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Method Development and Validation for the Simultaneous Estimation of Resveratrol and Quercetin in Bulk and Pharmaceutical Dosage Form by RP-HPLC

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Abstract

Resveratrol and Quercetin are the nutraneuticals which belong to the class of polyphenolic compounds that are widely used as antioxidants and anti obesity drugs. These compounds are obtained from natural sources so it is easy to develop a simple ,precise, economical and less time consuming method for its simultaneous estimation.The HPLC Waters instrument with a Sunfire C18(150x3.0mm I.D5µm particle size) column having a Rheodyne injector(20µl) using Methanol : Water : Formicacid : Triethylamine (10:70:15:5v/v) at a flow rate of 0.8ml/min the peaks of Resveratrol and Quercetin were eluted at retention time 1.24min and 2.14minutes respectively at the detection wavelength of 277nm.Concentration of both the drugs were found linear with a correlation coefficient of 0.996 and 0.999 and the average percent recovery was found to be 98.6% and all other validation parameters were found to be within the limits as per ICH Guidelines.

Keywords: Resveratrol, Quercetin, RP-HPLC, Nutraceutical ,ICH, Phytoalexins.

INTRODUCTION

Nutraceutical product is a food or fortified food product that not only supplements the diet but also assists in treating or preventing disease (apart from anemia), and provides medical benefits[5].So,a nutraceutical or 'bioceutical' is a pharmaceutical alternative which claims physiological benefits[6]. They exist in the same category as dietary supplements and food additives by the FDA, under the authority of the Federal Food, Drug, and Cosmetic Act.[5] Phytoalexins are a group of low molecular weight compounds in a large number of plants as a defence response to situations of stress and microbial infections[2] .Resveratrol is a phytoalexin that belongs to the group of compounds known as stilbenes, and is known to occur in grapes, grape products and in wine [1] . It is present in higher concentration in red grape varieties It is used for treatment of diseases like dermatitis, gonorrhea, fever, hyperlipidemia, atherosclerosis, and inflammation[1].Quercetin is a flavaonol that occurs widely in plants and is significantly present in red wine. The major biological action of quercetin are protection of LDL cholesterol against oxidation[1].There is a synergistic effect between ethanol and the grape polyphenols, quercetin and resveratrol, in inhibiting the nitric oxide synthase pathway involved in the damage of vascular walls and DNA.Further, resveratrol, quercetin have been associated with a reduced risk of cancer.[1]

The main aim of this work is the development and validation of a rapid and reliable reverse phase HPLC method for the Simultaneous estimation of Resveratrol and Quercetin from its bulk and Pharmaceutical dosage form(Capsule).The retention time of the drugs were obtained by injecting the individual samples f the drugs,Its standard mixture and the formulation through a C18 column under isocratic conditions with a fixed flow rate of the pump.AS per the ICH guidelines all the validation parameters were performed and the results were found to be within the limits.

MATERIALS AND METHODS

Reagents and Chemicals:

The reference standard of Resveratrol and Quercetin were purchased from Sami Labs Limited, Karnataka. The commercial product of Resveratrol and Quercetin combination was purchased from the market. HPLC grade Methanol and Acetonitrile were obtained from Sigma Aldrich, India. Triethylamine was received from Thermo Fischer Pvt Ltd and Formic Acid from Fischer Scientific Pvt Ltd.

Instrumentation:

The HPLC Waters instrument was used which consisted of Waters 515 solvent delivery system using a Sunfire C18 column(150X3.0mm5µm) and Waters 2489 UV detector.The software used was Empower 2.The mobile phase was sonicated using the Ultra Sonic Sonicator.The detetion wavelength was fixed utilizing UV-1650PC,Shimadzu.

RESULTS AND DISCUSSION

Preparation of Mobile Phase:

Methanol was dissolved in water along with addition of Formic Acid and Triethylamine in the ratio of 10:70:15:5and was sonicated for 10 minutes.

Preparation of Standard Solution :

10mg of Resveratrol and Quercetin was made upto 10ml with 99.7% methanol. From the above stock 1-5mg of stock was taken and made up with water to obtain a concentration of 10-50µg/ml and 5-25µg/ml respectively. Repeat the same procedure for the mixture. The calibration curve was obtained by plotting the ratio of peak area of drug versus concentration.

