

Development and Validation of an High Performance Thin Layer Chromatography Method for the Simultaneous Estimation of Embelin and Conessine in Palash Beejadi Churna

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Soni *et al.*: High Performance Thin Layer Chromatography Method on Embelin and Conessine

Herbs are the main source of polyherbal formulations. The potency of polyherbal formulations depends on their ingredients. The evaluation parameters for polyherbal formulations are based on chemical, physical and microbiological assessment. The aim of the present study was to formulate and standardize Palash beejadi churna and develop a simple, accurate and reproducible high performance thin layer chromatography method for simultaneous estimation of phytoconstituents in the formulation. Embelin and conessine were selected as the markers for development of high performance thin layer chromatography method. The chromatographic analysis was performed on pre-coated thin layer chromatography plates with silica gel G60 F254 using toluene:ethyl acetate:formic acid:ammonia:diethyl amine (3:3:1.5:0.5:2.5 v/v/v/v/v) as solvent system. Both the markers were scan at 520 nm. The retention factors values of conessine and embelin were 0.46 and 0.87, respectively. Linearity for conessine and embelin was observed in the ranges 2000-6000 ng/spot. Limit of detection and limit of quantification values of conessine and embelin were found to be 624.5 and 768.5, 1892 and 2328 ng/spot, respectively. The average percent recovery of conessine and embelin was found to be 98 % and 101 %, respectively. The standard deviation and percent relative standard deviation for of intraday and interday precision of both the markers were found to be <2 % respectively. The high performance thin layer chromatography method was validated as per the International Council for Harmonisation guideline and found to be simple, precise, specific, sensitive and accurate that can be used for routine quality control of raw material as well as formulation containing any of these markers.

Key words: Conessine, embelin, standardization, high performance thin layer chromatography, validation

Physicians frequently recommend herbal remedies that combine a few markers. Because they work synergistically in combination to reduce toxicity, polyherbal formulations have a superior therapeutic impact than single medication treatments^[1].

It is crucial to pharmacognostically standardise polyherbal formulations so that the product's quality is assured when tested and demonstrates the proper therapeutic efficacy. The Ayurvedic Pharmacopoeia of India (API) provides the standard value and assessment protocol for the raw drug^[1,2].

Palash Beejadi Churna (PBC) of Rasoddaratantra, mentioned in the Ayurvedic Formulary of India, PBC is a frequently prescribed medicine for Krimiroga^[3]. PBC was prepared following the procedure as given in the Ayurvedic Formulary of India^[4]. The

composition of churna was described in Table 1^[5-10].

High-Performance Thin-Layer Chromatography (HPTLC) is an extremely valuable quantitative investigation method that combines the rules of chromatography with speed and automation. HPTLC plays a significant role in today's logical world. HPTLC is an effective separation tool for quantitative separation methods with high sample throughput^[1,2].

According to a review of the literature, there are several methods available for estimating embelin and

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Accepted 25 October 2024

Revised 02 August 2024

Received 13 July 2023

Indian J Pharm Sci 2024;86(5):1845-1851

conessine individually and in combination with other phytoconstituents. Moreover, there is no HPTLC method available for simultaneous estimation of embelin and conessine as shown in fig. 1.

As a consequence, the aim of the present study is to develop and validate HPTLC method for the simultaneous estimation of embelin and conessine simultaneously in PBC. Additionally, these two markers are also used in other Ayurvedic formulations, including PBC and Krimi kuthara rasa.

MATERIALS AND METHODS

PBC was prepared following the procedure as given in the Ayurvedic Formulary of India. The ingredients viz., Palash (*Butea monosperma*) seed, Dravya (*Holarrhena pubescens* (Bunch-Ham) Wall. ex G. Don) seeds, Vidanga (*Embelin ribes* Burm.f.) fruits, Nimba (*Azadirachta indica*. Juss.) seed, Chirata (*Swertia chirayita* (Roxb.) Buch. Ham) whole plant was purchased from Yucca Enterprises, Mumbai, Maharashtra, India^[3].

Experimental condition of HPTLC:

Preparation of reference standard solution (embelin and conessine): Prepared 500 µg/ml solution with 0.5

mg of each marker weighed and transferred to 10 ml volumetric flask, added approximately 1 ml chloroform to volumetric flask and the contents of the flask were then dissolved, sonicated and mixed^[7,8].

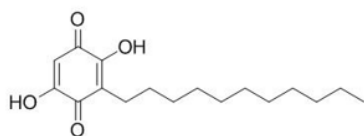
Preparation of sample solution: Accurately weighed 1 g of PBC was transferred to 100 ml of beaker, 10 ml chloroform was added to the beaker and was covered with aluminium foil and placed for 24 h. After 24 h it was filtered with 5 mm Whatman filter paper and dried. The dried extract was dissolved in chloroform, sample solution of PBC was prepared^[3].

