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Research Article

Development and Validation of a Spectrofluorimetric Method for the Estimation of Rifabutin in Bulk and Formulation

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ABSTRACT

A simple, accurate, precise, sensitive and cost-effective spectrofluorimetric method was developed and validated for the estimation of quercetin (quercetin) in bulk and formulation. The relative fluorescence intensity of quercetin was measured in distilled water at an excitation wavelength of 444 nm and an emission wavelength of 538 nm. Proposed method was found to be linear over the range of 100 to 4000 ng/ml with correlation coefficient 0.9999. Proposed method was validated using different analytical method validation parameters viz. Accuracy, precision, LOD, LOQ, robustness and ruggedness using QC standards as per the ICH guidelines. The percentage recovery was found to be 100.01 % and percentage RSD values were found to be less than 2 for accuracy and precision studies. The detection and quantification limits for the proposed method were found to be 4.38 and 13.29 ng/ml, respectively. A simple, accurate, precise, sensitive yet cost-effective spectrofluorimetric method was developed for the estimation of quercetin in bulk and formulation. The said spectrofluorimetric method was found to be economic as it comprises water as a solvent.

INTRODUCTION

Quercetin chemically is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy4h-1-benzopyran-4-one, the major representative of the flavonol subclass of flavonoids, is a common dietary component[1]. Quercetin (Fig.1) is a plant flavonol and it is found in many fruits, vegetables, leaves, and grains. It acts as antioxidant. It is a non-specific protein kinase enzyme inhibitor[2-4]. It has also been reported to have estrogenic activities by activating estrogen receptors[5-6]. It is used in treatment of heart diseases, exercise induced respiratory problems, high cholesterol, diabetes, asthma, gout, cancers such as lung cancer, ovarian cancer and pancreatic cancer[7-8]. Considering the therapeutic importance of quercetin some of the analytical methods were reported from all over the globe but till today, there is no single spectrofluorimetric

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method available for the estimation of quercetin using cost effective, non-reactive and biocompatible solvents. Therefore, a simple, sensitive, accurate and precise spectrofluorimetric method was developed for the estimation of quercetin in bulk and its formulation.

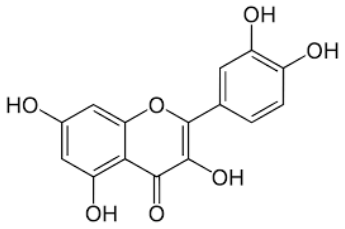

Fig. 1: chemical structure of quercetin

MATERIALS AND METHODS

Quercetin was purchased from TCI chemicals (India) Pvt. Ltd. HPLC grade Ethanol, water was used for study.

Instruments used

The spectrofluorimetric study was carried out with a shimadzuRF-5301 fluorimeter to determine levels of fluorescence in the quercetin. A xenon 150 w lamp was used as a light source. Quartz cells having 48 mm height, 10 mm path length with 0.5mm slit width were used for fluorescence measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the weighing purpose.

Preliminary analysis

A preliminary analysis was carried out to determine the excitation and emission wavelength of quercetin. Various solvents like distilled water, methanol, acetonitrile, isopropyl alcohol and their combinations were used to determine appropriate media for quercetin. Quercetin showed maximum fluorescence intensity in Ethanol: Water (75:25 %v/v) as a media. Initially, quercetin solution of 100 ng/ml strength was prepared in ethanol. Prepared solution was scanned spectrofluorimetrically to obtain the excitation and emission wavelengths. The scanning was performed over 250 nm to 800 nm range and excitation and emission wavelength were found to be 444nm and 538nm (figure.2) respectively.

