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Validation of Newly Developed Analytical Method for Standardization of Apigenin Using RP-HPLC Method in Prepared Extract

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ABSTRACT

In this study, a simple, precise and accurate analytical method was developed and validated for identification of apigenin using RP-HPLC method in prepared extract. Spectrophotometric determination was performed on a Perkin-Elmer UV-VIS Double Beam Spectrophotometer to know the maximum absorbance of the compounds. Chromatographic separation was performed using Merck C18 analytical column (5 µm, 250 mm × 4.6 mm, I.D). Phosphate buffer at acidic pH and acetonitrile in the ratio of 30:70 v/v was considered to be suitable solvent system. The effluents were detected by means of UV detector at 268 nm. The calibration curves were linear at a range of 10–50 µg/ml with significant correlation coefficient of 0.9996. The retention time was found to be at 3.53 min. The method was validated according to ICH guidelines and was found to contain the % RSD values below 2% which shows that the method was precise, specific and accurate.

Keywords: Apigenin, Spectrophotometric method, Method development and validation, RP-HPLC method, ICH guidelines.

INTRODUCTION

Apigenin is a plant derived flavanoid and most powerful chemo preventive and anticancer agents. It is a flavone abundantly found in fruits and vegetables, exhibits anti-proliferative, anti-inflammatory, and anti-metastatic activities [1-5]. The chemical

name of apigenin is 5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one with a molecular formula $C_{15}H_{10}O_5$ and molecular weight is 270.24 g/mol. It's highly soluble in ethanol, concentrated sulphuric acid but poorly soluble in water [6]. Literature survey revealed that there is an increasing need to develop a simple, accurate and economic RP-HPLC method for the identification of apigenin [7-14]. In the present study a novel, precise and accurate method has been developed to obtain accurate and reproducible results with least Relative Standard Deviation (RSD) compared to all other existing methods and validated as per ICH guidelines.

MATERIALS AND METHODS

Plant material and reagents

The plant parts were collected from the local market and were shade dried for 15 days. After shade drying they are separately powdered and stored in well closed airtight containers for further use. Apigenin (API) standard was purchased from Sigma-Aldrich Laboratories Ltd., Bangalore. HPLC grade methanol, acetonitrile and potassium di-hydrogen orthophosphoric acid were purchased from E. Merck (India) Ltd., Worli, Mumbai, India.

Extraction

The shade dried and coarsely powdered plant parts were extracted with 80% aqueous methanol by maceration at room temperature for 72 h [6-10]. After completion of extraction, the extracts were filtered, concentrated to dryness. The residues were then stored in desiccator.

Apparatus and chromatographic conditions

Analysis using UV was performed on a Perkin-Elmer UV-VIS Double Beam Spectrophotometer. Data acquisition was made with software named as lambda 25 nm and 268 nm is used as wavelength for the study. HPLC analysis was performed on a chromatographic system of Waters 2695 equipped with an auto injector with UV/Visible detector (UV-2489). A chromatographic separation was achieved on Merck C18 analytical column (5 µm, 250 mm × 4.6 mm I.D). Data acquisition was made with Empower 3 software. Analytical Balance (BSA224S-CW, Sartorius), Ultra Sonicator (Fast Clean) from Shimadzu were used for the study.

Preparation of standard and sample solutions

As the compound is freely soluble in ethanol standard stock solutions of API were prepared in ethanol at a concentration of 100 µg/ml. The aliquots of API were prepared by using mobile phase.

Accurately weighed and transferred 100 mg of the extract into a 100 ml clean dry volumetric flask containing 10 ml ethanol. The solution was sonicated for about 10 min and then made upto volume with mobile phase. 10 ml was pipette out from stock solution into separate 100 ml volumetric flask and made upto the mark with mobile phase.

Validation of HPLC method

The method was validated as per ICH guidelines for linearity, accuracy, precision, sensitivity, robustness, ruggedness and sensitivity [15].

