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A Novel Validated HPTLC Method for Simultaneous Estimation of Ascorbic acid and Quercetin as Biomarkers in Amalth Guava Leaf Extract Capsules

Suganthi A[1], Neethu Johnson[*] and T. K. Ravi

Department of Pharmaceutical Analysis, Sri Ramakrishna Institute of Paramedical Sciences, College of Pharmacy affiliated to The Tamilnadu Dr M G R Medical University, Coimbatore – 641044

Abstract

The main objective of the study is to develop a validated simple, precise, economical HPTLC method for the simultaneous estimation of ascorbic acid and quercetin in herbal formulation. The analysis was performed on 20 cm × 10 cm HPTLC silica gelG60 F_{254} plates using mobile phase composed of chloroform: methanol: formic acid, 9.5:3:1 (v/v/v). The quantification was done at 251nm. The calibration curve showed the linearity ranges from 30-105 ng/band and 15-52.5 ng/band for ascorbic acid and quercetin with the correlation coefficient > 0.999. The LOD for ascorbic acid and quercetin were found to be 0.121 ng/band and 0.083 ng/band and LOQ 0.368 ng/band and 0.254 ng/band, respectively. The recovery values close to 100 with low standard deviation proves the accuracy of the method. The developed HPTLC method was found to be simple, sensitive, accurate, reproducible and rugged and is applicable for the standardisation of ascorbic acid and quercetin in guava extract containing herbal capsule dosage form as well as other herbal formulations as a quality control tool in herbal research.

Keywords: HPTLC, simultaneous estimation, ascorbic acid, quercetin

1. Introduction

The use of herbal medicines continues to expand promptly across the world with many people now resorting to these products for treatment of various health issues in different countries. Herbal medicines are, therefore, often viewed as a balanced and modest approach to healing. India is a country which plays a major role in the production of standardized therapeutic effective ayurvedic formulations [Martins, 2014]. The World Health Organization (WHO) estimates that herbal medicine is still the main stay of about 75- 80% of the world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side-effects [Yadav and Dixit, 2008]**.** Population growth in the developing world along with increasing alertness in the developed nations has greatly extended the demand for plant products [Kalyan and Suparna, 2014]. Obesity is an epidemic disease that threatens the healthcare resources by increasing the incidence of diabetes, heart disease, hypertension, and cancer [George, 2004]. Diabetes associated with obesity, is a great and a growing risk to public health. The susceptibility of diabetes is 80 times greater among obese adults than non-obese.

Susceptibility of coronary heart diseases increases 2–3 times more in obese adults [Agha M and Agha R, 2017]. Herbal medicines are mainly used to treat obesity. The quality control of crude drugs and herbal formulations is of supreme importance in qualifying their acceptability in modern system of medicine. But one of the major problems faced by the herbal drug industry is non availability of inflexible quality control profile for herbal material and their formulations. Hence it is necessary to control the quality by standardizing the herbal formulation using its active phytochemical constituents [Sheetal and Singh, 2008]. Psidium guajava L. is a fruit-bearing tree commonly known as guava, which belongs to the family Myrtaceae. The leaves and bark of guava tree have a long history of medicinal uses. In India, decoction of the leaves and bark of guava is used to cure diarrhoea, dysentery, vomiting and sore throats, and to regulate menstrual cycles. Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fibre and fatty acids. Guava fruit is higher in vitamin C content. The leaves of guava are rich in flavonoids, particularly quercetin [Kamath *et al*., 2008].Guava leaf extract veg capsules is an ayurvedic formulation from Amalth life care Pvt. Ltd. which is available as 500 mg capsule. This formulation is marketed for maintaining blood sugar and cholesterol levels. Quercetin and ascorbic acid as shown in figure 1 and 2 are the two main active phytoconstituents present in guava leaf extract. Ascorbic acid is the vitamin which is rich in plants [Lakhanpal and Rai, 2007]. Human vitamin deficiencies lead to gastrointestinal problems, cardiovascular and nervous disorders, and growth inhibition [Priya and Ishwarlal, 2021]. Therefore, the concentration of vitamins in biological objects, food products and pharmaceutical formulations should be controlled. Quercetin, a flavonoid which is commonly available and possess antidiabetic, anticancer activities [Anand *et al*., 2016]. Literature survey revealed HPLC [Gazdik *et al*., 2008] and HPTLC [Trineeva, 2018, Kondawar, 2011 Kunle, 2012 Ali Moahammd, 2012] methods were reported for ascorbic acid, quercetin individually as well as with other combinations. Till date no method reported for the simultaneous estimation of ascorbic acid and quercetin in herbal formulation. Hence there is need for development of validated simple, easier, cost effective method for the simultaneous estimation. Therefore the main objective of the study is to develop a novel, sensitive, validated and economical HPTLC method for the simultaneous estimation of ascorbic acid and quercetin.

