



## Quantitative Analysis of Plumbagin in Root Extract of *P. zeylanica* linn Using HPLC

Ankita P. Jain\*, Purnima D Hamrapurkar, Sneha M Labana, Dnyanoba. M. Madrewar, Abhijeet A. Sonandkar

Department of Pharmaceutical Analysis, K. M. Kundnani College of Pharmacy, Cuffe Parade, Colaba, Mumbai, Maharashtra, India.

\*Corresponding author's E-mail: [anki.jain105@gmail.com](mailto:anki.jain105@gmail.com)

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### ABSTRACT

The Plumbagin selected for the research work as it showing antitumor activity and the plant *Plumbago zeylanica* is widely used medicinal plant in many countries for. A rapid, specific, sensitive, accurate and precise method has been developed for quantitative analysis of Plumbagin in Root extract of *P. zeylanica* collected from two geographical sources and its application on marketed formulation. Chromatographic separation of Plumbagin from its other plant constituent carried out on Thermo Hypersil BDS C18 column (250×4.6 mm i.d., 5µ particle size) using 5mM Ammonium acetate (pH 3.8): Acetonitrile [40:60 %v/v]. Flow rate was 1 ml/min and detection carried out at 268 nm (PDA detector). The method was linear in the range of 0.15-1.2µg/ml. The correlation coefficient was 0.9991. The limit of detection and limit of quantification was 0.05µg/ml and 0.15µg/ml respectively. Symmetry factor < 2 and NTP > 3000 showing system suitability of method. Refrigerated & bench top stability studies carried out as per ICH guidelines. Peak of Plumbagin was fully resolved which infers the specificity of method.

**Keywords:** Application, Plumbagin, Quantification, Stability studies, Validation.

### INTRODUCTION

Present study is based on semi-climbing shrub *Plumbago zeylanica* Linn belonging to family Plumbaginaceae is commonly known as 'Chitraka', which is a widely accepted ethnomedicine around the world including India, Africa, Pakistan, Bangladesh, Sri Lanka, and Australia.<sup>1</sup> *P. zeylanica* is very popular throughout India and Asia as a remedy for skin diseases, infections and intestinal worms. The plant has been found significant in different clinical condition, especially inflammation, leprosy, scabies, ringworm, dermatitis, ulcers, hemorrhoids, and hookworm.<sup>2-4</sup> Owing to its established therapeutic properties, the roots of *Plumbago zeylanica* have been used for research work.

### Active constituent – Plumbagin

Plumbagin, 5-hydroxy-2-methyl-1, 4-naphthoquinone, is an organic compound with the chemical formula  $C_{11}H_8O_3$ . Plumbagin, a potential novel agent in the control of Prostate cancer<sup>5</sup>, Squamous Cell Carcinomas (SCC)<sup>6</sup> and have antibacterial, antioxidant<sup>8</sup> and wound healing activity.<sup>9</sup> Structure of Plumbagin is showing in Figure 1.

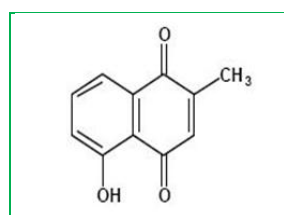


Figure 1: Plumbagin

Literature survey reveals that the TLC<sup>13</sup>, HPTLC<sup>14, 16</sup> and HPLC<sup>15,16</sup> methods are reported for the estimation of plumbagin. But these methods suffer from drawbacks

such as tediousness<sup>13, 14</sup>, expensive<sup>13</sup>, less sensitivity<sup>14-16</sup>, and poor resolution.<sup>16</sup>

Hence an attempt has been made to study the analytical aspects of *Plumbago zeylanica* (root) using 'Plumbagin' as characterizing compound for quantitative analysis of Plumbagin in plant extract and its application on marketed formulation.

### MATERIALS AND METHODS

#### Working Standard

The working standard of Plumbagin was purchased from Acquaint technologies & service (Hyderabad, India).