Preparation of Sample Solution:

To determine the amount of Resveratrol and Quercetin in capsules,5 capsules were individually taken, weighed and the weight to be taken was calculated. The contents of the capsule were emptied , weighed separately and from it 10mg of the drug was extracted using the mobile phase.

The concentration range of 10-50µg/ml was prepared and injected.

Selection of Detection Wavelength:

For both the reference standards of Resveratrol and Quercetin 10 mg of drug was taken and dissolved in 99.7% of methanol to obtain a concentration of 1000µg/ml.From this stock solution further dilution was done by pippeting 1ml of the above stock and was dissolved in water. From the secondary stock,serial dilutions were prepared at a concentration range of 10-50µg/ml and 5-25µg/ml respectively.Accordingly, these solutions were scanned in the UV range of 200-400nm to check maximum absorbance. From the overlain spectrum and the overlay spectrum,obtained by overlapping of the individual drug spectra 277nm was the isobestic point .Thus from the overlay spectra of both the drugs the detection wavelength was fixed as 277nm for the simultaneous estimation of these drugs.

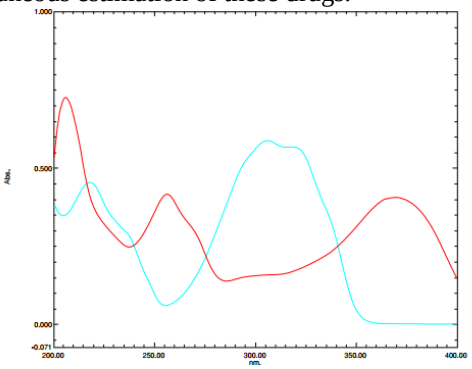

Fig.1 Overlay Spectrum of Resveratrol and Quercetin.

Optimization of Chromatographic conditions

Before fixation of the mobile phase for accurate detection of drugs several trials were performed using mixtures of organic phases,aqueous phases and acids with the standard solutions. Thus the final mobile phase for this thesis was found to be Methanol:Water:Formic Acid:Triethylamine (10:70:15:5).This particular ratio provides a sharp peak with good resolution.Stationary phase used was the Sunfire C18(150X3.0)5µm column.Eventhough Formic acid and Triethyamine were used there was no change in pH done because better results were obtained in mobile phase pH itself. Flow rates of 0.5ml/min,0.6ml/min,0.7ml/min were tried and finally 0.8ml/min was fixed for the study.

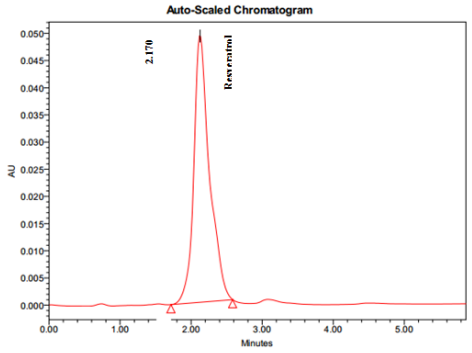

Fig.2.Standard Chromatogram of Resveratrol

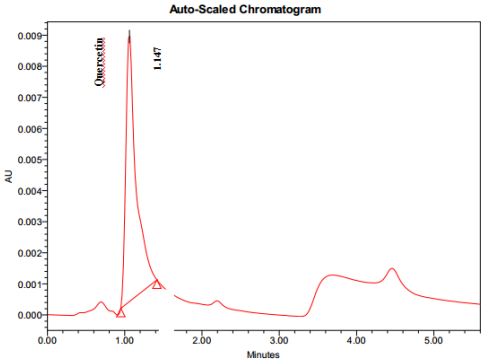

Fig.3.Standard Graph of Quercetin

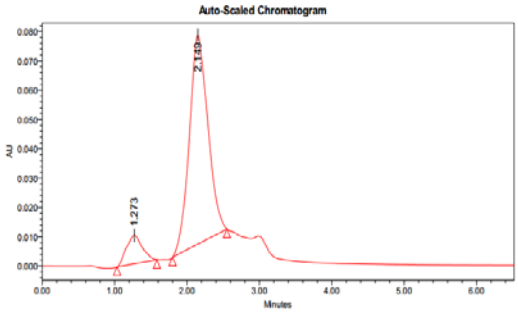

Fig.4.Chromatogram of Formulation

Table 1. Fixed Chromatographic Conditions

S.No	Paramaters		Specification
1.	Stationary Phase	:	Sunfire C18(150X3.0)5µm
2.	Mobile Phase	:	Methanol: Water: Formic Acid: Triethylamine
3.	Solvent Ratio	:	10:70:15:5
4.	Flow Rate	:	0.8ml/min
5.	Injection Volume	:	20µL
6.	Detection Wavelength	:	277nm
7.	Tempertaure	:	25ºC

