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Research Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF BOSWELLIC ACID AND MYRISTICIN IN COMMERCIAL HERBAL FORMULATION

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ABSTRACT

Objective: The aim of the current study was to developed simple, rapid, precise, accurate and specific HPLC method for simultaneous estimation of two major constituents i.e. boswellic acid and myristicin in rheumatoid arthritic herbal formulation.

Methods: An optimal condition of separation and detection were achieved on a Phenomenax C_{18} column (5µm 250×4.6 mm), with mobile phase composition of Acetonitrile: Water (85:15). The effluent was detected at 205 nm at the flow rate of 1.0 ml/min. Validation of the method was performed in order to demonstrate its selectivity, accuracy, precision, repeatability and recovery.

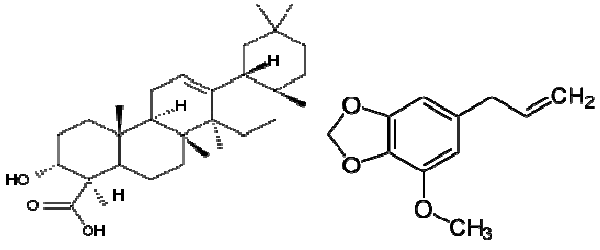
Results: All calibration curves showed good linear correlation coefficients (r^2> 0.995) within the tested ranges. Linearity was observed over concentration range of 1-20µg mL^{-1} for both the markers boswellic acid and myristicin. Two markers in market formulation were quantified with respect to boswellic acid (0.029 %w/w) and myristicin (0.03%w/w).

Conclusion: A method has been developed for the simultaneous determination of two markers in laboratory prepared mixtures and market formulation.

Keywords: Boswellic acid, Myristicin, UV detection, Column liquid chromatography

INTRODUCTION

In recent years, there has been great demand for plant derived products in developed countries. In order to have a good coordination between the quality of raw materials, in process materials and the final products, it has become essential to develop reliable, specific and sensitive quality control methods using a combination of classical and modern instrumental method of analysis. Standardization is an essential measurement for ensuring the quality control of the herbal drugs. Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease affecting multiple tissues but principally attacking the joints to produce a nonsuppurative proliferative synovitis. RA is not a condition of wear and tear that occurs with aging or injury. It is an autoimmune disease in which body's own immune system mistakenly attacks healthy tissue, causing inflammation that damages joints. [1,2]



Boswellic Acid **Myristicine**

Boswellia serrata (Family- Burseraceae), an oleo-gum-resin, is a medium size tree lavishly full-fledged in dry undulating parts of India, Northern Africa, and the Middle East. Strips of Boswellia bark are peeled away, yielding a gummy oleo-resin. [3,4] *B. serrata* has been used for a variety of therapeutic purposes such as cancer, inflammation, arthritis, asthma, psoriasis, colitis and as an anti-hyperlipidemic. *B. serrata* is an oleo-gum-resin. Its essential oil is a blend of mono, di and sesquiterpenes and gum portion consist of pentose and hexose sugars with oxidizing and digestive enzymes. Chemically resin is pentacyclic triterpenoid in nature in which boswellic acid is the dynamic moiety. Boswellic acids selectively hamper leukotrienes synthesis by inhibiting 5-lipoxygenase enzyme in an enzyme directed, non-redox, non-competitive mechanism. [5,6]

Nutmeg (*Myristica fragrans*), whose seed is widely used as a spice, is a tropical, dioeciously evergreen tree native to the Moluccas or Spice Island of Indonesia. Nutmeg has a characteristic pleasant fragrance and is slightly warm taste. It is used to flavour many kinds of baked goods, confections, puddings, meats, sausages, saucers, vegetables, and beverages. [7,8] It is also used as components of curry powder, teas and soft drinks, or mixed in milk and alcohol. [9] The main constituents of *Myristica fragrans* have been found to be alkyl benzene derivatives (myristicin, elemicin, safrole, etc.), terpenes, alpha-pinene, beta-pinene, myristic acid, trimyristin, neolignan (myrislignan), and macelignan. Medicinally, it is used as an anti-diarrheal agent for patients with medullar carcinoma of the thyroid. The effectiveness of the treatment may be due to the inhibition of prostaglandin synthesis in the mucosa and sub mucosa of the colon. It is sometimes used as a stomachic, stimulant, carminative as well as for intestinal catarrh and colic, to stimulate appetite, to control flatulence, and has a reputation as an emmenagogue (to promote and regulate menstrual flow) and abortifacient. It has also been found useful as tonic for the heart and brain and also in sexual and general debility. [9,10]

