RESEARCH ARTICLE

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Validation of RP-HPLC method for simultaneous estimation of hesperidine and quercetin

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Abstract

A new RP-HPLC method was developed for the simultaneous estimation of hepseridine and quercetin was validated as per ICH guidelines. The chromatogram for was found to be satisfactory on symmetry C-18 (4.6×150mm, 5µ Hypersil column) using mobile phase composed of Methanol-Water (70:30, pH adjusted to 3.0 with orthophosphoric acid) at a flow rate of 1.0 ml/min and the detection wavelength of 269.7 nm. The retention time of hesperidine was found to be 2.547 min and that of quercetin was found to be 2.106 min. The system suitability parameters proved that the proposed method is suitable for estimation of both the drugs under study. The linearity for hesperidine was studied from 1 to 5 µg/ml concentrations and for quercetin was studied from 1-5µg/ml concentrations. The precision of the method was good and the recovery of drugs was found to be within the acceptance limits of 80-120%. The LOD and LOQ for hesperidine were found to be 0.084 µg/ml and 0.256 µg/ml respectively. The LOD and LOQ for quercetin were found to be 0.054 µg/ml and 0.166 µg/ml respectively. The proposed RP HPLC method was found suitable for the simultaneous estimation of hepseridine and quercetin and is simple, selective, reproducible and accurate with good precision and can be successfully applied to routine analytical purpose.

Keywords: Hesperidine, quercetin, HPLC, validation, simultaneous estimation

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Introduction

Quercetin (Figure 1) is a natural flavonoid found in foods and natural supplement products. It is an antioxidant, like many other phenolic heterocyclic compounds[1]. Quercetin is a specific quinone reductase 2 (QR2) inhibitor, an enzyme (along with the human QR1 homolog) which catalyzes metabolism of toxic quinolines[2].

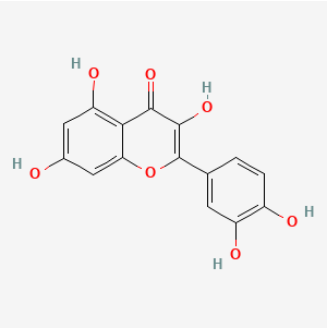

Figure 1. Structure of quercetin

Hesperidin (Figure 2) belongs to flavanone compounds, one of the flavonoids subclasses. It has been used as supplement and has been known for its antioxidant, anti-inflammatory, anticancer, antiviral effects, protective cardiovascular disorders and neurodegenerative properties[3,4].

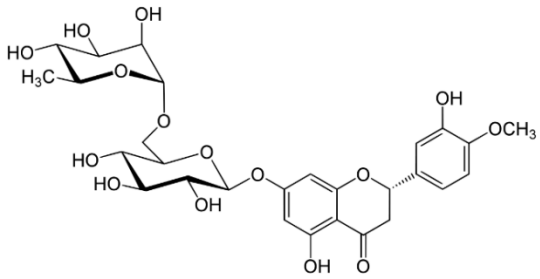

Figure 2. Structure of hesperidine

Several methods for individually estimating of quercetin and hesperidin have been reported over years by several researchers[5-10]. The presence of multiple flavonols in extracts and other herbal products has been the core of treatment of diseases with herbal formulations. Quercetin and hesperidine have been widely found flavonols in several plant extract and literature points no direct HPLC method for the simultaneous estimation of these two phytochemicals either in extract or on formulations. The present work is aimed to develop and validate a simple, accurate, fast, precise, efficient and reproducible RP-HPLC method for simultaneous estimation of hesperidine and quercetin and applicability in routine analysis of dosage forms.

Material and Methods

Hesperidine and quercetin pure drug were purchased from Yucca enterprises, Mumbai. The pure sample was used without further purification. HPLC grade water was purchased from Merck.

Preparation of mobile phase

An isocratic mixture of methanol 700 ml and 300 ml water (pH adjusted to 3.0 with orthophosphoric acid) was used as the mobile phase for elution of the components of the drug

sample. The solvent mixture was degassed in ultrasonic water bath for 15 minutes and filtered through 0.45µ filter by vacuum filtration. The mobile phase mixture was also used as the diluent for preparation of the working standard solutions of the drug.

