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Development and Validation of HPTLC Method For Assay of Bioactive Marker isolated from the bark of Albizzia lebbeck Linn.

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Abstract: A rapid and sensitive high performance thin layer chromatography (HPTLC) method was developed and validated for determination of Bio-active marker isolated from bark of *Albizzia lebbeck* (shirish). Purity of marker was checked on HPLC equipped with PDA dectector (Make-Jasco).Marketed syrup containing Albizzia lebbeck and other herbs was assayed for content of this marker by developed HPTLC method. The label claim states syrup contains 820mg/100mL of Albizzia lebbeck. A new HPTLC method has been developed for the assay of marker. The mobile phase consisted of Chloroform and Methanol (2:8 v/v). The marker was spotted on TLC plates which are aluminum sheets precoated with Silica Gel $60F_{254}$. The densitometric determination of the marker was performed at wavelength 279 nm. The linearity is observed in the range of 100 to 500ng/band with r^2=0.943. The method has been validated as per ICH guidelines Q2(R1). The method is found to be specific, accurate, precise and sensitive. This method is fast and has advantages of high throughput, economy due to low solvent requirement.
Keywords- Shirish, HPTLC, Marker Quantitation.

Introduction

Standardization of Herbal Drug

Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility. It is the process of developing and agreeing upon technical standards. Specific standards are worked out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular herbal medicine. Hence standardization is a tool in the quality control process.[1] The key steps in herbal standardization include, Identifying the key beneficial compounds for optimized product performance. Determining the optimum level of marker components. In cases where the marker compound has not been clearly identified, a herbal material can be concentrated to yield higher degree of all the plants compounds in a smaller volume than the plant itself. Albizzia lebbeck, also known as tree of happiness is extensively used in various traditional medicines. In Chinese system of medicine, it is used for relieving stress, anxiety & depression. Whereas in Ayurveda (Indian system of medicine) it is said to be

Vishaghana i.e. destroying the toxins present in body. It is mainly used in allergic conditions such as allergic rhinitis, allergic asthma, urticaria etc. It possesses anti-histaminic and mast cell stabilizing property by virtue of which it is supposed to work as anti-asthmatic drug.[2] It also possess anti-inflammatory and analgesic activity,[3] antidiarrheoal,[4] antiulcer,[5] antiallergic,[6] antioxidant,[7] immunomodulatory,[8] antimicrobial.[9] Due to above mentioned facts this plant was selected for further studies. Marketed sample contains *Albizzia lebbeck*(shirish) as one of its component which is claimed to inhibit degranulation of mast cells, synthesizes reaginic type antibodies and has a pharmacological action like Disodium Cromoglycate. [10,11]

In the present work, Aqueous Methanolic extract of Albizzia lebbeck was used for isolation of marker compound in our lab and HPTLC method was developed and validated for its quantitation. The present work describes the simple, accurate, precise, sensitive HPTLC method for determination of marker. The method was validated according to the ICH guidelines. [12]

Experimental Section

1. Equipments

Samples were applied on the plate using Camag 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Scanning was performed using Camag TLC scanner 3, operated by winCATS software (Version 1.4.3, Camag). Deuterium lamp was used as a radiation source. All weighing was done on Shimadzu balance (Model AY-120).

2. Reagent and Chemicals

Methanol (AR grade), Chloroform (AR grade).

3 Chromatographic Conditions

Samples were applied on the 5cm x 10cm plate which is aluminium plates precoated with silica gel 60 F_{254}, as a band with 4 mm width using Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (for 10 x 10 cm) and a densitometric scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software (Version 1.4.3, Camag). Slit dimensions were kept as 3.00 x 0.45 mm. Chamber saturation time was 15 minutes and the migration distance was 90 mm. Mobile phase used was Methanol:Chloroform (8:2 v/v ratio).

4. Marketed Formulation

An Ayurvedic syrup Broncorid by Dabur contains Shirish 820 mg/100mL was used to perform assay of the marker.

5.Preparation of Standard Stock Solution of Marker Isolated in Lab:

a) Standard stock solution

10 mg of marker was accurately weighed and dissolved in 10 ml of Aqueous Methanol to obtain stock solution of marker (1000µg/ml).

b) Working standard solution

50 µg/ml solution was prepared from the standard stock solution.

