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Research Article

Analytical Method Development and Validation For Estimation Of Resveratrol And Glycyrrhetinic Acid By RP-HPLC Method

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ABSTRACT
A RP-HPLC method was developed and validated for the simultaneous estimation of Resveratrol (RES) and Glycyrrhetinic acid (GA) in bulk and pharmaceutical dosage form. Chromatographic separation was achieved and optimized using Waters HPLC, Inertsil ODS 3V (150 x 4.6 mm x 5µm) with a gradient mobile phase composed of 0.2 % orthophosphoric acid and acetonitrile at ratios of 60/40, 60/40, 20/80, 20/80, 60/40, 60/40 for 0.01, 8, 15, 20, 25 and 30 minutes respectively with a flow rate of 1.0 mL/minute. The maximum wavelengths of UV detection were 307 nm and 250nm with retention times of 3.7 and 18.6 minutes respectively for RES and GA. Analytical parameters such as accuracy, linearity, precision, robustness, ruggedness were determined according to ICH Q2 B guidelines. The detector response was linear in the range of 25-100 µg/ml for both RES and GA. Correlation of coefficient and regression equation for RES and GA were found to be (R2=1, y = 71472x-7104.7) and (R2 = 0.9999, y = 10740x + 1548.4) respectively. 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 RES and GA. The validated method was applied to quantify the contents of RES and GA incorporated into a microemulsion formulation. The developed method is extensively precise, accurate and sensitive and can be successfully applied for routine quantification of RES and GA loaded formulations.

KEYWORDS: Resveratrol; Glycyrrhetinic acid; RP- HPLC; gradient

INTRODUCTION

Resveratrol (trans- 3,4', 5- tri hydrostilbene) is a potent antioxidant present in high concentrations in grape seed and wine possessing cardio-protective, chemo-preventive and anti-tumor activities [1,2].The main product of liquorice metabolism is the aglycone 18-ß-glycyrrhetinic acid (GA) . GA exhibits corticosteroid and mineral-corticoid activity due to the presence of the α,ß-unsaturated ketone group. GA interacts with mineral-corticoid and glucocorticosteroid receptors and exhibits anti-inflammatory properties [3,4,5]. Abe H *et al* studied the effects of glycyrrhizin (GL) and its aglycone, glycyrrhetinic acid (GA) on the growth and differentiation of mouse melanoma B16F10 cells [6]. In order to fully characterize RES and GA present in the microemulsion formulation, a

suitable and validated method is essential to assess the drug content. Published literature reveals HPLC methods and few bioanalytical methods for the quantitation of RES [7,8,9] . In several publications, sensitive and reproducible analytical methods have been developed using High-Performance Liquid Chromatography (HPLC)[10,11,12,13,14] . Several HPLC and UV methods have been reported for the estimation of RES individually[15,8,16,17,18]. Similarly, literature search reveals many studies for individual estimation of GA from glycyrrhiza glabra extract[19,20,21,22]. However, a method for simultaneous estimation of RES and GA in formulations is unavailable till date. This research work focuses on method development and validation for identification and quantification of RES and GA in a microemulsion formulation. All the procedures followed, parameters considered for methods trials and specification are in accordance with the ICH guidelines.

MATERIALS AND METHODS:

Instrumentation and Chromatographic conditions

Chromatographic techniques were performed using Waters HPLC systems. All HPLC systems were equipped with a column compartment with temperature control and an on-line degasser. Data acquisition, analysis, and reporting were performed by Empower2 (Waters) chromatography software. Method was developed using Inertsil ODS 3V (150 x 4.6 mm x 5µm) column. Gradient mobile phase composed of 0.2 % orthophosphoric acid and acetonitrile at a ratio of 60/40, 60/40, 20/80, 20/80, 60/40, 60/40 for time 0.01, 8, 15, 20, 25, 30 minutes respectively with a flow rate of 1.0 mL/min. Detection of RES and GA was carried out at 307 and 250nm respectively.

Special Precautions

All analysis were carried out in dim light, protected from light so as to avoid photodegradation of resveratrol.

Chemicals and reagents

RES was obtained as gift sample from Sami labs Ltd and GA was purchased from Yucca Laboratories, Pvt Ltd Mumbai. The purities of Resveratrol and GA were >99.5% and 96% respectively. HPLC grade Acetonitrile and orthosphosphoric acid were purchased from M/s SD fine-chem limited (Mumbai, India). Deionized water used in all the experiments was passed through a Milli-Q water purification system (18.2 MΩ/cm) Millipore.

