HPTLC Method for the Quantification of Plumbagin in Three Plumbago Species

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Abstract: Plumbagin is a naturally occurring naphthoquinone, found in abundance in the genus Plumbago. A simple, sensitive, selective, precise and robust HPTLC quantitative method of analysis of plumbagin has been developed and validated. The quantity of plumbagin in the crude extracts, obtained from the three common species of Plumbago namely, Plumbago zeylanica, Plumbago rosea and Plumbago capensis has been determined. Total Plumbagin content of P. rosea, P. capensis and P. zeylanica was found to be 0.569, 0.429 and 0.247 mg%, respectively. Densitometric analysis of Plumbagin was carried out in the absorbance mode at 265 nm. This method gave compact spots at Rf = 0.78 corresponding to plumbagin. The linear regression analysis data for the calibration plots for Plumbagin had shown good linear relationship with R² = 0.998±0.0035 in the concentration range of 20-80 ng/spot. The method was validated for robustness and precision. The limit of detection is noticed to be of 2 ng and is statistically tested for repeatability by inter day and intra day precision tests as per ICH guidelines and its updated international convention. Since this method resolves and quantifies Plumbagin effectively, it can be used to quantify the concentration of Plumbagin in the herbal preparations.

Key words: Plumbago, robustness, precision, plumbagin, HPTLC

INTRODUCTION

P. zeylanica is a semi climbing shrub that grows throughout Asia and Africa. This plant is a perennial, sub-scented shrub found wild in peninsular India and in low elevations in Taiwan (Anonymous, 1969). The whole plant and its roots have been used as folk medicine in the treatment of rheumatic pain, dysmenorrhoea, carbuncles, confusion of the extremities, ulcers and elimination of intestinal parasites (Li, 1998). P. capensis Thumb, a small scendent shrub, indigenous to S. Africa is a lesser known species in ethnopharmacognosy. P. rosea also called P. indica L. a shrubby perennial found growing throughout India often as a cultivated plant or as an ornamental plant. It is reported to be wild or indigenous in Sikkim and Khali hills (Chiu and Chang, 2003). The root bark of P. rosea are important indigenous medicine but less commonly used than those of P. zeylanica. Plumbagin is present in families of Plumbaginaceae and Drosieraceae (Botanical Dermatology Database, 1991a,b). Pure plumbagin is used primarily to Botanical dermatology databases exploit its properties as a superoxide generator, an antibiotic and an anti neoplastic agent. P. zeylanica is grown as a perennial herb in the plains of Bengal and Southern India. Extracts of the root have been reported to be a powerful poison when given internally or applied to the Ostium uteri leads to abortion (Prema kumara et al., 1977). When administered to hyperlipidaemic rabbits, plumbagin reduced serum cholesterol and LDL cholesterol by 53 to 86% and 61 to 91% (Sharma and et al., 1999). Anti- helicobacter activity of P. zeylanica has also been reported (Wang and Huang, 2005). In the present

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study, a HPTLC method has been developed for the determination of Plumbagin in the hydroalcoholic extracts of three Plumbago species. The proposed method has been validated as per ICH guidelines (ICH, 1994 and 1996) and its updated international convention (ICH, 2000).

MATERIALS AND METHODS

Plant Material
Air dried roots of P. capensis, P. rosea and P. zeylanica, collected from TAMPCOL farm, Chennai, was identified by botanists at CSDMRI, Chennai and a voucher specimen was deposited at CARISM herbarium. The roots of the three species have been used for the study as Plumbagin was reported to be accumulated mostly in roots and widely used for medicinal purpose (Van der Vijver, 1972).

Chemicals and Reagents
Standard Plumbagin was procured from Hi-media, India Pvt. Ltd. All other chemicals used were of analytical grade (Merck, India) and the HPTLC plates used were from E-Merck, Germany.

Preparation of Extracts
Air dried roots (100 g) of three species was extracted with 50% methanol (Hydroalcohol) by static cold maceration process. The extraction yield (%w/w) was calculated as ratio of the weight of the concentrated extract to the weight of the dried root.

Analytical Procedure
Chromatographic Conditions
Chromatography was performed on a 10x10 cm preactivated HPTLC Silica gel 60F254 plate. Samples and standard were applied to the plate as 6 mm wide band with an automatic TLC applicator Linomat V with N2 flow (CAMAG, Switzerland). 8 mm from the bottom. Densitometric scanning was performed on CAMAG scanner III at 265 nm. The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 3x0.45 and 40 mm sec⁻¹ scanning speed was employed. The mobile phase consisted of toluene:ethyl acetate:methanol (8:1:1) and 10 mL of mobile phase was used per chromatography. Linear ascending development was carried out in 10x10 cm twin glass chamber saturated with the mobile phase.

Method Validation
Precision
System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration of 10 ng/spot of Plumbagin. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of relative standard deviation (RSD%) and standard error was found to be less than 2%. Method repeatability was obtained from RSD value by repeating the assay six times in the same day for intra-day precision. Inter-day precision was assessed by the assay of six sample sets on different days. Inter-and intra-day variation for determination of Plumbagin in three different species was carried out at three different concentration levels of 20, 40 and 80 ng/spot.

Robustness of the Method
By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like toluene: methanol (8:2, v/v) have been tried and chromatograms have been run. The plates are prewashed by methanol and activated at 60±5°C for 10 min prior to development. Time from spotting to chromatography and from chromatography to scanning is varied from 0, 15, 30 and 60 min. Robustness of the method was done at three different concentration level 20, 40 and 80 ng/spot.
Limit of Detection and Limit of Quantitation

In order to estimate the Limit of Detection (LOD) and Limit of Quantitation (LOQ), blank chloroform was spotted six times. The signal to noise ratio was determined. LOD was considered as 2:1 and LOQ as 8:1. LOD and LOQ were experimentally verified by diluting known concentrations of Plumbagin until the average responses were approximately 2 or 8 times the standard deviation of the responses for six replicate experiments.