Table.2 Analysis of formulation

S.NO	DRUG	AMOUNT		%LABEL CLAIM	%RSD
		LABELLED	FOUND		
1.	RESVERATROL	250	244.3	88%	0.75
2.	QUERCETIN	120	118.3	92%	0.88

Table.3(a) .Calculated Concentration and Percentage Recovery of Resveratrol

S.No	LQC		MQC		HQC	
	5		20		25	
	Calculated concentration(µg/ml)	Accuracy	Calculated concentration(µg/ml)	Accuracy	Calculated concentration(µg/ml)	Accuracy
1.	4.87	97.4	19.88	99.4	24.80	99.20
2.	4.88	97.6	19.78	98.90	24.90	99.60
3.	4.76	95.2	19.85	99.25	24.88	99.52
4.	4.77	95.4	19.88	99.40	24.75	99
5.	4.95	99	19.87	99.35	24.82	99.28
6.	4.87	97.4	19.85	99.27	24.90	99.6
Mean	98.51					
%RSD	0.67					

Table.3(b).Calculated Concentration and Percentage Recovery of Quercetin

S.No	LQC		MQC		HQC	
	5		20		25	
	Calculated concentration(µg/ml)	Accuracy	Calculated concentration(µg/ml)	Accuracy	Calculated concentration(µg/ml)	Accuracy
1.	4.88	97.6	19.85	99.25	24.50	98
2.	4.96	97.6	19.75	98.90	24.69	98.76
3.	4.89	99.2	19.86	99.30	24.89	99.56
4.	4.97	97.8	19.54	97.70	24.79	99.12
5.	4.88	99.4	19.97	99.85	24.66	98.64
6.	4.96	99.2	19.87	99.35	24.82	99.28
Mean	98.75					
%RSD	1.72					

METHOD VALIDATION:

The developed method's validation parameters were employed by ICH Guidelines.The retention time of Resveratrol and Quercetin was found to be 1.27minutes and 2.14minutes respectively. This study was confirmed as all the validation parameters of the reproduced results were found within the limits.

Linearity:

The calibration curve of Resveratrol and Curcumin were plotted by Concentration v/s Peak area and the regression equation was calculated. The linearity of Resveratrol was calculated using the concentration 5,10,15,20 and 25 and similarly for Quercetin as well. These concentrations were prepared by diluting the required volume of working standard with the mobile phase and the calibration curve was plotted. The correlation coefficient for both the drugs were found to be 0.996 and 0.999.

Accuracy:

The Recovery studies were performed to validate the accuracy of the newly developed method with different concentrations that is the lowest,middle and highest of pre analyzed sample solution of the capsule.

Precision:

For the calculation of Intraday Precision nine different solutions were prepared using 5-15µg/ml for Resveratrol and similarly for Quercetin with three replicates of each and their peak area was measured on the same day at different intervals of time.Similar procedure was followed for Interday Precision but on three different days in a week.