Linearity

Linearity was performed by making serial dilutions at a range of 10-50 µg/ml. Calibration curve was constructed by plotting concentrations against peak areas. Linearity was assessed by calculating slope, y-intercept and co-efficient of determination. The results obtained were sown in Table 1 and Figure 1.

Table 1: Linearity of apigenin

S. No.	Conc. (ppm)	Peak Area
1	10	185073
2	20	325581
3	30	485690
4	40	626758
5	50	784377

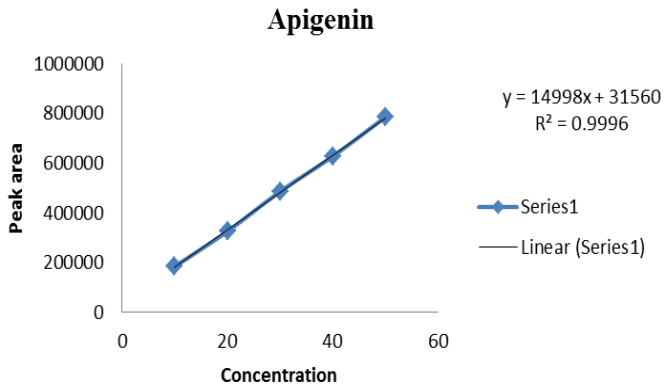

Figure 1: Calibration plot of apigenin

Accuracy

The accuracy of proposed method was determined at three concentration ranges (50%, 100% and 150%). All studies were carried out in triplicates and the results obtained were tabulated in Table 2.

Table 2: Accuracy results for apigenin

Level	Amount added (ppm)	Total amount (ppm)	Amount found	Amount recovered	% recovery
50%	5	15	14.87	4.95	99
100%	10	30	29.82	9.94	99.43
150%	15	45	44.66	14.88	99.24

Precision

The degree of closeness of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample under the prescribed condition was determined. The intra-day precision was performed by analyzing six replicate standard solutions on the same day, and inter-day precision was performed by analyzing a series of standard solutions for 3 consecutive days using the proposed HPLC method. The data obtained was represented in Table 3**.**

Table 3: Precision results for apigenin

Parameters	%RSD
Inter-day	0.78
Intra-day	0.075

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by a small, but deliberate variation in the method parameters and provides an indication of its reliability during normal usage. It was investigated under a change of conditions like deliberate changes in the change in flow rate and mobile phase ratio.

Ruggedness

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e., different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e., from laboratory to laboratory, from analyst to analyst). Ruggedness of the method was investigated under a variety of conditions including different analysts.

Sensitivity (LOD and LOQ)

Detection limit (LOD) and quantification limit (LOQ) were calculated from standard deviation (σ) and slope values (S) which were obtained from calibration curve. The values were calculated using non instrumental method. The formula used to calculate LOD and LOQ are as follows:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

Specificity

Specificity is the ability to assess unequivocally the analytes in the presence of components, which may be expected to be present. Typically these might include impurities, matrix, degradants etc. It is evaluated by injecting the blank, placebo and the control sample solution prepared as per the proposed method to check for the interference of any peak at the retention time of apigenin.

RESULTS AND DISCUSSION

Linearity was determined for apigenin in the range of 10-50 µg/ml. The correlation coefficient value was 0.9996. The regression equation was found to be y = 14998x + 31560 which is shown in Figure 1 and the results were presented in the Table 1. The percentage recovery of apigenin was found to be 99%, 99.43% and 99.24% from 50%, 100% and 150% sample solutions respectively. The percentage recovery was found to be within the range which indicates that the proposed method was more accurate when compared to existing methods. The results were displayed in the Table 2. Both inter-day and intra-day precision were carried out and the % RSD was found to be 0.674% and 0.922% respectively.

