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DEVELOPMENT AND VALIDATION OF A NOVEL AND SIMPLE RP-HPLC METHOD FOR ESTIMATION OF TANGERETIN IN *ORANGE PEEL* POWDER EXTRACTS

Sachin Bhusari*, Amit Rindhe[1] and Pravin Wakte[1]

*Assistant Professor, Pharmaceutical Technology Division, Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad - 431001, Maharashtra, India.

[1]University Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India.

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***Corresponding Author**
Dr. Sachin Bhusari
Assistant Professor, Pharmaceutical Technology Division, Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad - 431001, Maharashtra, India.

ABSTRACT

A rapid and sensitive reversed phase high-performance liquid chromatography (RP-HPLC) method was developed for the estimation of tangeretin. The method was validated according the Q2 (R1) guidelines of International Conference on Harmonization (ICH) with respect to system suitability, linearity, range, LOD, LOQ, accuracy, precision. The chromatographic analysis was performed on Shimadzu HPLC instrument using a C18, 250mmx4.6mm, 5 µm dimensions Column and mobile phase comprising Methanol and water were mixed in the ratio of 60:40 v/v at the flow rate of 1 ml/min. The column eluent was monitored at 327 nm. The total run time was 10 min and the average retention time of b tangeretin was found to be 7.498 min. The method showed excellent linear response with correlation coefficient values (R^2) of 0.999 which were within the limit of correlation coefficient (R^2 >0.999). The intra- and inter-day accuracy and precision values for all the analytes were within the acceptable range. The LOD and LOQ were 0.1325 and 0.9945 ng/ml. The developed method was found to be robust. The method was successfully used for the quantitative analysis of herbal plant containing *Orange peel* extract. Due to sensitivity, proposed method can be used industrially for routine analysis of *Orange peel* samples.

KEYWORDS: Tangeretin, HPLC method development, RP- HPLC.

INTRODUCTION

Citrus fruits such as mandarin, pomelo, orange, lime, lemon, and grapefruit have been recognized as having high contents of bioactive compounds.[1] Citrus fruits and their by-products are of high economic and medicinal value because of their multiple uses, such as in the food industry, cosmetics and folk medicine. *Orange peel* commonly known as *Citrus vulgaris, Citrus bigaradia, Citrus aurantium amara, Biga-rade orange, Bitter orange, Seville orange*. The orange peel is the fresh or dried outer part of the pericarp of *Citrus aurantium* Linn, belonging to family Rutaceae.[1-2] *Orange Peel* powder consists of variety of chemicals with wide range of activities. Tangeretin (4′, 5, 6, 7, 8-pentamethoxyflavone) is a natural polymethoxyflavone compound which exhibits antiproliferative, anti-invasive, antimetastatic, antioxidant, anti-tumor activity, neuroprotective action and anti-cancer activity s.[3-8]

Considering the therapeutic importance, various analytical methods for the determination of tangeretin were reported earlier. It includes thin layer chromatography-based methods, HPLC methods using either UV detection or electrochemical detection, and LC-MS.[9-13] Detailed literature survey revealed the fact that previously reported HPLC methods were somewhat complicated, less sensitive, or time-consuming. Keeping in view the above-mentioned drawbacks of earlier HPLC methods it is imperative to develop a simple, sensitive, and economic HPLC method for the determination of tangeretin in *Orange Peel powder* extracts.

MATERIALS AND METHODS

Chemicals and Reagent

Tangeretin was purchased from TCI Chemicals (India) Pvt. Ltd, Chennai. All the chemicals and reagent used were of at least analytical grade. HPLC grade methanol and water were used for the proposed study.

Instruments

Chromatographic analysis was performed using a Shimadzu [Japan] HPLC system was used in analysis. It was equipped with a model [2LC-10AD vp] pumps, autosampler [sil-10ADvp], column oven [CTO-10A (C) vp] and UV detector [UV SPD-10A (V) vp]. HPLC grade water was obtained from "Extrapure" water purification system (Lablink). Mobile phase was degassed by using Ultrasonicator (PCiAnalyticals). For weighing purpose, Vibra HT (Essae)

analytical balance was used. Analysis was carried out at 234 nm with a C18, 250mmx4.6mm, 5 µm dimensions at ambient temperature.

Optimization of RP-HPLC Method

Chromatographic conditions were optimized by injecting standard solution (10 ng/ml tangeretin into HPLC system and allowed to run in different mobile phases so as obtain optimum conditions for separation drug.

