Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 4, 2013

Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF BIO-ACTIVE MARKERS COROSOLIC ACID, ASIATIC ACID AND β-SITOSTEROL FROM LEAVES OF *LAGERSTROEMIA SPECIOSA* LINN. AND FROM MARKETED FORMULATION

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Received: 20 Aug 2013, Revised and Accepted: 27 Sep 2013

ABSTRACT

Objective: To develop simple, accurate and reproducible High Performance Liquid Chromatographic (HPLC) method for simultaneous quantification of Corosolic acid, Asiatic acid and β-sitosterol in the leaf extract of *L.speciosa*.

Methodology: The methanolic extract of the leaf powder of *L.speciosa* was separated on HPLC column (HyPurity C18, 100mm X 2.1mm, 5µ column) with gradient mixture of HPLC grade Acetonitrile and Water as mobile phase. The separated components were detected using Photo Diode Array (PDA) detector at 210 nm.

Results: Linear response was found in the concentration range of 0.5-600 µg/mL for Asiatic acid, 1.0-600 µg/mL for Corosolic acid and 2.0-500 µg/mL for β-sitosterol respecticvely. The relative standard deviation for inter-day and intra-day precision was found to be <2%. The validated method was successfully applied for quantification of the three components in a commercially available dietary supplement "Banaba" containing *L.speciosa* leaf extract.

Conclusion: A precise, accurate and reproducible HPLC method is developed for simultaneous quantification of three bio-active components from *L.speciosa*. The method can be used as quality control tool in standardization of raw materials and marketed formulations.

Keywords: HPLC; Corosolic acid; Asiatic acid; β-sitosterol.

INTRODUCTION

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed [1].

Lagerstroemia speciosa Linn. (Lythraceae), commonly known as Banaba or Tamhan, is a deciduous, tropical, flowering tree that grows widely in India, Philippines and South-east Asian countries. The tea from the leaves of *L.speciosa* has traditionally been used in the Philippines as a folk medicine for the prevention and treatment of diabetes [2],[3]. In addition, *L.speciosa* extracts are also known to possess weight loss [4] and anti-oxidant effects [5],[6].

In recent years, there has been a growing interest in the chemical composition and biological activities of *L.speciosa*. Among the various phytochemical found in leaves of *L.speciosa*, sterols and particularly triterpenoids are widely regarded as major biologically active components which may contribute to their reputed and diversified health benefits.

Scientists have identified different components of *L.speciosa* to be responsible for its activity. The leaves contain ellagic acid derivatives, ellagitannins, lagerstroemin, flosin B and reginin A. Lagertannins, β-sitosterol, stigmasterol, campesterol and some olefins also have been found in the leaf extracts [7]. Six pentacyclic triterpenes (oleanolic acid, arjunolic acid, Asiatic acid, maslinic acid, corosolic acid and 23-hydroxyursolic acid) were isolated from *L.speciosa* leaves [8]. Corosolic acid has been reported to have anti-diabetic activity [9],[10],[11]. β-sitosterol, the principal phytosterol appears to have important immunomodulatory activity in human and animal physiology [12]. Asiatic acid has been reported to have many biological effects such as acetyl cholinesterase inhibitory effect [13] and enhancement of cognitive functions [14].

In view of these wide therapeutic effects a need was felt for simultaneous quantitation of corosolic acid, asiatic acid and β-sitosterol in the leaves of *L.speciosa*. Literature survey revealed that HPLC and HPTLC methods have been reported for quantitative determination of corosolic acid in leaves of *L.speciosa* [15]. Also, HPLC and HPTLC methods have been reported for estimation of Asiatic acid and β-sitosterol from different plant sources such as *Centella asiatica* and *Boerhavia diffusa* respectively [16],[17]. However, no HPLC method has been reported for simultaneous estimation of Corosolic acid, Asiatic acid and β-sitosterol from leaves of *L.speciosa*.