Preparation of Stock Solutions, Calibration Curve (CC), and Quality Control Samples (QC)

Standard stock solutions were prepared by transferring 5mg each of RES and GA in separate 10 ml volumetric flasks, to which 5 mL of diluent (90%ACN) was added, sonicated for 5 min and volume was made up to 10 mL with diluent to obtain stock solution. Further, 1ml from each of the stock solutions of RES and GA were transferred to 10 volumetric flasks and volume was made up to 10 mL with diluents to generate a mixed stock solution. Working standard solutions of various concentration including 25,40,50,62.5,75 and 100ug/ml were prepared by pipeting out appropriate amount of mixed stock solution and volume was made up to 10 mL with diluent. Six replicate of resveratrol at concentrations 25,40,50,62.5,75 and 100ug/ml were considered as quality control (QC). Series dilutions were carried out in 10ml volumetric flasks to yield solutions having concentrations of 25,40,50,62.5,75 and 100ug/ml of both drugs. Solutions at each concentration were prepared in triplicates and 10 µl injections of each concentration were injected into the RP-HPLC system and chromatographed under above described conditions. RES and GA were evaluated at wavelength of 307 and 250 nm respectively using a PDA detector. Peak areas were recorded for all the peaks and peak areas were plotted against the concentrations to obtain the standard calibration curves.

Method validation

The method was validated according to ICH guidelines. Parameters studied for the validation included system suitability, limits of detection and quantification, linearity, accuracy, precision, specificity, robustness and ruggedness[22],[23].

System suitability

System suitability parameter was calculated before starting validation parameters to verify that the reproducibility and resolution of the chromatographic systems are adequate for the analysis to be done. Data from six injections of 10 µL of the working standard solutions of RES and

GA were used for the evaluation of the system suitability parameters like tailing factor, the number of theoretical plates, retention time and resolution factor.

Limits of Detection and Quantification

LOD and LOQ were calculated according to LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve [24].

Linearity

Six concentrations of the standard mixture, 25,40,50,62.5,75 and 100ug/ml were injected and chromatogram was recorded. The linearity point of each experiment was performed in triplicate according to the optimized chromatographic conditions. The peak areas of the chromatograms were plotted against the concentration of RES and GA to obtain the calibration curve and further co relation coefficient was calculated.

Accuracy

The accuracy of the method expresses the closeness of agreement between the true value and the value found. To determine the accuracy in sample preparation method of standard additions was used for measuring the recovery of the drugs. To the standard solution known amounts of standard RES and GA corresponding to 50%, 80 %, 100%, 125%, 150% and 200% were added. The accuracy was expressed as the percentage of analyte recovered by the proposed method[25].

Precision

Precision designates the closeness of agreement, that is, the degree of scatter between a series of measurements obtained from multiple sampling of the similar homogeneous sample and it was determined by repeatability (intraday) and intermediate precision (interday) for three consecutive days[26]. It is very important that the method developed should be precise. Six replicate of the sample prepared from the microemulsion were injected and Assay was calculated to measure the repeatability of retention times and peak area of standard and sample, in accordance with ICH guidelines. The intra-day and inter-day precision were determined by analyzing the samples of RES and GA. Determinations were performed on the same day as well as well as on consequent days.

Specificity

The retention times of the individual drugs, drug blend and drug in emulsion were determined and analyzed to identify if any drug-drug/ drug-excipient interaction is present.

Robustness

Robustness of the method was identified by evaluating the effect of small but deliberate changes in the chromatographic conditions[27]. The flow rate of the optimized method was 1.0mL/min. It was changed to 0.9mL/min and 1.1mL/min and chromatograms were obtained.

Ruggedness

Percentage RSD for the retention time and area was calculated by performing the analysis on different days to check for ant changes in the chromatograph.

Method Applicability

The new development method provided ease in determining the percent content of microemulsion fabricated by water titration method. The amount of drug initially loaded in the microemulsion was 1 %w/w. The total drug content in the RES and GA loaded microemulsion was analyzed by dissolving the carrier system in diluent.0.1 ml of microemulsion was diluted upto 10ml with ethanol. Appropriate dilutions were carried out to get standard RES and GA solution equivalent to 100ppm. Both the sample and standard solutions were analyzed by developed RP-HPLC method. The assay was performed in triplicate.