In the present investigation, we have developed a simple, optimized and validated HPLC method for the standardization of herbal formulation. Two biologically active markers were selected for quantification, boswellic acid for *B. serrata* and myristicin for *M. fragrance*. The method was validated on the basis of its selectivity, linearity, precision, accuracy, limit of detection and limit of quantification according to International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS

Materials

Boswellia serrata gum resin powder and nutmeg kernels were purchased from local market of Vadodara, Gujarat and authenticated in the Botany Department, M. S. University of Baroda, Vadodara. Boswellic acid (BA) and myristicin (MYR) were isolated according to the method described by Singh S.et al. Boswellic acid was purchased from M/S Natural Remedies private Ltd. Bangalore and Myristicin was purchased from Sigma-Aldrich, USA. LOSWEL Tablets (Dr. Vasishth's Ayu Remedies, Ahmadabad.) were purchased from local pharmacy. HPLC grade acetonitrile and methanol were procured from Qualigens fine chemicals, Mumbai.

Instrumentation

Chromatography was performed on Shimadzu chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a rheodyne 7725 injector valve with fixed loop at 20µl. Data acquisition and integration was performed using spinchrom software. Separation was carried out using a Supelco 516 C-18-DB, 5µm (25cm x 4.6mm). The UV detector was set at 205 nm.

Preparation of Standard solutions of BA and MYR

Accurately weighed 5 mg of standard Boswellic acid, 5 mg of standard myristicin were separately dissolved in 5 ml of methanol obtaining stock concentrations of 1000 µg/ml. Aliquots of each standard were mixed to obtain solutions in the range of 1-20 µg/ml in methanol.

Preparation of laboratory mixture

An accurately weighed portion of the Laboratory sample equivalent to 1 mg of BA and 1 mg of MYR was transferred to volumetric flask and sufficient methanol was added and sonicated for 10 min to get concentration of 100µg mL^{-1}. The solution was filtered through nylon filter paper 0.2µm in 10 ml volumetric flask and then diluted to volume with sufficient methanol to get stock solution. Different volumes of working sample solution were taken and diluted with methanol to get different concentrations of BA and MYR.

Preparation of marketed formulation for analysis

The 20 tablets were weighed, crushed to obtain a powder and powder equivalent to 1 tablet was weighed (1.32gm), extracted with methanol overnight. The resulting extract was filtered through nylon filter paper 0.2µm and from that dilutions were made such that final concentration was 40µg mL^{-1}.

Calibration

The contents of both the markers were determined using a calibration curve established with six dilutions of each standard, at concentrations ranging from 1-20µg/ml. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentrations of the markers injected. Peak identification was achieved by comparison of both the retention time (RT) and UV absorption spectrum with those obtained for standards.

Validation parameters

The method was validated according to ICH guideline for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification [11,12]. Selectivity was checked using an extract of formulation and a mixture of standards in order to optimize separation and detection. Linearity of the method was performed by analyzing a standard solution of markers by the method in the concentration range of 1-20 µg/ml. The accuracy of the proposed method was determined by a recovery study, carried out by adding known quantity of standard markers in the formulation extract. The samples were spiked with three different amounts of standard compounds prior to extraction. The spiked samples were extracted in triplicate and analyzed under the previously established optimal conditions. The obtained average contents of the target compounds were used to calculate the spike recoveries. Precision was determined by repeatability and interday and intraday reproducibility experiments. A standard solution containing both markers was injected six times; Formulation was also extracted six times to evaluate the repeatability of the extraction process. The mean amount and standard deviation (SD) value of both the markers were calculated. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of marker compounds were calculated at signal-to-noise ratio of approximately 3:1 and 10:1 respectively. To evaluate robustness of the method few parameters were deliberately varied. The parameters included variation of flow rate, different instruments and ACN of different manufacturer. The average value of % RSD less than 2 % reveals the robustness of the method. Several parameters were calculated for system suitability of HPLC method like Theoretical plates per meter, Symmetry factor/Tailing factor, Resolution etc.

RESULTS AND DISCUSSIONS

Selection and Optimization of chromatographic condition

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for BA and MYR was obtained with a mobile phase ACN: WATER (85: 15 v/v) at a flow rate of 1.0 ml/min to get better reproducibility and repeatability. Selecting 205 nm as the detection wavelength resulted in an acceptable responses and enable the detection of both the compounds used in this study. Complete resolution of the peaks with clear baseline was obtained (Fig. 2) and System suitability test parameters for BA and MYR for the proposed method are reported in Table 1. An HPLC fingerprint for the laboratory formulation and marketed formulation were developed.