Selection of wavelength: Since the approach was developed utilising a simultaneous estimation technique, it is crucial to detect both standards at the right concentrations using two separate wavelengths. After standards spotting and formulation, an HPTLC plate was scanned at both 254 and 366 nm.

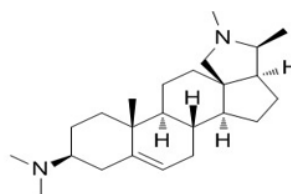
Optimised HPTLC condition: In order to analyse and separate conessine and embelin correctly in samples as well as in standards, adequate chromatographic conditions were developed by analysing numerous Thin Layer Chromatography (TLC) and HPTLC trials as shown in Table 2.

TABLE 1: COMPONENTS OF PBC

S. No.	Components	Part used in formulation	Scientific name	Major chemical constituents
1	Palash beejadi	Seed	<i>Butea monosperma</i>	Palmitic acid, oleic acid, linolenic, linalenic and stearic acid
2	Indrayava (kutaja)	Seed	<i>Holarrhena pubescens</i>	Conessine, norconessine, conimine, conessimine, isoconessimine and antidiysentericine
3	Vidanga	Fruit	<i>Embelin ribes</i>	Embelin, vilangin, quercitol, tannins and volatile oil
4	Nimbabija	Seed	<i>Azadirachta indica</i>	Azadirachtin, nimbosterol, nimbdiol, nimbin, nimolinin, nimbin and nimbidin
5	Chiryata (kiratatikta)	Plant	<i>Swertia chirayita</i> (Roxb.) Buch. Ham	1,3,7,8-tetrahydroxyxanthone, 1,3,8- trihydroxy-5-methoxyxanthone, 1,5,8- trihydroxy-3-methoxyxanthone, swertianin, sweroside, chiratanin, amarogentin and amaroswerin



(2,5-dihydroxy-3-undecyl-1,4-benzoquinone)



N, N -dimethylcon-5-enin-3β-amine

Fig. 1: Structure of embelin and conessine

TABLE 2: OPTIMISED HPTLC CONDITIONS

Parameters	Conditions
Mobile phase	Toluene:ethyl acetate:formic acid:ammonia:diethylamine (3:3:1.5:0.5:2.5)
Saturation time	15 min
Solvent	Chloroform
Wavelength	520 nm
Visualization in Ultraviolet (UV)	Iodine chamber is used for conessine at 254 and 366 nm for embelin

Method validation:

The method was developed in order to separate and quantify markers embelin and conessine in PBC. Appropriate conditions were developed in order to properly determine the markers, as it is necessary to evaluate whether the developed method is really accurate, precise, robust, etc., validation has to be performed. So, validation of the developed method was performed as per International Council for Harmonisation (ICH) guidelines Q2(R2). Specificity, precision, linearity, consistency, robustness, Limit of Detection (LOD), Limit of Quantification (LOQ) and system suitability were all accessed as part of the validation of the developed method^[11,12].

System suitability: System suitability testing was done to determine whether or not the established method is appropriate. Embelin and conessine standards were spotted six times using the same volume for each spot (8.0 µl/spot) of the 500 µg/ml solution.

Specificity: The specificity was determined by analysing standard and test sample in developed mobile phase. The spot of each standard in the sample was confirmed by the Retention factors (R_f) values and UV spectra of the separate bands of the standard peaks.

Linearity: Mixture of 500 µg/ml of the two markers i.e., embelin and conessine was prepared. Calibration was performed by deciding the range with respect to the concentration of markers present in the formulation. This stock solution was spotted on 10×10-dimension TLC plate in the volume of (8, 16, 24, 32 and 40) µl. Using the above result calibration graph was plotted using concentration vs. peak area to obtain a regression coefficient (R^2).

Accuracy: Accuracy was assessed by adding known amounts of standard to the sample matrix silica gel G60 F254. The accuracy was determined by relative recovery method. The percentage recoveries of both the markers were found at three different concentration levels of 80 %, 100 % and 120 %.

Precision: Precision was evaluated in terms of intraday

precision and interday precision. Intraday precision was determined by analysing the standard solution at different intervals within the same day. The Standard Deviation (SD) and Percent (%) Relative SD (RSD) was calculated. The interday precision was determined by measuring same concentration (500 µg/ml) at six times on three different days and mean, SD and % RSD was calculated.