RESULTS AND DISCUSSION

After conducting sufficient number of trials the developed method was optimized. It is evident that the proposed method is the specific, fast and accurate for simultaneous estimation of RES and GA, hence proving industry feasibility.

Method validation

System suitability

System suitability parameters such as retention time, number of theoretical plates, peak area, and resolution and peak asymmetry were determined. The results obtained are depicted in Tables 1 and 2.

Table 1: System Suitability Result For RES

Standard	Average	% RSD
Retention Time	3.65	0.36
Area	3565451	0.72
Theoretical Plates	3679	-
Asymmetry	1.18	-

Table 2: System Suitability Result For GA

Standard	Average	% RSD
Retention Time	18.97	0.09
Area	537701	0.24
Theoretical Plates	79805	-
Asymmetry	1.05	-

Limits of Detection and Quantification:

The estimated LOD for RES and GA was 0.36 *µ*g/mL and 0.46*µ*g/mL respectively, while the LOQ for RES and GA was 1.1 *µ*g/mL and 1.4 *µ*g/mL respectively.

Linearity

Linearity was evaluated over the concentration ranges of 25,40,50,62.5,75 and 100 *µ*g/mL each for RES and GA in the ratio 1:1, estimating the regression equation and the determination coefficient (R2) obtained from the least squares method. The coefficients of determination for the calibration curves of the RES and GA were 1 and 0.9999 respectively, presenting evidence that the data fits to the regression line linearly over the proposed concentration ranges. The results are presented in Tables 3 and 4. Data depicted in Figures 1 and 2 proves an excellent correlation between peak areas and concentrations.

Table 3: Linearity data of RES

Sr. No.	Concentration in *µ*g/mL	Peak Area	Average Peak area	Standard deviation
1	25	1784571 1769874 1782364	1778936.333	7925.410168
2	40	2853147 2864156 2864125	2860476	6347.11911
3	50	3536723 3586706 3572925	3565451.333	25816.0191
4	62.5	4465892 4456214 4456321	4459475.667	5556.965209
5	75	5369147 5321789 5321789	5337575	27342.15405
6	100	7145896 7145932 7156586	7149471.333	6161.508365

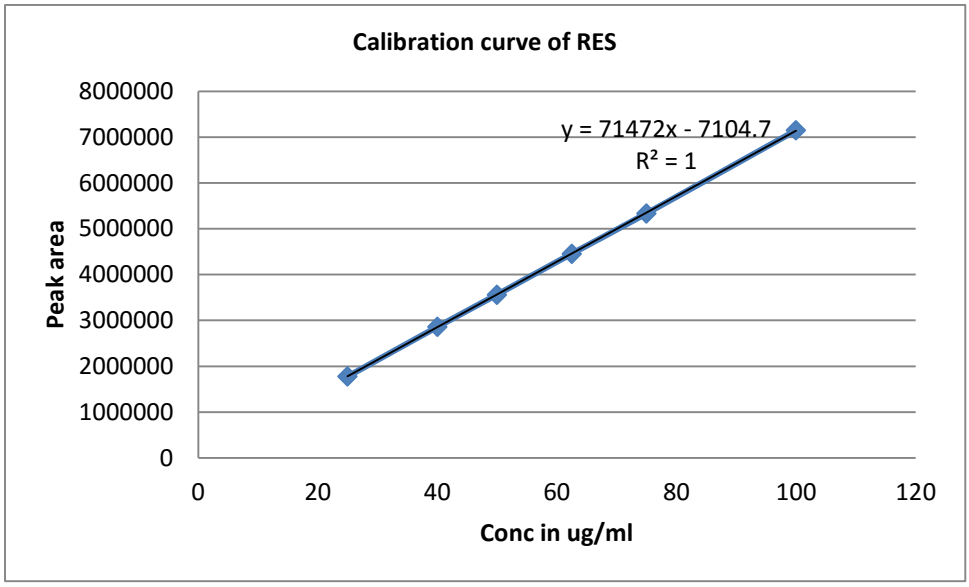

Fig. 1: Calibration curve of RES

Table 4: Linearity data of GA

Sl. No.	Concentration in µg/mL	Area	Average Peak area	Standard deviation
1	25	268457	267139.3	1518.944
		265478		
		267483		
2	40	430145	434288.3	3883.379
		434875		
		437845		
3	50	536564	537701.3	1319.481
		539148		
		537392		
4	62.5	674582	676272	1981.659
		675781		
		678453		
5	75	806457	804625.3	1966.636
		804872		
		802547		
6	100	1075587	1075035	2276.203
		1076985		
		1072534		