The % RSD value indicates a good degree of precision within the specified limit. The results of precision studies were shown in the Table 3. The LOD and LOQ value for apigenin was found to be 0.108 µg/ml and 0.329 µg/ml, respectively which resembles that the proposed method was sensitive. The relative standard deviation for the value of apigenin obtained under deliberately modified chromatographic conditions should be less than 2%. The results obtained in the present study also indicate the method is robust. The results obtained are shown in Table 4. Ruggedness of the method was investigated under a variety of conditions including different analysts. The results have shown that there is no significant change which indicates that the proposed method is having ruggedness. The chromatogram of apigenin was shown in Figure 2. The specificity of the proposed method was illustrated in Figure 3 which shows that there is no interference of any peak at the retention time of apigenin in the chromatogram of blank solution. Thus the proposed method was specific and selective (Figures 2 and 3).

Table 4: Robustness results for apigenin.

S. No	Parameters	Conditions	AUC	% RSD
1	Standard solution	Standard condition	484162	0.78
2	Flow change	0.8 ml/min	485358	0.37
		1.2 ml/min	485763	0.02
3	Mobile phase ratio	Phosphate buffer: ACN (35:65)	485422	0.03
		Phosphate buffer: ACN (25:75)	484962	0.01

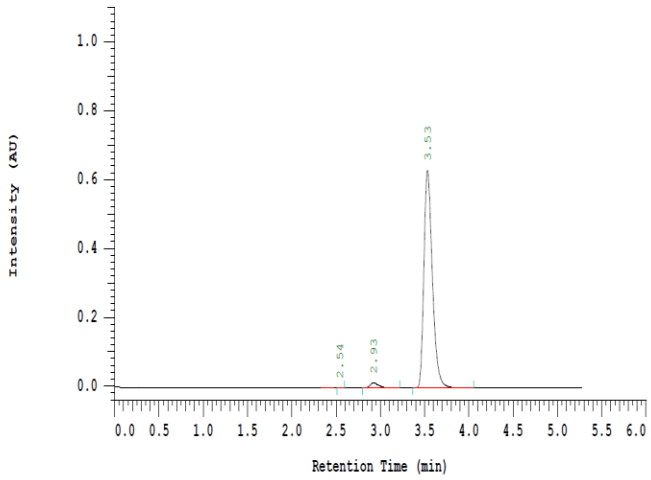

Figure 2: Typical HPLC chromatogram for apigenin

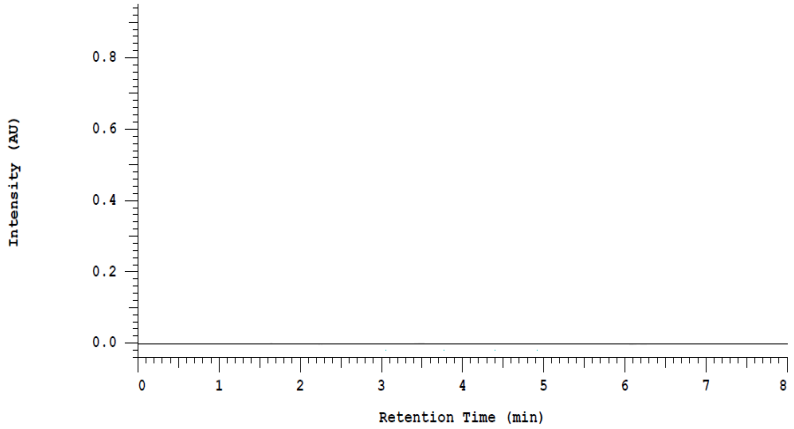

Figure 3: Typical HPLC chromatogram for blank

CONCLUSION

The results obtained from the present study were represented in respective tables. A new, simple, precise, specific and accurate analytical method for identification of apigenin using RP-HPLC method in prepared extract. The proposed method was validated as per the ICH guidelines. The % RSD values were also found to be less than 2% which indicates that the method is suitable for the identification of the compound. Thus the method has a scope of applying it in the quality control of medicinal plants as well as food ingredients by means of detecting adulterants.

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