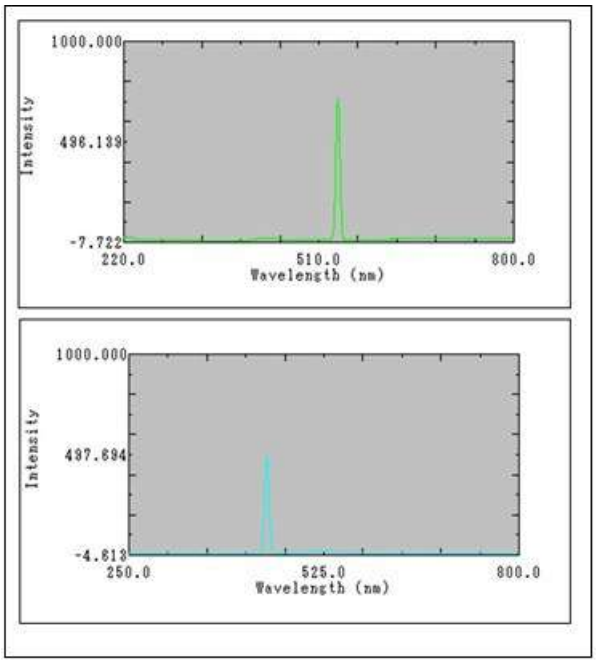

Fig. 2: Excitation and Emission Spectra of Quercetin

Preparation of Standard Stock Solution

Accurately weighed 10 mg of quercetin was transferred into the calibrated volumetric flask and dissolved in 10 ml water to achieve a stock solution of 1000 µg/ml (stock-I). Stock- I solution was suitably diluted with water to achieved further calibration standards.

Construction of Calibration Curve

Calibration curve was prepared by diluting the stock-I (1000 µg/ml) solution to achieve the seven different calibration standards representing 100, 200, 400, 600, 1000, 2000, 4000 ng/ml strength. The fluorescence intensity was measured at pre-defined excitation and emission wavelengths of 444 and 538 nm respectively. The calibration curve representing concentration vs. Fluorescence intensity was plotted using excel program of Microsoft office 2013. Above mentioned procedure was repeated three times, so that reproducible results can be obtained.

Spectrofluorimetric Method Validation

Validation is the process which provides a high degree of assurance, so as to produce a desired result and meeting its predetermined specifications and quality characteristics. Developed fluorimetric method for the estimation of quercetin was validated as per the ICH guidelines. Different validation parameters like linearity and range, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were calculated using predefined calibration standards and or quality control standards as described below[9-10].

Linearity and Range

Linearity of the proposed spectrofluorimetric method was calculated by using seven different calibration standards. After analysis of calibration standards, calibration curves in terms of concentration vs. Fluorescence intensity plots were developed and subjected to linear least square regression analysis. R square value was considered to be important factor for determining the linearity of the proposed method.

Accuracy

To determine the accuracy of the method, different quality control solutions were prepared independently from stock-I i.e., LQC: 150 ng/ml, MQC: 1500 ng/ml and HQC: 3900 ng/ml and analyzed at level of 80%, 100% and 120% of its predefined concentrations .to the predefined concentrations, different amounts of quercetin were added (standard addition method) and the accuracy was calculated on the basis of percent recovery.

Precision

The precision of the method was checked by preparing different quality control solutions independently from stock-I i.e. LQC: 150 ng/ml MQC: 1500 ng/ml HQC: 3900 ng/ml at three different time intervals in a day. Same procedure was followed on three different consecutive days so as to obtain inter-day variation. The fluorescence intensities for quercetin were recorded and the results were expressed as % relative standard deviation (%RSD).

Robustness

Robustness of the proposed spectrofluorimetric method was established by changing composition of the ethanol by ± 1.0 %. MQC samples of quercetin were prepared in ethanol with and analyzed at 444nm and 538 nm (excitation-emission wavelength of quercetin). The results were calculated in terms of % RSD.

Ruggedness

Ruggedness of the proposed method was studied by analyst variation. MQC samples of quercetin were prepared by three different analysts of the laboratory and were analyzed at 444nm and 538 nm. The results were calculated in terms of % RSD.

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

LOD and the LOQ of the drug were calculated by using the following equations as per ich guidelines.

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$

Where, SD= standard deviation of lower most concentration of calibration curve

S= slope of calibration curve

Estimation of Quercetin in Bulk and Marketed Formulation

The quercetin content in its marketed formulation (Health vit Quercetin) was estimated using pre-validated UV-Visible spectrophotometric method. Capsule formulation contents (labeled strength: 100 mg/capsule) were dissolved in 1 ml of co-solvent system to achieve a stock solution of 1500 ng/ml (n=5). Said solution was suitably diluted with co-solvent system and analyzed for the Budesonide content using proposed UV method.

