A NOVEL RP-HPLC METHOD FOR THE DETERMINATION OF BHARANGIN IN GHANTU BHARANGI CRUDE EXTRACTS

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ABSTRACT

An accurate, simple, reproducible and sensitive RP-HPLC method for the determination of bharangin has been developed and validated. The separation of bharangin and 2-nitroaniline (internal standard) was achieved on Supelcosil LC-18 (3µ, 150× 4.6 mm i.d.) column using UV detection at 388 nm. The mobile phase was consisting of methanol and 0.01M KH_2PO_4 buffer (pH 3.0, adjusted with *ortho*-phosphoric acid) (75:25, % v/v). The linear range of detection for bharangin was found to be 10-50 ng/ml. Intra-and inter-days assay relative standard deviations were less than 3.21. The method has been successfully applied to the determination of bharangin in various crude extracts. The method has been shown to be linear, reproducible, specific, and rugged.

Keywords: Bharangin, 2-Nitro aniline, HPLC.

INTRODUCTION

Bharangin (figure 1(a)) a novel diterpenoid quinonemethide isolated from the hexane extract of the root nodules of *Pygmacopremna herbaceae* (Roxb). It possesses (S) configuration at the chiral carbon atom from a consideration of its mass, UV, IR, NMR and CD spectrum(Sankaram *et al*., 1998). Fresh rootstock and roots along with ginger are given in asthma, rheumatism and dropsy. The root bark is used to cure toothache. The leaves are prescribed in fever, cough and rheumatism and their poultices are applied to boils.

It is an important drug used by the ayurvedic physicians either alone or as an ingredient in the compound preparations for the treatment of bronchitis, asthma, blood pressure, tumors, inflammation, hiccough, epilepsy, helminthiasis (Quing and Weixin, 1988; Quing *et al*., 1988; Quing *et al*., 1989; Gopalan *et al*., 1988).

The crude hexane, chloroform and aqueous extracts of this plant have been subjected to screening for different medicinal properties. The crude hexane extract was found to be exhibiting antifungal, antibacterial, anti-amoebic and blood sugar lowering properties. It is interesting to note that the major component bharangin exhibited all the biological properties of the crude hexane extract as well as cytotoxic properties against P-338 tumor cell line. Bharargin exhibit higher efficiency in curing of plasmids belonging to IncF, H_2 and X-groups (Lakshmi *et al.,* 1989; Murthy *et al.*, 2006).

High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is the typical analytical method utilized to quantitate terpenoids in extracts for use in developing formulations and pharmacokinetic studies. Literature survey reveals that there was no single analytical method was developed for the determination of bharangin in extracts.

In this paper we have presented a RP-HPLC method for the determination of bharangin using 2-nitro aniline as an internal standard (fig. 1b). The overall aim of the present study is to develop a simple, efficient, reliable and accurate method for the estimation/determination of bharangin in crude plant extracts of *Pygmacopremna herbaceae.*

MATERIALS AND METHODS

Standards and chemicals

2-Nitro aniline (IS) was obtained from E. Merck (India) Limited (Mumbai, India). All chemicals were analytical grade: potassium dihydrogen orthophosphate and *ortho*-phosphoric acid from S.D Fine-Chem. Ltd (Mumbai, India), methanol from Qualigens fine chemicals (Mumbai, India). The stock solutions of 0.01M KH_2 PO_4 was prepared with water and adjusted to pH 3.0 with *ortho* - phosphoric acid. All the above solutions were degassed in an ultrasonic bath (Sonicator), for 30 minutes before use. Water used for the preparation of mobile phase was obtained from All Quartz double distiller apparatus, Bhanu Scientific Instruments Company Pvt. Ltd.

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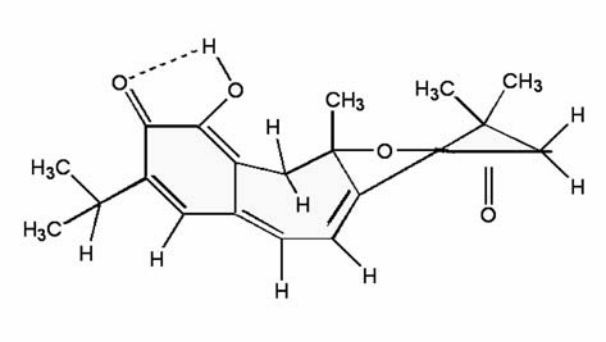

Fig. 1a: Structure of bharangin

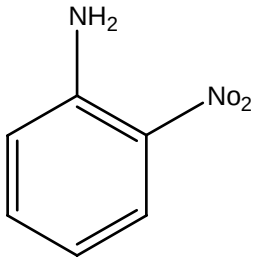

Fig. 1b: Structure of 2- nitro aniline

(Bangalore, India) and pH of the mobile phase was adjusted by using Digital pH Meter, Model DI 707 (Digisun electronics, Hyderabad, India).

