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Research Article

METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF RESVERATROL AND GALLIC ACID BY RP-HPLC

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ABSTRACT

In this study, a simple, fast, accurate, precise, reproducible, reverse phase high performance liquid chromatographic method have been developed and validated for the simultaneous estimation of Resveratrol and Gallic acid in pure drug form. The chromatography was carried out on a shimadzu LC-2010 ATVP prominence liquid chromatograph and using shimadzu SPD-10AVP UV-Visible detector, an C-18, Nucleodur C_{18} having dimensions 5µ (250×4.6 mm) column used as stationary phase, with a mobile phase consisting of Phosphate buffer pH 3 ±0.02 pH adjusted with ortho phosphoric acid and methanol and acetonitrile (50: 30: 20 v/v) at a flow rate of 1 ml/min and detection wavelength of Resveratrol and Gallic acid at 306 nm and 263 nm, respectively. The retention time of resveratrol was found to be 4.75 minute and retention time of gallic acid was 2.15 minute respectively. The proposed method has been validated for accuracy, precision, linearity, robustness and range were within the acceptance limit according to International Conference on Harmonisation (ICH) guidelines. The method was found to be linear with r^2 value of 0.998 for gallic acid and 0.999 for resveratrol. The calculated limit of detection (LOD) values were 0.689207 and 0.130961 µg/mL and limit of quantitation (LOQ) values were 2.088505 and 0.39685 µg/mL for resveratrol and gallic acid correspondingly. The developed method can be successfully employed for the routine analysis of Resveratrol and Gallic acid in API and Pharmaceutical dosage forms. **Objective:** The objective of present work was method development and validation of RP-HPLC for estimation of Resveratrol and Gallic acid.

Keywords: Resveratrol, Gallic acid and RP-HPLC.

INTRODUCTION

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a stilbenoid (Fig. 1a), a type of natural phenol, and a phytoalexin produced by several plants in response to injury or, when the plant is under attack by pathogens such as bacteria or fungi. Most resveratrol capsules sold in the U.S. contain extracts from an Asian plant called *Polygonum cuspidatum*. It is used in the treatment of cancer, heart diseases, Alzheimer and diabetes. Resveratrol can inhibit the phosphatidylinositol 3-kinase (P13K)/Akt pathway to regulate cell differentiation, growth, proliferation, and several other activities[12-14]. Gallic acid is a trihydroxybenzoic acid (Fig. 1b), a type of phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants. It is found both free and as part of hydrolysable tannins. It possesses various pharmacological activities like anti-cancer, anti-microbial, anti-fungal, antioxidant and anti-inflammatory. It has pro-oxidant property in concentration dependent manner which induces the apoptotic signaling pathway in cancerous cells. Moreover, it has been revealed that matrix metallo proteinase-2 (MMP-2) and MMP-9 proteolytic activities were inhibited *via* gallic acid[15-17].

The combination of these two drugs is not official in any pharmacopoeia; hence no official method is available for their simultaneous estimation. The aim of the present work was to develop simple, precise, accurate and reproducible RP-HPLC method for simultaneous determination of resveratrol and gallic acid. The proposed method was optimized and validated in accordance with ICH guidelines.

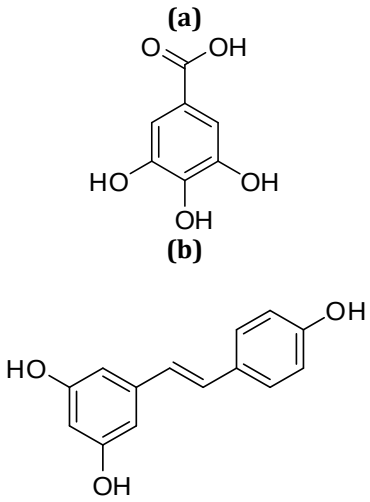

Fig. 1: Chemical structure
(a) Gallic acid (b) Resveratrol

MATERIALS AND METHODS

CHEMICAL AND REAGENTS

The drug Resveratrol (RES) was gifted from Lupin laboratories [Aurangabad, Bihar] and Gallic acid was procured as a gift sample from Symbiosis pharmaceuticals private limited [Sirmor, H.P.]. Acetonitrile [HPLC grade] and triethylamine were purchased from Thermo fisher scientific india private limited [Mumbai] and glacial acetic acid purchased from Fischer scientific [Mumbai].