6. Selection of Detection Wavelength

From the standard stock solution further dilution were done using Aqueous Methanol and scanned over the range 200nm-400nm and spectra was obtained. It was observed that Marker showed maximum absorbance at wavelength 279nm.

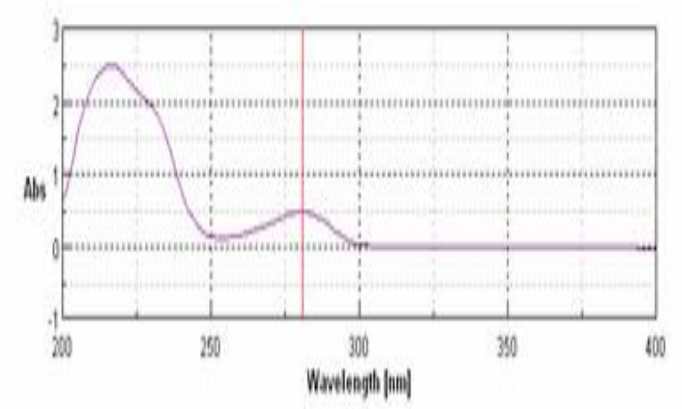

Fig.1 UV spectrum of Marker

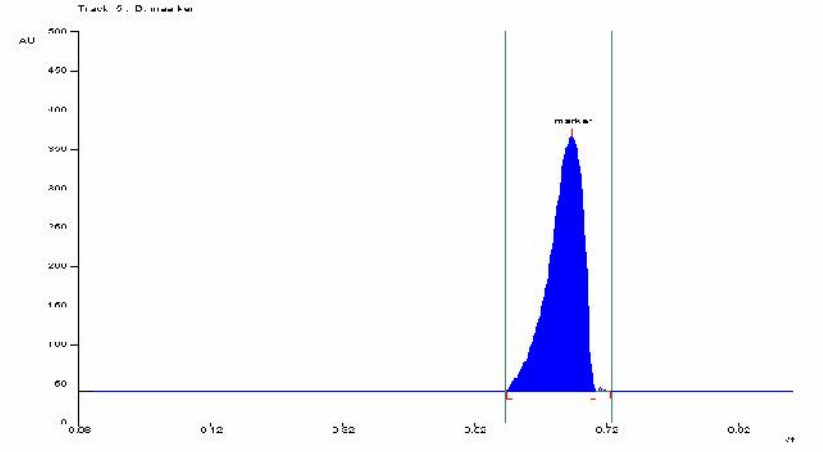

Fig 2: Representative Densitogram of Marker 300ng/band(Rf 0.65)

5. Validation Of Hptlc Method

Method Development and Optimization of Mobile Phase

Chromatographic separation studies were carried out on the working standard solution of marker (50µg mL^{-1}). Initially, various trials were carried out using neat solvents. Various preparations of chloroform:methanol were tried and Rf. between 0.2 to 0.8 and compact band with symmetric peak were obtained at Rf 0.65 with the optimized mobile phase.

5.1 Specificity

The specificity of the method was ascertained by peak purity profiling studies. Purity of the drug peak was ascertained by analyzing the spectrum at peak start, middle and at peak end. The peak purity was determined on TLC scanner 3 in the range of 200-400 nm using WinCats software (version 1.4.3).

5.2 Accuracy

To check accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample aqueous methanolic extract of *Albizzia lebbeck* at three different levels of 80, 100 and 120 %. Mean percentage recovery was then determined.

5.3 Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity was studied by analyzing five concentrations of 100-500 ng band^{-1} for the marker and process was repeated for five times each.

5.4 Precision

Precision of the system was evaluated by analyzing six independent standard preparations obtained from homogenous solution and % RSD value obtained was calculated to determine system and method precision.

5.5 Limit Of Detection and Limit of Quantitation

The detection limit 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. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

5.6 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness of the method was determined by making slight deliberate changes like chamber saturation time, time from spotting to development and time from development to scanning .

6.Results And Discussion

6.1 Linearity (Calibration Curve)

The calibration plot was found to be linear over the concentration range 100-500ng/spot for Marker with linearity equation y=6.302x + 770.6 a correlation coefficient(r^2) of 0.943.

