RESEARCH ARTICLE

Development and Validation of HPLC and Spectrophotometric Method for the Quantification of Quercetin in Calendula Flower Extract

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ABSTRACT

HPLC and Spectrophotometric methods are developed to estimate Quercetin in Calendula extract. Developed methods were statistically evaluated according to ICH guidelines. Retention time of Quercetin was found to be 12.06 min and absorption maxima comes out to be 374 nm. Quercetin content in calendula extract was calculated to be 0.190 mg/mL by UV spectrophotometric method. Accuracy data was found to be within the range which shows good recovery values for both methods. Sensitivity data gave limit of detection (LoD) 0.014 µg/mL and limit of quantitation (LoQ) 0.0043 µg/mL for high performance liquid chromatography (HPLC) and LoD 0.82 µg/mL and LoQ 2.47 µg/mL for UV spectrophotometric method. No specific changes in parameters were found showing the method robust and rugged. The repeatability, Inter-day and Intra-day precision of Quercetin gave RSD below 2% showing proposed method is highly precise. Validation parameters for HPLC and UV spectrophotometric method of Quercetin were determined with no mutual significant variance.

Keywords: Calendula *extract,* HPLC method, Quercetin, UV Spectrophotometric method, Validation.

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INTRODUCTION

Calendula officinalis Linn. is an annual or biennial plant with angular, hairy stem, bright yellow to orange flower heads with tubular florets. Its powdered form is found in yellowish brown colour with aromatic odor and a slightly bitter taste.[1,2] It is mentioned in Ayurveda, homeopathy, and Unani medicinal system of medicine indicating that leaves and flowers are antipyretic, anti-inflammatory, antiepileptic and antimicrobial.[3-8] Quercetin, (6.09 mg/g) is a flavonoid found in plants, fruits and vegetables. It helps in mental/physical performance and reduces infection possibility with various other medical solutions.[9,10] There are few methods available in literature for the estimation of the Quercetin from different plants.[11] However, very limited methods are available for the determination of Quercetin from *C. officinalis*. Muñoz *et al.* has developed HPLC method for quantification of total Quercetin in *C. officinalis* with the linearity between 1.0 to 5.0 mg/mL. The method is not sensitive at micrograms level.[12] Hamad *et al.* detected Quercetin along with other flavonoids from *C. officinalis* but the method developed has not been further validated.[13] Thus, there was a need for development sensitive method for estimation of Quercetin from *C. officinalis.* In the present study, authors have developed, validated and compared two methods for estimation of Quercetin and applied the developed methods for estimating Quercetin from *C.* officinalis extract.

MATERIALS AND METHODS

Reagents and Chemicals

All the chemicals and solvents were of HPLC grade and employed without any purification. All the solutions were prepared in HPLC grade water. If not specified, all solutions were filtered through a 0.2 µm Ultipor® N66® Nylon 6, 6 membrane filter (Pall Life Sciences, USA) prior to use. The calendula flower extract was obtained from Kshipra Biotech Private Limited. Quercetin standard was supplied as a gift sample from Tokyo Chemical Industry (TCI), Japan. Methanol was procured from Thermo Fisher Scientific, India, O- phosphoric acid was purchased from Merk Ltd., Mumbai.

Instruments

(i) HPLC system of Shimadzu equipped with an auto-sampler and SPD-10AVP UV-vis detector with column Phenomenex Luna, C_{18} bonded with 5 µm (4.6 x 250 mm) particle size,

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coupled with LC-solution software was used for recording and processing of chromatographic data.

(ii) Spectrophotometric measurements were made on a Shimadzu 1700 double beam UV Visible spectrophotometer with a fix slit width of 1 nm coupled with computer loaded with Shimadzu UV Probe software of version 2.31.

Chromatographic Conditions

Mobile phase consisting of Methanol and 0.5% v/v solution of O-phosphoric acid was prepared, filtered through 0.45 µm membrane filter and degassed by an ultrasonic bath before each use. Best resolution and sensitivity of the method was at 370 nm at a flow rate of 1.0 mL min^{-1} with isocratic elution (50:50 v/v) and 30 °C. The column was equilibrated for at least 30 min with the mobile phase flowing through the system. Each solution was prepared diluent (Methanol: Water (8:2) (v/v)) and was injected in triplicate.