#### Plant material and formulation

The Chitraka root of *P. zeylanica* was procured from the Local Market, Mumbai (MH) and Damoh (MP). It was identified and authenticated by Agharkar Research Institute, Pune and voucher number allotted for Mumbai variety: 'R-166' and for Damoh variety: 'R-169'.

Commercially available Chitraka tablets were procured from ayurvedic medical store.

#### Equipments

The HPLC system employed for method development and validation was Jasco PU 2089 Plus Quaternary Gradient HPLC pump and Jasco MD-2018 Plus Photo Diode Array Detector with ChromNav software as data integrator. Thermo BDS Hypersil C<sub>18</sub> Column (250×4.6 mm, particle size 5 µ) was used.

#### Chemicals and Solvents

Ammonium acetate, Acetic Acid was of analytical grade and supplied by Qualigens Fine Chemicals (Mumbai, India). Acetonitrile and methanol used of HPLC grade

procured from Merck (Darmstadt, Germany). Milli-Q system (Millipore) used to make HPLC grade water.

## Experiment work

### Preparation of standards

10 mg of working standard Plumbagin was accurately weighed and dissolved in 10mL methanol to get a stock solution of 1mg/mL. Further dilutions were made in Methanol to get standard of (0.05, 0.15, 0.3, 0.6, 0.75, 0.9, and 1.2) µg/mL

### Preparation of sample

The roots of *P. zeylanica* were powdered and passed through sieve of 40 mesh size. Powder was extracted with methanol by cold maceration for 24hrs. The extract obtained was filtered and concentrated to get dried extract by evaporating the solvent. 10mg of the dried extract was dissolved in 10mL methanol to prepare 1 mg/mL solution, suitably diluted and used for further studies.

The marketed formulation of *P. zeylanica* root powder was extracted with methanol by cold maceration for 24hrs. The extract obtained was filtered and concentrated to get dried extract by evaporating the solvent. 10mg of the dried extract was dissolved in 10mL methanol to prepare 1mg/mL solution, suitably diluted and used for further studies.

### RP-HPLC Conditions

The mobile phase used was 40: 60 (% v/v) mixture of 5mM ammonium acetate buffer (pH-3.8), adjusted with glacial acetic acid): Acetonitrile. Flow rate was adjusted to 1 ml/min. Column was equilibrated for 20 min with mobile phase before injection. The injection volume was 20µl and detection was carried out at 268nm.

### Preparation of Mobile Phase

385.5 mg of ammonium acetate was accurately weighed into 1000 ml beaker and dissolved with HPLC grade water; pH was adjusted to 3.8 with glacial acetic acid. This buffer and Acetonitrile were mixed in 40: 60 (%v/v) and filtered through 0.45 µ filter under vacuum filtration. It was degassed in ultrasonic bath for 10 min before use.

## RESULTS AND DISCUSSION

### Optimization of Chromatographic conditions

The main aim for the development of chromatographic method was to get the reliable method for the quantification and separation of Plumbagin in the root extract of *P. zeylanica* from other chemical constituents present in the extract. For this purpose the efficiency of two different reverse – phase column C18 and C8 was evaluated but C8 column being less hydrophobic as compared to C18, retention of drug on C8 column was prolong. Hence C18 column was selected. Best separation was achieved on this column and therefore it was used further.

Phosphate buffer having buffering capacity at required pH range i.e. pH 1.1 – 3.1 was tried. But buffer concentration used was high but reproducibility was not good. Then ammonium acetate having a pH range 3.8 to 5.8 was tried showing good reproducibility with lowest concentration of buffer (5mM) gave satisfactory results. Hence mobile phase composition consisting of 5mM Ammonium acetate buffer (pH-3.8): Acetonitrile in ratio of 40:60%v/v was optimized. pH adjusted with glacial acetic acid. Flow rate set at 1 ml/min. This mobile phase composition resulted in symmetric peak with acceptable tailing factor ( $\leq 2$ ) and theoretical plates more than 3000.