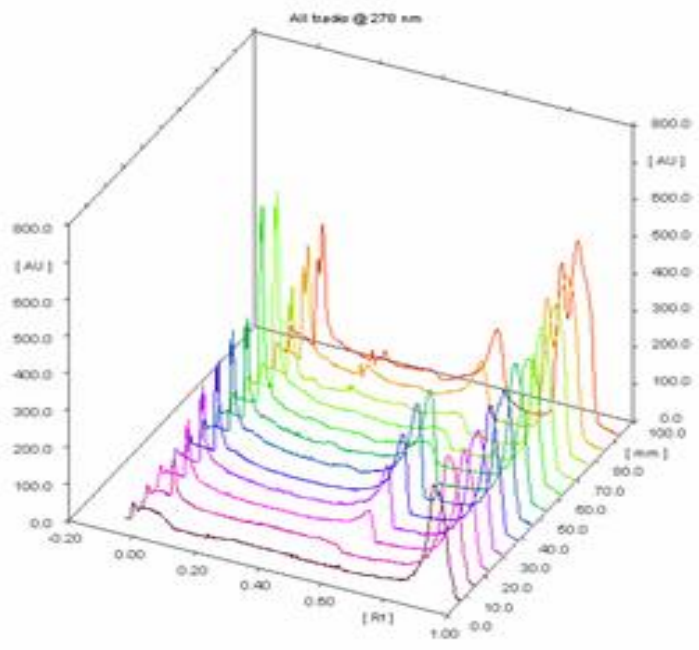

Fig 3. Chromatogram depicting Linearity of Marker. a) track 1 methanol b) track 2 to7 depicting linearity of marker c) track 8 to 10 depicts extract d) track 11-12 are for marketed formulation(Broncorid)

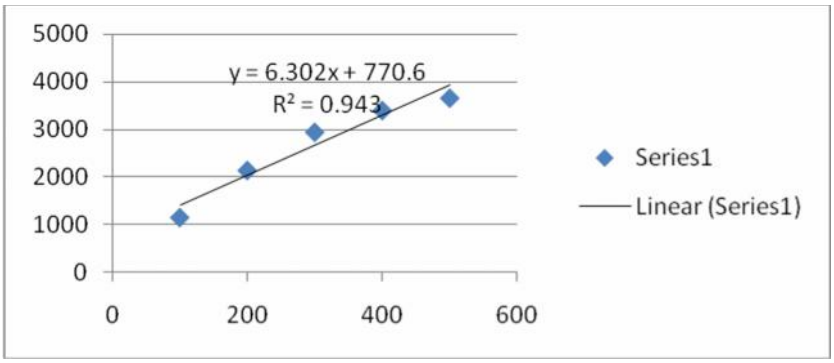

Fig 4. Linearity for Marker

6.2 Precision

The RSD of the inter-day and intra-day precision experiments are NMT 1% and 1.5% respectively. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were less than 2% respectively as recommended by ICH guidelines.

6.3 Robustness

For robustness studies, there were no significant changes in Rf and peak areas, which was demonstrated that the developed HPTLC method is robust.

6.4 Specificity

The peak purity of Marker was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9989 and r (M, E) = 0.9986. A good correlation (r = 0.9981) was also obtained between the standard and sample spectra of Marker.

6.5 Accuracy

Accuracy was tested by standard addition method. The amount recovered was in the range of 98 to 102%.

6.6 LOD and LOQ

Limit of detection was calculated using the formula

LOD = 3.3 * Std. Dev. of lowest response in the range / slope.

LOD was found to be 29.9ng/band. The formula for Limit of Detection was LOQ = 10 * Std. Dev. of lowest response in the range / slope. LOQ was found to be 90.6ng/band.

6.7 Summary Of Validation Parameter

Table1. Summary Of Validation Parameter

Sr. No.	Validation Parameter	Results
		Marker
1.	Linearity	Y=6.302x + 770.6 r^2 =0.943
2.	Range	100-500 ng/spot
3.	Precision	%RSD
	A) Intraday precision	1.4%
	B) Interday precision	0.94%
4.	Accuracy	% recovery
	80%	91
	100%	97
	120%	90
5.	LOD	29.9 ng/spot
6.	LOQ	90.6 ng/spot
7.	Robustness	Robust
8.	Percentage of marker in extract	1.16%
9.	Percentage of marker in marketed formulation	2.6%

6.8 Conclusion

The Method used for quantitation of marker is simple, Marker is isolated by extraction process of maceration of bark powder and column chromatography. This HPTLC method can be used for quality testing of bark sample.

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