Preparation of standards

Stock solutions of bharangin and IS (2-Nitro aniline) for generating standard curves were prepared by dissolving 10 mg of each compound in methanol to yield concentrations of 1 mg/ml. Working standard solution of bharangin and 2-nitro aniline was obtained by diluting 40 times both bharangin (25µg/ml), and 2-nitroaniline (25 µg /ml) respectively.

A 0.01M solution of Potassium dihydrogen ortho-phosphate (pH: 3.0) was prepared by dissolving 0.680 g of potassium dihydrogen orthophosphate in 800 ml water and diluting to 1000 ml with water. The pH was adjusted to 3.0 with *ortho*-phosphoric acid.

Instrumentation

Chromatography was performed on HPLC equipment consisting of LC-10AS pumps, SPD-10A UV-Visible detector, and an injector equipped with a 20-µl sample loop (Rheodyne, USA). Analytical separation was on column packed with 3µm Supelcosil LC-18 stationary phase (Supelco, USA). The dimension of the separation column was 150 x 4.6 mm i.d with 3 µm particle size. Data and chromatograms were collected using C-7RA chromatopac software system (Shimadzu, Japan). The pH of the solution was adjusted by using Digital pH Meter, Model DI 707 (Digisun electronics, India). Dissolution of compound was enhanced by sonication on Bandelin sonerex (Bandelin, Berlin). UV spectra of bharangin for selecting the working wavelength of detection were recorded on Cintra 5 UV-Visible spectrophotometer (GBC Scientific equipments, Australia).

Chromatographic conditions

The mobile phase consisted of methanol, 0.01M KH_2PO_4 buffer (pH: 3.0 adjusted with *ortho*-phosphoric acid) (75:25, %v/v). Prior to use, the mobile phase was degassed by sonication. Between the samples the injection needle was washed with methanol. The mobile phase was pumped through the system at a flow rate of 1.0 ml/min. All experiments were carried out at ambient temperature of 23°C. The U.V wavelength was set at 388 nm. The retention times of bharangin and 2- nitro aniline were 4.6 and 2.4 min.

Calibration of standards

Calibration standards were prepared by spiking working standard solutions of bharangin in to methanol to yield concentrations of 10, 20, 30, 40 and 50 ng/ml. To the above solutions, 25 ng/ml of working standard solution of 2-nitro aniline was added and final volume made up to 5 ml. Standard curves were prepared daily and constructed by linear regression analysis of the bharangin/internal standard peak area ratio versus the respective concentration of bharangin. Another stock solutions separately prepared for quality control. All the standard curves were checked using quality control.

Search for internal standard

Several compounds, whose structure and solubility is resembles to standard, were added to standard solution of bharangin and next to the crude extracts, and the resulting mixture was subjected to analysis according to the procedure.

Method of validation

Method of validation was performed in terms of sensitivity and specificity, linearity, LOQ, LOD, precision, accuracy, robustness and system suitability.

Specificity

The specificity of the method was evaluated with regard to interference due to presence of any other compounds. Three different samples were injected and studied with respect to other compounds.

Linearity

To establish the range of linearity between compound concentration and detector response, the compound concentrations of 10, 20, 30, 40, and 50 ng/ml were injected and studied five replicates.

Table 1: Precision (C.V) and accuracy (relative error) of intra-day assay measurements of bharangin at UV detection 388 nm.

S. No	Nominal concentration (ng/ml)	Measured concentration (ng/ml) ± S.D	% C.V	%Relative error
1	6.3	6.10± 0.05	0.893	-3.18
2	50	50.10±0.52	0.998	+0.01
3	100	99.92± 3.01	3.009	-0.08
4	200	200.1 ± 3.80	0.184	+0.02

Table 2: Precision (C.V) and accuracy (relative error) of inter-day assay measurements of bharangin at UV detection 388 nm.