Preparation of Standard Solution

Hesperidine (1 mg) and Quercetin (1 mg) were accurately weighed and transferred into a 10 ml clean, dry volumetric flask and about 5 ml of diluent was added and sonicated to dissolve the drugs completely and the volume was made up to the mark with the same solvent to obtain the standard stock solution. 1 ml of the stock solution was pippetted out from the above stock solution into a 10 ml volumetric flask and the volume was made up to the mark with the diluent to obtain the working standard solution. A series of dilutions of the working standard were prepared to obtain concentrations of 1 to 5 µg/ml of hesperidine and 1 to 5 µg/ml of quercetin. These solutions were analyzed using the same chromatographic conditions as used for sample analysis to generate the calibration curve.

Selection of detection wavelength[11]

The spectra of hesperidine and quercetin were obtained in methanol using UV spectrophotometer. The isosbestic point (wavelength at which the two drugs exhibit same absorbance) was used as the detection wavelength for the two drugs. This wavelength was found to be 269.7 nm.

Optimization of chromatographic conditions and method development

Chromatographic conditions

Column: Octadecylsilane (ODS) (4.6 x 150mm, 5µm, Hypersil)

Flow rate: 1.0 ml per min

Wavelength: 269.7 nm

Injection volume: 20 µl

Column oven temperature: Ambient

Run time: 10 min

Several trials of solvent combinations were made to obtain the best possible combination to elute out both hesperidine and quercetin at with sufficient resolution and reasonable retention times. The trial 1 was performed by using mobile phase containing water and methanol in the ratio of 40:60 v/v. The trial 2 was performed by using mobile phase containing acetonitrile and methanol in the ratio of 40:60 v/v. The trial 3 was performed by using mobile phase containing water (pH adjusted to 5.0 with orthophosporic acid) and methanol in the ratio of 50:50 v/v. The trial 4 was performed by using mobile phase containing water (pH adjusted to 3.0 with orthophosphoric acid) and methanol in the ratio of 50:50 v/v. The trial 5 was performed by using mobile phase containing water (pH adjusted to 3.0 with orthophosphoric acid) and methanol in the ratio of 30:70 v/v.

Validation of the method[12]

The developed method was validated for various parameters like linearity, specificity, precision, accuracy, LOD & LOQ in compliance with ICH guidelines.

System Suitability

The working standard solution was injected six times into HPLC system as and the chromatographic study was performed as per the developed and optimized conditions. The system suitability parameters were evaluated from standard chromatograms obtained by calculating the % RSD of retention times, theoretical plates and peak areas from six replicate injections.

Linearity

Dilutions of the working standard were prepared at five different levels (1 -5 µg/ml for hesperidine and 1-5µg/ml for quercetin) by appropriate dilution with the mobile phase as discussed in section 4.2.2.

Each level of dilution was injected into the chromatographic system in six replicates and the peak area and retention time was measured. A calibration curve was plotted taking concentration on X-axis and peak area on Y-axis.

Specificity

Solutions of standard and samples were prepared as per test procedure and injected into the HPLC system. A study to establish the interference of blank was conducted. The diluent was injected into HPLC system as per the test procedure.

Accuracy

Accuracy of the method was determined by performing recovery studies using regular addition method. The recovery studies were carried out at three concentration levels (50%, 100%and 150%). Each level was repeated six times. For both the drugs, recovery was performed in the same way. The percentage recovery and standard deviation of the percentage recovery were calculated.

Preparation of Sample Solution:

Accurately weighed quantity of 1 mg hesperidine and 1 mg quercetin was transferred into a 100 ml clean dry volumetric flask and about 25 ml of diluent was added and sonicated to dissolve the drug completely. The volume was made up to the mark with the diluent. 5 ml of this solution was further diluted to 50 ml in a volumetric flask with the diluent.

Preparation of 50% Solution

A quantity of 0.5 ml of working standard was accurately pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluent. This solution was added to the above sample solution.

Preparation of 100% Solution

A quantity of 1 ml of the working standard solution was accurately pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluent. This solution was added to the above sample solution.

Preparation of 150% Solution

A quantity of 1.5 ml of the working standard solution was accurately pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluent. This solution was added to the above sample solution.

The sample solution with the added (50%, 100% and 150%) solutions were injected into the HPLC system. The amounts added, amounts estimated and the individual recovery and mean recovery values were calculated.

Precision

The precision of the method was validated in terms of repeatability (intra-day precision) and inter-day repeatability (intermediate precision). The working standard solution was injected in six replicates in the HPLC system and the peak area for all six injections in HPLC was measured on same day (repeatability) as well as different days (ruggedness). Similar procedure was repeated for all the concentrations of the linearity range. The % RSD for the area was calculated.

Robustness

In order to evaluate the robustness of the method, deliberate changes in the flow rate were made and the drug was assayed using the proposed conditions.