APPARATUS

Absorbance measurements were made on shimadzu UV-1800 UV/Visible spectrophotometer with 10 mm matched quartz cells. Chromatography was performed on shimadzu HPLC equipped with LC-10 ATVP pumps, SPD-10AVP UV-Visible detectors and rheodyne injector with a 50µL loop. Separation was performed on Nucleodur C_{18} column having dimensions 5µ (250×4.6 mm). Data acquisition and integration were performed using LC-solution version 6.42 software.

MATERIALS AND METHODS

Chromatographic conditions

The method development for analysis of resveratrol and gallic acid was performed using various solvents. Final separation was achieved using a mobile phase consisting of buffer: methanol: acetonitrile (50: 30: 20 v/v) pumped at a flow rate of 1 ml/min. The eluent was monitored using a shimadzu SPD-10AVP UV-Visible detector at a wavelength of 263 and 306 nm for gallic acid and resveratrol respectively. The mobile phase was vacuum filtered through 0.22 µm nylon membrane filter followed by degassing in an ultrasonic bath prior to use.

Preparation of solutions

Preparation of buffer solution

The buffer solution was prepared by dissolving 7.0g of potassium di hydrogen ortho phosphate in 1000ml of HPLC grade water and pH 3.0 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator.

Preparation of blank solution

Acetonitrile and methanol in ratio of 50: 50 was used as blank solution.

Preparation of standard solution

The quantity of powder equivalent to 10 mg of resveratrol and gallic acid were weighed and transferred into 10 ml volumetric flask , 5 ml of diluent was added and sonicated for 15 minutes and the volume was made upto the mark with diluent. From this further dilution was made to get the final concentrations of resveratrol and gallic acid.

Validation of the developed method

The proposed analytical method was validated for system suitability, linearity and range, precision, limit of detection [LOD], limit of quantitation [LOQ] and accuracy in accordance with International Conference on Harmonisation (ICH) guidelines for analytical procedures Q2[R1][1-2]

System suitability

System suitability factors were studied to verify the system performance study. Six replicate samples of both the drugs of concentration 10 µg/ml were analysed using the developed method. Various parameters such as theoretical plate count, tailing factor, percent relative standard deviation [%RSD] of peak area and retention/ capacity factor were taken into consideration for testing system suitability.

Specificity

Specificity is the ability of a method to discriminate the analyte response in the presence of other drugs, excipients, degradants and their potential impurities. In addition, specificity to show that there is no- elution of key analytes is demonstrated by a peak purity assessment using PDA or MS, or by comparing the results of the sample to those obtained by a second well characterized technique.

Accuracy and precision

To determine the precision, triplicate injections of selected concentrations were analyzed, and the values of relative standard deviations (%RSD) were calculated. In order to demonstrate applicability and accuracy of the

proposed method, recovery tests are also carried out by analyzing the synthetic mixtures of the resveratrol and gallic acid. Parameters are concerned with the repeatability of method of a same concentration at different time periods, the recoveries from these sample mixtures were calculated for each compound.

Linearity

Linearity of the method was established by triplicate injections of the solutions containing the drugs in the range of 1-10 µg/ml for resveratrol and gallic acid separately at their λ_{max} 306 and 263 nm. Resveratrol and Gallic acid, standard area was plotted against the concentration. The calibration curves were constructed and the acceptable fit to the linear regression was demonstrated and reported by the necessary parameters like regression coefficient, correlation coefficient, standard deviation and mean.