S. No	Nominal concentration (ng/ml)	Measured concentration (ng/ml) ± S.D	% C.V	%Relative error
1	6.3	6.01± 0.057	0.945	-4.77
2	50	50.02 ± 0.59	1.102	+0.08
3	100	98.95± 3.19	3.210	-0.15
4	200	200.01 ±3.98	1.981	+0.005

Table 3: Purity studies of bharangin from plant crude extracts

Name of the crude extract	Added amount (mg)	Observed amount (mg) ± S.D	Purity (%)
Hexane	10	0.35 ±0.41	3.5
Ethyl acetate	10	0.052 ±0.74	0.52

Limit of detection (LOD)

The LOD can be defined as the smallest level of analyte that gives a measurable response. The LOD is based on S/N ratio typically 3.0 for HPLC methods. Five replicate of analyte was measured.

Limit of quantification (LOQ)

Limit of quantification was defined as the lowest concentration at which the precision expressed by relative standard deviation (RSD) is less than 20% and accuracy expressed by relative difference in the measured and true value was also less than 20%.

Five replicates of samples analyzed and quantified.

Recovery

Percentage recoveries of bharangin from spiked blank were determined and represented as mean ± standard deviation by injecting quality control samples of five replicates.

Precision and accuracy

To assess the precision of the method, intra-day and inter-day (days 7) measurements of bharangin were completed with computation of the coefficient of variation (C.V.%) for replicate samples (n=5) using concentrations of 6.3, 50, 100 and 200 ng/ml. Both intra-day and inter-day samples were calibrated with standard curve concurrently prepared on the day of analysis. Accuracy (% bios) was calculated as percent deviation (relative error) from the nominal concentration.

System suitability

It is defined as tests to measure that the method can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. For this, parameters like plate number (N), tailing factor (k), resolution (R) and relative retention time (α), HETP, capacity factor (k^l), plates per meter and peak symmetry of samples were measured.

Robustness

The optimum HPLC conditions set for this method have been slightly modified for samples of bharngin dissolved in crude extracts as a means to evaluate the method ruggedness. The small changes include: the mobile phase ratio, the flow rate, the detection wavelength, the sonication time, the filtration system and the column.

Analysis of crude extracts

10 mg of bharangin was quantitatively transferred to a 10 ml volumetric flask and dissolved in methanol, and the final volume was made up to the mark with methanol. Then, this solution was subjected to sonication for 1 hr. After getting clear solution, the solution was filtered through 0.2µm membrane filter. Further dilutions were provided.