## Method Validation

### System Suitability

The working standard solution was prepared and injected 6 times into the HPLC system and system suitability parameters were evaluated. Tailing factor, theoretical plates, RSD of retention time and peak area is given in Table 1. All these parameters were found to be well within the limits.

### Limit of Detection and Limit of Quantification

LOD and LOQ were determined by injecting progressively low concentration of standard solutions found to be 0.05µg/ml and 0.15µg/ml respectively. The LOD and LOQ values reveal that the developed method shows very good sensitivity.

### Linearity and range

The Linearity of peak areas versus different concentration was evaluated for Plumbagin ranging from 0.15-1.2µg/ml. Each set of linearity solutions were injected with optimized chromatographic conditions for five times. The calibration plot was obtained by plotting mean peak area against standard concentration. The equation of the calibration plot was found as  $y = 73298x - 504.76$ . The plot was linear in concentration range of interest with correlation coefficient 0.9991. Linearity data and curve are given in Table 2 and Figure 2 respectively.

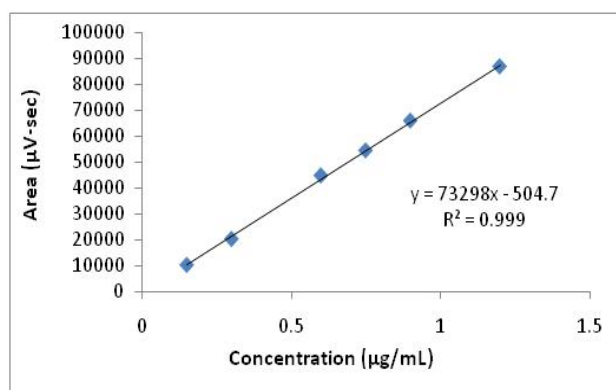


Figure 2: Calibration curve for plumbagin

### Precision and Accuracy

Precision and accuracy studies were carried out at three different concentrations namely 0.6, 0.75, 0.9µg/ml for intraday and interday studies. The results given in Table-3

stands for a quite good trueness of the proposed method particularly considering interday and intraday analysis.

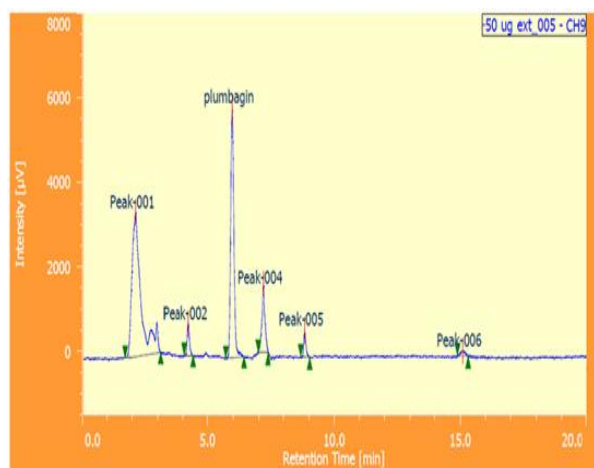
The % RSD was found to be within the specified limits (not more than 2) for intra and inter day precision.

**Table 1:** System Suitability Results

Sr. No.	Area ( $\mu\text{V}\cdot\text{sec}$ )	Retention Time (mins)	NTP	Tailing Factor
1	86458	6.105	15328	1.19
2	87514	6.031	15627	1.17
3	86272	6.042	15531	1.18
4	86771	6.012	15384	1.17
5	87154	6.001	15498	1.18
6	86315	6.071	15587	1.18
<b>MEAN</b>	<b>86747.3</b>	<b>6.047</b>	<b>15525.4</b>	<b>1.18</b>
<b>SD</b>	<b>500.06</b>	<b>0.039</b>	<b>93.4</b>	<b>0.007</b>
<b>%CV</b>	<b>0.58</b>	<b>0.65</b>	<b>0.60</b>	<b>0.64</b>