Preparation of Standard Stock Solution for HPLC Method

Stock solutions of Quercetin reference standard (1 mg/ml) was freshly prepared by dissolving in diluent and sonicated for 10 minutes and stored at 2–8°C until used. The standard solution was prepared by dilution of the stock standard solution with Methanol: Water (8:2) (v/v) to obtain 10 µg/mL. This solution was used for optimization of proposed method to achieve the calibration curve.

Preparation of Sample of Calendula Flower Extract for HPLC Method

Accurately weighed quantity of 2.5 mg of calendula flower extract was transferred to 25 mL volumetric flask. Sufficient diluent was added to dissolve the extract and after sonicating for 30 minutes volume was made upto the mark with diluent to obtain 100 µg/ml. Solution was filtered through a 0.2 µm Ultipor® N66® Nylon 6, 6 membrane filter and injected in HPLC system.

Preparation of Standard Stock Solution for Spectrophotometric Method

10 mg Quercetin was weighed accurately, and 10 ml methanol was transferred into a volumetric flask, then further taken 5ml from above stock solution and put in 50 mL volumetric flasks and volume adjusted with methanol up to mark and sonicated for 5 minutes. This solution was further diluted with methanol, to obtain various dilutions from 2–12 µg/mL.

Preparation of Sample of Calendula Flower Extract for Spectrophotometric Method

A one gm amount of Calendula flower extract was taken in thoroughly cleaned culture tubes containing 5ml of methanol and culture tubes were tightly closed. These culture tubes were shaken on water bath shaker for 24 hours. at room temperature. Then every sample was centrifuged at 15,000 rpm and supernatant was extracted and filtered. Post-dilution, the filtrates were determined spectrophotometrically.

Procedure for UV Spectrophotometric Method

All the samples were found to be stable during each kind of experimental measurements (spectra was unchanged up to about 24 hours). Each measurement was done at room temperature. Absorption spectra of the samples were recorded between 200–400 nm using a 1.0 cm quartz cell in UV-visible spectrophotometer and absorbance of these solutions was recorded at 374 nm against methanol as blank.

Optimization of HPLC Method

After various trials of different chromatographic parameters, optimum mobile phase was found to be Methanol**:**0.5% v/v solution of O-phosphoric acid with isocratic elution at flow rate of 1 mL/min. Optimal resolution and sensitivity was obtained for Quercetin at 370 nm. Typical chromatogram with optimized condition gives sharp and symmetric peak with retention time of 12.06 min. Developed chromatogram of is shown in (Figure 2).

Validation of Optimized Methods

The analytical method validation presents that the method's features satisfy the requirements of the application province. Those were validated in the limelight of International Council for Harmonisation (ICH) Guidelines for linearity, range, accuracy, precision, robustness, ruggedness, limit of detection, limit of quantification and sensitivity.

Linearity

It is an analytical procedure (within a given range) to obtain test results directly proportional to the concentration of analyte in the sample. For HPLC method, linearity was checked by diluting standard stock solution at six different concentrations. The calibration plot was generated by replicate analysis (n = 3) at all concentration levels. With six different concentrations in triplicate, a calibration curve was derived in the designated range of 1–10 µg/mL for Quercetin. The chromatographic response was linear over the same range. For UV spectrophotometric method, a calibration curve was plotted over a concentration range of 2 to 12 µg/mL at 374 nm. Accurately measured working stock solution of Quercetin at 374 nm (2,4,6,8,10 and 12 mL) was transferred to separate series of 10 mL volumetric flask and diluted up to the mark with methanol. The absorbance of all solutions was taken at their respective wavelength. The linear regression equation was calculated by the least-squares method using GraphPad Prism 9.0.0 program for both methods.

Precision and Accuracy

The precision expresses the closeness among a series of arrangements obtained from multiple sampling of the same homogeneous sample. The intermediate precision of the method was confirmed by intraday and inter-day analysis, repeated thrice. The absorbance was determined and %RSD was also calculated. Accuracy data is obtained by employing recovery experiments by determining %mean recovery of sample at three levels (80–120%). At each level, three determinations were performed. Accuracy precision for the developed method was measured in terms of % R.S.D.

Specificity

Specificity for HPLC method was determined by evaluating the peak purity of sample which was determined by spectral

comparison of calendula flower extract peak at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions.