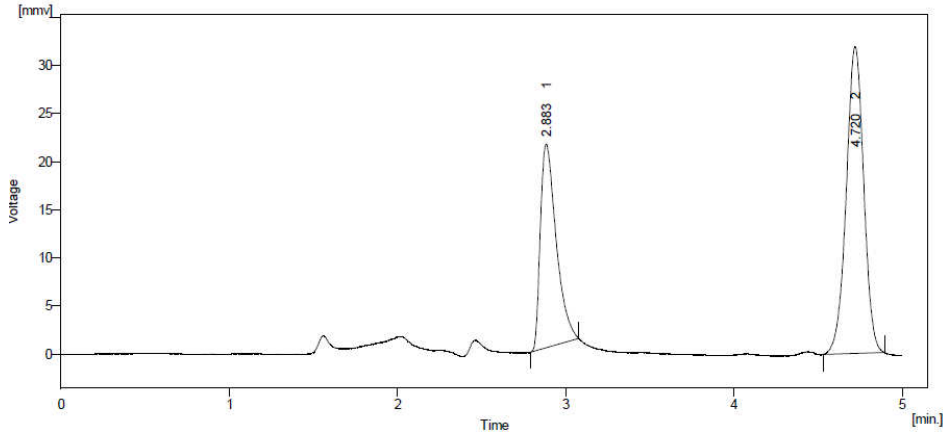


Fig. 1: Chromatogram of Standard Solution of BA (2.88 min) and MYR (4.73 min) at 205 nm

Table 1: System suitability parameters of chromatogram for Boswellic acid and Myristicin

Parameters	Data obtained (n=6)	
	BA±RSD	MYR±RSD
Retention Time (min) ± SD	2.88±0.03	4.73±0.04
Theoretical plates per meter ± SD	52760±2569	133303±9568
Symmetry factor/Tailing factor	1.756±0.04	1.391±0.05
Resolution	6.23±0.14	

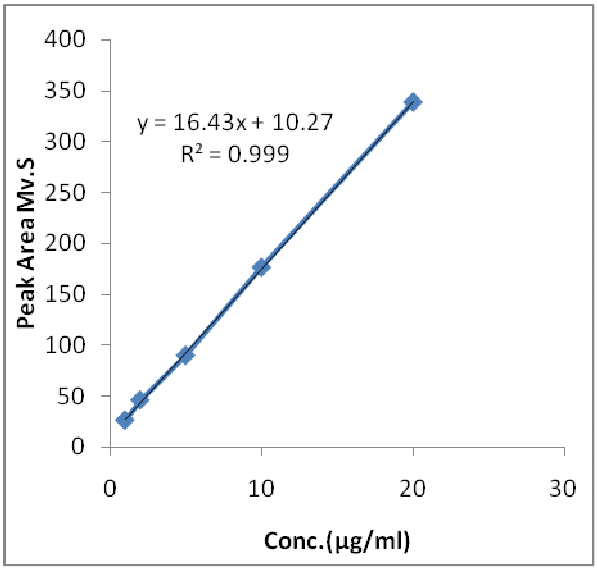


Fig. 2: Calibration curve of BA at 205nm

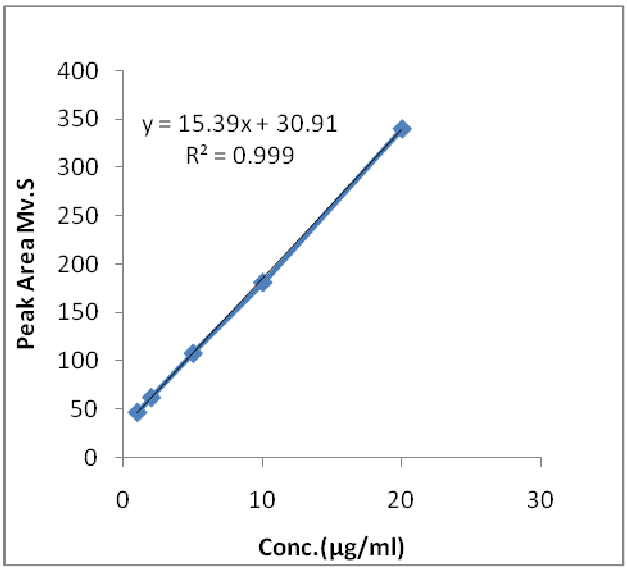


Fig. 3: Calibration curve of MYR at 205nm

Validation of the Proposed Method

The proposed method has been validated for the simultaneous determination of BA and MYR in commercial Polyherbal formulation using following parameters.