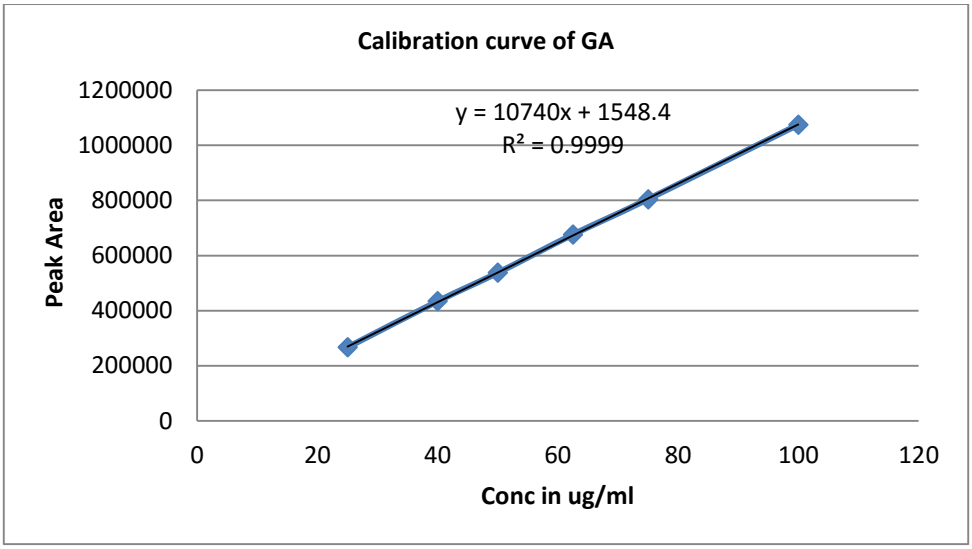

Figure 2: Calibration curve of GA

Accuracy

The accuracy of the method was validated by determining the percent recovery of the RES and GA as depicted in Tables 5 and 6 respectively. The results suggest that all the recoveries are well within the acceptable range, thus indicating the method to be accurate for simultaneous estimation of RES and GA.

Table 5: RES Accuracy

Level in %	Standard Area	Solution 1 Area	Solution 2 Area	Solution 3 Area	% Accuracy of Solution 1	% Accuracy of Solution 2	% Accuracy of Solution 3	Mean % Accuracy	% RSD
50	3565451	1747811	1735689	1743682	98.3	97.6	98.1	98.0	0.36
80		2841578	2838954	2798564	99.9	99.8	98.4	99.3	0.84
100		3541258	3525981	3498547	99.6	99.2	98.4	99.0	0.61
125		4352487	4298795	4299574	98.7	97.5	97.5	97.9	0.70
150		5292589	5214789	5248736	99.2	97.8	98.4	98.4	0.71
200		7156893	7046578	7065894	100.7	99.1	99.4	99.7	0.85

Table 6: GA Accuracy

Level in %	Standard Area	Solution 1 Area	Solution 2 Area	Solution 3 Area	% Accuracy of Solution 1	% Accuracy of Solution 2	% Accuracy of Solution 3	Mean % Accuracy	% RSD
50	537701	268457	263865	264489	100.8	99.3	99.3	99.3	1.06
80		425242	425189	425098	99.8	99.7	99.7	99.7	0.05
100		531943	530158	559956	99.8	99.5	99.5	99.6	0.17
125		658894	657845	651258	99.7	99.6	98.6	99.3	0.61
150		791572	790124	791258	99.1	98.8	99.0	98.9	0.15
200		1055871	1052487	1051489	99.1	98.8	98.7	98.8	0.21

Precision

The percentage RSD vales of the assay in precision study were calculated and are depicted in Table 7. The results indicate that the method is precise.