Sensitivity

Sensitivity of both the methods was determined by calculating LoD and LoQ using following equation:

$$LoD = 3.3\ \sigma/S$$

$$LoQ = 10\ \sigma/S$$

Where σ = Standard deviation of the response; S = slope of the calibration curve

Robustness

Only the most critical parameters were exchanged to measure the extent of method robustness, and the chromatographic profile was observed and recorded.[15-17] The studied parameter was changed in wavelength and flow rates for HPLC method. For UV spectrophotometric method change in wavelength was studied. The deviations in the results of peak area were expressed as % RSD.

Ruggedness

Ruggedness was determined by analyzing the same samples of Quercetin by two different analysts and the respective percentage recovery results were shown as %RSD. The ruggedness of the methods was studied by changing the experimental conditions. In the present work, two different analysts performed both the methods with same set of parameters and mean, standard deviation and relative standard deviation was calculated.

Determination of Quercetin Content in Calendula Extract UV Spectrophotometric Method

One g of Calendula extract was taken in thoroughly cleaned culture tubes containing 5 mL of methanol and tightly closed test tubes. These tubes were kept on water bath shaker at 25°C for 24 hours at room temperature. Then every sample was centrifuged 15,000 rpm, and the supernatant was withdrawn to be filtered. The filtrates were diluted and obtained spectrophotometrically.[14]

RESULTS AND DISCUSSION

Reversed-phase chromatography (C8, C18) separates the non-ionic and ion-forming non-polar to medium polar substances. Also, most ionizable pharmaceutical compounds may be separated on octadecylsilane reversed-phase columns. Hence, octadecylsilane was selected for estimation of the Quercetin. Firstly, HPLC analysis was done for the blank sample, and chromatogram was obtained (Figure 1). Figure 2 describes the chromatogram of the Quercetin (10 µg/mL), which has a retention time of 12.065 minutes. For UV Spectrophotometric method, 12 µg/mL of Quercetin in methanol as solvent was scanned in the 200-400 nm range. Absorption maxima of Quercetin were found to be at 374 nm similar to literature as shown in Figure 3.

Calibration Curve (Linearity)

The chromatographic response was linear over an analytical range of 1–10 µg/mL for HPLC and absorbance was linear over 2-12 µg/ml for UV Spectrophotometric method for estimation of Quercetin. The linear regression equation was calculated by the least-squares method using GraphPad Prism 9.0.0 program. The variance of slope and intercept were reported in Table 1 for HPLC method and Table 2 for UV Spectrophotometric method. The intercept is not significantly varying from zero; hence no interference is found to estimate the Quercetin by both the methods. Further, the slope and intercept were within the confidence interval. Overlay spectra of Quercetin have been illustrated in Figure 4.

Accuracy

Accuracy assessment is performed utilizing recovery experiments, determination of %mean recovery of the sample at three levels (80–120%). Three determinations were performed at each level. Percent mean recovery was calculated as shown in Table 3 for HPLC method and Table 1 for UV Spectrophotometric method. The accepted limits of recovery

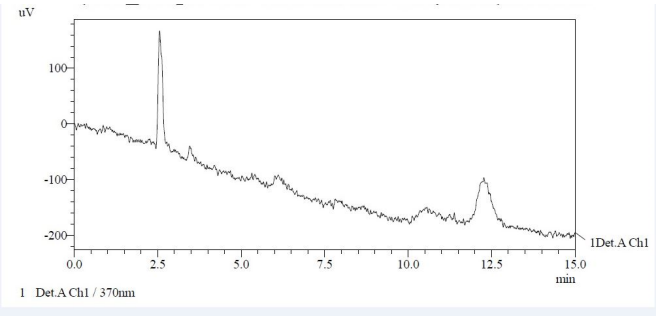

Figure 1: Chromatogram of Blank Sample

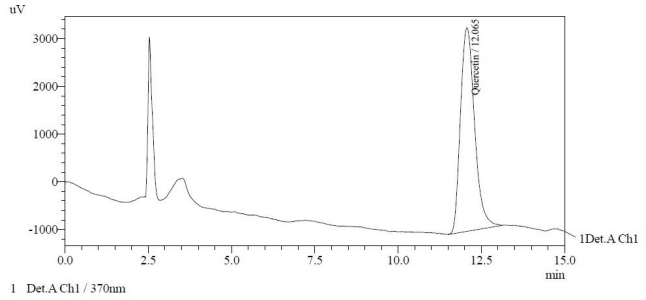

Figure 2: Chromatogram of Quercetin Standard (10 µg/ml)

