Premnine HCl Estimation in Selected Formulations of ‘Dashmul’ and in Chloroform Extract of Premna integrifolia L. by a Selective, Validated and Developed HPTLC Fingerprint Method

Attarde DL*, Pal SC† and Bhambar RS‡

1Department of Pharmacognosy, Mahatma Gandhi Vidyamandir’s Pharmacy College, Panchavati, Nashik, Maharashtra, India
2Department of Pharmacognosy, RG Sapatkal College of pharmacy, Kalyani Hills, Trimbakeshwar, Nashik, Maharashtra, India

Abstract

HPTLC technique developed as validated method for estimation of Premnine HCl in P. integrifolia chloroform extract (PI-AK) and in selected marketed formulations ‘Dashmul arishtha’, ‘Dashmul kadha’ in 3 x 3 batches as per ICH guidelines. Premnine HCl (Pr-S) was isolated as per literature from P. integrifolia and focused first time as standard bioactive marker for quantification. Developed mobile phase Toluene: Acetone: Diethylamine (7:2:1) for Pr-S gave Rf 0.59 at λ max 283 nm in densitometric scan, focused for specificity, fingerprint and estimation study in PI-AK and selected formulation successively. Linearity assessed in range of 8 to 16 µg /band with regression coefficient of 0.9983, LOD 0.742 µg/Band, LOQ 2.225, also robust for Pr-S. Accuracy for % recovery performed on extract as well on formulation, further subjected for precision study with application one way ANOVA for finding F value, found within limit therefore no significance of variance. A rapid and selective HPTLC method shows good linearity, recovery and high precision, useful method for analysis of Pr-S and as quality control parameter for raw material as well formulation as per foremost need of WHO, FDA and Pharmacopoeia.

Keywords: Premna integrifolia; HPTLC; Dashmul arishtha; Dashmul kadha; Premnine HCl

Introduction

Premna integrifolia Linn. (syn. P. corymbosa auct., P. obtusifolia R., P. spinosa Roxb., P. serratifolia) is thorny deciduous shrub belonging to Verbenaceae, common along India, Andaman costs, tropical and subtropical Asia, Africa Africa and the specific Islands, known as Agnimanth, Arni, Girikarnika in Sanskrit, Malbau in Malay language useful for inflammation, brochitis, dyspepsia, piles, constipation, fever and root forms an ingredients of ‘Dashmul’ which is renowned traditional Ayurvedic remedy tonic for liver, uterus, kidney, also detoxifies and strengthens body. Decoction of root useful for convulsion, rheumatism, and neuralgia [1-3].

Literature survey shows three novel diterpenoids isolated from root bark and simultaneous quantification of it by HPTLC method [4,5] a verbascoside iridoid glycosides from leaves [6], two alkaloid premnine and ganianaraine isolated from root [7], volatile constituents isolated from flower buds [8], flavonoids Luteolin 7-O-methyl ether and Apigenin 5,7-O-dimethyl ether isolated from leaves [9], identification of volatile constituents of leaves and roots analysed by GC-MS [10], stem bark and wood alcoholic extracts shows cardiotonic effects and β adrenergic effects [11] and also antioxidant effects [12], antimicrobial activity of root extracts [13], wood extract for antiarthritic activity [14], methanol extract of root evaluated for immunomodulatory effects [15].

Premnine alkaloid shows raise in blood pressure by contracting blood vessels but decreases force of contraction of heart, and dilates pupil [7].

In this original research Premnine alkaloid isolated as per literature and its salt as Premnine HCl first time here focused as unique characteristic bio-active marker for qualitative and quantitative analysis in chloroform extract of P. integrifolia and in its marketed formulation as ‘Dashmul arishtha’ and ‘Dashmul kadha’ with view to develop standardise parameter that will serve as crucial quality control parameter for drug.

Experiment

Equipment

HPTLC Instrument (CAMAG, Switzerland): Linomet Syringe V, TLC scanner V, Digistore- Reprostar 3, Win CATS version 1.4.2. Software, Twin trough Chamber, Pre-coated silica gel 60 F , aluminium plates (0.2 mm thick, Merck, Germany).

Chemicals and solvents

Premnine HCl- isolated as per literature [7].

Analytical grade solvents: Ethanol, Conc. HCl, Pet. Ether (60-80°C), chloroform, Toluene, Acetone, Diethyl amine (Merck), Dragentoff’s reagent (Freshly prepared).

Plant material

P. integrifolia shrubs are located in Trimbakeshwar forest area of Nashik District, herbarium was prepared of flowering branch. It was deposited to Botanical Survey of India, Pune for identification purpose. Certificates were issued as i.e., Ref.: BSI /WRC/Tech./2012/DVR-1 dt. 02/1/2012 for Premna integrifolia L.(Verbenaceae). Stem branches were sliced into small pieces, dried under shade for about 20 days, powdered, passed through sieve 25/30 no.-600 µ. Dried powder packed in air tight container and stored to cool, dry condition for further use.