Specificity

The specificity of the method was ascertained by analyzing standard Plumbagin and extracts. The spots for plumbagin in sample was confirmed by comparing the Rf and spectra of the spot with that of sample. The peak purity of Plumbagin was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot.

Estimation of Plumbagin in Herbal Extract

To determine the content of plumbagin in the herbal extract, 500 mg of hydroalcoholic extract is made up to 25 mL with chloroform. As napthoquinones are reported to be easily soluble in chloroform, chloroform has been used as the solvent (Zhong et al., 1984; Siddhu and San karam, 1971). The resulting solution is centrifuged at 3000 rpm for 15 min and the supernatant is analyzed for plumbagin content. Forty microliter of the filtered solution is applied on the TLC plate followed by development and scanning. The analysis is repeated for six times and the possibility of interference from other components of extract in the analysis is studied.

RESULTS AND DISCUSSION

Development of the Optimum Mobile Phase

The TLC procedure was optimized with a view to develop a stability indicating assay method. Both the standard and the sample were run in different solvent systems. The mobile phase consisting of toluene: ethyl acetate: methanol (8:1:1) gave better resolution. The spot at Rf 0.78 was identified as Plumbagin with the help of chromatogram of the standard compound. To improve the resolution of the spots, the plate was run in the same mobile phase twice.

Calibration Graph

Linearity was found over the concentration range of 20-80 ng/spot with R²±SD = 0.998±0.0035 (Table 1). Peak area and concentration were subjected to linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data has shown a good linear relationship over the concentration range of 20-80 ng/spot. The linearity of calibration graphs and adherence of the system to Beer’s law are validated by high value of correlation coefficient and the SD for intercept value is noticed to be less than 2%. No significant difference is observed in the slopes of standard curves (ANOVA; p<0.05).

VALIDATION OF THE METHOD

Precision

The repeatability of sample application and measurement of peak area have been expressed in terms of RSD% and was observed to be <2% for plumbagin. The results show that there was no

<table>
<thead>
<tr>
<th>Linearity range (ng/spot)</th>
<th>R²±SD</th>
<th>Slope±SD</th>
<th>Confidence limit of slope</th>
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<tbody>
<tr>
<td>Plumbagin 20-80</td>
<td>0.998±0.0035</td>
<td>0.28±0.007</td>
<td>0.275-0.286</td>
</tr>
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significant intra-and inter-day variation has been observed in the analysis of Plumbagin at three different concentrations level 20, 40 and 80 ng/spot. The RSD % for intra-and inter-day analysis is found to be <2% in all the cases (Table 2). The standard deviation of peak areas was calculated for each parameter and RSD% was less than 2% in all the cases. The low values indicate robustness of the method (Table 3).

**LOD and LOQ**

The LOD was found to be 2 ng/spot for Plumbagin with signal/noise ratio of 2.1 while the LOQ was seen to be 4 ng/spot for Plumbagin with signal/noise ratio of 8.1.

**Specificity**

The peak purity of Plumbagin was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., r (start, middle) = 0.999825 and r (middle, end) = 0.996673. Good correlation was (R= 0.9995) also obtained between standard and sample overlay spectra of plumbagin (Fig. 1).

**Estimation of Plumbagin in Hydroalcoholic Extract**

The spot at RF = 0.78 corresponding to plumbagin was observed in the chromatogram of the extracts along with other components. There was no interference from other components present in the chromatogram (Fig. 2-4). Total Plumbagin content was found to be 0.569% w/w and 0.429% w/w in *P. rosea* and *P. capensis* while *P. zeylanica* had 0.247% w/w of Plumbagin (Fig. 2-4).

<table>
<thead>
<tr>
<th>Table 2: Intra and inter day precision of HPTLC method (n = 6)</th>
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<tbody>
<tr>
<td>Amount (ng/spot)</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>20</td>
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<tr>
<td>40</td>
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<td>80</td>
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<th>Table 3: Robustness testing</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>Mobile phase composition</td>
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<tr>
<td>Time from spotting to chromatography</td>
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<td>Time from chromatography to scanning</td>
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Fig. 1: Spectra of Plumbagin in *P. rosea* (Violet), *P. capensis* (Brown) and *P. zeylanica*
Fig. 2: HPTLC chromatogram of *Plumbago capensis*

Fig. 3: HPTLC chromatogram of *P. rosea*

Fig. 4: HPTLC chromatogram of *P. zeylanica*
CONCLUSIONS

The developed HPTLC method was precise, specific, accurate and robust for determination of Plumbagin in the three species of Plumbago namely P. rosea, P. capensis and P. zeylanica. A comparative study of this bio marker compound has shown that it is present in higher amount in the species P. rosea while the concentration was low in the hydroalcoholic extract of P. zeylanica. The activity of a plant extract is always influenced by the quantity of active principle present in the extract. Since Plumbagin is used in the variety of different chronic diseases, it is very essential to develop a standardization method from which one can optimize its quantity in the herbal formulations. Moreover, HPTLC being an easier and cheaper compared to its ally technique HPLC, was taken for this study to develop a chromatography method for Plumbagin quantification. In polyherbal formulations, hydroalcoholic extracts have been widely used. So, a method has been developed to ascertain the quantity of Plumbagin in the hydroalcoholic extract. So, it is necessary to choose or develop a right extraction procedure so that active ingredients are present in right quantity.

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