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Development and Validation of a HPTLC Method for Determination of psoralen in *Psoralea corylifolia* (Bavachi)

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Abstract: A new, simple, sensitive, accurate and precise high-performance thin-layer chromatographic method for quantification of psoralen from *Psoralea corylifolia* was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F- 254 as the stationary phase. Linear ascending development with toluene–ethyl acetate 7.5: 2.5 (v/v) as the mobile phase was performed at room temperature (25 ± 2°C) in a twin-trough glass chamber saturated with mobile phase vapour. Compact bands (Rf 0.47 ± 0.02) were obtained for psoralen. Scanning was performed in absorbance mode at 299 nm. Linear regression analysis of the calibration plots showed good linear relationship between peak area and peak height (r^2 = 0.99828 and 0.99649) in the concentration range 10–100 ng/ spot. The method was validated for precision, recovery, robustness, specificity, and detection and quantification limits. The limits of detection and quantification were 8.65 and 26.2 ng/ spot, respectively. The average recovery of the method was 99.73 %. The amount of psoralen in seed powder extract was found to be 0.928%. This method can be used as quality control method for checking the purity of *Psoralea corylifolia* seed powder, extract and its formulation.
Key Words: psoralen, HPTLC, *Psoralea corylifolia,* method validation.

Introduction

Dry fruits of Leguminous plant *Psoralea corylifolia* Linn. (syn: *Cullen corylifolium* Linn.) is one of the most popular Traditional Chinese Medicine and officially listed in Chinese Pharmacopoeia (1). It's an annual herb growing throughout the plains of India. The plant is of immense biological importance and it has been widely exploited since ages for its magical effect against several skin diseases like psoriasis, leucoderma and leprosy (2). It is reported to contain essential oil, coumarins, alkaloids, flavonoids and terpenoids (3, 4). The literature also records therapeutic action of *Psoralea corylifolia* against various diseases such as asthama, diarrhea, alopecia areata (5), impotence, menstruation disorder and uterine hemorrhage (6). It shows antitumor (7), anti-allergic (8), antioxidant (9),insecticidal (10) and antimicrobial activity (11). The drug has also been reported for the treatment of enuresis, various kidney problems (12), depression (13, 14), osteoporosis and bone fractures (3, 15), lumbago and tuberculosis (16).

Psoralen (7H-Furo (3, 2-g) (1) benzopyran-7-one) is the major and most active furanocoumarin present in *Psoralea corylifolia* which promotes pigmentation (17, 18). Psoralen has been found to intercalate into DNA, where they form mono and di adducts in the presence of long wavelength UV light and thus are used for the treatment of hypo- pigmented lesions of the skin like leucoderma (19).

Literature revealed that a number of methods like UV spectrophotometry, HPLC, HPTLC of estimation of psoralen from *Psoralea corylifolia* are available which are either tedious or lack sensitivity and specificity and are not cost effective. Thus a simple, very sensitive, cost effective validated HPTLC method for estimation of psoralen from the extract of *Psoralea corylifolia* was developed. HPTLC is a valuable tool for the investigation of herbal products with respect to different aspects of their quality. The advantage of HPTLC over other techniques is that large number of samples can be simultaneously analyzed using small

volume of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis. Mobile phases having pH 8 and above can be employed. Suspensions or turbid samples can be directly applied. It facilitates automated application and scanning in situ and also repeated detection (scanning) of the chromatogram with the same or different parameters (20). Moreover, HPTLC results are not only reported as peak data but can also be presented and communicated as images (21).

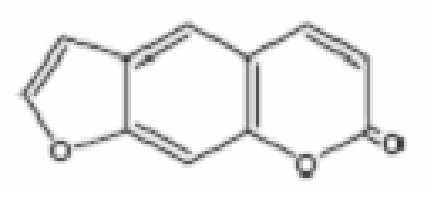


Fig.1: Chemical structure of Psoralen

Experimental

Materials and Methods

All chemicals were of AR grade and were purchased from S.D. Fine Chemicals, Mumbai. Chemicals such as methanol, toluene, ethyl acetate were used.

Plant material

The seeds of *Psoralea corylifolia* were procured from local market and authenticated for its botanical identity by Agharkar Research Institute, Pune. The seeds were dried and powdered and kept in an air tight container.