**Table 2:** Linear Regression data for Calibration Curve

Concentration ( $\mu\text{g}/\text{mL}$ )	MEAN Peak Area	$\pm$ S.D.	% C.V
0.15	10461.4	105.64	1.01
0.3	20463	343.29	1.68
0.6	44873.4	422.11	0.94
0.75	54425.4	630.78	1.15
0.9	65896.4	642.49	0.98
1.2	86715.6	667.01	0.77
<b>Slope</b>	<b>73298</b>		
<b>Y-Intercept</b>	<b>504.76</b>		
<b>Correlation Co-efficient</b>	<b>0.9991</b>		



**Figure 3:** Chromatogram for extract

**Table 3:** Precision and Accuracy data

Actual Concentration ( $\mu\text{g}/\text{ml}$ )	Precision		% Accuracy	
	Measured Concentration ( $\mu\text{g}/\text{ml}$ ), $\pm$ S.D.; % RSD		Intra-day	Inter-day
	Intra-day	Inter-day		
0.6	0.608, $\pm$ 0.008; 1.38	0.608, $\pm$ 0.007; 1.10	101.38	101.25
0.75	0.748, $\pm$ 0.007; 0.98	0.748, $\pm$ 0.007; 0.94	99.69	99.70
0.9	0.912, $\pm$ 0.009; 0.95	0.911, $\pm$ 0.008; 0.93	101.30	100.25

**Table 4:** Stability Results of Standard Plumbagin

Concentration ( $\mu\text{g}/\text{ml}$ )	Precision			%Stability		
	Measured Concentration ( $\mu\text{g}/\text{ml}$ ), $\pm$ S.D.; % RSD			Fresh solution	Refrigerated solution	Bench top solution
	Fresh solution	Refrigerated solution	Bench top solution			
0.6	0.609 $\pm$ 0.003;0.56	0.604 $\pm$ 0.007;1.2	0.598 $\pm$ 0.010;1.72	101.43	100.63	99.72
0.75	0.757 $\pm$ 0.008;1.08	0.751 $\pm$ 0.008;1.07	0.748 $\pm$ 0.010;1.42	100.96	100.26	99.68
0.9	0.906 $\pm$ 0.008;0.86	0.891 $\pm$ 0.009;0.96	0.889 $\pm$ 0.008;0.97	100.65	99.02	98.74

### Specificity

Figure 3 showing the specificity of the method for the quantitative Analysis of plumbagin in the plant extract.

The method was quite selective for Plumbagin since there was no other interfering peak around the retention time of Plumbagin. The baseline did not show any significant peak.

### Solution stability

The refrigerated (1 week) and bench top (3 days) stability of the standard solutions was tested. The stability of solutions was determined by comparing results of the freshly prepared standard solutions. The results are mentioned in Table-4, which indicate that standard solution is stable in refrigerated conditioned and also in ambient condition but results more good for the refrigerated solution. We can conclude that we can put extract in refrigerated condition for quality control analysis.

### Quantification of Plumbagin in the root extract

All sample solutions (50µg/mL) of different extract prepared in methanol was injected under the same chromatographic conditions and the chromatograms were recorded in triplicate. The amount of plumbagin present in extract was determined by compare the peak area from the standard. The results are furnished in Table-5. No interfering peaks were found in the chromatogram indicating that the determination of the plumbagin content was free from interference by other constituents present in the extract.

**Table 5:** Content of Plumbagin in two varieties of *P. zeylanica* extract Marketed formulation

Name of the sample	Quantity found in extract (100mg)	Plumbagin in sample % w/w of (100gm of powder)
<i>P. zeylanica</i> (Mumbai)	1.63 mg	0.30%
<i>P. zeylanica</i> (Damoh)	1.89 mg	0.37%
Marketed formulation	2.17mg	0.50%

### CONCLUSION

The proposed PR-HPLC method is simple, rapid, accurate, reproducible, selective and economic and can be used for routine quality control analysis of *Plumbago zeylanica* Linn. (Root) powder and quantitative determination of Plumbagin in root powder.

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