness studies express the consistent results and the suitability of the method at 10 µg/mL analytes concentration. Therefore, it was

Table 2: DOE run results for RSV analyte concentration 10 µg/mL (*n*=3)

Run	% Acetonitrile	pH of Ammonium acetate solution	Flow Rate	Retention Time	Peak Area	$Tf_{(10\%)}$	No. of theoretical plate
1	42	4.3	0.8	7.36	2135101	1.13167	8929.48
2	48	4.3	0.8	5.81	2131014	1.04967	8257.07
3	42	4.7	0.8	7.55	2137127	1.13367	8977.8
4	48	4.7	0.8	5.9	2147420	1.06533	8116.41
5	42	4.3	1	5.94	1717889	1.14733	7439.61
6	48	4.3	1	4.68	1715807	1.095	7384.86
7	42	4.7	1	6.03	1714542	1.15133	8141.02
8	48	4.7	1	4.62	1717140	1.104	7233.58

considered realistic for routine usage in any laboratory. Results from the analysis of robustness studies found that the quadratic effect of factors and the responses were significant (ρ <0.05). The regression coefficient (r^2) close-to 1 and low relative error was shown that DOE runs were statistically significant.

In Figure 2, the desirability of the optimized factors was found to be 0.9532, which shows the aptness of this method. The desirability values near to the 1 indicate that the method was very strong even in the deviation of the factors.

Validation of the optimized method

System Suitability

System Suitability data were expressed as percentage standard deviations of peak area, Peak Area, Tailing F (10%) and total no. of theoretical plate count. All the parameters were within the acceptance criteria (Table 4a).

System specificity

As RSV is dissolved in methanol, methanol is used as a blank and run has been carried out in an optimized chromatographic condition. It represents the specificity of the chromatogram of RSV from blank methanol. The overlay chromatogram of blank methanol and RSV at different concentration is as shown in Figure 3. The retention time of RSV with the optimized method was found to be 5.8 min.

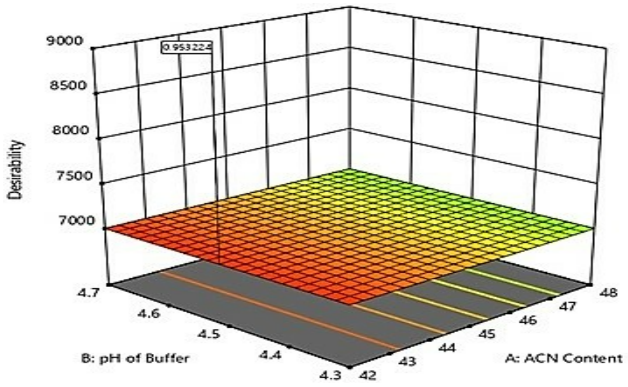

Figure 2: The 3-D plot is indicating the desirability of optimization of factors.

Linearity

The calibration plot of peak area against the RSV concentration in the range of 0.1–10 µg/mL was found to be linear. The coefficient of determination (R^2) = 0.9989 and linear regression slope was y = 208908x – 7378.6 (Figure 3).

Intra-day and Inter-day accuracy and precision

For the Intra-day and Inter-day accuracy estimations, RSV analyte concentration at three different levels as such as 0.1, 1 and 10 were used. Intra-day and Inter-day accuracy results lie in the acceptance criteria of 80-120 %. For intra-day and inter-day precision studies, RSV at 10 µg/mL (n=6) was injected and the precision was found to be below 2 (Table 4 b and 4c).

Limit of Detection and Limit of Quantification (LOD and LOQ)

LOD, the lowest detectable concentration by HPLC was found to be 0.28 µg/mL while LOQ, the lowest concentration that could be quantified was found to be 0.87 µg/mL.

Applicability of the method for the quantification of RSV in the nano-formulation

An optimized formulation had particle Size 196.9 nm, with polydispersity index 0.26 and zeta potential was found to be -64.21 mV. The % entrapment efficiency of the formulation was found to be 79.27±5.54%. The overlay chromatogram of PLGA-alendronate blank nanoparticles and RSV loaded PLGA-Alendronate nanoparticles is as indicated in Figure 4.