Linearity

Linear correlation was obtained between peak area Vs concentrations of BA and MYR in the concentration ranges of 1-20 µg/ml for both the markers. Regression parameters are mentioned in table 7.8 and the calibration curves of these two markers at 205 nm are shown in Fig. 2 & Fig. 3.

Range

Range is the interval between upper and lower concentration (amount) of analyte in sample in sample for which it has been demonstrated that the analytical method has suitable level of precision accuracy and linearity. The linear response was observed over a range of 1-20 µg/ml for both the markers and the calibration curves of these two drugs at 205 nm are shown in Fig. 2 & Fig. 3.

Precision

The low %RSD values of Intraday (0.327) for BA and (0.590) for MYR and Interday (0.580) for BA and (0.625) for MYR, reveal that the proposed method is precise (Table 2).

Table 2: Precision of the Intra-day and Inter-day HPLC measurement for BA and MYR

Marker	Intra-daily[a] RSD (%)	Inter-day[b] RSD (%)
BA	0.327	0.580
MYR	0.590	0.625

a Sample were analyzed six times a day

b Sample were analyzed once a day over six consecutive days

LOD and LOQ

LOD values for BA and MYR were found to be 0.342µg/ml and 0.6295µg/ml, respectively and LOQ values for BA and MYR were found to be 1.038µg/ml and 1.907µg/ml, respectively (Table 3). These data show that the proposed method is sensitive for the determination of BA and MYR.

Accuracy

The recovery experiment was performed by the standard addition method. The summary of the results and average mean of recovery data for each level of both markers was within accepted range as shown in Table 4. The low value of standard deviation indicates that the proposed method is accurate.

Table 3: Regression parameter, Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) of the proposed HPLC method.

Marker	Concentration range (µg/ml)	Rt (min) [a]	Regression equation	R^2	LOD (µg/ml)	LOQ (µg/ml)
Boswellic acid	1-20	2.88±0.03	y = 15.39x + 30.91	0.999	0.342	1.038
Myristicin	1-20	4.73±0.04	y = 16.43x + 10.27	0.999	0.6295	1.907

[a]Mean ±SD (n=6)

Table 4: Recovery study of BA and MYR in market formulation

Excess drug added to analyte (%)	Theoretical Content (µg mL^{-1})	*Amount Found (µg mL^{-1})		*Recovery(%) ± S.D.	
		B.A.	MYR	B.A.	MYR
80	9 :9	8.95	8.85	99.44±0.15	98.33±0.28
100	10:10	9.87	9.79	98.7±0.24	97.9±0.34
120	11:11	10.25	10.89	93.18±0.34	99.0±0.41

*n=6

Robustness

To evaluate robustness of the method few parameters were deliberately varied. The parameters included variation of flow rate, change in pH of buffer, different instruments and ACN of different manufacturer The average value of % RSD for determination of BA and MYR less than 2 % revealed the robustness of the method. The selected factors remained unaffected by small variation of these

parameters, which demonstrates that the method developed was robust as shown in Table 5.

Table 5: Robustness of the method for BA and MYR

Factor	Retention time	
A. ACN	**BA**	**MYR**
Brand-I	2.88	4.73
Brand-II	2.89	4.732
Mean ±SD	2.885±0.007071	4.731±0.001414
B. Instrument	**B.A**	**MYR**
I	2.88	4.73
II	2.885	4.723
Mean ±SD	2.8825±0.003536	4.7265±0.00495

Quantification of markers present in marketed formulation

The two markers were found in market formulation and they were quantified with respect to Boswellic acid (0.029 % w/w) and Myristicin (0.003% w/w). The chromatograms of a mixture of market formulation (Fig 4) and laboratory mixture (Fig 5) showed complete separation of the two markers. The results obtained are shown in Table 6.