2. Materials and methods

2.1. Chemicals and reagents:

All the solvents and reagents used in the experiment were of analytical grade. Biomarkers, ascorbic acid and quercetin were procured from HIMEDIA laboratories Pvt. Ltd., India. The herbal formulation containing guava leaf extract was procured from local market, India.

2.2. Apparatus

HPTLC system with Linomat V automatic sample spotter as application device, 100 µL Hamilton syringe, glass twin trough chamber (20 cm × 10 cm) (CAMAG), TLC Scanner 3 linked to WinCATs Software, pre-coated silica gel 60 F254 were used for this study. Temperature and humidity were maintained during the experiment.

2.3. Quantitative estimation of ascorbic acid and quercetin

2.3.1. Preparation of stock solutions of ascorbic acid and quercetin

The stock solution of ascorbic acid and quercetin were prepared by accurately weighing 25 mg of standard ascorbic acid and quercetin in two separate 25 ml standard flask, dissolved using methanol by sonicated for 15 minutes. Then the volume of each flask was made upto 25 ml using methanol to get a concentration of 1000 µg/ml of ascorbic acid and quercetin, respectively. From each solution 10 ml was transferred into 100 ml standard flask and the volume was made upto the mark with methanol to get 100 mcg/ml of the mixture.

2.3.2. Sample extract Preparation:

Twenty capsules were weighed carefully, transferred the contents and the empty shells were weighed once again for further calculation. From the extract powder formulation a total of 2.5 g of was weighed accurately, transferred to a 100 mL standard flask, sonicated for 15 minutes similar to standard and filtered. The filtrate was used for the analysis.

2.3.3. Procedure

Analysis was performed on 20 cm × 10 cm HPTLC silica gelG60 F254 plates. Before starting the analysis, HPTLC plates were cleaned by predevelopment with methanol by ascending method. HPTLC plate was immersed in a CAMAG glass chamber (20 cm × 10 cm), containing 30 ml methanol (HPLC grade) as solvent system. The chamber was covered with glass lid and left till development of the plate to the top with methanol. After complete development, the plate was removed from TLC glass chamber and dried in an oven at105°C for 5 min.

Again the chamber was saturated with the mobile phase consisting of 9.5 ml chloroform, 3 ml methanol and 1 ml formic acid. Sixteen spots were applied from 1 to 14 µl including two dummy in the form of band of standard mixture by means of a CAMAG Linomat 5 (automated spray-on applicator) equipped with a 100 mL syringe and operated with the settings band length 6 mm, distance between band 8 mm, distance from the plate side edge 15 mm, and distance from the bottom of the plate 15 mm). After development plate was dried and the chromatogram was recorded at the selected wavelength.

2.3.4 Method validation [ICH 1996]

The linearity of the method was checked by spotting 10-120 ng/band ascorbic acid and quercetin 5-75 ng/band. Correlation coefficient values were determined by plotting concentration against peak area.

Accuracy was determined by the technique of standard addition method. Percentage recovery and relative standard deviation (%RSD) were determined.

Precision was accessed by measurement of repeatability, intra and interday precision. Repeatability was determined by applying 45 ng/band of ascorbic acid and 22.5 ng/band on pre-coated plates. Intra day precision was determined by spotting 45 and 75 ng/band of ascorbic acid and 22.5 and 37.5 ng/band of quercetin on the plate for six times and the response for each spot was measured. Inter-day precision was carried out by spotting 45 and

75 ng/band of ascorbic acid and 22.5 and 37.5 ng/band of quercetin for two days and the % RSD were calculated.

