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Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF QUERCETIN AND RUTIN IN AGANOSMA DICHOTOMA [ROTH] K. SCHUM

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ABSTRACT

Objective: A simple, specific, accurate and precise high performance liquid chromatography method was developed for the simultaneous estimation of quercetin and rutin in *Aganosma dichotoma*.

Methods: The chromatographic separation was achieved by using C_{18} column, 150 x 4.6mm i.d., 5µ Hibar Lichrospher, mobile phase containing acetonitrile:25mM ammonium acetate pH 3 (40:60 v/v). The flow rate was 1 ml/min and the absorbance was monitored at 259 nm. The retention time of quercetin and rutin was found to be 4.30 min and 1.71 min respectively.

Results: The proposed method was validated in terms of the analytical parameters such as accuracy, linearity, precision, robustness, limit of detection (LOD), limit of quantification (LOQ) were determined based on the International Conference on Harmonization (ICH) guidelines. The detector response was linear in the range of 1-5 µg/ml, 0.1-0.5 µg/ml for quercetin and rutin respectively.

Conclusion: The proposed method was successfully applied for the simultaneous estimation of both the constituents in *Aganosma dichotoma.* This study established a quantitative method for the simultaneous determination of quercetin and rutin from *Aganosma dichotoma*.

Keywords: *Aganosma dichotoma*, Simultaneous estimation, Flavonoids, Quercetin, Rutin.

INTRODUCTION

Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. To date about 300 varieties of flavonoids are known. Flavonoids, as a major active constituent, display a remarkable role in various pharmacological activities including anti-allergic, anti-inflammatory and anti-oxidant effects [1-3]. Quercetin and rutin possess antioxidant activity and reduce low density lipoproteins oxidation [4].

Quercetin and rutin are some important flavonoids known for its anti-inflammatory, anti-allergic, anti-thrombic, hepatoprotective, anti-spasmodic and anti-cancer properties [5, 6].

Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization [7]. Many flavonoid containing plants are diuretics or antispasmodics and some flavonoids have antitumor, antifungal and antibacterial properties as well as antihepatotoxic activity [8]. High performance layer chromatography (HPLC) has recently emerged as a preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high throughput screening. HPLC method is a suitable method for estimation of chemical constituents present in plant materials [9-12]. The proposed method is optimized and validated as per the International conference on harmonization (ICH) guidelines [13].

The literature survey showed that there was no report about the simultaneous estimation of flavonoid constituents of *Aganosma dichotoma.* Quantitative estimation of these compounds is important for current research and a variety of methods are required for this and in the present study the quantification of main flavonoids which was found to be characteristic for *Aganosma dichotoma* was reported. A sensitive, accurate and specific HPLC method was developed and validated for the simultaneous estimation of quercetin and rutin in the hydro alcoholic and methanolic extract of *Aganosma dichotoma*.

MATERIALS AND METHODS

Materials and reagents

Quercetin 96% and rutin (98%) were purchased from Sigma-Aldrich, Bangalore, India Methanol and Acetonitrile were of HPLC grade from Qualigens fine chemicals, Mumbai, India. All the reagents and chemicals used were of analytical and HPLC grade. Water (HPLC grade) was obtained from Milli Q RO system.

Plant Material

The whole plant of *Aganosma dichotoma* was collected during the month of March 2013, from the Western Ghats, Kottayam region, Kerala.

The plant was botanically identified, confirmed and authenticated by Dr. Gunasekaran, Field botanist, Coimbatore, Tamilnadu, India. The plants were cut and dried in tray drier at 50°C for 48 hrs. The dried samples were powdered and used for the study.

Preparation of standard solution

The standard stock solution (1 mg/ml) of quercetin and rutin were prepared by dilution in methanol. These stock solutions were stored in light resistant containers. The dilute standard solutions of concentration (1-5 µg/ml of quercetin and 0.1-0.5 µg/ml of rutin) were prepared from above stock solution and used for calibration curve of quercetin and rutin.

Preparation of sample solution

About 50 g of the powdered sample was weighed and extracted with the selected solvents by Soxhlet apparatus for 24 h. The extract was collected and filtered; the filtrate was dried at 50°C under reduced pressure in a rotary evaporator (BUCHI Rota vapor). The dried extract (1 mg/ml) was dissolved in the mobile phase. After filtering through Whatmann filter paper No.42, the extract was injected directly.

Instrumentation and Chromatographic conditions

The simultaneous estimation of quercetin and rutin was performed on a Shimadzu liquid chromatographic system equipped with LC-2010AT VP solvent delivery system (pump), SPD M-10A photodiode array detector and Rheodyne 7725i injector with 20 µl loop volume,

Class VP 6.01 data station for data collection and processing (Shimadzu technologies, Japan). The mobile phase, acetonitrile and ammonium acetate (40:60) was pumped with a flow rate of 1 ml/min. The elution was monitored at 259 nm. Peak identity was confirmed by spectrum and retention time comparison. All the analysis was performed at ambient temperature.