Limit of Detection

LOD is the smallest concentration of the analyte which can produce a measurable response. It can be calculated using the signal to noise ratio and the slope of the calibration curve by the following formula:

$$LOD = \frac{3.3\ \sigma}{S}$$

LOQ is the smallest concentration of the analyte, which can produce a response that could be absolutely quantified. It can be calculated using the signal to noise ratio and the slope of the calibration curve by the following formula:

$$LOD = \frac{10\ \sigma}{S}$$

Where σ is the standard deviation of the calibration curve; S is the slope of the calibration curve.

Results and Discussion

The standard chromatograms obtained for hesperidine, quercetin and the combination are presented in Figure 3-5.

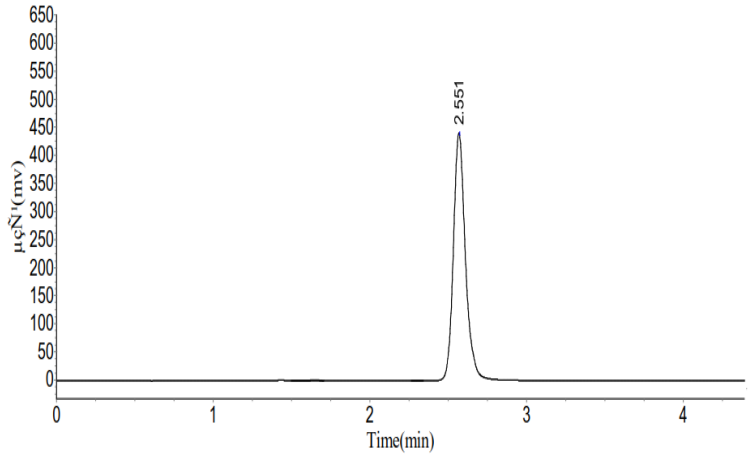

Figure 3. Chromatogram of Hesperidine

Hesperidine eluted out from the sample at averaged of **2.547 minutes** (Retention time, R_t)

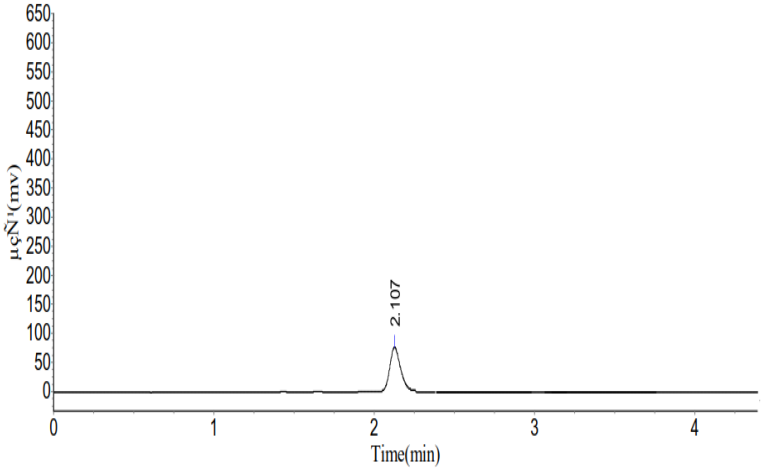

Figure 4. Chromatogram of Quercetin

Quercetin eluted out from the sample at average time of **2.106 minutes** (Retention time, R_t)

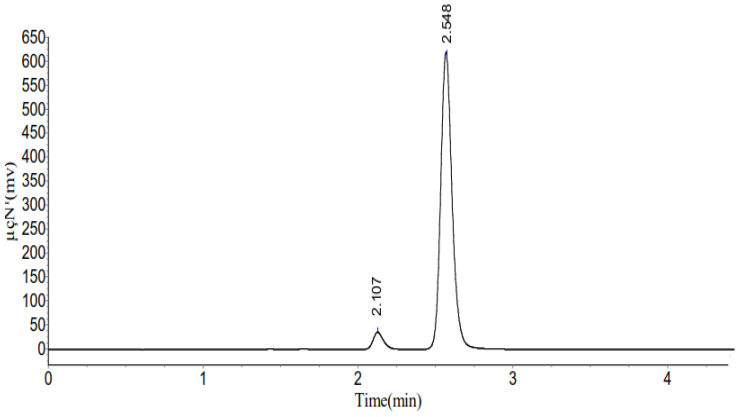

Figure 5. Chromatogram of hesperidine and quercetin combination

Validation of the method

System Suitability

Six replicate injections of the working standard were made to HPLC system and the % RSD of retention time and the number for theoretical plates was observed. The % RSD of retention time was less than 2% and the number of theoretical plates was found to be sufficiently satisfactory for both hesperidine and quercetin. Hence the selected system parameters were found to be suitable for the simultaneous estimation of these drugs.