*Corresponding author: Attarde DL, Department of Pharmacognosy, Mahatma Gandhi Vidyamandir’s Pharmacy College, Panchavati, Nashik, Maharashtra, India, Tel: 9730075256; E-mail: dakhsha511@rediffmail.com

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Isolation of premnine HCl alkaloid

As per literature [7] stem powder (2 kg) charged in soxhlet assembly in several batches and exhaustively extracted with ethanol. Alcoholic extract concentrated and poured syrupy mass with mechanical stirring into 1% warm HCl and kept for 2 hr on magnetic stirrer. Filtered precipitated black resinous mass. And extracted with chloroform, each chloroform extract fraction washed with 1% HCl and washing returned to acidic alcoholic fraction. pH of acidic alcoholic fraction now adjusted to 9 with dilute ammonia solution and exhaustively extracted with chloroform. Chloroform extract concentrated, dried and yield noted. It was dissolved in few ml of chloroform and gasoline added dropwise to precipitate out blackish resinous mass and filtered. This is repeated several times for purification. This yellowish semi purified alkaloid fraction obtained was divided into two fraction. One fraction subjected for isolation as follow, dissolve into dry ether and few drops of absolute alcohol saturated with HCl gas were added to it drop by drop and stirred continuously. Yellowish precipitate obtained was filtered, dissolved in alcohol and recrystallized this isolated Premnine HCl melting point taken, recorded as 212-214°C, matched with reference value of 211-213°C, as per literature no [7]. It was further confirmed as alkaloid with Dragendorff’s test. It was designated as Pr-s.

Preparation of purified chloroform extract of P. integrifolia

From above procedure second portion of purified chloroform extract was dissolved in few ml of 1% alcoholic HCl, allow to stand for 2 hr and then dried to solid mass, yield noted and designated as PI-AK.

Selected Marketed Formulation and Treatment

‘Dashmul arishtha’: Manufacture I: selected 3 batches, Manufacture II: selected 3 batches,

‘Dashmul kadha’: Manufacture III: selected 3 batches, batch no., volume, label claim noted and designated with codes. Coded batches were concentrated using rotary evaporator, made hydro alcoholic. pH adjusted to 9 with dilute ammonia solution and extracted with chloroform in several batches. All chloroform extracts were concentrated and dried separately and further dissolved in 1% alcoholic HCl batch wise and allow to stand for 2 hr and then dried to solid mass, yield noted and designated as for Manufacturer I, II, III batches as: DA1, DA2, DA3, SA4, SA5, SA6, BA7, BA8 and BA9 respectively.

Sample preparation for HPTLC

Standard Pr-S solution: 2 mg of standard Pr-S was dissolved in 1 ml of alcohol in volumetric flask (2000 ppm), sonicated for 15 min.

PI-AK solution: 20 mg of purified chloroform extract PI-AK was dissolved in 1 ml of methanol (20000 ppm), sonicated for 15 minutes, filtered through whatman filter paper No.1.1

Marketed formulations solution: Coded batches as mentioned and treated above-prepared as 20 mg/ml each in methanol separately and designated as DA1, DA2, DA3, SA4, SA5, SA6, BA7, BA8 and BA9.

HPTLC condition

Stationary phase: Pre-coated Silica Gel G 60 F_254 HPTLC Plates

Mobile Phase: Toluene: Acetone: Diethylamine (7:2:1)

Derivatizing Reagent (Visualising agent): Dragendorff’s Spray Reagent

Wavelength: 283 nm (λ max for standard Premnine HCl),

Band application point -X-8 mm, Y-15 mm, Sample Band length-8 mm,

Chamber saturation Time: 10 min, Solvent Run -80 mm, Lamp: Deuterium,

Scanning slit width 6 × 0.45 mm, Scanning speed 20 mm/s, Area Temperature-22+/−2°C Applicator Syringe-100 µl, Sample Application speed 0.2 µl/s.

Spray gas- Nitrogen Inert gas, Scanner- TLC scanner 5 (version 1.14.26),

Photo documentation-Digistore-Reprostar 3.

HPTLC method

The HPTLC analysis was performed using above chromatographic condition, 5, 10, 15 µl of Pr-S applied on pre-coated TLC Silica gel 60 F_254 plates as band length of 8 mm using Linomat 5 syringe, spots bands were air dried, mobile phase Toluene: Acetone: Diethylamine (7:2:1) poured to CAMAG twin trough chamber and allowed to saturate for 10 min. than developed till 80 mm, dried with dryer, scanned over TLC scanner 5 (1.14.26) with absorption/remission mode at scan speed 20 mm/s at 254 nm initially. Spectral scan done in between 200-400 nm with 100 nm/s speed, spectra of Pr-S wavelength noted, again plates were rescanned at 283 nm in detection scanner mode. Rf (Retention factor), AUC (Area Under Curve) for standard Pr-S noted, used the data for further detection.