LOD and LOQ

Limit of detection and limit of quantification of the developed method were calculated from the standard deviation of the y-intercepts and slope of the calibration curve of resveratrol and gallic acid using the following formula:

Limit of detection = 3.3 σ/S

Limit of quantitation = 10 σ/S

Where σ - Standard deviation (SD) of the y-intercepts

S – Slope of the calibration curve

Robustness and Ruggedness

Robustness is a measure of the performance of a method when small, deliberate changes are made to the specified method parameters such as in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions *i.e.* different analysts (*i.e.* from analyst to analyst from, laboratory to laboratory). Acceptance criteria for ruggedness, the Percentage relative standard deviation for the area of five standard injections should not be more than 2%.

Method validation

System suitability

The developed method has produced theoretical plate above 2000 for resveratrol and gallic acid with tailing factor less than 2. Similarly, the percent relative standard deviation [%RSD] of peak area and retention time of resveratrol and gallic acid were less than 2, which ensure the suitability of the developed method. The results of the system suitability study were summarised in Table 2 and Fig.

RESULTS AND DISCUSSION

Method development and Optimization of the method

Method development for resveratrol and gallic acid was started with a different combination of solvents with different ratios. Several trials were conducted with different isocratic or gradient mobile phases. However, finally a combination of methanol: buffer: acetonitrile 30:50:20 v/v has shown good resolution for resveratrol and gallic acid. To get a adequate separation increase and decrease in the flow rate was applied and finally 1.0 ml/min shows good resolution between the peaks of resveratrol and gallic acid.

Chromatographic conditions

The analytical conditions were selected, keeping in mind the chemical nature of resveratrol and gallic acid. Several trails were taken using different conditions for the method development. Column selection has been done on the basis of back pressure, peak shape, theoretical plates and day-to-day reproducibility of the retention time. After evaluating all these factors, the chromatographic separation was carried out on Nucleodur C_{18} column having dimensions 5µ (250×4.6 mm) using a mobile phase methanol: buffer: acetonitrile in the ratio of 30:50:20 v/v, the flow rate 1.0 ml/min and the injection volume was 20 µl, the detection was carried out at 263 and 306 nm for gallic acid and resveratrol. The peak retention time of resveratrol and gallic acid were found to be 4.75 min and 2.15 min respectively. Hence this method was finalised as an optimized method for the estimation of resveratrol and gallic acid. The optimised chromatographic condition was shown in table 1.

Specificity

Study was performed by injecting blank. Specificity was carried out to determine whether there is any interference of any excipients and impurities in retention time of analytical peak. Specificity chromatogram was shown in Fig. **3**, where the retention time of resveratrol does not interfere with the retention time of the gallic acid.

Accuracy

The percentage recovery of the sample was within 100±2% which ensures the accuracy of the developed method. Accuracy was assessed by using a minimum of three different

concentrations of standards. Results of recovery studies were summarised in Table 3

Precision

The developed method has shown percent relative standard deviation [% RSD] less than 2 for intra-day and inter-day precision study, which ensures precision of the developed method. The %RSD was found to be within the specified limits. Results of the precision study were summarised in Table 4 and 5.

Linearity

The linearity was established by the least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 1-10 µg/ml for both the drugs. Standard areas of resveratrol and gallic acid were plotted against the concentration in the mobile phase, and linear regression analysis performed on the resultant curves and was confirmed by the high value of the correlation coefficients 0.998 for both drugs.

Results of resveratrol and gallic acid were summarised in Table 6.

Robustness

As per ICH, the prepared solution was analysed as per the proposed method with a slight variations in optimized conditions. The variations made were ±5 ºC in the temperature of column, ±0.1mL/min in the flow rate and ±5 nm in the wavelength. Results revealed that small changes could not hamper the suitability of the method (listed below Table 7 and 8).

Ruggedness

Ruggedness was determined by using the data obtained by the analysis performed by two different analysts and acceptance criteria for ruggedness, the Percentage relative standard deviation for the area of standard injections should not be more than 2%. Results were shown in Table 9.

LOD and LOQ

Limit of detection [LOD] and limit of quantification [LOQ] was estimated from the standard deviation of the y-intercepts and slope of the calibration curve of resveratrol and gallic acid. The LOD and LOQ for resveratrol were found to be 0.689207 and 2.088505 µg/ml respectively and LOD and LOQ for gallic acid were found to be 0.130961 and 0.39685 µg/ml respectively.