RESULTS AND DISCUSSION

Construction of Calibration Curve

Quantification of unknown samples by any instrumental method of analysis needs a

reproducible calibration curve and a mathematical equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. To establish linearity of the proposed method, seven different calibration standards were prepared from the stock solution and analyzed at excitation wavelength 444 nm and emission wavelength 538 nm by spectrofluorimeter. Least square linear regression analysis was performed for the obtained spectrofluorimetric data using MS-excel 2013. Calibration curve was repeated five times for reproducibility. Various concentrations and their fluorescence intensities with mean ± standard deviation were reported (table 1).

Table 1: calibration standard data for quercetin

S. No.	Concentration (ng/ml)	Fluorescence intensity
1	100	21.54±0.5344
2	200	43.18±0.3762
3	400	85.47±0.5464
4	600	121.54±0.4641
5	1000	200.34±0.8805
6	2000	406.19±0.4746
7	4000	812.15±0.6183

SPECTROFLUORIMETRIC METHOD VALIDATION

Linearity and Range

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven point calibration curve of quercetin was plotted covering a range of 100-4000 ng/ml. Different concentrations and the respective mean fluorescence intensities values are depicted in table 1. Calibration curve when subjected to least square regression analysis yielded an equation; y=0.2025x +1.3959 with correlation coefficient 0.9999 (figure 3). From the linearity study, it was revealed that, developed method was linear over the concentration range of 100 to 4000 ng/ml.

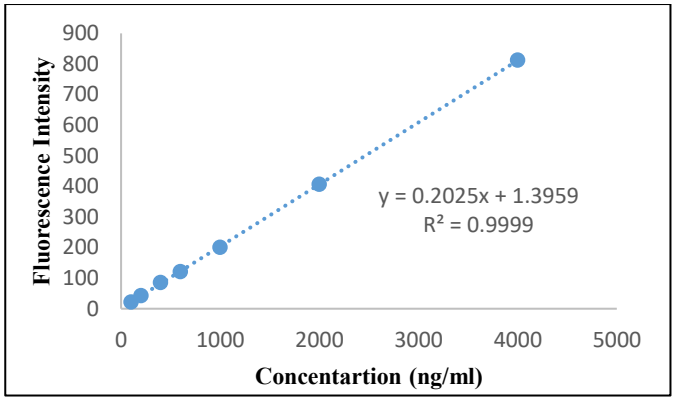

Fig. 3: calibration curve for quercetin

Accuracy

Accuracy is the closeness of test results to the true value obtained by method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For quercetin, accuracy was determined using recovery studies. At 80, 100 and 120 % standard addition, mean recovery of quercetin was found in between99.93 to 100.16 %. The relative standard deviation (% RSD) was found to be less than 2 as shown in table 2. From the results of accuracy studies, it was predicted that developed method is highly accurate.

Table 2: Accuracy data of Spectrofluorimetric method for Quercetin

S. no.	Concentration (%)	Origin level (ng/ml)	Amount added (ng/ml)	% recovery	Mean % recovery	% RSD
1	80	150	120	99.87	99.93	0.6342
2	80	150	120	100.59		
3	80	150	120	99.33		
4	100	1500	1500	99.95	100.16	0.4357
5	100	1500	1500	100.66		
6	100	1500	1500	99.87		

7	120	3900	4680	99.84	99.96	0.1873
8	120	3900	4680	100.18		
9	120	3900	4680	99.87		

Precision

Precision is defined as closeness of agreement among the individual test result when the method is applied repeatedly to multiple sampling of homogeneous sample. Precise analytical method leads to accurate results. Intra- and inter-day precision of spectrofluorimetric method was established at 150, 1500 and 3900 ng/ml levels of quercetin. The results expressed in terms of mean fluorescence intensity values, % assay and % RSD for the intra- and inter-day precision study are demonstrated in table 3 and table 4 respectively. Percent RSD values of intra-day precision study were found to be in between 0.10 and 1.68 whereas those of inter-day precision study were in between 0.12 and 1.66 overall, % RSD values of less than 2 demonstrated that developed spectrofluorimetric method is precise and reproducible.