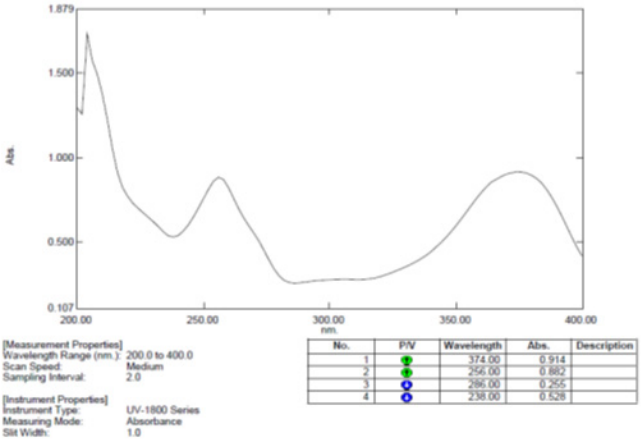

Figure 3: Absorbance maxima of Quercetin by UV Spectrophotometric method

are 90–120%. Obtained recovery study data indicates that within the range, Quercetin indicates good recovery values for both methods.

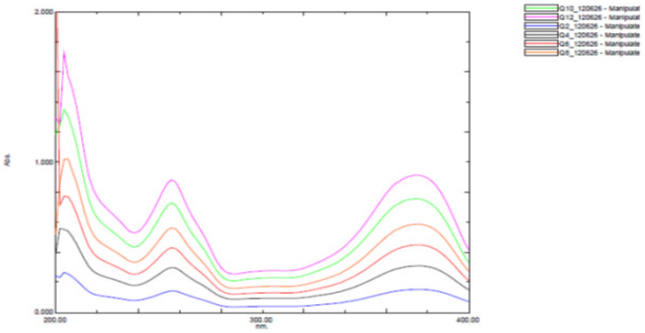

Figure 4: Overlay spectrum of Quercetin by UV spectrophotometric method

Table 1. Validation parameters for RP-HPLC method of Quercetin

Parameters	*Quercetin*
Absorption maxima (nm)	370
Linearity range (µg/mL)	1-10
Coefficient of determination (r^2)	0.998
Correlation coefficient (r)	0.999
Regression equation (Y^a)	Y=39002x + 298.49
Slope (m)	39002
Intercept (c)	298.49
LoD (µg/mL)	0.014
LoQ (µg/mL)	0.043
Precision	
Repeatability (%RSD)	0.53
Intra-day (%RSD)	1.38
Inter-day (%RSD)	1.27

Table 2: Validation parameters for UV spectrophotometric method of Quercetin

Parameters	*Quercetin*
Absorption maxima (nm)	374
Linearity range (µg/mL)	2-12
Coefficient of determination (r^2)	0.9990
Correlation coefficient (r)	0.9994
Regression equation (Y^a)	Y=0.075x – 0.001
Slope (m)	0.075
Intercept (c)	0.001
LOD (µg/ml)	0.817
LOQ (µg/ml)	2.474
Precision	
Repeatability (%RSD)	0.63
Intra-day (%RSD)	0.55
Inter-day (%RSD)	0.53
Accuracy	
80%	100.593±0.463
100%	98.278±0.481
120%	100.844±0.429
% Quercetin in Calendula extract (mg/ml) (Mean ± SD)	0.190±0.001

Precision

The method's precision was demonstrated by repeatability, intra- and inter-day variation studies. The mean, standard deviation and percentage of relative standard deviation were calculated and presented in Tables 1 and 2. Repeatability, inter-day and intra-day replicates of Quercetin gave an RSD below 2.0%, revealing that the proposed method is highly precise.

Repeatability

It is the concordance of a pattern of measurements of the same quantity during experiments under designated conditions (analyst, apparatus, instrument, and day) in rapid succession. For HPLC method, standard solution of Quercetin (1.4 µg/mL) was prepared and analyzed 6 times as per the proposed method and %RSD was calculated (Table 1). For UV Spectrophotometric method, standard solution of Calendula extract at 374 nm (8 µg/mL) was prepared and analyzed 6 times as per the proposed method and % RSD was calculated and presented in Table 2.