Preparation of Mobile Phase

The mobile phase Methanol and water were mixed in the ratio of 60:40 v/v and filtered through membrane filter (Millipore Nylon disc filter of 0.45 µ). This filtered mobile phase was sonicated for 10 min in ultrasonic bath.

Preparation of standard stock solution

Stock solutions 1 mg/mL (Stock-I) of tangeretin were prepared in HPLC grade methanol and filtered through 0.45-m nylon membrane syringe filter.

Preparation of standard calibration curve

Stock I were diluted suitably with methanol and mixed together to achieve 7 calibration standards (CAL STD) of tangeretin: CAL STD-1: 1 ng/ml, CAL STD-2: 2 ng/ml, CAL STD-3: 4 ng/ml, CAL STD-4: 8 ng/ml, CAL STD-5: 12 ng/ml, CAL STD-6: 16 ng/ml, CAL STD-7: 20 ng/ml. All the solutions were injected into HPLC column and the peak area of each solution was measured. The standard calibration curves of peak area Vs concentration (ng) were plotted.

Method Validation

Developed method was validated as per ICH guidelines. Various analytical method validation parameters viz. system suitability, linearity, range, LOD, LOQ, accuracy, precision and stability were assessed.[15-16]

System Suitability

Before performing the main analysis, the system suitability test was carried out using freshly prepared standard working solution of 1.5 ng/ml of tangeretin. During the test, five replicates of above mentioned solution were analyzed for retention time, peak area and the theoretical plates. Acceptable upper limit of % RSD for peak area and retention time was set at 2

whereas acceptable lower limit of number of theoretical plates was set at 2000. System was considered to be suitable only when obtained values were within the set limits.

Validation Parameter

Linearity

Linearity of the proposed method was calculated by using seven different CAL STDs. After analyzing CAL STDs, calibration curves representing concentration vs. peak area were plotted and linear regression analysis was performed.

Accuracy (% Recovery)

To ensure the accuracy of method, recovery studies were performed by standard addition method using 80%, 100% and 120% levels of drug concentrations. Percent recovery was calculated from the amount found and the actual amount added.

Precision

The precision of the developed method was evaluated by performing Intra-day and Inter-day studies. Intra-day precision study was carried out by analyzing five replicates of three different concentrations (1.5, 10 and 19.5 ng/ml of tangeretin) at morning, afternoon and evening time of the same day. Similarly, inter-day precision study was carried out by analyzing the samples on three consecutive days. Intra- and inter-day precision results were expressed in terms of % RSD.

Robustness

Robustness of the proposed HPLC method was evaluated by making slight, deliberate change in chromatographic parameters viz. column temperature, flow rate of mobile phase and the mobile phase composition. Modified chromatographic conditions for the assessment of robustness were ±1^{o}C deviation in column temperature, ±0.5 ml/min deviation in flow rate of mobile phase and ±1 unit deviation in volume of methanol. For the robustness study, a solution (10 ng/ml) was repeatedly (n=5) analyzed for retention time and peak area of tangeretin using above mentioned modified chromatographic conditions. Results of the robustness study were expressed in terms of % RSD. Proposed method was considered to be robust only when the % RSD values for both retention time and peak areas were below 2.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable accuracy and precision. LOD and LOQ were calculated using following formula

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$

Where SD = standard deviation of response (peak area) and S = slope of the calibration curve.

Application of proposed HPLC method

Proposed HPLC method was used for the estimation of tangeretin in different type of extraction techniques viz. Soxhlet Assisted Extraction (SAE) and Ultra-sonic assisted extraction technique (UAE).

Soxhlet Assisted Extraction (SAE)

Soxhlet assisted extraction (SAE) technique was used for the extraction of tangeretin. 100 gram of powdered *Orange Peel* was placed in a Whatman extraction thimble (Merck, India) which was inserted into a Soxhlet apparatus. The material was exhaustively extracted with 300 ml ethanol. SAE was performed for 6-8 hrs. After the extraction process completion, the sample present in the round bottom flask was collected and concentrated using rotary vacuum evaporator and analyzed for tangeretin content by using HPLC.