Table1. System suitability parameters

System suitability parameters	Peak Area AUC x 10^5	Retention Time	No.of Theoretical plates
Quercetin			
Mean	2.132	2.1065	4155.83
R.S.D	**-**	0.001	**-**
Hesperidine			
Mean	2.972	2.547	5664.5
R.S.D	**-**	0.002	**-**

Linearity of Hesperidine

Area of hesperidine peak and concentration were submitted to linear regression analysis to obtain the regression equation and correlation coefficient. Table 2 presents the data of linearity for hesperidine. The regression equation was found to be **Peak area = 551014 (concentration) + 11330** with a correlation coefficient of 0.997. Figure 6 represents the linearity curve for hesperidine. The linearity range was found to be from 1 to 5 µg/mL.

Table 2. Linearity data of hesperidine

Concentration (µg/ml)	**Area under curve**						**Mean**
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	
1	583461	583421	583455	584379	584461	584415	583932
2	998929	987654	998901	997765	998669	998813	996789
3	1683391	1683121	1683312	1662391	1683356	1683264	1679806
4	2177658	2177291	2165801	2177489	2177797	2111687	2164621
5	2772309	2771625	2772254	2771937	2772256	2772084	2772078
Correl Coeff (r^2)	0.997						
Slope (m)	551014						
Intercept (c)	11330						

Linearity of quercetin

Area of quercetin peak and concentration were submitted to linear regression analysis to obtain the regression equation and correlation coefficient. Table 3. presents the data of linearity for quercetin. The regression equation was found to be **Peak area = 43644 (conc) – 808.5** with a correlation coefficient of 0.999. Figure 7 represents the linearity curve for quercetin. The linearity range was found to be from 1 to 5 µg/mL.

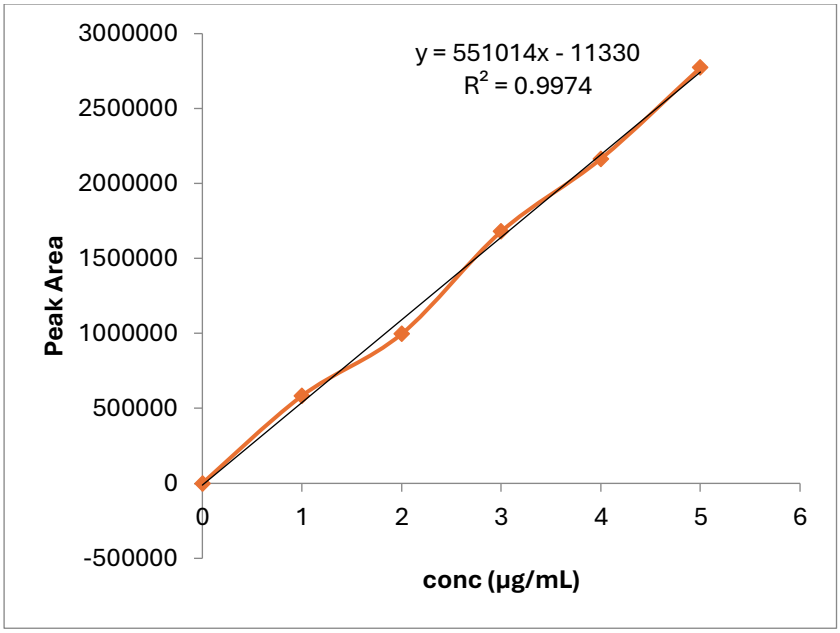

Figure 6. Linearity graph for hesperidine

Table 3. Linearity data of quercetin

Concentration (µg/ml)	Area under curve						Mean
	R1	R2	R3	R4	R5	R6	
1	40571	40552	40564	40522	40445	40551	40534.2
2	86142	86105	86128	86044	86092	86132	86107.2
3	120113	119657	119692	119566	120639	119698	119894
4	162284	163210	164256	163288	163685	164264	163498
5	207855	207763	207821	207611	207732	207831	207769
Correl Coeff (r^2)	0.998						
Slope (m)	41186						
Intercept (c)	1.1587						