Intraday and Intraday Precision

The intra-day and inter-day variations to determine Quercetin were carried out 6 times on the same day and three consecutive days with concentration of 1.4 µg/mL of Quercetin for HPLC method and 8 µg/mL for UV spectrophotometric method. %RSD was calculated and represented in Table 3 for HPLC method and Table 2 for UV Spectrophotometric method.

Robustness

Robustness for HPLC and UV spectrophotometric method in Tables 4 and 5 indicated a small change in the conditions not influencing the Quercetin determination. Identified changes

Table 3: Results of recovery study of Quercetin by HPLC method

Level of addition	*% Recovery*	*Statistical Analysis*		
		Mean	*SD*	*%RSD*
80%	100.69	100.49	0.4711	0.4688
	100.83			
	99.95			
100%	106.51	105.82	0.68323	0.6456
	105.80			
	105.15			
120%	91.74	92.18	0.6847	0.7428
	92.97			
	91.83			

Table 4: Robustness parameter of Quercetin studied for HPLC method

Parameters	*Alterations*	*%RSD for Area (n=6)*
Change in flow rate	-2%	1.36
	Normal	1.27
	+2%	1.28
Detection wavelength	-2 unit	1.48
	Normal	1.38
	+2 unit	0.51

Table 5: Robustness parameter of Quercetin studied for UV spectrophotometric method

Parameters	*Alterations*	*%RSD for Area (n = 6)*
Detection wavelength (of 8µg/ml)	-4 unit (370)	0.968
	Normal (374)	0.620
	+4 unit (378)	0.933

Table 6: Statistical comparison of the results.

	HPLC method	*UV Spectrophotometric method*
Quercetin content Mean ± SD	100.48 ± 0.47	100.59 ±0.46
	$t_{calculated}$ = 0.289 $t_{theoretical}$ = 2.92	$t_{calculated}$ = 0.289 $t_{theoretical}$ = 2.92

Results obtained are average of ten experiments for each; SD, standard deviation.

were not observed in both methods' parameters, demonstrating robust methods.

Ruggedness

Ruggedness was found by analyzing the same Quercetin samples by two different analysts and the respective percentage recovery was noted and the results were indicated as %RSD. Percent RSD was found to be 1.86 and 1.08 for Analyst 1 and 2, respectively for HPLC method and 0.2 and 0.25 for Analyst 1 and 2 for UV spectrophotometric method. It was observed that there were no marked changes in the HPLC parameters demonstrating that the HPLC methods developed are rugged.[15,16,17]

Sensitivity

The LoD and LoQ of the method were obtained on standard deviation of the response and the calibration curve slope (s) at approximate LoD and LoQ levels. The LoD was found to be 0.014 µg/mL, and LoQ was found to be 0.0043 µg/mL, respectively for HPLC method, and LoD was found to be 0.82 µg/mL and LoQ was found to be 2.47 µg/mL respectively for UV spectrophotometric method and which showed that sensitivity of the method was high.

Specificity

Till the specificity, the procedure is applied to analyte of interest and checked by evaluating the calendula flower extract samples for any interfering peaks. It was determined concerning interference where other extract constituents are present. Other extract constituents did not involve with the Quercetin peak.

Determination of Quercetin Content in Calendula Extract by UV Spectrophotometric Method

It was found that in solution of 2 mg/mL of Calendula extract in methanol contained 0.190 ± 0.001 mg/mL of Quercetin as described in Table 2.

Statistical Comparison of the Results of the Developed Four Methods

Validated methods were employed to analyze the Quercetin content in Calendula extract without interfering with other constituents. Results obtained were compared statistically by Student's *t*-test. Calculated values of the Student's *t*-values at 95% confidence level (*p-value*=0.7864), indicating no significant differences among the results of these developed methods, represented in Table 6.

CONCLUSION

The developed RP-HPLC method was robust, specific, sensitive, precise, accurate, and reliable and can be applied for routine analysis in research institutions. It can perform as a quality control method for quantifying total Quercetin, to become a tool for quality control of *C. officinalis* extract.

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