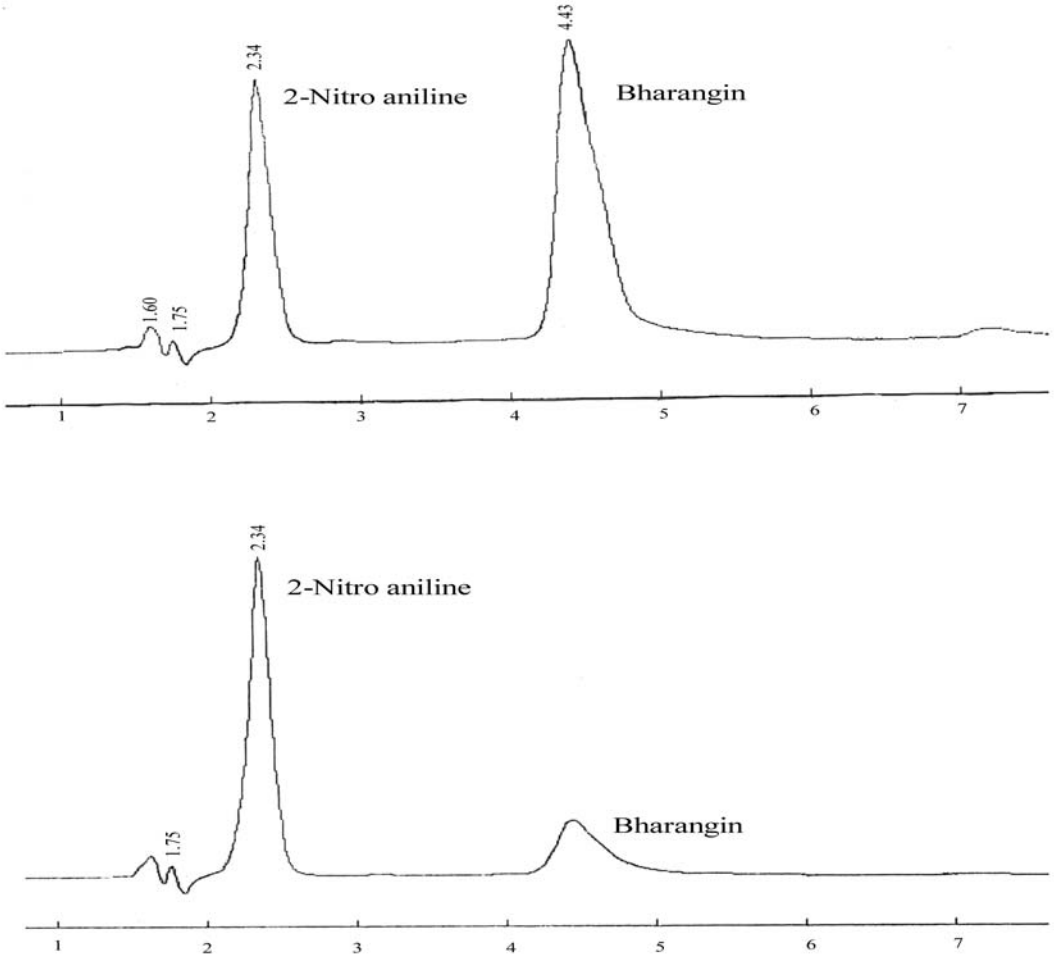

Fig. 2: HPLC chromatograms of blank spiked with 100 ng/ml of bharangin and 50 ng/ml of 2- nitro aniline (IS)

RESULTS AND DISCUSSION

Choice of stationary phase and mobile phase

For the selection of stationary phase several columns such as C-8 and C-18 were used, among which non-polar C-18 analytical chromatographic column was chosen as the stationary phase for the separation and determination of bharangin molecules.

For the mobile phase a number of eluting systems were examined. The use of methanol, water and acetonitrile in any proportion (50:50, 60:40, 70:30) resulted in prolonged retention time and tailing.

In order to reduce tailing we can try with acetic acid also. Therefore, the use of buffer with lower pH was found to be essential. For optimization of the concentration of buffer (0.001, 0.005, 0.01, 0.02), pH of buffer (3.0, 3.2, 3.5, 4.0, 5.0) and composition of mobile phase (50:50, 60:40, 70:30, 75:25, 80:20), many different mixtures consisting of methanol and 0.01M KH_2PO_4 buffer were examined at different conditions. The choice of the optimum composition based on chromatographic response factor. A composition of 75:25 (%, v/v) for methanol and 0.01M KH_2PO_4 buffer, pH 3.0, provided an efficient separation of bharangin with sufficient retention time. A flow rate of 1.0 ml/min was found to be optimum from the studied range 0.5-1.5 ml/min as comprise between an optimum retention time, baseline stability and noise.

Internal standard approach

In order to minimize the contribution of sample preparation, injection variation and column deterioration to the final results, the internal standard mode of quantification applied. For this several compounds were tested. Amongst 2-nitro aniline was considered as internal standard, because it fulfills the requirements for good internal standard; it possess similar physicochemical

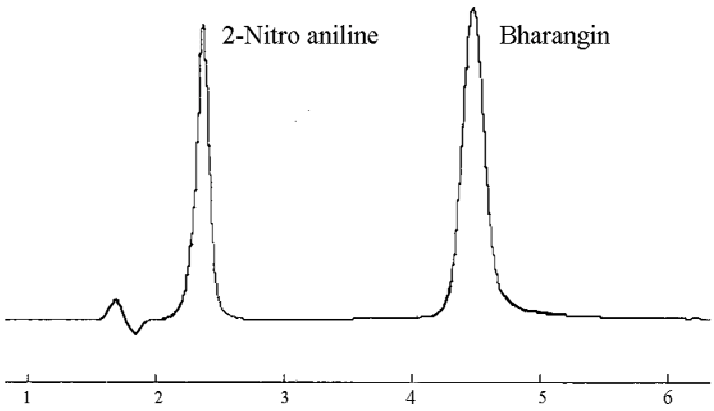

Fig. 3: HPLC chromatogram of bharangin in crude extracts (A= Hexane and B=Ethyl acetate),

properties, go through the all steps of procedures, and elute close to analyte.

