Simultaneous Quantification of Furanocoumarins from Aegle marmelos Fruit Pulp Extract

Prashant B. Shinde* and Kirti S. Laddha

Medicinal and Natural Product Research Laboratory, Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga, Mumbai 400 019, India

Received 19 December 2013; revised 28 May 2014

Aegle marmelos Correa (Rutaceae) is a prominent species in the Sub-Himalayan region, also found in central and south India, and has been widely used as remedy for diarrhea and dysentery. Unripe fruits show antiviral activity and prove to be a potent hypoglycemic agent. This study, a high-performance liquid chromatography (HPLC) method for the simultaneous quantification of major furanocoumarin components marmelosin, psoralen and bergapten in the extract from fruit pulp of A. marmelos has been developed. Components were found in the range of 5–6.5, >0.4–0.75 and >0.25 mg/g of dried fruit weight, respectively. The method was validated for linearity, precision, accuracy, robustness, limit of detection and limit of quantification. Linearity was determined over the range of 5–40 μg/ml (r > 0.965). Intra- and interday precision showed a relative standard deviation of <2.5%. The accuracy of the method was determined by a spike recovery study, and the average recoveries were 99.46, 101.04 and 100.8% for marmelosin, psoralen and bergapten, respectively. The proposed HPLC method was found to be simple, precise and specific and can be used simultaneously for routine quality control of raw materials of A. marmelos fruit extracts and their products, and also other products containing these markers.

Introduction

Aegle marmelos, also known as Bengal quince or bael, is traditionally used as a remedy for diarrhea and delivered as tonic. Additionally, unripe fruit pulp shows significant hypoglycemic activity (1). Bael pulp can be processed into nectar or squash, jelly, powder or toffee for both food and therapeutic uses. Fruit pulp can also be used in the preparation of traditional Indian drink ‘sharbat’ and a preserved food called murabba, which is generally used to treat stomach disorder (2). Different chemical constituents such as alkaloids, coumarins and steroids have been isolated and identified from different parts of the plant such as leaves, fruits, wood, root and bark (3). Roots and fruits contain coumarins such as scoparone, scopoletin, psoralen, bergapten, umbelliferone, marmesin, marmelosin and skimming. Fruits, in addition, contain xanthotoxol, alloimperatorin and alkaloids such as agelicine and marmeline (4). Among these, marmelosin (C_{16}H_{14}O_{4}), a prenyl furanocoumarin, shows antidiabetic and anthelmintic activity (5, 6). Psoralen (C_{12}H_{9}O_{3}) and bergapten (C_{12}H_{9}O_{3}) are used in psoriasis, leukoderma and other skin diseases because of its ability to increase skin tolerance to the light. Although there are proven medicinal properties, few data are available on quantitative estimation of concentration of these compounds in the fruits (1, 7). There is need to standardize and develop an easy, quick and validated method for quantitative estimation of marmelosin, psoralen and bergapten.

Experimental

Standards and sample

Accurately weighed 5 mg each of reference standard marmelosin, psoralen and bergapten (Natural Remedies, Bangalore, India) having >99% purity was dissolved in 10 mL of HPLC grade methanol to get 500 μg/mL stock solutions. A standard solution of concentration 5–30 μg/mL was prepared by appropriate dilution of stock solution with mobile phase. Bael fruits were collected from the Nasik district, Maharashtra, India. A 10 g of powdered bael fruit pulp was extracted with 50 mL methanol using a Soxhlet apparatus for 3 h at 70°C.

HPLC instrumentation

HPLC analysis was performed with a Jasco (Hachioji, Tokyo, Japan) system consisting of a binary pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i) equipped with a 20-μL loop and a UV-visible detector (UV-1575). Compounds were separated on a 250 × 4.6 mm i.d., 5-μm particle, Hibar LiChrocron Purospher Star RP-18 end-capped column (Merck, Darmstadt, Germany) with 65 : 35 (%, v/v) acetonitrile-water containing 0.1% acetic acid as isocratic mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20 μL, and the detection wavelength was set at 300 nm (appropriate common UV absorption maxima of these compounds). HPLC was performed at room temperature, and chromatographic data were collected using Borwin software.

Calibration curve of standards

Calibration standard solutions of six concentrations for marmelosin, psoralen and bergapten (concentration range 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 μg/mL) were obtained by appropriate dilutions of stock solution. A calibration curve was obtained by plotting concentration versus area under curve (AUC).