LOD: LOD was carried out based on calibration curve method. A specific calibration curve was studied using sample containing an analyte in the range of Detection Limit (DL). The SD of y-intercepts of regression lines used as the SD. It was calculated as per ICH guidelines, equation as shown below,

$$\text{LOD} = 3.3 \times \text{SD} / \text{slope of calibration curve}$$

LOQ: LOQ was carried out based on calibration curve method. A specific calibration curve was studied using sample containing an analyte in the range of Quantification Limits (QL). The SD of y-intercepts of regression lines used as the SD. It was calculated as per ICH guidelines, equation as shown below,

$$\text{LOQ} = 10 \times \text{SD} / \text{slope of calibration curve}$$

Robustness: To determine the robustness of the developed method, small changes were made to some of the developed conditions in order to see the capacity of developed method to remain unchanged. Changes were made in parameters like saturation time (± 2) and in the detection wavelength (± 2) in order to calculate SD and % RSD thus to prove the reliability of method.

RESULTS AND DISCUSSION

The identification of the markers in the PBC was done using different compositions of the mobile phase and was optimised depending on the polarities of the solvents. Standard conessine and embelin with the R_f values of 0.46 and 0.83, respectively, at the selected wavelength of 520 nm it was found that the toluene:ethyl acetate:formic acid:ammonia:diethyl amine as a mobile phase with the ratio (3:3:1.5:0.5:2.5 v/v/v/v/v) shows a good and sharp peak when the complete chamber saturation is done for 15 min with the mobile phase.

It was found that a good and well-defined peak was shown in fig. 2 and fig. 3.

The developed method was validated by parameters like linearity, precision, specificity, accuracy, and robustness according to the ICH Q2(R2) guidelines. Specificity was established by comparing standard drugs with the sample formulation and by collating the retardation factor and the spectrum of the standard with the sample formulation. The developed method was found to be specific, as shown in Table 3. The linearity of conessine and embelin was established in the concentration range of (8-12) μl in the range of 1200-6000 $\mu\text{g}/\text{band}$, and it was found to be linear in the range with a R^2 0.998 for conessine and 0.997 for embelin as shown in Table 4. The calibration curves for conessine and embelin are shown in fig. 4-fig. 7. A recovery study for accuracy was performed at three different levels; 80 %, 100 %, and 120 % by spiking the standards, and the % recovery and % RSD were calculated. The accuracy results are shown in Table 5. A precision study was performed by analysing intraday and interday variations. It was carried out at one concentration in each of the six replicates. The procedures were found to be precise. Intraday was performed by repeating one concentration six times in

the interval of time calculation shown in Table 6, and interday was performed by repeating one concentration in a day in six replicates as shown in Table 7. Robustness was performed by the minor changes in the wavelength of 520 ± 2 and the saturation time of 15 ± 2 min as shown in Table 8. Quantification of standard in the formulation was done by the R^2 equation by the area of the standard in formulation as shown in Table 9.

It is necessary to be sure about the quality of the formulation in order to ensure the quality of PBC (Table 10). Standardisation was carried out. A proper method was developed for the quantification of conessine and embelin, which were 4862 $\mu\text{g}/\text{spot}$ and 6320 $\mu\text{g}/\text{spot}$ respectively. The linearity of conessine and embelin was found in the range of (1200-6000) ng/spot . The accuracy of conessine and embelin was in the range $98\% \pm 102\%$, as per ICH guidelines. LOD and LOQ of conessine were 624 and 768, apparently for embelin it was 1892 and 2328. The robustness study for both of these markers was done by comparing wavelength and saturation time ± 2 to their desired data. So, we conclude that all validation parameters were as per ICH guidelines, and simultaneous estimation of conessine and embelin was followed by the HPTLC method.