The LOD and LOQ were calculated using the equation,

$$LOD = 3.3 \times \sigma/S$$

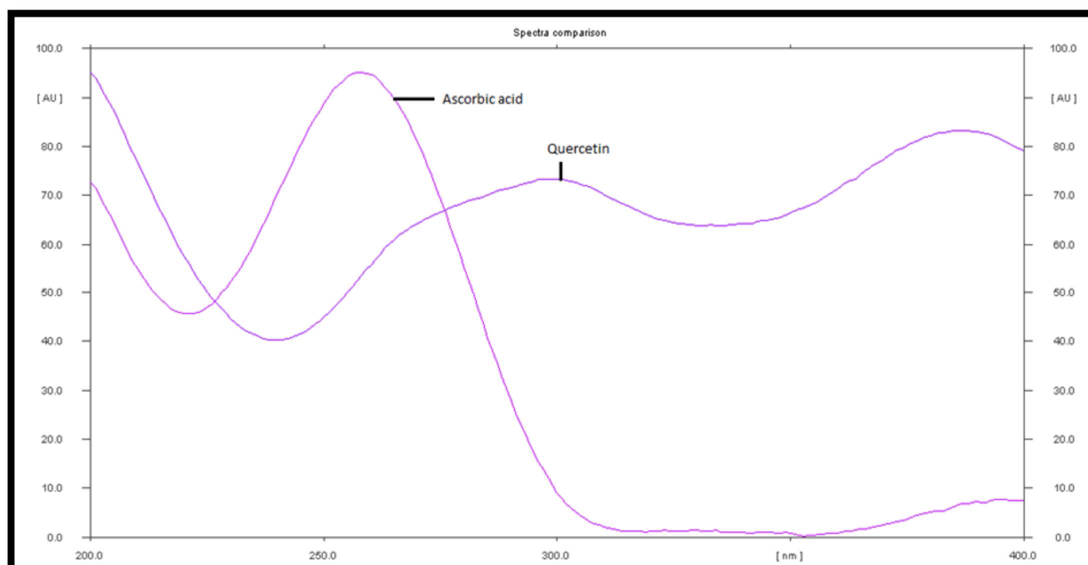
$$LOQ = 10 \times \sigma/S$$

Where, σ is the standard deviation of y intercepts of regression line, and S is slope of calibration curve.

3. Results

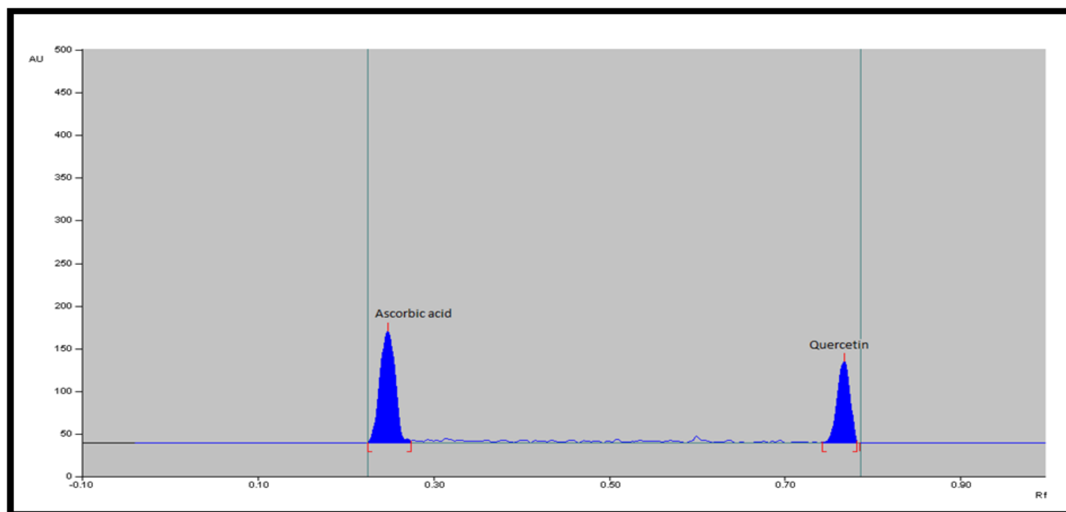
In order to develop an effective solvent system for the separation of ascorbic acid and quercetin, the analysis was tried on normal phase HPTLC plates using various combinations of solvents in different proportions, but good separation of the compounds could not be achieved.Finally the mobile phase system consisted of chloroform: methanol: formic acid, 9.5:3:1 (v/v/v) gave the best resolution, with symmetrical and reproducible peaks, of ascorbic acid (R_f = 0.25) and quercetin (R_f = 0.77), respectively. The plates were visualized at two different wavelengths 254 and 366 nm as the compounds were found to absorb at variable spectrum range. In addition, this helped in identification of the bands of ascorbic acid and quercetin in the sample herbal extract capsule was confirmed by overlaying their UV absorption spectra with those of the standards (Fig.1). The HPTLC densitograms of standards are shown in (Fig. 2).