DISCUSSION

The LOD and LOQ value for the resveratrol and gallic acid obtained demonstrate the suitability of the system for the analysis of the drugs; system suitability parameter may fall within ±2% range during routine performance of the method. To study the accuracy and reproducibility of the proposed method recovery experiment study were carried out, in this a fixed amount of pre analyzed sample taken and standard drug was added at 80%, 100%, and 120% level. Each level was repeated for three times. The mean recovery of resveratrol was in the range of 101.40-102.0 and that of gallic acid was in range of 98.43-101.02. Recovery data states that the proposed method is accurate and reproducible. These results concluded that the proposed method is better in comparison to previously reported method. In present method retention time of the resveratrol was 4.75 min which is least as compare to other method reported by Zhang et al., 2013[3]; Singh et al., 2014[4]; Cvetkovic et al., 2015[5]; Singh et al., 2016[6]; Kumar et al., 2016[7]; that state the retention time of resveratrol is 11.5 min, 3.26 min, 2.535 min, 10 min, 4.34 min respectively and retention time of gallic acid was 2.15 min which is also least as compare to other methods reported by Zhang et al., 2013[3]; Gurav et al., 2013[8]; Fernandes et al., 2015[9]; Damle et al., 2015[10]; Pratima et al., 2017[11] that state the retention time of gallic acid is 5.883 min, 3.60 min, 8.5 min, 3.1 min and 6.4 min respectively. This shown that the run time of resveratrol and gallic acid is less than previous reported method. The LOD & LOQ value of resveratrol is 0.689207 µg/ml and 2.088505 µg/ml respectively, previously reported by Zhang et al., 2013 [0.007 & 0.023 µg/ml respectively]; Singh et al., 2014 [0.002 & 0.007 µg/ml respectively]; Cvetkovic et al., 2015 [0.125 & 0.413 µg cm^{-3} respectively]; Singh et al., 2016 [0.006 & 0.008 µg/ml respectively] and Kumar et al., 2016 [0.95 & 2.87 ng/ml respectively]. The LOD and LOQ value of gallic acid was found to be 0.130961 µg/ml and 0.39685µg/ml respectively, better than methods reported by Zhang et al., 2013 [0.032 & 0.098 µg/ml respectively]; Gurav et al., 2013 [0.0178 & 0.0539 µg/ml respectively]; Fernandes et al., 2015 [1.22 & 1.82 µg/ml respectively]; Damle et al., 2015 [0.15 and 0.49 µg/ml respectively]; Pratima et al., 2017 [0.31 and 0.91 µg/ml respectively]. There results exhibited that the less sample is required for the quantitation and quantification of the drug. These data represent that this method is better than existing method and have less runtime compared to the previously reported method.

CONCLUSION

The HPLC method developed for the analysis of mixture of Gallic acid and Resveratrol in their

pharmaceutical preparations is stability indicating, precise, accurate and with a short run time. Method gives good peak resolution of resveratrol and gallic acid within short analysis time (<10 min) and high percentage of recovery shown that method is free from interference of excipients. The % RSD of each parameter lies below the limit of 2% proven the suitability. Method was fully validated showing satisfactory data for all the method validation parameters tested. The developed method can be conveniently applied for the routine estimation of Gallic acid and Resveratrol.

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Table 1: Optimised chromatographic condition for estimation of RES and gallic acid

Parameters	Condition
Mobile phase	methanol: buffer: acetonitrile in the ratio of 30:50:20 v/v
Diluent	acetonitrile: methanol [50:50 v/v]
Column	Nucleodur C_{18} column having dimensions 5µ (250×4.6 mm)
Column temperature	40 ºC
Detection wavelength	263 and 306 nm for gallic acid and resveratrol respectively
Injection volume	20 µl
Flow rate	1.0 ml/min
Run time	7 minutes

Table 2: System suitability of developed method

Parameters	Name of drug		Acceptance criteria
	Gallic acid	Resveratrol	
Retention time, min	2.15	4.75	-
Theoretical plates [N]	19421.239	34606.842	>2000
Tailing factor	1.322	1.169	<2