In this research work, a simple, precise and accurate HPLC method has been established for simultaneous quantitation of corosolic acid, asiatic acid and β-sitosterol in the dried leaf powder of *L.speciosa* Linn. Further, the proposed method has been validated as per ICH guidelines and applied as a quality control tool for standardization of a commercially available anti-diabetic dietary supplement containing leaf extract of *L.speciosa*. Such a study would not only facilitate standardization of the raw material and commercial products but also facilitate future pharmacological studies and quality control.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol, acetonitrile and water were procured from E.Merck, Mumbai, India. Reference standards of Asiatic acid (purity >95%) and β-sitosterol (purity >97%) were purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany). Corosolic acid reference standard (purity >90%) was purchased from Natural Remedies, Banglore, India.

Plant material

Leaves of *L.speciosa* were collected from the wild tree found in Mumbai. Herbarium samples of *L.speciosa* were prepared in duplicate and authenticated by Botanical Survey of India (BSI), Pune, India. A voucher specimen numbered NJ-1 has been retained in the herbarium section of BSI, Pune for future reference. The leaves were washed with water to remove any dust particles, dried in shade, powdered and then sieved through BSS mesh size 85 and stored at 25°C in an airtight container.

Dietary supplement "Banaba capsules" were procured from Sushrut Ayurved Industries, Dharwad, Karnataka, India.

Preparation of stock solutions

Standard stock solutions of pure compounds were prepared separately by dissolving 10 mg of each compound in 10 mL of methanol to get concentration of 1000 µg/mL.

For calibration curve, aliquots of 0.5-600 µg/mL, 1.0-600 µg/mL and 2.0-500 µg/mL were prepared from the above stocks for asiatic acid, corosolic acid and β-sitosterol respectively.

Also three quality control levels (LQC, MQC, HQC) each of Asiatic acid (1.0, 100, 400 µg/mL), Corosolic acid (2.0, 100, 400 µg/mL), and β-sitosterol (3.5, 100, 400 µg/mL) were prepared for precision, accuracy and ruggedness studies.

Sample Preparation

About 5 gm of dried leaf powder of *L.speciosa* was weighed into a round bottom flask. 30 ml of methanol was added to the flask and the mixture was refluxed on a boiling water bath for about 30 min. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The same operation was performed twice and filtrate obtained was combined together and made up to 100 ml with methanol. This solution was further used for assay.

Dietary supplement: For analysis of the Banaba capsule, contents of 20 capsules were combined and 5 gm was accurately weighed into a round bottom flask. 30 ml of methanol was added to the flask and the mixture was refluxed on a boiling water bath for about 30 min. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The same operation was performed twice and filtrate obtained was combined together and made up to 100 ml with methanol. This solution was further used for assay.

Chromatography

HPLC was performed on Shimadzu UFLC Prominence system. The system was equipped with binary pumps (LC – 20 AD), autosampler (SIL – 20 AC HT) and Degasser (DGU – 20 A_3). The column oven (CTO – 20 AC) temperature was maintained at 40°C throughout the analysis. The detection was carried out using SPD – M 20 A photo diode array detector.

The method involves use of a C18 column (Thermo Electron Corporation, HyPurity 100 mm X 2.1 mm) with 5µ particle size of stationary phase. Different compositions of solvents were tried as mobile phase in both isocratic and gradient mode. Finally a gradient of acetonitrile:water was selected which gave a good resolution between the sample components. The concentration of acetonitrile was increased from 20% to 70% in the first five minutes and was maintained at the same level till 7 minutes. The concentration of the organic solvent was then increased to 100% in 10 minutes and was maintained at the same level till 14 minutes to allow complete elution of the components of plant sample from the column. Passing of 100% organic solvent ensured that no traces of the plant or formulation sample remained in the column which might have otherwise interfered with the next sample analysis and also may decrease the life of the column. The flow rate was maintained at 1 mL/min and the separated components were detected at 210 nm.