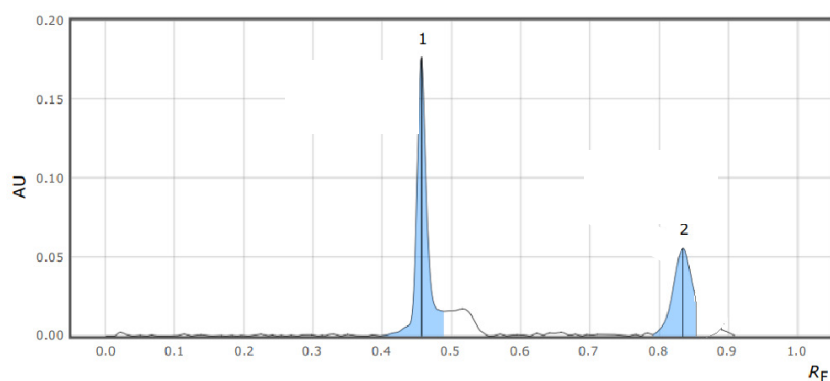


Fig. 2: HPTLC densitograph of standards
Note: 1: Conessine and 2: Embelin

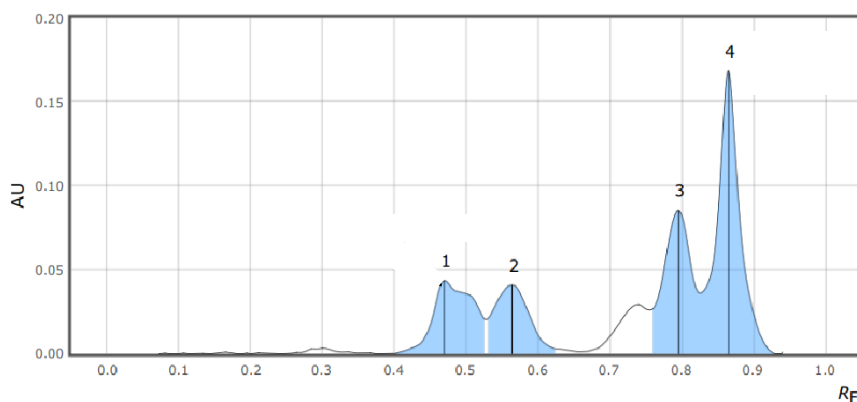


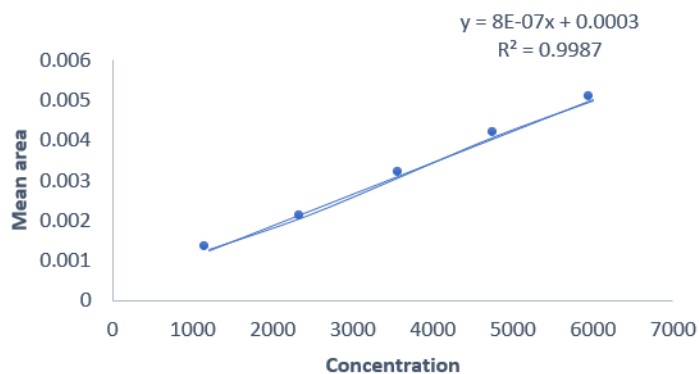
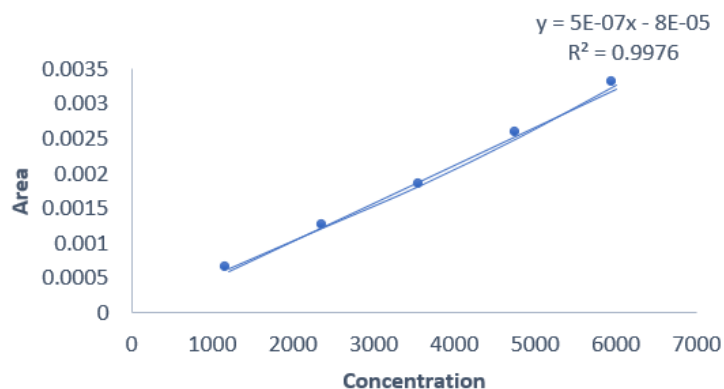
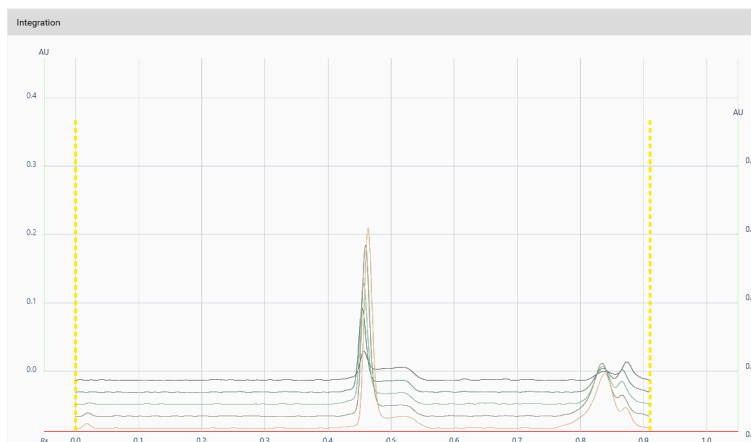
Fig. 3: HPTLC densitograph of PBC
Note: (1 and 2): Conessine and (3 and 4): Embelin

TABLE 3: SCANNING RESULT OF SPECIFICITY FOR BOTH THE MARKERS AND FORMULATION

Sample	R_f values	
	Conessine	Embelin
Mixture	0.465	0.835
Formulation	0.456	0.829