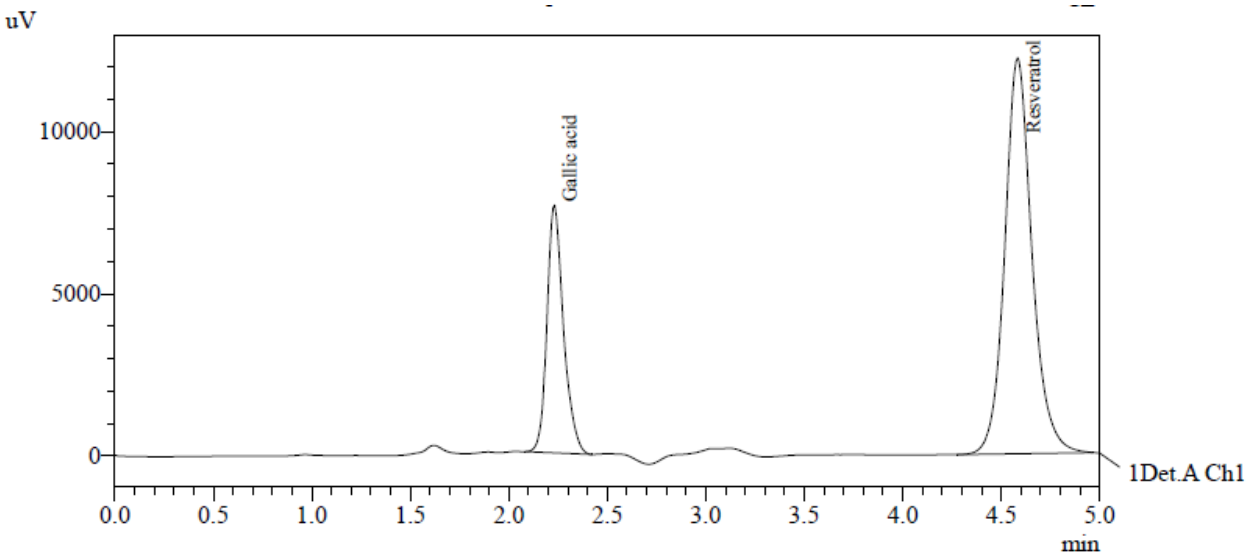

Fig. 2: System suitability

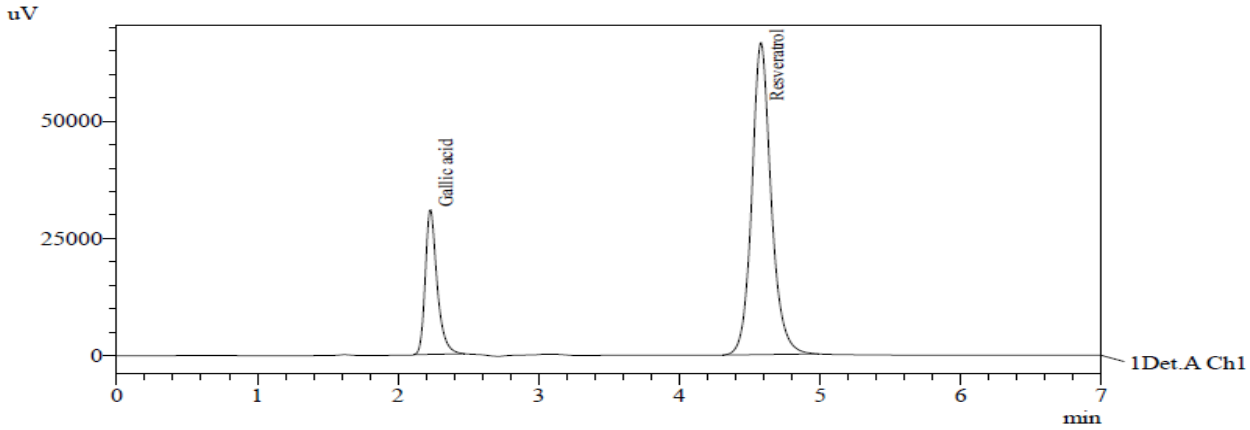

Fig. 3: Specificity

Table 3: Accuracy data

S. No.	Conc. (%)	Injection	Gallic acid		Resveratrol	
			Area	RSD (%)	Area	RSD (%)
1.	80	Replicate 1	179854	0.753457	638717	0.234976
		Replicate 2	177519		636121	
		Replicate 3	179858		638717	
2.	100	Replicate 1	226016	0.128251	815502	0.327891
		Replicate 2	226595		820178	
		Replicate 3	226270		815574	
3.	120	Replicate 1	267000	0.63215	990053	0.58518
		Replicate 2	264002		980052	
		Replicate 3	264204		990053	

*n=3

Table 4: Intraday precision for resveratrol and gallic acid

S. No.	Intraday			
	Morning		Evening	
	Gallic acid	Resveratrol	Gallic acid	Resveratrol
1.	210381	796649	212017	797207
2.	211468	796356	211353	797780
3.	211303	796481	212589	796870
Average	211050.7	796495.3	211986.3	797285.7
SD	585.7869	147.0249	618.5704	460.0721
%RSD	0.277557	0.018459	0.291797	0.057705

*n=3

Table 5: Interday precision of resveratrol and gallic acid

S. No.	Interday			
	Day 1		Day 2	
	Gallic acid	Resveratrol	Gallic acid	Resveratrol
1.	206635	793703	207008	797261
2.	207128	797346	205883	796297
3.	207008	797261	205883	796297
Average	206923.7	796103.3	206258	796618.3
SD	257.0921	2079.184	649.5191	556.5657
%RSD	0.124245	0.26117	0.314906	0.069866

*n=3

Table 6: Linearity data for resveratrol and gallic acid

Concentration	Mean peak area	
	Resveratrol	Gallic acid
1	114874	44585.67
2	277324.3	79458
3	435451.3	135976
4	639311.3	184451