Validation of the Method

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method [18].

Selectivity and Specificity

During the experiments an UV scan ranging from 200 to 400 nm in the time window of the analytes using PDA detector was performed with the aim of revealing eventual interfering compounds and evaluating the selectivity of the method. Specificity of the intended method was established by comparing the HPLC retention time and absorption spectra of target peaks from the analysed samples with those of the reference compounds.

System suitability

System suitability experiment was performed by injecting six consecutive injections (10 µg/mL) of each bioactive marker, namely asiatic acid, corosolic acid and β-sitosterol during the start of the method validation. Values with % CV of ≤2% were accepted.

Calibration curves

Linearity of the components was determined in triplicate at eight different concentrations for Asiatic acid and seven different concentrations for corosolic acid and β-sitosterol respectively. Calibration curve was plotted as mean peak area versus concentration. The linear regression equation was obtained using a least-square method and used to estimate the concentration of the three components in the analysed samples. RSD of standard peak areas for solutions of the same concentration were less than 2%, indicating there was no statistically significant variation.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Sensitivity of the method was evaluated by determining the values of LOD and LOQ. Stock solution of each standard was serially diluted with methanol to prepare the series of samples with least concentration and injected into the HPLC system. The limit of detection (LOD) and quantification (LOQ) for asiatic acid, corosolic acid and β-sitosterol were determined by measuring the signal to noise ratio (S/N). LOD and LOQ was considered at S/N of 3:1 and 10:1 respectively.

Precision

Variability of the method was studied by analyzing quality control samples of asiatic acid (1.0, 100, 400 µg/mL), corosolic acid (2.0, 100, 400 µg/mL) and β-sitosterol (3.5, 100, 400 µg/mL) on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy values within the range of 85 – 115% and % CV of ≤2% were accepted.

Recovery

Recovery tests were carried out to further investigate the accuracy of the method by adding three different concentration levels of the mixed standard solutions to known amounts of L. speciosa samples and dietary supplement prior to extraction. The resultant samples were then extracted and analyzed with the described method. The mean percentage recoveries were calculated using the formula: Recovery (%) = [(amount found – original amount) / amount added] x 100. Values within the range of 85 – 115% were accepted.

Ruggedness

Ruggedness of the method was studied by determining the effects of small variations of mobile phase composition (±2%), and flow rate (1.00 ± 0.05 mL/min). Effect of these deliberate changes on the response (area) and retention time of QC samples of Asiatic acid, corosolic acid and β-sitosterol was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of ±5% were accepted.

Stability

The stability of the stock solutions of all the three standards was evaluated by storing the solutions in refrigerator at 2-8°C for 72 hours and then comparing the results against freshly prepared stocks for each standard. Samples in triplicate were also subjected to bench top stability at 0.0 h and 6.0 h respectively. Values within a difference range of ±5% were accepted.

Estimation of Corosolic acid, Asiatic acid and β-sitosterol in *L.speciosa* leaf powder and dietary supplement containing *L.speciosa* leaf powder

The extract of *L.speciosa* and Banaba capsules were injected seven times separately and analysed using the optimized chromatographic conditions. Peak areas were recorded for each analyte of interest and the amount of all the three analytes (corosolic acid, asiatic acid and β-sitosterol) was calculated by use of the calibration plot.

RESULTS AND DISCUSSION

Optimization of the Chromatography

Initial trial experiments were conducted to select a suitable mobile phase for accurate analysis of the standards. Of the various mobile phases tried in both isocratic and gradient mode, acetonitrile:water in gradient mode gave the best resolution between the three

analytes asiatic acid, corosolic acid and β-sitosterol. These standards were also resolved from other components present in the sample extract enabling simultaneous quantification. HPLC chromatograms corresponding to the three standards, *L.speciosa* leaf extract and marketed formulation are represented in Figure 1 to 3. All the analytes exhibited the UV maximum absorption at 210 nm and hence this wavelength was chosen for the detection of components eluting out from the column.