Table 7: Method precision

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Average	% RSD
GA	97.0	97.9	96.8	96.6	97.1	97.3	97.1	0.47
RES	97.5	97.2	97.4	97.8	98.1	97.6	97.6	0.32

Specificity

The retention times of individual drugs RES and GA were found to be 3.7 and 18.6 minutes respectively**.** The retention time of Res and GA in the drug blend was 3.69 and 18.9 minutes respectively. Both the drugs were resolved very well in the chromatogram. Further, the microemulsion was diluted with appropriate diluent and 10µL of sample was injected into the RP-HPLC wherein RES and GA were eluted at 3.67 and 18.7 minutes respectively. Respective HPLC chromatograms are represented in Figures 3, 4, 5, 6. No change was observed in the chromatogram. This suggests that both the drugs are excellently resolved by the method without any drug-drug / drug-excipient interaction.

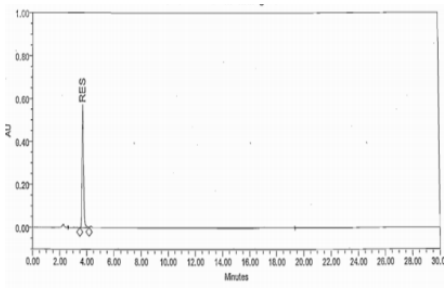

Fig 3: Chromatograph of RES

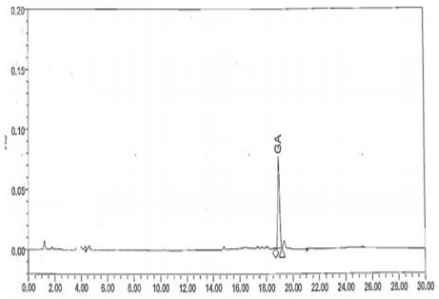

Fig 4: Chromatograph of GA

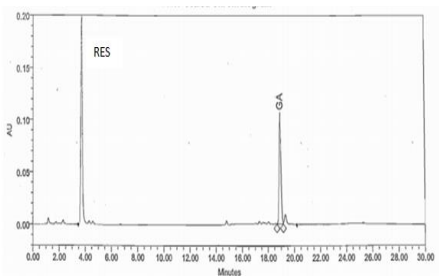

Fig 5: Chromatograph of Drug blend

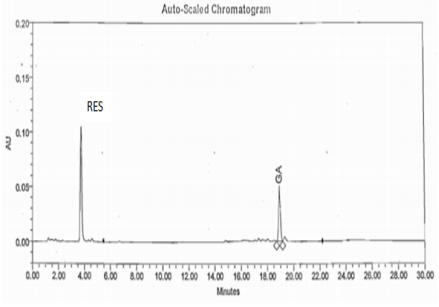

Fig 6: Chromatograph of Dosage form

Robustness

After relevant changes were made in the method, no drastic differences observed in the chromatograms, thereby proving that the method is robust. The results are presented in Table. 8

Table 8: % RSD at different flow rates

Flow= 0.9 ml/min			
Content	**RT % RSD**	**Area % RSD**	**Sample Area % RSD**
GA	0.05	0.36	0.45
RES	0.12	0.45	0.78
Flow= 1.1 ml/min			
Content	RT % RSD	Area % RSD	Sample Area % RSD
GA	0.08	0.79	0.36
RES	0.24	0.12	0.88

Ruggedness

The comparative % RSD of retention time, area of peak, %RSD of area and retention time was been calculated and is presented in Table 9. Based on the data, it is evident that the method is rugged.

Table 9: % RSD of the drugs on different days (Ruggedness)

Day 1			
Content	RT % RSD	Area % RSD	Sample % RSD
GA	0.58	0.12	0.45
RES	0.41	0.89	0.10
Day 2			
Content	RT % RSD	Area % RSD	Sample % RSD
GA	0.16	0.88	0.41
RES	0.30	0.32	0.56

Method Applicability

The method developed in this work was used to determine the content of RES and GA in microemulsion. The obtained assay values were 95.82% and 98.16% of RES and GA respectively. Hence the developed gradient RP-HPLC method is suitable for determination of RES and GA concentrations in bulk and pharmaceutical dosage forms.

CONCLUSION

This study proves that the developed gradient RP-HPLC method is fast, reproducible, accurate and simple. By adopting this method RES and GA can be eluted within 19 minutes. It can be concluded that the method will be useful for routine quality control of formulations containing both drugs.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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