5	782271	227060.3
6	999616	258898.3
7	1150763	319302.3
8	1310713	362381
9	1482006	407100.3
10	1665535	447366.3
Slope	172,828.64	45375
y-intercept	64,771.07	2906
Correlation coefficient	001.00	0.998

Table 7: Robustness data for Resveratrol

Parameters	Resveratrol		
	Retention time	%amount found*	%RSD
Temperature plus* (45 ºC)	4.416	102.3	0.894659
Temperature minus* (35 ºC)	4.588	102.2	1.233173
Flow rate plus* (1.1 ml/min)	4.759	102.3	0.55671
Flow rate minus* (0.9 ml/min)	4.895	102.8	0.968204
Wavelength plus* (311 nm)	4.452	98.2	0.260532
Wavelength minus* (301 nm)	4.433	102.1	1.963336

*n=3

Table 8: Robustness data for gallic acid

Parameters	Gallic acid		
	Retention time	%amount found*	%RSD
Temperature plus* (45 ºC)	2.184	98.68	0.695691
Temperature minus* (35 ºC)	2.194	101.73	1.017611
Flow rate plus* (1.1ml/min)	2.412	102	1.352822
Flow rate minus* (0.9 ml/min)	2.434	100.41	1.291932
Wavelength plus* (268 nm)	2.186	101.98	0.382344
Wavelength minus* (258 nm)	2.203	102.53	0.41396

*n=3

Table 9: Ruggedness data for resveratrol and gallic acid

S. No.	Area			
	Analyst 1		Analyst 2	
	Gallic acid	Resveratrol	Gallic acid	Resveratrol
1.	296386	949290	304720	982772
2.	299630	942019	303946	967160
3.	298815	986852	306061	986626
4.	298567	968447	302116	949362
5.	307706	987140	305294	991040
6.	303662	970681	302376	983862
Average	300794.3	967404.8	304085.5	976803.7
SD	4139.142	18719.38	1586.597	15688.45
%RSD	1.376071	1.93501	0.52176	1.606101

*n=6

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