Method Validation

Selectivity and Specificity

During the UV scan no appreciable difference was found in the spectra of reference standards and the analysed samples. Hence, the method demonstrated a high degree of selectivity.

System suitability

System suitability tests are used to verify whether the resolution and reproducibility of the chromatographic system are adequate for the analysis. For Asiatic acid, corosolic acid and β-sitosterol the %CV values for area and retention time was found to be <2% indicating that the system was suitable to carry out further analysis.

Linearity

The method was found to be linear from 0.5-600 µg/mL for asiatic acid, 1.0- 600 µg/mL for corosolic acid and 2.0-500 µg/mL for β-sitosterol respectively. The correlation coefficient was found to be ≥0.995 for all the three components. Results of regression analysis are summarized in Table 1.

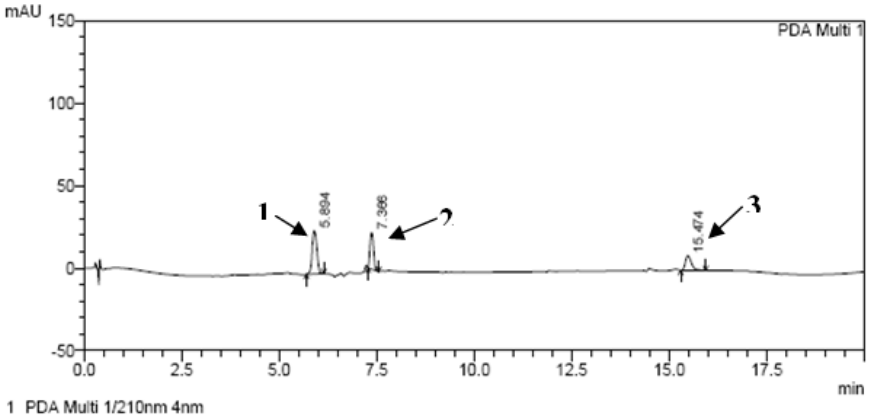

Fig. 1: Typical HPLC chromatogram of mixed standards

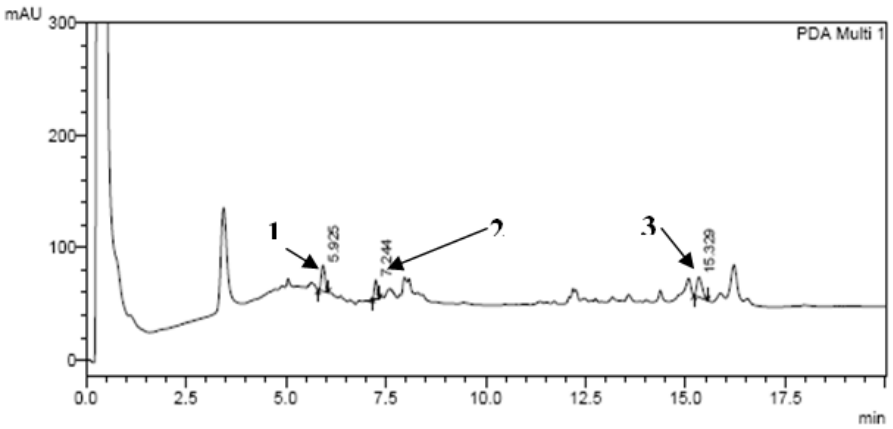

Fig. 2: HPLC chromatogram of leaf extract of *L.speciosa*

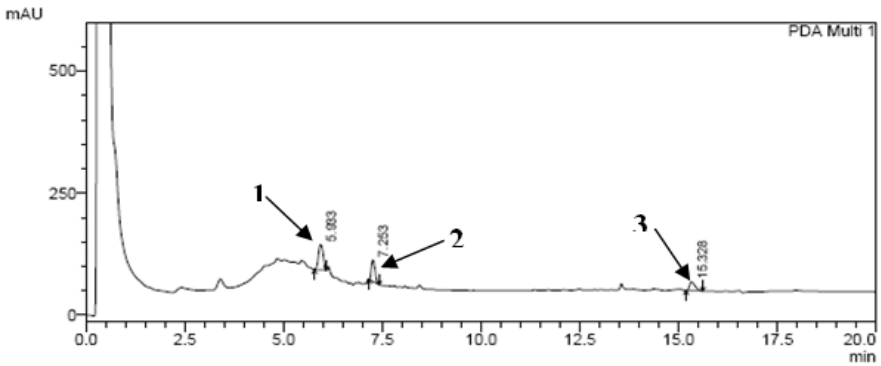

Fig. 3: HPLC chromatogram of marketed formulation "Banaba"

Key: 1 – Asiatic acid; 2 – Corosolic acid; 3 – β sitosterol

Sensitivity

Sensitivity of the method was affirmed in terms of LOD and LOQ for asiatic acid, corosolic acid and β-sitosterol respectively. The results are represented in Table 1. The values for both LOD and LOQ were low when compared to other published works, [15,16,19] which indicated that the method is capable of detecting and quantifying trace amount of the three components in plant samples.

Precision

In the repeatability study intra-day and inter-day precision of the HPLC method were investigated using replicate injection (*n*=3) of quality control samples of all the three standards. The developed method was found precise with % CV<2%.

Stability

Stock solution stability study of all the three standards stored for the period of 72 hours at 2-8°C showed % CV <2% with the % mean difference within ±5%. During bench top stability study, similar results were obtained. Stability studies showed that asiatic acid, corosolic acid and β-sitosterol were found stable for at least 6.0 h at room temperature and 72 hours at 2-8°C of storage condition.