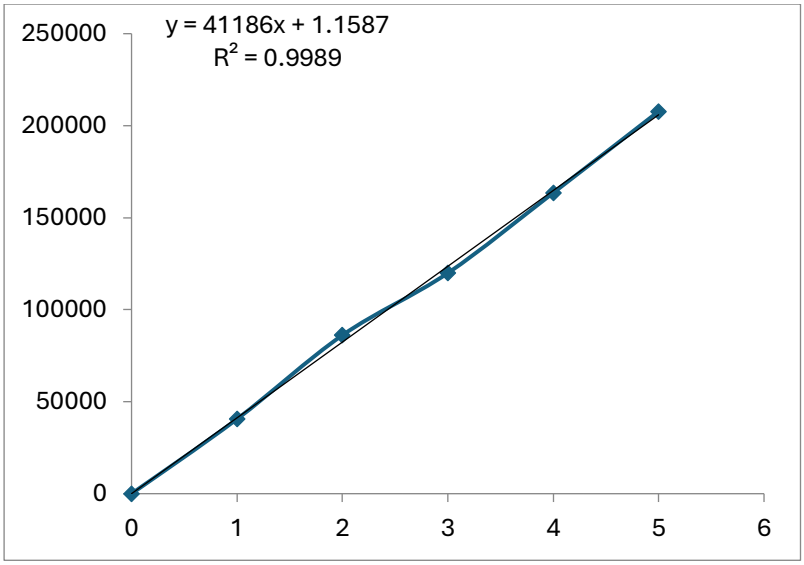

Figure 7. Linearity graph for quercetin

Accuracy

Accuracy of the method was determined by recovery analysis of spiked samples. Pre-analyzed sample was spiked with 50%, 100% and 150% of standard drug solution. The spiked sample was recovered within a range of 99.4-100.17% for hesperidine and 98.5 to 100.4% for quercetin suggesting that the method was accurate in estimating the concentration of the drugs of mixture.

Table 4. Recovery study data of hesperidine and quercetin

Conc. of drug in preanalyzed sample µg/ml)	Conc. of drug added to final (µg)	Mean Recovery	% Recovered (mean)
Hesperidine			
1	0.5	1.500	99.889
1	1	2.010	99.333
1	1.5	2.510	99.200
Quercetin			
1	0.5	1.493	99.544
1	1	1.967	98.333
1	1.5	2.490	99.600

Precision

Precision depicts the ability of the method produce the same results irrespective of the instrument used, or day of analysis or even the analyst performing the analysis. The results of repeatability and intermediate precision are reported in table 5 and table 6 respectively.

Table 5. Repeatability of the developed method

Concentration (mg/ml)	Hesperidine (1 µg/mL)	Quercetin (1 µg/mL)
	Retention time (min)	Retention time (min)
Mean	2.529	2.083
SD	0.041	0.038
%RSD	1.633	1.863

Table 6. Intermediate precision data of the developed method

Concentration (mg/ml)	Hesperidine (1 µg/mL)	Quercetin (1 µg/mL)
	Retention time (min)	Retention time (min)
Mean	2.535	2.093
SD	0.015	0.004
%RSD	0.596	0.235

The results reveal that the % RSD in both the repeatability and intermediate precision studies was less than 2%, thereby ascertaining that the developed method will produced consistent results.

Robustness

A few deliberate changes in flow rate were made for studying its effect on the results obtained by the method. The method was able to adjust to the changes with no significant change in the retention time of the eluted components (Table 7).

Table 7. Effect of flow rate on retention time of hesperidine and quercetin

Flow rate (ml/min)	Retention time (min)*	Standard deviation	% RSD
Hesperidine			
0.9 (-0.1)	2.573	0.001	0.058
1.1 (+0.1)	2.53	0.002	0.075
Quercetin			
0.9 (-0.1)	2.125	0.001	0.071
1.1 (+0.1)	2.084	0.001	0.088

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

The LOD and LOQ were calculated using the slope of the calibration curve obtained in the linearity study. The lowest amount that could be detected (LOD) for hesperidine was found to be 0.084 μg/mL while the LOD for quercetin was found to be 0.054 μg/mL. The lowest concentration of drug that could be quantified with accuracy (LOQ) for hesperidine was calculated to be 0.256 μg/mL whereas for quercetin the LOQ was calculated to be 0.166 μg/mL.

Conclusion

The investigation resulted in the development of a new RP – HPLC method for the simultaneous estimation of hepseridine and quercetin. The method is simple, selective, reproducible and accurate with good precision and can be used for routine pharmaceutical analysis. The method was found to be highly effective in the analysis of fixed dose combination, and extracts of the phytomolecules under investigation.

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