DISCUSSION

Most of the analytical methods reported in the literature were for the quantification of RSV in plant extract and wines. The RSV is increasingly gaining its importance both as nutraceuticals and pharmaceutical formulations and hence, it is essential to have a reproducible analytical method. In the current study, we have developed a RP-HPLC method for RSV and further validated by the various parameters under ICH Q2(R1) guidelines. The 2^3 Full factorial design using DOE software

Table 3: Analysis of robustness study

	R^2	Model *P*-value	Predicted	Observed	Relative Error
Retention Time (R1)	1	0.013 **	5.98	5.87	1.344
Peak Area (R2)	1	0.0108 **	1927005.08	1600440.66	16.94
$Tf_{10\%}$ (R3)	0.9988	0.0030 **	1.1	1.12	1.818
Theoretical Plate Count(R4)	0.9626	0.0177	8059.08	8248.44	2.349

Table 4: Method validation results as per ICH guidelines with acceptance criteria

Table 4a - System suitability test using RSV concentration 10 µg/mL

Parameters	Mean		SD	% RSD	Acceptance criteria
Peak Area	141504.16		1212.64	0.85	% RSD < 2.5
$Tf_{10\%}$	1.12		0.007	0.62	% RSD < 2.5
Total theoretical plate count	8139.93		32.39	0.39	Total no.> 2000

Table 4b - Intra-day and inter-day accuracy (RSV conc. 0.1, 1, 10)

Initial conc. (µg/ mL)	Intra-Day		Inter-day		Acceptance criteria
	Observed conc. (µg/ mL)	Mean Recovery (%)	Observed conc. (µg/ mL)	Mean Recovery (%)	
0.1	0.12	120	0.12	120	80-120%
1	1.02	102	0.8	80	
10	9.78	9.78	10.3	103	

Table 4c - Intra-day and inter-day precision (RSV conc. =10 µg/mL)

Parameters	Intra-day			Inter-day			Acceptance criteria
	Mean	SD	% RSD	Mean	SD	% RSD	
Peak Area	1534791.8	9765.79	0.63	1600440.66	9287.95	0.58	% RSD
							< 2.5
$Tf_{10\%}$	1.124	0.006	0.55	1.12	0.000	0.03	% RSD
							< 2.5%
Total Theoretical Plate	8157.78	34.98	0.42	8248.44	70.77	0.85	> 2000

RSD -Relative Standard Deviation; $Tf_{10\%}$ - tailing factor at 10% peak;

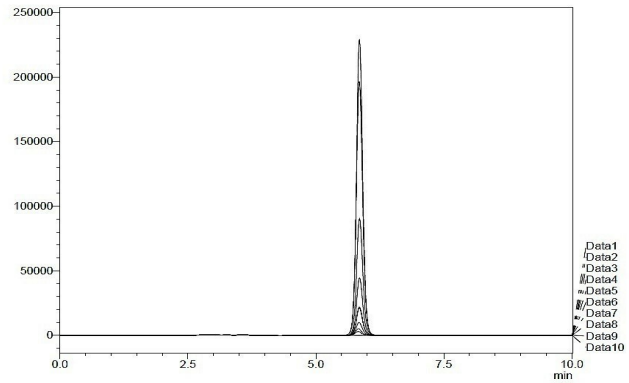

Figure 3: Overlay HPLC Chromatogram of RSV. Overlay data 1: Blank Methanol; 2: RSV 0.1 µg/mL; 3: RSV 0.2 µg/mL; 4: RSV 0.4 µg/mL; 5: RSV 0.8 µg/mL; 6: RSV 1 µg/mL; 7: RSV 2 µg/mL; 8: RSV 4 µg/mL; 9: RSV 8 µg/mL; 10: RSV 10 µg/mL.