Recovery

The recovery values for all the three components were within acceptable limits (85.0 to 115.0%). This indicated that the method was reliable and accurate.

Table 1: The regression data, linearity, LOD and LOQ and retention times for three bioactive compounds by HPLC

Compounds	Retention time	Regression equation	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Asiatic acid	5.894	y = 4540.x + 1048	0.9997	0.5-600	0.3	0.5
Corosolic acid	7.366	y = 2588.x + 8834	0.9998	1.0-600	0.2	0.5
β-sitosterol	15.474	1618.x + 6114	0.9995	2.0-500	0.3	1.0

Ruggedness

Proposed method was not influenced by the factors considered for ruggedness study. Change in flow rate and mobile phase composition affected the retention time of the three analytes but the results were satisfactory since % CV was <2% with % mean difference <5%.

Assay

The assay value for samples of *L speciosa* leaf powder was found to be 0.32%, 0.06 % and 0.78% for Corosolic acid, Asiatic acid and β-sitosterol respectively, while for the dietary supplement it was found to be 0.92 %, 0.29% and 0.33% for Corosolic acid, Asiatic acid and β-sitosterol respectively. The method is specific for all the three components because it resolved all standards well in the presence of other phytochemicals in *L. speciosa*. The proposed HPLC method was found to be suitable for qualitative and simultaneous quantitative analysis of corosolic acid, asiatic acid and β-sitosterol in the methanolic extract of *L. speciosa*.

CONCLUSION

A precise, accurate and reproducible HPLC method is validated for simultaneous quantification of three bioactive markers Corosolic acid, Asiatic acid and β-sitosterol. Proposed HPLC method can be used as an analytical tool for quality evaluation of plants and formulations containing Corosolic acid, Asiatic acid and β-sitosterol as chemical markers. It is an efficient method to screen *L.speciosa* leaf samples in order to assess its quality and authenticity. Hence, it can be demonstrated that HPLC is a powerful practical tool for comprehensive quality control of plant raw materials and its formulations.

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