Table 6: Quantification of markers present in marketed Polyherbal formulation

Marker	Qty. of Marker per 1 tablet
B.A.	0.029%
MYR	0.003%

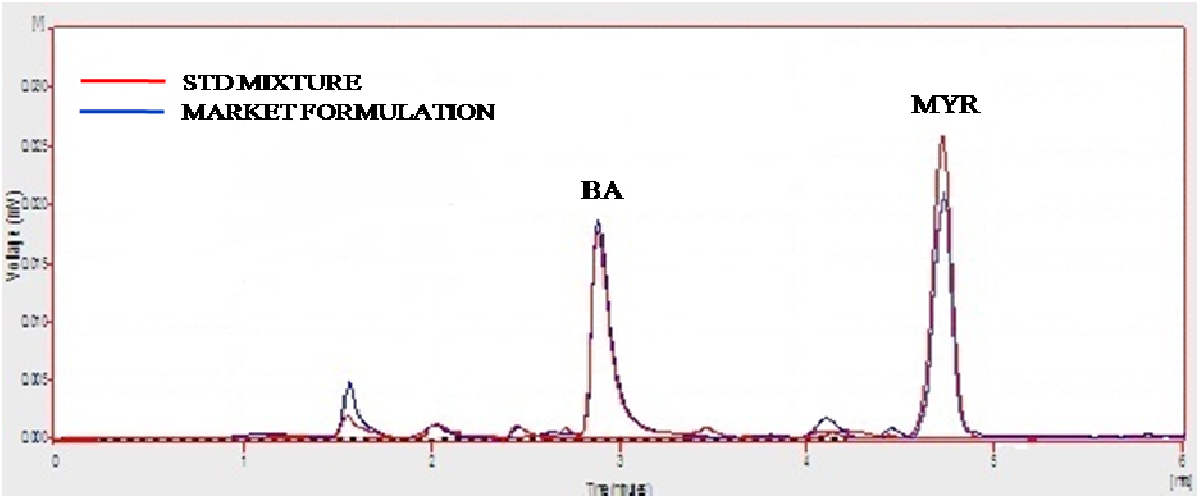


Fig. 4: Chromatograms of Mixture (BA+MYR) and Market Formulation

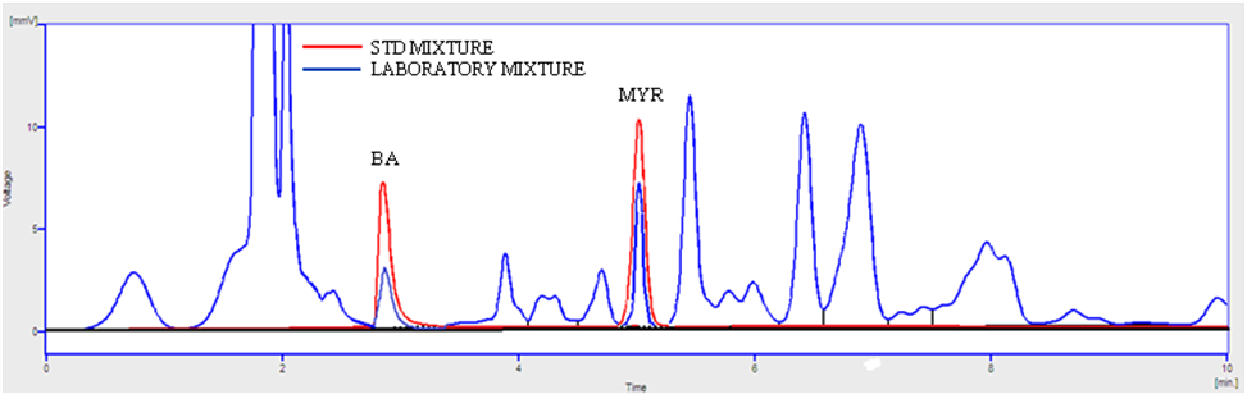


Fig. 5: Chromatograms of Mixture (BA +MYR) and Laboratory formulation

CONCLUSION

Herbal formulation being multicomponent system lack adequate analytical methods ascertain there quality and consistency. A concept of simultaneous estimation of more than one biologically active phytoconstituents in a single set of analytical method is found to be beneficial in the present case; therefore an attempt was made to develop method for simultaneous estimation of Boswellic acid and Myristicin using HPLC.

The developed HPLC method was found to be simple, precise and reliable. The method was applied to commercial polyherbal formulation and was found to be applicable for quantification of biological markers. The method had good resolution and thus can be used for analysis of multi component formulations also.

The quantification results indicated that Boswellic acid and Myristicin content varied significantly in different formulations. Thus, this method can provide a scientific and technical platform to the products manufacturers for setting up the quality control standard as well as to the public quality and safety assurance of proprietary herbal medicines and other herbal preparation containing *B.serrata* and *M.fragrance*.

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