Ultrasound assisted extraction technique (UAE)

The extraction of *Orange Peel* powder was conducted using a tunable ultrasonic bath (PCi^{TM} Analytics, 230V AC, 50 Hz, Mumbai, Maharashtra, India). 10 gram of *Orange Peel* powder was mixed with 100 ml of ethanol in a beaker. The extraction of *Orange Peel* powder was carried out by placing the beaker in an ultrasonic bath with the fixed power of 150W. The beaker was immersed in the ultrasonic bath and extracted for 30 min. To avoid the overheating produce by the ultrasound waves the water in the ultrasonic bath was circulated at 25°C. The sample was then collected and concentrated using rotary vacuum evaporator and analyzed for the content of tangeretin by using HPLC.

RESULTS AND DISCUSSION

Optimization of RP-HPLC Method

Resolution was considered to be the most important criteria for the method and was imperative to achieve good resolution among the both compounds. Based on pKa and solubility of both the compounds, various compositions of mobile phase were tried and best resolution was obtained with mobile phase consisting of water and methanol in the ratio of 60:40 v/v. Better resolution of the peaks with clear base line was found. Detection was carried out at 327 nm. Optimized chromatographic conditions are given in Table 1. Under these conditions retention time for tangeretin was found to be 7.498 min (Fig. 2).

Table No. 1: The optimized chromatographic conditions.

Separation variable	Optimized conditions
Chromatography	Shimadzu HPLC system
Column	C18, 250mmx4.6mm, 5 µm
Mobile phase	Methanol: Water (60:40 v/v)
Flow rate	1 mL/min
Total Run Time	10 Min
Temperature	Ambient
Detection wavelength	327nm
Retention time	7.498 min

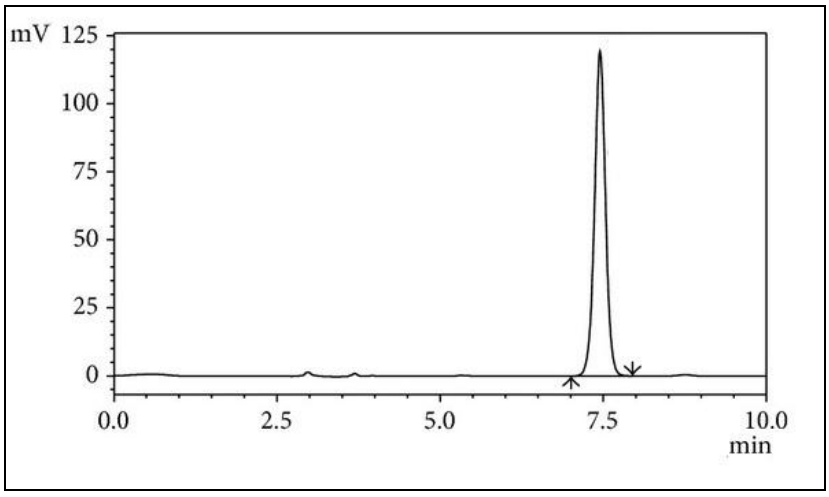

Fig. 2: A typical RP-HPLC chromatogram of Tangeretin.

System suitability

During system suitability test, RSD of all parameter were calculated to evaluate the suitability of the developed method. From the results, it was found that %RSD for retention time and peak area was less than 2 and the number of theoretical plates were more than 2000 (Table 2). On the basis of obtained results, it was found that system is suitable for the analysis.

Table No. 2: System suitability parameters for Tangeretin.

Sr.No.	Parameter	Acceptance criteria	Results		
			Tangeretin	%RSD	Status
1	Retention Time	%RSD ≤ 2%	7.499	0.045	Passed
2	Area	%RSD ≤ 2%	18649	1.021	Passed
3	Theoretical plates	≥ 2000	5711	1.254	Passed

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 tangeretin (1-20 ng/ml) were constructed. Different concentrations and peak area values are depicted in Table 3. Calibration curve when subjected to least square regression analysis yielded an equation; y = 12455x – 304.73 with correlation coefficient 0.9999 (Fig. 3). From the linearity study, it was revealed that, there is a linear relationship between response and amount of drug within the range 1-20 ng/ml.