Table 3: Intra-day precision data of Spectrofluorimetric method for Quercetin

		Day 1			Day 2			Day 3		
S. No	Concentration Range (ng/ml)	Amount (Mean)	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	150	147.10	98.06	1.0222	148.33	98.88	1.4817	150.07	100.04	1.6806
2	1500	1497.61	99.84	1.3873	1509.05	100.60	1.2728	1487.81	99.18	0.3137
3	3900	3901.64	100.04	0.1043	3904.75	100.12	0.1384	3899.51	99.98	0.1347

Table 4: Inter-day precision data of Spectrofluorimetric method for Quercetin

		Day 1			Day 2			Day 3		
S. No	Concentration Range (ng/ml)	Amount (Mean)	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	150	148.50	99.00	1.3948	150.80	100.53	1.3287	148.69	99.13	1.6615
2	1500	1498.16	99.87	0.9913	1508.84	100.58	0.6724	1525.27	101.68	0.7032
3	3900	3901.97	100.05	0.1258	3901.43	100.03	0.1562	3901.61	100.04	0.1311

Robustness

Robustness of an analytical method is the measure of its capacity to remain unaffected by small but deliberate change in method parameters. It is an important parameter of analytical method as a small, un-intentional change in method parameters like solvent composition, buffer strength, pH etc. May occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the method. Therefore, robust analytical method is preferred. Robustness of proposed spectrofluorimetric method was performed by changing the pH of water. After analysis, it was found that change in pH of water did not affect the performance of method. Percent RSD values were found to be in between 0.46 and 0.89 (table 5). Percent RSD values below 2 depicted that proposed spectrofluorimetric method is robust in nature.

Table 5: Robustness data of Spectrofluorimetric method for Quercetin

S. No.	Concentration (ng/ml)	Mobile phase composition (% v/v)	Amount	% RSD
1	1500	74:26	1490.08	0.89
2	1500	75:25	1502.14	0.46
3	1500	76:25	1589.29	0.79

Ruggedness

Ruggedness of analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions, such as different laboratories, different analyst, different instruments, different lots of reagent, different temperatures etc.. In order to determine the ruggedness of proposed spectrofluorimetric method, quercetin solutions were prepared and analyzed by different analysts. Sample analysis and data processing resulted into % RSD values between 0.65 and 0.98. Results of ruggedness studies revealed that proposed spectrofluorimetric method was rugged as it showed % RSD values less than 2 (table 6).

Table 6: Ruggedness data of Spectrofluorimetric method for Quercetin

S. No.	Concentration (ng/ml)	Analyst	Amount	% RSD
1	1500	I	1510.49	0.76
2	1500	Ii	1506.09	0.65
3	1500	Iii	1524.16	0.98

Limit of quantitation (LOQ) and limit of detection (LOD)

Limit of quantification (LOQ) represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. Limit of detection (LOD) of an individual analytical procedure is the lowest amount of an analyte in a sample which can be detected but not necessarily quantitated as an exact value. From the standard deviation of lower most concentration and the slope of the calibration curve, LOD and LOQ of proposed spectrofluorimetric method was found to be 4.38 and 13.29 ng/ml respectively (table 7). Lower LOQ value indicated that proposed method is sensitive enough to quantify the quercetin content of samples at its lower level.

Table 7: LOD & LOQ data for Spectrofluorimetric method for Quercetin

1	LOD	4.38 ng/ml
2	LOQ	13.29 ng /ml

Estimation

The developed spectrofluorimetric method was successfully applied for estimation of quercetin in marketed formulation. By proposed method, quercetin content in the capsules was found to be 101.68 ± 0.57 %.

CONCLUSION

A simple, accurate, sensitive and precise spectrofluorimetric method for the estimation of Rifabutin was developed and validated. The proposed method was found to be robust and rugged in nature with high accuracy and precision. Proposed method was successfully used for the estimation of Rifabutin in its marketed formulation.

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