Chromatography

HPTLC was performed on 20 cm × 10 cm TLC aluminium plates coated with 200-µm layer thickness of silica gel 60F 254 (E. Merck, Germany). Samples were applied as 6 mm width bands using Camag 100 microlitre sample syringe (Hamilton, Switzerland) with a Camag Linomat 5 applicator (Camag, Switzerland). A constant application rate of 150 nL s−1 was used. Linear ascending development with toluene–ethyl acetate 7.5:2.5 (v/v) as mobile phase was carried out in a twin trough glass chamber (Camag) (20 x 10 cm) previously saturated with mobile phase vapour for 20 mins (optimized chamber saturation time) at room temperature (25 ± 2°C). The development distance was 80 mm. After development plates were air- dried. Scanning was performed using Camag TLC scanner 3 at 299 nm in the absorbance mode and operated by winCATS software (version 1.4.1). The source of radiation was a deuterium lamp emitting a continuous UV spectrum in the range 190– 400 nm. The slit dimensions were
5 mm × 0.45 mm and the scanning speed was 100 mm/ s.

Calibration

A stock solution (50 µg/ mL) of psoralen was prepared in methanol and dilution was done to obtain a solution of 5ug/mL which was used for further analysis. Different volumes of the diluted solution (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µL) were applied in duplicate to a plate to furnish 10 -100 ng/ spot of psoralen. Peak area data and the corresponding amounts were treated by linear least-square regression analysis.

Method Validation

This method was validated as per the ICH guidelines (22), the method validation parameters checked were as follows:

Precision

The precision of the system was determined by measuring repeatability of sample application and measurement of peak areas for six replicates of the same band (60 ng/ spot psoralen). To evaluate intra-day precision, six samples at three different concentrations (40, 60, and 80 ng/ spot) were analyzed on the same day. The inter-day precision was studied by comparing assays performed on three different days. The precision of the system and method were expressed as relative standard deviation
(% RSD) of peak area.

Robustness

Robustness was studied in triplicate at 40 and 60 ng/ spot by making small changes to mobile phase composition, mobile phase volume, and duration of mobile phase saturation. The effects on the results were examined by calculation of RSD (%) of peak areas. Mobile phases prepared from toluene and ethyl acetate in different proportions (7.2:2.8 and 7.8:2.2 v/v) were used for chromatography. Mobile phase volume and duration of saturation investigated were 20 ± 2 mL (18, 20, and 22 mL) and 20 ± 5 mins (15, 20, and 25 mins), respectively.

Limits of Detection and Quantification

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated using the formula, 3.3σ (standard deviation)/slope of the calibration plot for psoralen. The quantification limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the formula, 10σ (standard deviation) /slope of the calibration plot.

Accuracy, as Recovery

The samples were spiked with extra 50, 100, 150% of the standard psoralen (standard addition method) and the mixtures were analyzed by the proposed method. Three determinations were performed at each level. This was to check the recovery of psoralen at different levels.

Specificity
The specificity of the method was assessed by analyzing and comparing the Rf values and spectra of the psoralen band from a sample with that from a standard.

Analysis of Psoralen in Herbal Extract
Preparation of the methanolic extract of *P. corylifolia*: Accurately weighed 2.5-g dried seed powder of *Psoralea corylifolia* was defatted with petroleum ether (60- 80 º C) and extracted with methanol (4 x 25 mL) under reflux and dark brown extract was concentrated in vacuum via rota vaccum drier until pourable. Further this extract was taken in the tarred evaporating dish and evaporated to the constant weight. This dark brown sticky extract was then used for further analysis.

Preparation of the sample solution: The methanolic extract (10 mg) was accurately weighed and transferred to a 10 mL volumetric flask containing 5 mL methanol. The mixture was sonicated for 15 mins and diluted to 10 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 10 mins and the supernatant was analyzed for drug content. The supernatant solution (5 µL) was applied to a TLC plate followed by development and scanning. The analysis was repeated in triplicate.

Results and Discussion

Development of the Optimum Mobile Phase
The TLC procedure was optimized for quantification of psoralen in herbal extract. TLC procedure was optimised with varying ratios of toluene and ethyl acetate. The mobile phase toluene: ethyl acetate with ratio (7.5: 2.5 v/v) gave good resolution, dense, compact and well-separated spots of psoralen as well as a well- defined peak at Rf value of 0.47. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible Rf values, better resolution, and symmetrical peak shape for the compound.