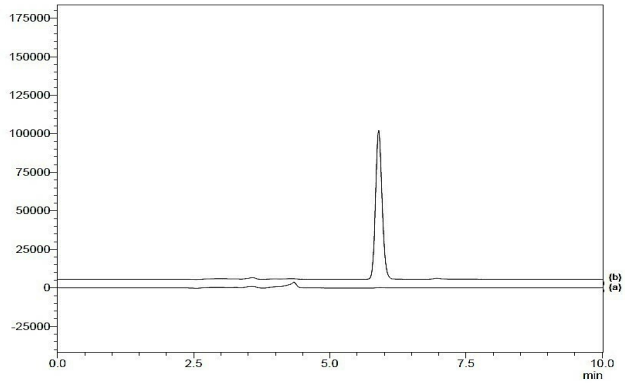

Figure 4: HPLC Chromatogram of (a) PLGA-ALN blank nanoparticles and (b) and the supernatant of RSV loaded PLGA-Alendronate nanoparticles.

defines the robustness of the method. This software has been used earlier for the development and validation of an analytical method for pharmaceuticals and nutraceuticals.[18] The desirable value was near to 1, indicates that the developed method is ideal. Further, the developed analytical method was employed to calculate RSV content and subsequent %EE in the bone-targeted RSV nano-formulation. The bone-targeted RSV loaded PLGA nanoparticles were developed using Alendronate as targeting ligand. The nano-formulation intended to prolong circulation, half-life and thereby increasing the pharmacokinetic profile of RSV with improved efficacy. The optimized RP-HPLC method was consistent and reproducible for the quantification of RSV in the developed formulation. Therefore, it is concluded that this

analytical method can be used to estimate RSV content in various nano-formulations and dosage forms.

The present work can be extended to estimate RSV in plasma and other biological fluids for pharmacokinetic profiling associated with the various formulations. With the increasing potential for RSV containing nutraceuticals, the present method is found to be statistically significant for the analysis of RSV in dietary supplements. It leads to fulfil the GMP criteria for the development of RSV containing dietary supplements and to match the "labelled claim" content.

CONCLUSION

An RP-HPLC method was successfully validated for the quantitative determination of RSV. The following method is under the acceptance criteria and statistically found to be significant. Thus, this method can be used for the quantification of RSV in nano-formulation development and to maintain quality control of RSV containing nutraceuticals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RSV: Trans-resveratrol; **RP-HPLC:** Reverse Phase High-Performance Liquid Chromatography; **FDCA:** Food Drug and Cosmetic Act; **USFDA:** United State Food and Drug Administration; **GMP:** Good Manufacturing Practices; **DOE:** Design of Experiment; **PDA:** Photo-diode Array; **%RSD:** Percent Relative Standard Deviation; **LOD:** Limit of detection; **LOQ:** Limit of Quantification; **PLGA:** Polylactic-Co-Glycolic Acid; **ALN:** Alendronate; **%EE:** %Entrapment Efficiency.

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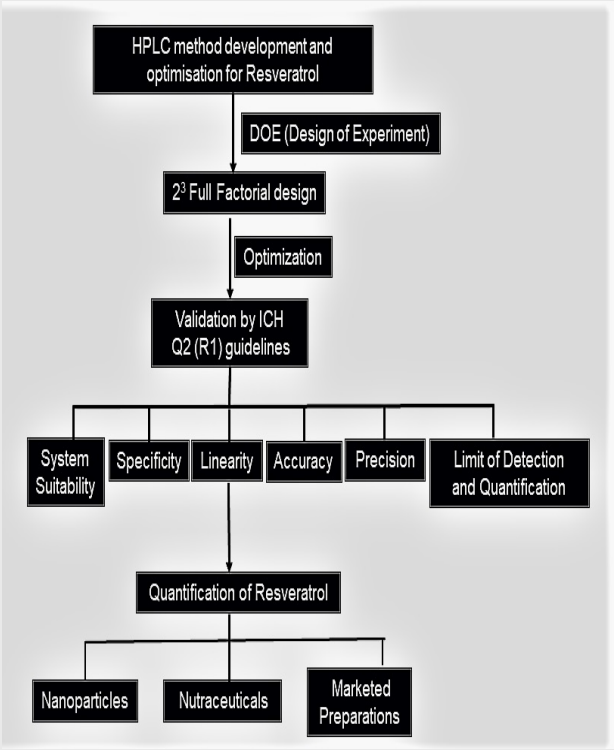
PICTORIAL ABSTRACT



SUMMARY

An RP-HPLC method was applied for the quantification of Resveratrol in bone-targeted drug delivery system. It can also be used in various marketed RSV containing nutraceuticals maintain quality control as per regulatory requirement.

About Authors

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