Method of validation

All three standards was analyzed in triplicate for each calibration solution. Linearity was established using correlation coefficients across the ranges of the calibration curves for all three compounds.
According to the ICH guidelines, precision is the closeness of values between a series of measurements obtained from multiple sampling of the same sample under the prescribed condition (8). Precision was determined as the intraday variation of results obtained from the analysis of six different concentrations of standard solutions on the same day. Interday precision was determined by triplicate analysis of the solutions on three successive days as suggested in the ICH guidelines (9). The relative standard deviations (RSDs) of retention time ($R_t$) and AU Co of three analytes were calculated to measure precision, repeatability and stability.

For limits of detection (LODs) and limits of quantification (LOQs), standard solutions were further diluted in methanol to obtain 1 $\mu$g/mL concentration. LOD and LOQ are amounts for which signal-to-noise ratios (S/N) were 3 and 10, respectively, according to the ICH guidelines. The LOD and LOQ of the method are calculated as follows:

\[
\begin{align*}
\text{LOD} &= 3 \left( \frac{\text{SD}}{\text{a}} \right), \\
\text{LOQ} &= 10 \left( \frac{\text{SD}}{\text{a}} \right),
\end{align*}
\]

where SD is the standard deviation of the intersection and $a$ is the average slope, obtained from calibration curves of the linearity study (10).

To access accuracy, which was evaluated as the percentage recovery of all analytes, known amounts of the calibration standards were added to 1 mL of the pre-analyzed bael fruit pulp extract and then analyzed in duplicate as described above. The total amount of each compound was calculated from the corresponding calibration plot, and the recovery of each compound was calculated by using following equation:

\[
\text{Recovery}(\%) = \left( \frac{\text{Amount found} - \text{Amount contained}}{\text{Amount added}} \right) \times 100.
\]

Ruggedness and robustness studies were carried out by performing analysis of the sample and standard in triplicate by making some changes in the standard procedure set for the experiments. Parameters changed are changes in wavelength, change in flow rate and changes in the analyst as reported in Table IV.

Estimation of marmelosin, psoralen and bergapten in the bael fruit pulp extract

To quantify coumarins in the $A. \ marmelos$ fruits pulp extract, an accurately measured 1 mL of stock solution (200 $\mu$g/mL) of the extract was transferred into a 10-mL volumetric flask and the volume was made up with methanol. The solution was filtered using a glass-sintered funnel. Filtered solution was injected for HPLC analysis. The analysis was repeated in duplicate.

Results and Discussion

Different mobile phases (acetonitrile–water, methanol–water, acetonitrile–methanol) were used to optimize the chromatographic condition for the simultaneous determination of three compounds. The results showed that isocratic elution of acetonitrile–water (65 : 35) efficiently separated three compounds under study as shown in Figure 2. One of the absorption maxima ($\lambda_{max}$) of marmelosin, bergapten and psoralen was found to lie between 295 and 305 nm, so the UV detector was set at 300 nm. Chromatographic profile of the standard and extract are shown in Figure 2A and B.

System performance

The calibration curve shows good linear regression. The calibration plots for marmelosin, psoralen and bergapten were linear in the range of 5–30 $\mu$g/mL. The regression equations were $y = 42,439.1x - 10,410.0$ ($R^2 = 0.9655$), $y = 83,999x$ ($R^2 = 0.971$) and $y = 85,761x + 24,461$ ($R^2 = 0.993$), respectively, for
marmelosin, psoralen and bergapten. Figure 2 displays RP-HPLC chromatograms of three marker compounds.

Intra- and interday RSDs of AUC were 2.5%, showing good precision (Tables I and II). Recovery of marmelosin, psoralen and bergapten was an average of 99.46, 101.04 and 100.8%, respectively, with RSD 2.5%, indicating the closeness of theoretical value with the amount found (Table III).

The LOD (S/N = 3) and LOQ (S/N = 10) for all three analytes are below 1 and 3, respectively. The LOD and LOQ were 0.98 and 3.35 mg/mL for marmelosin, 0.69 and 2.90 mg/mL for psoralen and 0.84 and 2.82 mg/mL for bergapten, respectively (Table III).

The ruggedness and robustness study shows satisfactory results with RSD, not exceeding 2.5% (Table IV).

**Conclusion**

**Estimation of marmelosin, psoralen and bergapten**

These results revealed that the method enables rapid, precise and accurate simultaneous detection and quantification of marmelosin, psoralen and bergapten. When the method was subsequently used for the analysis of methanolic extract of *A. marmelos*, the amounts of marmelosin, psoralen and bergapten were found to be 5–6.5%, >0.4–0.75% and >0.25 mg/g, respectively. The method described in this study may be used for other matrices with slight modification.

**Acknowledgments**

The authors are thankful to University Grant Commission, New Delhi, India, for providing financial assistance.

**References**