Validation of the Method
Linearity
A good linear relationship (correlation coefficient r^2 = 0.9982) was obtained between psoralen peak area and concentration in the range 10- 100 ng/ spot (Table 1 and Fig. 2).
Precision
% RSD for the repeatability of sample application (60 ng/ spot) and measurement of peak areas were 0.092 % and 0.088 % respectively. Measurement of the peak area at three different concentrations resulted in low values of % RSD (<1%) for inter and intra-day variation, which suggested the precision of the method was excellent. (Table 2).

Robustness
The RSD (%) of the peak area were calculated, in triplicate, for changes in mobile-phase composition, mobile-phase volume, and duration of saturation for 40 and 60 ng/ spot. The low values of RSD (%) obtained after introducing small deliberate changes in the method indicated the method was robust (Table 3).

LOD and LOQ
The limits of detection and quantification were 8.65 and 26.2 ng, respectively, indicating the sensitivity of the method was adequate.

Recovery
The proposed method when used for quantification of psoralen from seed extract after spiking with standard afforded average recovery of 99.73 % (Table 4).

Specificity
There were no interfering spots by the other constituents of the plant at the Rf values of the standard psoralen (Rf = 0.47). The spectrum of standard psoralen and the corresponding spot present in the sample matched exactly and was found to be overlapping, indicating no interference by the other plant constituents (Fig. 3 and 4).

Estimation of psoralen in the Extract
A single spot at Rf 0.47 was observed in the chromatogram obtained from
psoralen isolated from the extract with other components. There was no interference
with the analysis from other components present in the extract, which appear in the chromatogram at significantly different Rf values (Fig. 5 and 6). The amount of psoralen in dry methanolic extract of *Psoralea corylifolia* was found to be 0.928 %.

Conclusion
This developed and validated HPTLC method for the determination of psoralen is simple, reliable, precise, specific, and robust with high recovery. Statistical analysis proves the method is reproducible and selective for analysis of psoralen. Because the proposed mobile phase effectively resolves psoralen, the method can be used for both qualitative and quantitative analysis of psoralen in herbal extracts without interference of other constituents. The method is economical, because it uses a small amount of solvent and sample clean-up is minimal. The proposed method can be used for quality control of *Psoralea corylifolia* plant and also for routine analysis of psoralen.

Table 1. Regresssion analysis or calibration plots. (n= 2)

Linearity Range	10- 100ng/ spot
Coefficient of Determination (Height)	0.99649
Coefficient of Determination (Area)	0.99828
Regression Equation (Height)	y = 2.492 + 1.24 x
Regression Equation (Area)	y = -68.03 + 39.42 x
Slope (Height)	1.24
Slope (Area)	39.42
Intercept (Height)	2.492
Intercept (Area)	-68.03

Table 2. Intra-day and inter-day precision of the method (n = 6)

Amount (ng/ spot)	Intra- day precision			Inter- day Precision		
	Mean area	SD	% RSD	Mean area	SD	% RSD
40	1431.75	1.469	0.102	1445.73	3.277	0.2266
60	2466.32	2.703	0.1096	2448.19	3.429	0.14
80	2953.58	2.677	0.090	2971.60	4.361	0.1467

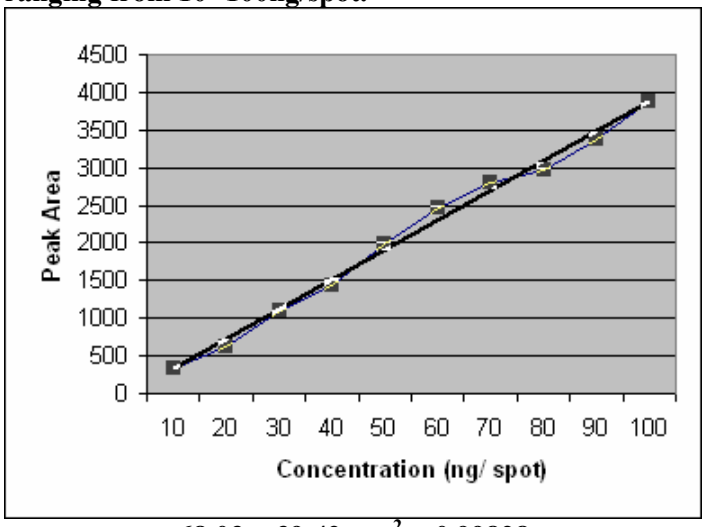
Table 3. Robustness of the method.