RESULTS AND DISCUSSION

Method development and validation

Upon application of the developed method, well-separated peaks were obtained for both quercetin and rutin (Figure 1). Quercetin and rutin were identified in *Aganosma* herb extracts. The quantitative analysis revealed that rutin (72.08 + 0.18 mg/g) predominated in the hydro alcoholic extract and 57.8 + 0.22 mg/g in the methanolic extract of *A. dichotoma* whereas quercetin were determined in lower quantities (4.87 + 0.16 mg/g) in hydro alcoholic extract and 3.60 + 0.20 mg/g in the methanolic extract of *A. dichotoma*. The chromatograms of hydro alcoholic and methanolic extracts containing quercetin and rutin contents were given (Figure 2, Figure 3). For validation of analytical methods, the guidelines of the International Conference on the Harmonization have recommended the accomplishment of linearity, accuracy tests, precision, detection and quantitation limit and robustness of the method.

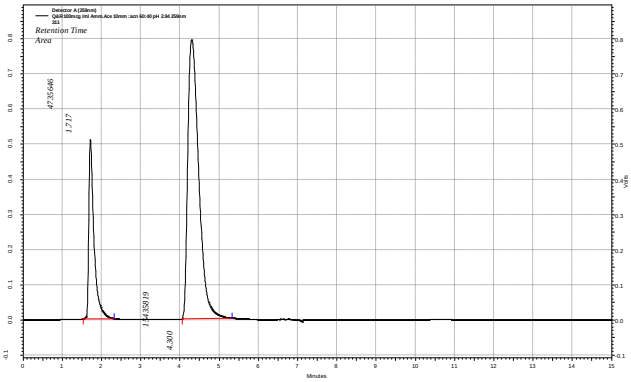

Fig. 1: Typical HPLC Chromatogram of Quercetin and Rutin standard solutions

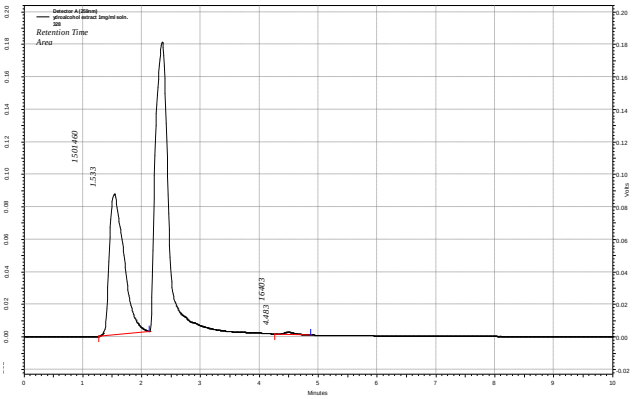

Fig. 2: Typical HPLC Chromatogram of hydro alcoholic extract containing Quercetin and Rutin

Linearity and range of the developed method

For linearity study, five solutions in the range of 1-5 µg/ml for quercetin and 0.1-0.5 µg/ml rutin were analyzed. Each concentration was made and analyzed in triplicate. The peak areas obtained against each concentration of the analytes were used to build a linear regression equation and to determine value of correlation coefficient (Table 1). Good linearity was observed over the above-mentioned range with linear regression equation Y= 88980x- 5306 for quercetin and Y = 41670x+ 333.33 for rutin (x is concentration of analytes in µg/ml and Y is peak area). The value of correlation coefficient was found to be 0.997 for quercetin and 0.995 for rutin. The results indicate that the method is linear over the concentration range studied (Figure 4, Figure 5).

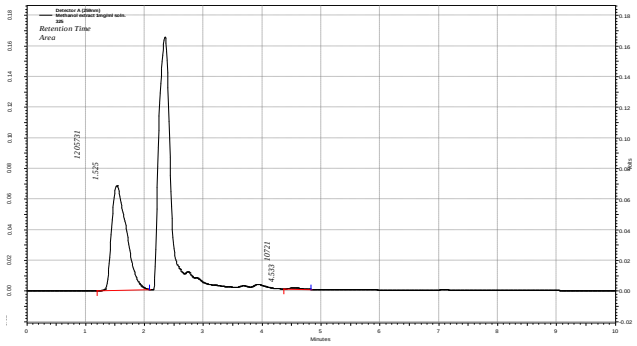

Fig. 3: Typical HPLC Chromatogram of methanolic extract containing Quercetin and Rutin

Table 1: Linearity and range for quercetin and rutin by HPLC

Sl.No.	Concentration of Quercetin (µg/ml)	Concentration of rutin (µg/ml)	Peak area	
			Quercetin	Rutin
1	01	0.1	88915	4167
2	02	0.2	154416	9334
3	03	0.3	262559	13501
4	04	0.4	355976	16668
5	05	0.5	440992	20835