Figure 1: UV spectrum of Ascorbic acid and quercetin



UV spectrum of Ascorbic acid and quercetin using HPTLC system showing isoabsorptive point at 251 nm

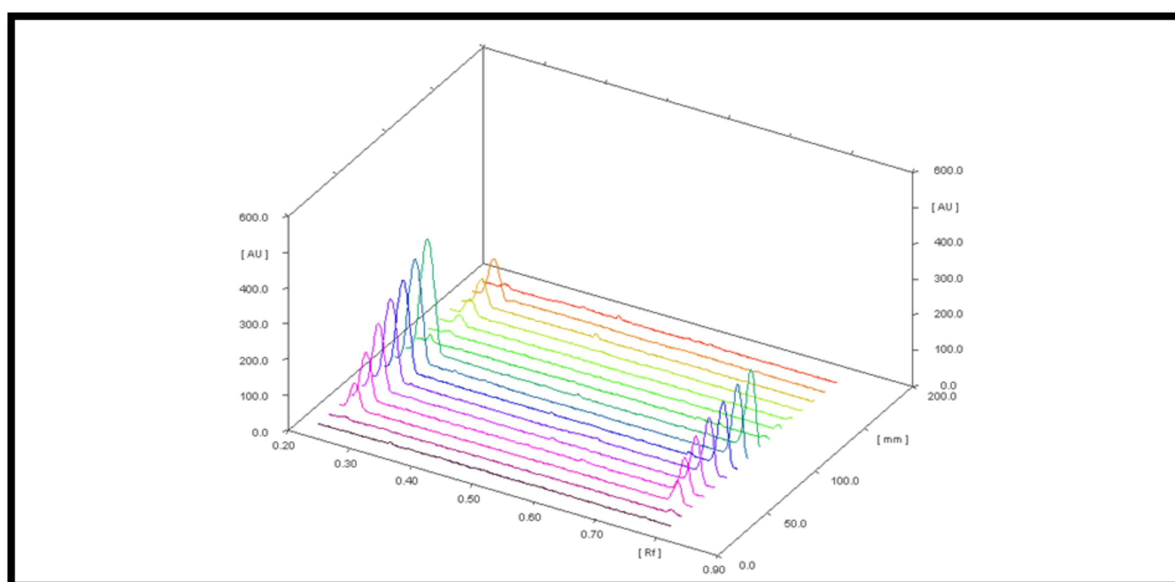
Figure 2: Chromatogram of Ascorbic acid and quercetin



The peak response of concentration 45 ng/band of ascorbic acid (R_f = 0.25) and 22.5 ng/band quercetin (R_f = 0.77)

Linearity of ascorbic acid and quercetin was validated by the linear regression equation and correlation coefficient. The six-point calibration curves for ascorbic acid and quercetin were found to be linear in the range of 30–105 ng/band and 15–52.5 ng/band. Regression equation and correlation coefficient for the reference compound were: Y = -1082.910 + 57.283x (0.9994) for ascorbic acid, Y = -341.914 + 84.819x (0.9991) for quercetin, which revealed a good linearity response for developed method. The 3D overlay spectrum of ascorbic acid and quercetin was shown in figure 3.

Figure 3: Overlay 3D spectrum of Ascorbic acid and Quercetin



3D overlay spectrum of ascorbic acid and Quercetin at 251 nm for linear concentration range 30–105 ng/band and 15–52.5 ng/band.

To study the variability of method, the intra-day precision and inter-day precisions (expressed in terms of %RSD) were calculated for two different concentration (45 and 75 ng/band) by measurement of peak area for ascorbic acid as well as quercetin (22.5 and 37.5 ng/band) and were observed below 2 which demonstrated the good precision of proposed method. Lower limits of detection (LOD) and limit of quantification (LOQ) were shown in Table 1. Good recoveries were obtained by the fortification of the sample at two different concentrations of ascorbic acid and quercetin. It is evident from the results that the percent recoveries for ascorbic acid and quercetin after sample processing and applying were close to 100% indicated the accuracy of the method. The summary of validation parameters are given in Table 1.