Parameters	% RSD		Mean % RSD
	40 ng/ spot	60 ng/ spot	
Mobile phase composition			
Toluene : Ethyl acetate (7.2 : 2.8 v/v)	0.785	0.562	0.673
Toluene : Ethyl acetate (7.8 : 2.2 v/v)	0.592	0.431	0.5115
Mobile phase volume (mL)			
18	0.541	0.415	0.478
20	0.23	0.338	0.284
22	0.427	0.499	0.463
Duration of Saturation (mins)			
15	0.287	0.342	0.314
20	0.192	0.205	0.198
25	0.298	0.214	0.256

Table 4. Recovery Studies (n = 3)

Amount of psoralen added (ng)	Amount of psoralen found (ng)	Recovery (%)	Average Recovery (%)
25	24.97	99.88	99.73
50	49.11	98.22	
75	75.83	101.10	

Fig. 2. Calibration curve of peak area versus standard psoralen concentration ranging from 10- 100ng/spot.



$y = -68.03 + 39.42\ x$; $r^2 = 0.99828$

Fig. 3. Spectra of psoralen standard

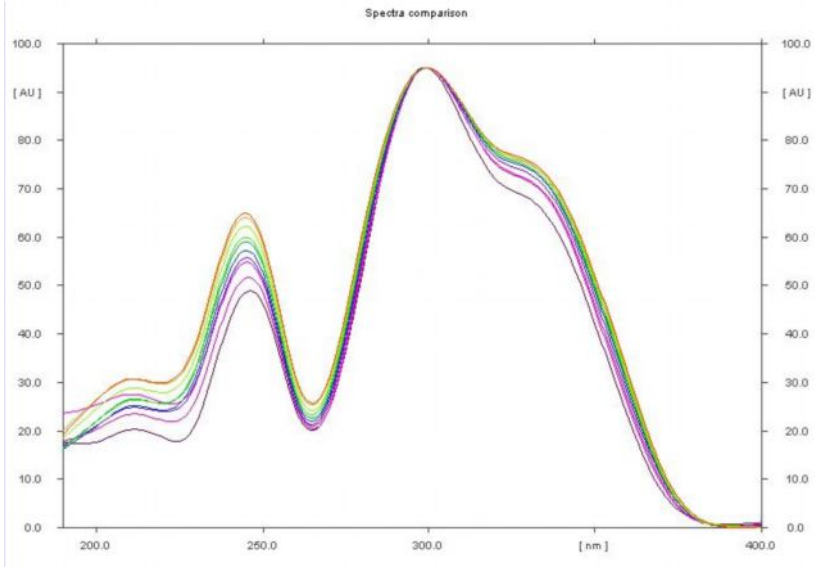


Fig. 4. Spectra of psoralen from sample

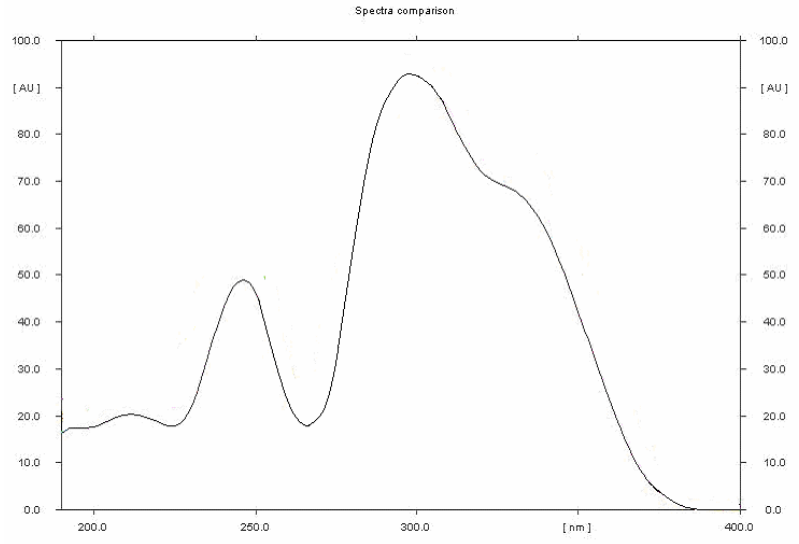


Fig. 5.Chromatogram of psoralen standard

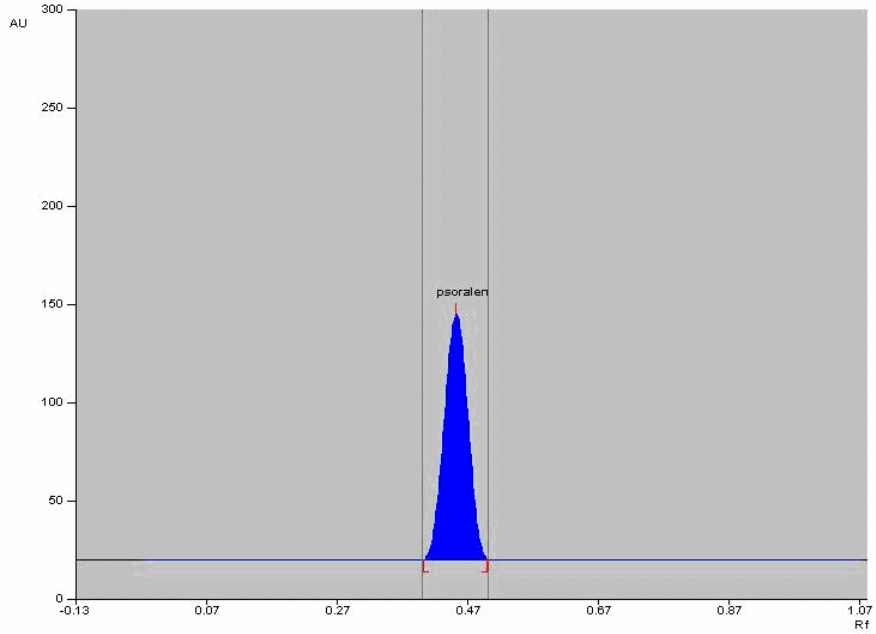
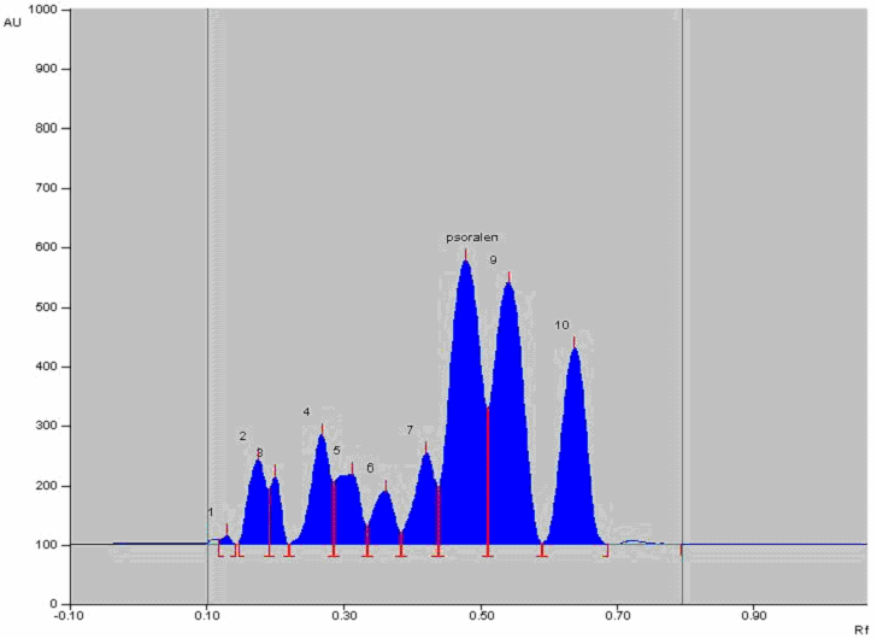


Fig. 6.Chromatogram of methanolic extract of *Psoralea corylifolia.*



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