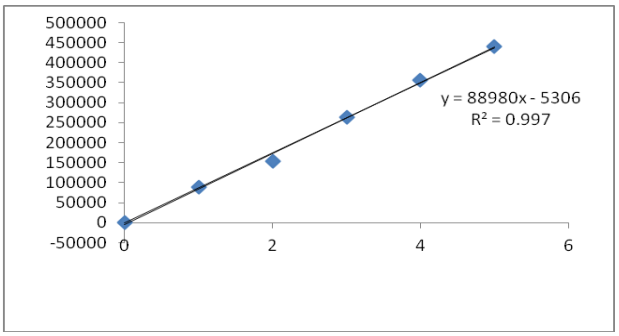

Fig. 4: Calibration curve of Quercetin by HPLC

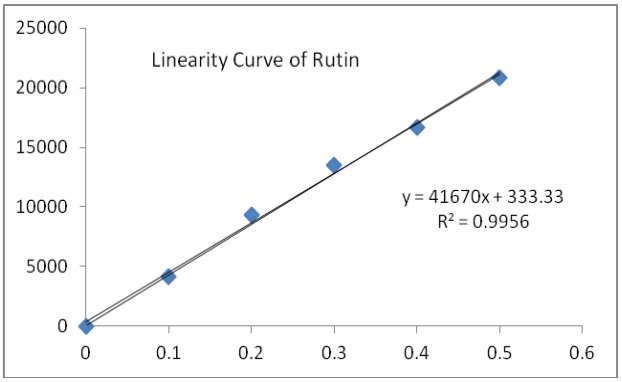

Fig. 5: Calibration curve of Rutin by HPLC

Accuracy of the developed method

This study was performed by adding known amounts of quercetin and rutin to the placebo solution. Three level of solutions were made having concentrations of 1, 2 and 3 µg/ml for quercetin and 0.1, 0.2 and 0.3 µg/ml for rutin. The recovery range for quercetin and rutin were found to be 99.3 to 101.1 % and 101.3 to 102 % respectively (limit 98 to 102%). The relative standard deviation ranged from 0.185 to 0.529 % for quercetin and from 0.054 to 0.075 % for rutin (Table 2).

Table 2: Recovery and accuracy data

Compounds	Recovery		
	Amount Added (µg/ml)	Recovery (%)	RSD (%)
Quercetin	1	99.3	0.185
	2	100.3	0.44
	3	101.1	0.529
Rutin	0.1	101.7	0.054
	0.2	102.0	0.075
	0.3	101.3	0.06

Precision of the developed method

Repeatability was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of about 1 mg/mL, performed on the same day and under same experimental conditions. The results of quercetin and rutin determinations in the working standard solution with the relative standard deviation were calculated (Table 3). Intermediate precision studies include the estimation of variations in analysis when a method is used within laboratories, on different days. The RSD values obtained for quercetin and rutin were 0.97 and 1.268% respectively.

Table 3: Precision studies for Quercetin and Rutin

Compound	Conc. (µg/ml)	N	Inter day		Intra day	
			Mean	%RSD	Mean	%RSD
Quercetin	1	6	0.99	0.925	1.00	0.525
	3		3.01	0.512	3.08	1.419
	5		5.12	1.376	5.02	0.932
Rutin	0.1	6	0.10	1.309	0.10	0.834
	0.3		0.30	1.447	0.29	1.410
	0.5		0.48	1.630	0.50	1.132

Limit of detection and quantification

LOD were calculated by using the following equations.

LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = the standard deviation of the response, S = Slope of the calibration curve. The LOD value was found to be 100 ng/ml and the LOQ value was found to be 300 ng/ml for the simultaneous estimation of quercetin and rutin.

Table 4: Robustness study of the proposed HPLC method

Parameter	Conditions	Retention Time	
		Quercetin	Rutin
Flow Rate (ml/min)	0.9	4.85	1.93
	1.0	4.30	1.71
	1.1	3.95	1.60
Mobile Ratio (v/v)	65:35	6.00	3.00
	60:40	4.30	1.71
	55:45	3.65	2.60
Absorbance λ-max (nm)	264	4.33	1.74
	259	4.30	1.71
	254	4.29	1.75

Robustness of the developed method

The robustness of the proposed method was evaluated by deliberately changing the chromatographic conditions such as solvent ratio, flow rate and absorbance. The results showed that varying the chromatographic conditions had no appreciable effects on the chromatographic parameters (Table 4).

CONCLUSIONS

Using this method, quercetin and rutin could be determined simultaneously, and the validity of the method was also verified. The proposed analytical method for simultaneous estimation of quercetin and rutin in the extracts of *Aganosma dichotoma* is accurate, precise, linear, robust, reproducible and within the range. The results shows that *Aganosma dichotoma* contains considerable amounts of flavonoids which demonstrates that the plant could be considered as a potential source of natural health-promoting antioxidants for medicinal and food applications. This study established a quantitative method for the simultaneous determination of quercetin and rutin from *Aganosma dichotoma*.

Table 5: System suitability studies for estimation of quercetin and rutin by HPLC

S. No.	Parameters	Quercetin	Rutin
1	Linearity range	1 - 5 µg/ml	0.1 - 0.5 µg/ml
2	Regression equation	Y = 88980x – 5306	Y = 41670x + 333.33
3	Correlation coefficient	0.997	0.995
4	Asymmetric factor	1.0	1.0
5	Tailing factor	1.01	1.1
6	Theoretical plates	2546	4025
7	Resolution	4.69	
8	LOD (ng/ml)	100	
9	LOQ (ng/ml)	300	

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