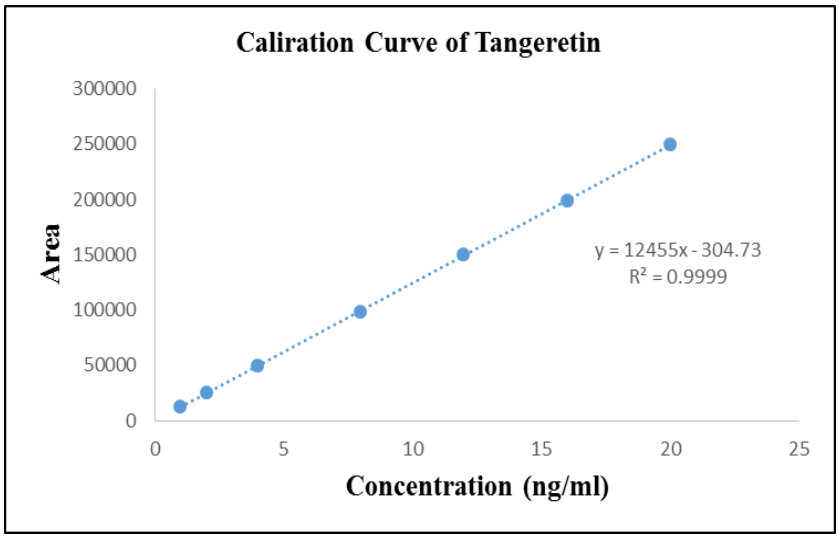

Table No 3: Calibration standard data for Tangeretin.

S. No	Conc. (ng/mL)	Peak Area
1	1	12484 ±0.64
2	2	25315 ±0.35
3	4	49215 ±0.25
4	8	98245 ±0.59
5	12	149358 ±0.49
6	16	198245 ±0.68
7	20	249658 ±0.42

Accuracy (% Recovery)

Accuracy is the closeness of test results to the true value obtained by proposed 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 tangeretin, accuracy was determined using recovery studies. At 80, 100 and 120% standard addition, mean recovery of tangeretin was found to be in between 99.75 to 100.25%. The relative standard deviation (% RSD) was found to be less than 2 (Table 4). From the results of accuracy studies, it was concluded that the proposed method is accurate.

Table No. 4: Recovery studies of Tangeretin.

Sr. No.	Sample	Spiked level	Theoretical Concentration (ng/mL)	Practical Concentration (ng/mL)	% Recovery	Mean % Recovery	% RSD
1	Tangeretin	80%	1.2	1.197	99.75	99.98 ±0.26	0.689 ±0.49
		100%	10	9.994	99.94		
		120%	23.4	23.459	100.25		

Precision

Precision was studied by analysis LQC, MQC and HQC STDs of the tangeretin at concentrations covering the entire calibration range**.** The results expressed in terms of % RSD for the intra- and inter-day precision study (Table 4 and 5).Percent RSD values of intra-day precision study were found to be in between 0.9541 to 1.2021, whereas inter-day precision was found to be in between 0.8421 to 1.0212. It was concluded that the analytical technique showed good repeatability.

Table 5: Intra-day precision data for Tangeretin.

Sr.No.	Tangeretin			
	Amount present (ng/ml)	Amount recovered (ng/ml)	% Assay	% RSD
1	1.5	1.492	99.46	1.1021
2	10	9.994	99.94	0.9854
3	19.5	19.521	100.10	0.9541

Table 6: Inter-day precision data for Tangeretin.

Sr.No.	Tangeretin			
	Amount present (ng/ml)	Amount recovered (ng/ml)	% Assay	% RSD
1	1.5	1.490	99.33	1.0212
2	10	9.993	99.93	0.9524
3	19.5	19.510	100.05	0.8421

LOD and LOQ

LOD and LOQ of proposed HPLC method was found to be 0.1325 and 0.9945 ng/ml. Lower LOQ value indicated that proposed method would be sensitive enough to quantify the tangeretin content of samples at its lower level.

Estimation of Tangeretin in *Orange Peel* powder extracts

Proposed HPLC method was successfully used for the estimation of tangeretin content in different type of extracts sample of *Orange Peel powder*. Analytical standard of tangeretin and the various different sample of *Orange Peel* where analyzed by using proposed HPLC method showed the presence of tangeretin at the average retention time of 7.498 min. By proposed HPLC method, tangeretin content in Soxhlet assisted extraction and Ultrasound assisted extraction technique of *Orange Peel* Powder was found to be 0.025 ± 0.41 and 0.040 g/100g powder respectively.

CONCLUSION

In the present investigation, a simple, accurate, precise and robust RP-HPLC method has been developed for the estimation of tangeretin in raw materials, herbal extracts, and dosage forms. The present method demonstrated a rapid analysis run time (< 10 min). The method was validated according the Q2 (R1) guidelines of International Conference on Harmonization (ICH). The proposed method is robust enough to reproduce accurate and precise results under different chromatographic conditions. The proposed method was successfully applied for the estimation of tangeretin in commercially available dosage forms, bulk, naturally available and extracts.

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