TABLE 4: LINEARITY OF CONESSINE AND EMBELIN

Standard	R^2 value	Equation
Conessine	0.998	$y=0.0000008x+0.0003$
Embelin	0.997	$y=0.0000005x-0.00008$

**Fig. 4: Calibration curve of conessine****Fig. 5: Calibration curve of embelin****Fig. 6: Linearity of standards**

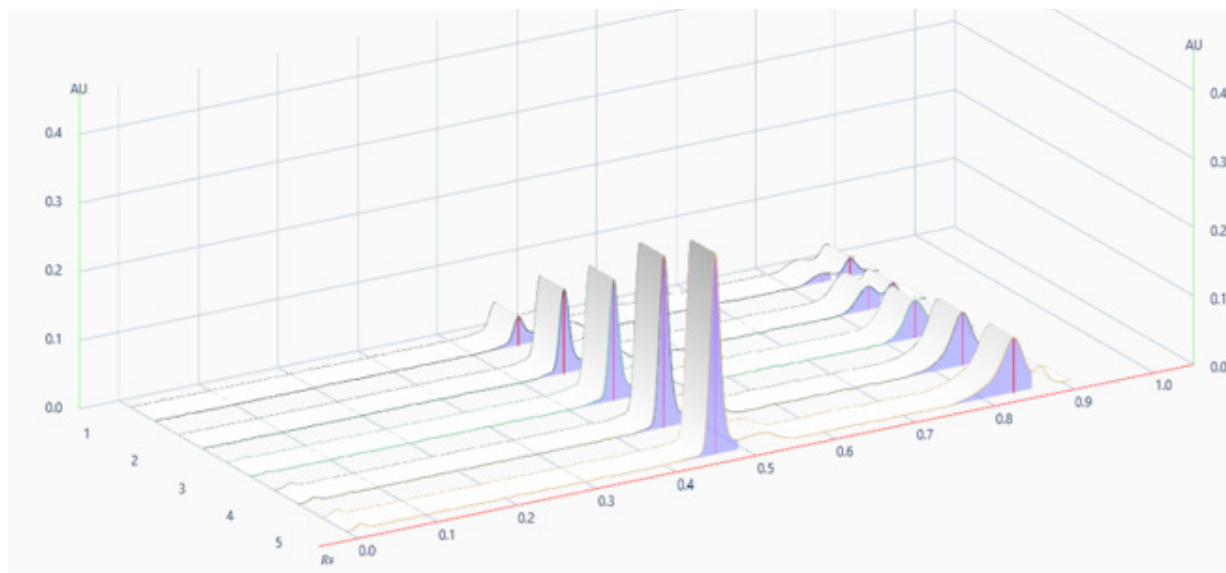


Fig. 7: Overlay of standards

TABLE 5: ACCURACY OF CONESSINE AND EMBELIN

Spike (%)	Recovery (%)	% RSD
Conessine		
80	0.9801	1.249
100	0.9806	0.363
120	1.02	0.622
Embelin		
80	0.9801	0.673
100	1.01	0.817
120	1.01	0.363

TABLE 6: INTERDAY PRECISION OF CONESSINE AND EMBELIN

Standard	Mean±SD	% RSD
Conessine	0.00215±0.00008	0.421
Embelin	0.00122±0.00001	0.956

TABLE 7: INTRADAY PRECISION OF CONESSINE AND EMBELIN

Standards	Mean±SD	% RSD
Conessine	0.002148±0.00001	0.801
Embelin	0.001248±0.00002	1.785

TABLE 8: ROBUSTNESS STUDY OF CONESSINE AND EMBELIN

Parameters	Changes	% RSD	
		Conessine	Embelin
Wavelength	(350±2) nm	0.719	1.235
Saturation time	(15±2) min	0.718	1.682

TABLE 9: QUANTIFICATION OF CONESSINE AND EMBELIN

Standard	Quantification (µg/spot)
Conessine	4862
Embelin	6320

TABLE 10: SUMMARY OF VALIDATION PARAMETERS

Parameters	Conessine	Embelin
R _f value	0.46	0.83
Scanning wavelength (nm)	520	520
Linearity range (ng/spot)	1200-6000	1200-6000
Regression equation	y=0.0000008x+0.0003	y=0.0000005x-0.00008
R ² value	0.9987	0.9976
Intercept	0.0003	0.00008
LOD (ng/spot)	624	1892
LOQ (ng/spot)	768	2328

Acknowledgements:

The authors are thankful to the management of Parul Institute of Pharmacy and Research, Parul University, Vadodara for providing facilities to carry out this research work.

Conflict of interest:

The authors declare no conflict of interests.

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