Table 1: Validation data for the developed HPTLC method

Parameters	Values	
	Ascorbic acid	Quercetin
Linearity	30-105 ng/band	15-52.5 ng/band
Correlation co-efficient	0.9994	0.9991
LOD	0.121 ng/band	0.083 ng/band
LOQ	0.368 ng/band	0.254 ng/band
Precision (%RSD*)		
a) Intraday	0.38-1.0	0.5-1.07
b) Interday	0.38-1.07	0.50-1.07
c) Repeatability	1.23	1.43
Stability	2 hours	
% Recovery±RSD*	97.93 ± 0.44	97.22 ±0.78

*Mean of six determinations

The content of ascorbic acid and quercetin was estimated in the powder extract capsule formulation by the proposed method. The peaks of ascorbic acid and quercetin were identified by comparing with the standard R_f values. The result of quantification was shown in Table 2.

Table 2: Standardization of Amalth guava extract capsules

Herbal FORMULATION	Average amount found (mcg/2.5 g)	%RSD*
Ascorbic acid	21.65	1.35
Quercetin	6.35	1.79

*Mean of six determinations

4. Discussion

It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the simultaneous quantification of bioactive marker, ascorbic acid and quercetin in herbal formulation. One of the biomarkers, ascorbic acid is highly polar and water soluble. Reversed-phase TLC has been used for the separation and identification of vitamins. The mobile phases frequently used include mixed organic or aqueous–organic solvents. Most studies have been performed using methanol, ethanol, butanol, acetone, chloroform, ammonia, pyridine, benzene, or toluene as one of the components of the mobile phase with adsorbents such as silica gel, cellulose, and surface-modified silica gel. No simultaneous normal phase HPTLC methods have been reported for the quantification of ascorbic acid in herbal formulation. Quercetin is non-polar and soluble in methanol. The challenging task was the selection of mobile phase for the simultaneous estimation. Various combinations of solvent systems like toluene-methanol, toluene-ethanol, ethyl acetate-methanol, chloroform-ethyl acetate-acetic acid, chloroform-methanol, chloroform–ethyl acetate– methanol, chloroform–ethyl acetate– methanol–triethyl amine, chloroform-methanol-triethyl amine-formic acid, and methanol–hexane in different proportions were tried, but good separation of the compounds could not be achieved. This may be due to wide polarity and functionality differences among these two compounds. The developed HPTLC method was validated for different parameters like specificity, linearity, accuracy, precision, robustness, LOD and LOQ. The specificity of the method was ascertained by analyzing the standard compounds and samples for the interference of other components. The bands for ascorbic acid and quercetin were confirmed by comparing the R_f values and spectra of the bands with that of standards. Absence of any interfering peak indicated that the method was specific. The purity of bands was confirmed by overlaying the absorption spectra at the start, middle, and end position of the bands. RSD of the samples varied from 0.3% to 1.0% for ascorbic acid and quercetin under analysis.

Good recoveries were obtained by the fortification of the sample at two different concentrations of ascorbic acid and quercetin. It is evident from the results that the percent recoveries for ascorbic acid and quercetin after sample processing and applying were in the range of 97.2–97.90%. The mobile phase with a slight differences in their composition, i.e.chloroform- methanol- formic acid with three different ratios were used, 8.5:3:2, 9.5: 3: 1.5, 9.5: 2.5:1 and 9.5:3:1(v/v/v) were used and developing distance was checked varying between 7 and 8 cm and no considerable effect on the analysis was recorded. Also different TLC plate lots of the same manufacturer had no influence on the chromatographic separation. The instrumental precision was represented as repeatability of sample application and measurement of peak area was found to be 0.25 and 0.27 for ascorbic acid, 0.77 and 0.79 for quercetin. The good sensitivity of the proposed method for the quantification of ascorbic acid and quercetin is shown in table 1. The content of ascorbic acid and quercetin was estimated in the powder extract capsule formulation of Guava Leaf Extract by the proposed method is given in Table 2. Moreover the proposed HPTLC method has high degree of precision and will provide fast and cost-effective quantitative control for the standardization of guava leaf extract formulation. Hence it is concluded that the developed HPTLC method could be recommended for quality assurance and biomarkers-based standardization of formulations containing guava leaves as well as other herbal formulations after modifying suitable extraction procedure.

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Conflict of interest statement

We declare that we have no conflict of interest.

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