Specificity and fingerprinting of Pr-S as standard in PI-AK and in DA1, SA4, BA7 formulations

For specificity and fingerprinting study sample applied as PI-AK (track 1 to 3: 3 µl each), Pr-S (track 4,5,10 µl), DA1(track 6,7,5, 7 µl), SA4 (track 8,9,5, 7 µl), BA7 (track 10, 11,5, 7 µl), respectively sequentially and plate developed as above and scanned in detection at 283 nm λ max for Standard Pr-S. Rf obtained for it is noted, identified and marked in all samples track. The identified and selected bands subjected for spectral scan between 200-400 nm and over laid spectra for confirmation of specificity for standard. The purity of the bands was confirmed at start, middle and end position of chromatogram. Then finally plate derivatized by dipping into freshly prepared Dragendorff’s spray reagent, dried, colour visualized as identity for alkaloids at Pr-S RF and photo documented. Specificity and fingerprinting chromatogram are as shown in Figure 1a and 1b.

Figure 1a: Overlay spectra of Premnine HCl with PI-AK, DA1, SA4, and BA7.
Figure 2: Linearity Curve of Premnine HCl standard.

**Linearity assessment for Pr-S standard and its estimation in PI-AK and in selected marketed formulation**

For linearity samples applied as Pr-S: 4, 4.5, 5, 6, 7, 8 µg (8 to 16 µg/band), for estimation on same plate sample applied as PI-AK- 2 µl, DA1, DA2, DA3- each 8 µl, SA4, SA5, SA6- each 6 µl, BA7, BA8 and BA9 each 7 µl over track 1 to 16 respectively with band length 8 mm. As per above condition chromatogram developed till 80 mm in above mobile phase, scanned at 283 nm and recorded at Rf 0.59 for Pr-S, repeated thrice and average AUC noted. Plates were derivatized with Dragendorff’s reagent for confirmation of Pr-S in samples. Standard linearity curve prepared by plotting Area Under Curve vs conc. in ng as shown Figure 2 and using calibration curve of concentration of Pr-S in PI-AK and in marketed formulations DA1, DA2, DA3, SA4, SA5, SA6, BA7, BA8 and BA9 calculated as shown in Tables 1 and 2, Figures 3a-3j.

**Validation of HPTLC method**

As per International Conference on Harmonization Guidelines-Q2 (R1), 2005 the above estimation method was validated for various parameters as follows.

Linearity for Pr-S standard was assessed in range of 8-16 µg/band. The calibration curve established by plotting Peak area (AUC) vs. Conc. in µg/band, regression equation with slope, intercept and coefficient of correlation was calculated.

Using following formula, the Limit of detection (LOD) and limit of quantification (LOQ) were calculated as:

\[ \text{LOD}=3.3 \times \sigma/S \]

\[ \text{LOQ}=10 \times \sigma/S \]

Where \( \sigma \)=standard deviation of the response; S=slope of the regression line.

**Repeatability precision**

The repeatability of the method was assessed by three concentration 8, 12, 16 µg/band in triplicate for Standard Pr-S. The percentage relative standard deviation was calculated and expressed as Table 3.

**Accuracy (% recovery)**

The accuracy of the method was calculated by recovery experiments over known concentration on Extract–PI-AK and formulation SA4 at 80%, 100% and 120% spike of Standard Pr-S in triplicate for each experiment and analyzing it with %RSD. As per Table 4a, 3D graph of accuracy study shown in Figures 4a and 4b.

**Intermediate precision**

Precision study for Standard Pr-S done for three concentration as 8,
Concentration\(^2\) (µg/band) & Average Concentration\(^2\) (n=3) Intraday (µg /band) & Intra day %RSD & Average Concentration\(^2\) (n=3) Interday (µg /band) & Inter day %RSD \\ 
8 & 7.78 & 1.9842 & 7.79 & 0.6465 \\ 
12 & 12.22 & 0.5664 & 12.02 & 1.4634 \\ 
16 & 15.79 & 1.6727 & 15.88 & 0.5044 \\ 

T- Therotical, a- Obtained, % RSD- Relative Standard Deviation.

Table 3: Intermediate Precision Study for Standard Premnine HCl.

(a) PI-AK- Premna integrifolia chloroform extract, (b) SA4-Manufacture-2 -coded Batch, (c) Pr-S- Premnine HCl Standard

Table 4a: Accuracy (%Recovery) for PI-AK extract and SA4 extract with spike of Standard Premnine HCl (Pr-S) at 80%, 100% and 120% level.
12, 16 µg/band, done in triplicate for intraday (Three times a day with 2 hr' interval) and inter day precision

(Three day consecutively).