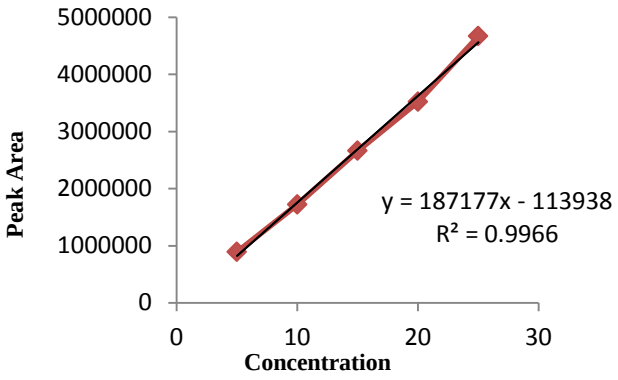

Fig.5.Calibration Curve of Resveratrol

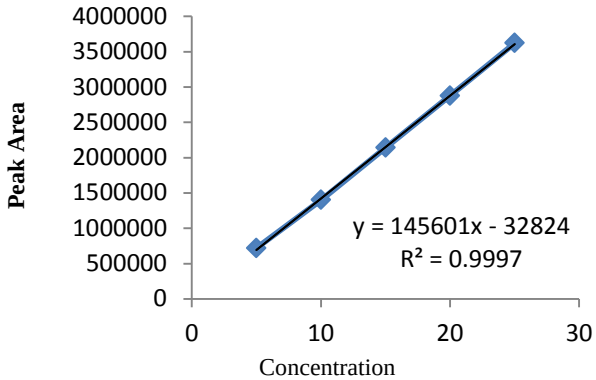

Fig.6.Calibration Curve of Quercetin

Table.4a. Repeatability of Quercetin and Resveratrol

S.No	PEAK AREA		%RSD	
	RESVERATROL	QUERCETIN	RESVERATROL	QUERCETIN
1.	3597909	2755787	0.9	0.7
2.	3647752	2797568		
3.	3597780	2784589		
4.	3667800	2808678		
5.	3656821	2793210		
6.	3677520	2799870		

Table 4b. Data Representing Interday and Intraday Precision Of Resveratrol

CONCENTRATION	PEAK AREA		%RSD	
	INTERDAY	INTRADAY		
5µg/ml	892185	892185	0.502	0.624
	901082	881082		
	900101	891101		
10µg/ml	1745163	1745163	0.713	0.810
	1718700	1728700		
	1729680	1759680		
15µg/ml	3667800	3667800	1.021	1.132
	3598990	3588990		
	3609895	3649895		

Table 5. Data Representing Interday and Intraday Precision Of Quercetin

CONCENTRATION	PEAK AREA		%RSD	
	INTERDAY	INTRADAY		
5µg/ml	708398	708398	0.730	0.850
	709496	717897		
	718398	718796		
10µg/ml	1502118	1502118	0.852	0.941
	1518072	1510389		
	1527789	1529887		
15µg/ml	2042700	2042700	1.05	1.367
	2072662	2076983		
	2030543	2098963		

Limit Of Detection and Limit Of Quantification:

The limit of Detection and the limit of Quantification of the developed method is established by injecting the lowest concentration of the standard solution using RP-HPLC method.

LOD=3.3X SD/Slope and
LOQ=10X SD/Slope.(Table.5)

Specificity:

The specificity of the developed method was confirmed by peak purity test of the analyte solution by UV detector. Peaks obtained in the standard and sample at working concentrations are because of drugs as mobile phase has no peaks in retention time of Resveratrol and Quercetin. The peak purity was observed to be 0.996 and 0.999.These results prove that the observed peak of analyte was pure and the excipients in the formulation does not cause any interference in the peaks and so the method is specific.

System Suitability:

System Suitability was studied by injecting six consecutive replicates of the standard solution and the hplc parameters like Resolution, Tailing Factor,Capacity Factor and Theoretical Plates were calculated.The results obtained were found to be in the acceptable limits.(Table.5).

Robustness:

Even on change of temperature, flow rate,pH and detection wavelength for the developed method there was no dramatic changes noted in the retention time of the drugs so it can be claimed to be robust.

Table.5. The Validation Parameters

S.No	Parameters	Resveratrol	Quercetin
1.	Retention Time	1.23	2.14
2.	Linearity	0.996	0.999
3.	LOD(µg/ml)	0.034	0.059
4.	LOQ(µg/ml)	0.103	0.181
5.	Recovery	0.67	1.72
6.	Tailing Factor	0.95	0.56
7.	Precision	0.81	0.82

DISCUSSION

The newly developed method was found to be simple ,reliable,robust ,economical ,precise and less time consuming.By reverse phase HPLC an isocratic method was developed with an elution time of less than 5 minutes. Very less ratio of organic phase is used as major proportion of water is present in the mobile phase proving it to be an economical method. As per the ICH Guidelines of validation all the parameters like Accuracy,Precision,LOD,LOQ and System sutability were found within the limited range and is thus acceptable.Thus,we can strongly determine that the proposed method development can be employed for other pharmaceutical dosage forms and also for Bioanalytical studies especially using Plasma.

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