METHOD OF VALIDATION

Specificity

The HPLC chromatograms recorded for the impurities in crude extracts (mixture of the compound-impurities) showed almost no peaks within a retention time range of 8 min. fig. 2 illustrates bharangin with its internal standard (a mixture containing about 100 ng/ml of bharangin and 50 ng/ml of 2-nitro aniline). The fig. 2 shows that bharangin is clearly separated from its internal standard and these are well separated from each other. Thus, the HPLC method presented in this study is selective for bharangin.

Linearity

Fig. 2 illustrates HPLC chromatograms of blank spiked with 100 ng/ml of bharangin and 50 ng/ml of internal standard 2-nitro aniline. The total eluting time was less than 10 min. The regression lines relating standard concentrations of bharangin and peak ratios were calculated using weighted regression analysis (weight + $1/(\text{concentration})^2$], the calibration curves were linear in the studied range.

The mean ± standard deviation (SD) for the slope, intercept and correlation coefficient of standard curves (n=5) were 0.0153, $5x10^{-5}$ and 0.9999.

Limit of quantification and limit of detection

It was found that below 5 ng/ml, the back calculated values failed to meet the acceptance criteria. Hence 5 ng/ml levels were five times injected. It was found that the relative standard deviation was 2.75% and accuracy, defined as the deviation between the true values expressed, as a percentage RSD was 3.9% at this concentration. So 5 ng/ml was established, as the lowest limit of quantification and 3 ng/ml was LOD.

Recovery

Percent recoveries of compounds from spiked blank were found and are represented as mean ± standard deviation. For bharangin, the recovery was 99.62 % and for 2- nitro aniline, at a concentration of 25 ng/ml, the recovery was 99.92.

Precision and accuracy

Intra-assay precision of the method was shown in table.1. This was estimated by assaying the quality control samples (6.3, 50, 100 and 200 ng/ml) five times in the same analytical run. The % C.V was less than 3.21 and the % relative error less than –3.0.

Inter-assay precision of the method was shown in table.2. This was estimated by assaying the quality control samples (6.3, 50, 100 and 200 ng/ml) for replicate samples (n=5).

System suitability

For system suitability, five replicates of standard samples were injected and studied the parameters like plate number (N), tailing factor (k), resolution (R) and relative retention time (α), HETP, capacity factor (k^1), plates per meter and peak symmetry of samples.

Robustness

Method shows that the percent recoveries of bharangin was good under most conditions and did not show a significant change when the critical parameters were modified. The tailing factor for bharangin was always less than 1.0 and the components were well separated under all the changes carried out. Considering the modifications

in the system suitability parameters and the specificity of the method, as well as carrying the experiment at room temperature may conclude that the method conditions are robust.

Application of the method to crude plant extracts

The validated method was applied to the assay of bharangin in crude extracts of *Pygmacopremna herbaceae*. The purity of bharangin in various extracts was found to be 3.5% in hexane extract and 0.52% in ethyl acetate extract at R.S.D. of 2.81. A representative chromatogram of bharangin in extracts was illustrated in figure 3 and precision and accuracy assay measurements of bharangin in crude extracts were shown in table 3.

Under the presently prescribed conditions, the recoveries of bharangin and 2-nitroaniline were found to be 99.62% and 99.42% respectively. A very low concentration of buffer (0.01M potassium dihydrogen orthophosphate, pH adjusted to 3.0 with *ortho*-phosphoric acid) was used to reduce the tailing of bharangin.

Till now there is no single analytical method was developed for the determination of bharangin. So this method is very useful for determination of bharangin. In the present method, a Supelcosil LC-18 has been used and the buffer pH in the mobile is 3.0, which is within the limits (pH 2-8) specified by the manufacturers (Supelco, USA).

The observation of C.V. less than 3.5 for both inter-day and intra-day measurements also indicates high degree of precision.

In the present method, we have established a linearity range of 10-50 ng/ml; this linearity range covers all the strengths of bharangin. Hence this method can be applied for quantifying the low levels of bharangin in extracts and other pharmacokinetic studies if necessary.

CONCLUSIONS

Development of new analytical methods for the determination of an ayurvedic compound in crude plant extract is more important in pharmacokinetic, toxicological and biological studies.

This paper describes a new, sensitive, specific and validated reverse-phase high-performance liquid chromatographic method for the determination of bharangin has been developed. It has been shown to be accurate precise and sensitive. There was no evidence instability of bharangin. The method can be used for the determination of bharangin in various crude extracts and for the any biological and pharmacokinetic studies of bharangin. The method described herein was simple and validated assay procedures that can readily be used in any laboratory for the quantitative determination of bharangin in crude extracts.

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