Precision study also checked for extract and formulation as for PI-AK and SA4 (selected for recovery and precision study) done separately in triplicate for 80%, 100% and 120% triplicate for intraday and inter day precision with 3 × 3 model for each experiment and analyzing it with % RSD.

Further result obtained were subjected for one way analysis of variance and with-day mean square compared to between -day mean square by F test (Table 4b).

Robustness

Mid concentration of 12 µg/band in triplicate of standard Pr-S subjected for robustness study using variability like wavelength 283-5 nm, slit width change as 6 × 0.3 mm, scan speed change as 40 mm/s, mobile phase change of saturation time as 12 min., and mobile phase change in composition as Toluene: Acetone: Diethylamine (7:2:1). Table 4 summarise the validation parameter.

Resolution of Pr-S peak in PI-AK extract and formulations while estimating are precise, checked for peak purity of the bands at start, middle and end position of chromatogram as shown in Figures 3a-3j, though co-eluted compound as peak no.1 are observed in resolution middle and end position of chromatogram as shown in Figures 3a-3j, may be having structural similarity with standard Premnine HCl, but it is not alkaloid as do not give Dragendorff’s reagent gave orange red spot for Pr-S all sample at Rf 0.59 that confirms presences and helped for fingerprinting of Pr-S bioactive alkaloid in PI-AK and in selected formulations as shown in Figures 1a and1b. The calibration curve of Pr-S was found to be linear in range of 8-16 µg/band with good regression coefficient 0.9983. Table 1 summarize the validation parameter.

Estimation analysis of Pr-S as bio active marker for Extract PI-AK and formulation as shown in Table 2: Variability observed between manufactures and even within batches. Pr-S was selected as standard marker due to its bioactive properties and also specific content of Premna species that it can selectively justify presence of raw material in formulation.

Resolution of Pr-S peak in PI-AK extract and formulations while estimating are precise, checked for peak purity of the bands at start, middle and end position of chromatogram as shown in Figures 3a-3j, though co-eluted compound as peak no.1 are observed in resolution chromatogram of Figures 3f-3k, may be having structural similarity with standard Premnine HCl, but it is not alkaloid as do not give Dragendorff’s test. And the peak area scanned for these peak and peak corresponds to standard marked as separate peak No. and integrated with red mark line as shown in Figures 3f-3k, noted as different with peak area by Camang -TLC scanner 5 software, therefore that value corresponds to Premnine only consider for calculation. Precision study with repeatability performed over low, mid high concentration over Premnine HCl expressed as % RSD inter and intraday as per Table 3. Accuracy study with % recovery at three concentration level performed in PI-AK and in one of selected formulation SA4 shows result as per Table 4a.

Accuracy with % recovery at 80, 100,120% level with spike of Pr-S subjected for intraday and inter day, results at each level subjected

Figure 4 a and b: Graph 3D resolution chromatogram for PI-AK and SA4 respectively for Accuracy (%Recovery) with spike of Standard Premnine HCl at 80%,100% and 120% level. (One Way ANOVA).

Table 4b: Precision study for PI-AK and SA4 with spike of Standard Premnine HCl at 80%,100% and 120% level. (One Way ANOVA).
to one way analysis of variance and the F value for each level were determined as per Table 4b.

F value as ratio of BMC/WMC, compared with tabulated F value which is 5.14 and all calculated values are below it [16,17], therefore there was no significant difference between intra and inter day variability, suggesting good intermediate precision of the method.

Robustness study with change in wavelength by 5 nm, slit width, scan speed for mid concentration, for Pr-S shows %RSD value as 2.003, 1.4321, 1.756 respectively. Changes in mobile phase saturation time for 10 to 12 min and composition change as 7.2: 1.8:1, 6.8:2.2:1 do not show change in Rf from 0.59 and average ng values found as 12.29, 12.34, 12.24 and % RSD 1.74,1.24,1.76 respectively. Therefore, shows no significant changes in values so method is robust.

Conclusion

With present research study of development, validation and estimation conclusion is drawn as, HPTLC method is simple, precise, rapid, robust and selective for estimation of Pr-S alkaloid in Dashmul-P. integrifolia and its marketed formulations.

Pr-S selected as standard marker due to its bioactive properties and that has proven fingerprinting and specificity parameter as unique content in Premna species that it can etch presence of raw material in formulation.

Although variance is observed in % content of Pr-S alkaloid between manufactures and even in batches but method is precise and accurate even on extract and formulation through validation study therefore can be recommended as method for qualitative and quantitative analysis for quality control parameter for ‘dashmul’ P. integrifolia and its formulation.

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