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TLC densitometric fingerprint development and validation of Berberine as markers in poly-herbal Unani formulations

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Abstract

Sat-e-gilo (Tinospora cardifolia) and its poly herbal formulations have been in use in Indian traditional medicine since antiquity. However, there has been no attempt to standardize the polyherbal formulations containing Berberine as the main ingredient in terms of its active principal or marker compound. Biologically active Berberine is identified as the marker compound for the crude drugs and its formulations. In the present study, the method for fingerprints of the formulations in the form of densitogram following charring of the chromatographic plate was developed that could be useful for marker-based quality assurance of the polyherbal products containing Tinospora cardifolia. Accuracy was validated by analysis of spiked blank and standard addition samples and precision by performing replicate analyses on a single day and on different days. Recoveries from spiked blank and standard addition sample were 98.03-100.37%. Repeatability for sample, each of which was analyzed six times on a single plate, was 0.034967 % relative standard deviation. The intra and inter precision was 0.045 and 0.047% relative standard deviation for a sample analyzed in duplicate once per plate on same day and the different days over a seven-day period. The content of Berberine was found to be 11.75 ± 0.0208– 12.46 ± 0.0264 mg/gm in lab formula tions. A survey was made of Berberine content compared with label values for two marketed products, using the new method. The method was shown to be simple, precise and accurate suitable for routine analysis of Berberine in a manufacturing quality-control or regulatory agency laboratory.

Key Words: Densitometry, HPTLC, Berberine, Unani formulation, Fingerprint, Giloe

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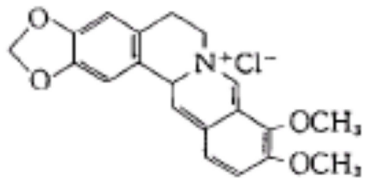
INTRODUCTION

Herbal drugs have been in use by different civilizations in different parts of the world for Centuries to fight a large number of diseases. Many of these are in common use even today. *Tinospora cardifolia* is large, glabrous, succulents, climbing shrub belonging to the family Menispermaceae. It is distributed throughout tropical indian subcontient, Sri lanka and China, ascending to an altitude of 1,200 mtrs.

Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices, and composition is important to ensure quality and optimum levels of active principles for their bio-potency. Recently, the concept of marker-based standardization of herbal drugs is gaining momentum. Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization [1]. Being the major active principles largely responsible for the bio-potency of Giloya, Berberine is recognized as the marker compounds. There are some reports on the application of TLC (Thin layer chromatography), GC (gas chromatography) and HPLC (High performance liquid chromatography) methods for the analysis of Berberine, but attempts to apply these techniques for the profiling of Berberine in polyherbal formulations are not available.

TLC and HPTLC (High performance thin layer chromatography) are methods commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutriments). These flexible and cost-effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including a great variety of post-chromatographic derivatization reagents [2].

Habb-e-Bukhar is a polyherbal unani formulation, consisting of four ingredients of plant origin and it is widely used Daf-e-Humma, Moauriq and Humma-e-Hadda which is official in Unani pharmacopoeia. The major active components are Berberine and quinine. From such polyherbal formulations separation, identification and estimation of chemical components is very difficult. Habb-e-Bukhar is used as antipyretic. It is given in fever due to elephantiasis and malaria [3].
Berberine is an important anti-inflammatory drug for heart and intestinal disorders widely, owing to its antitumor promotion active and anti-lipase effective [4].



Structure of berberine chloride.

In Hindi, the plant is commonly known as Giloya, Giloe, Amrita, Gulancha. Giloya is the hindu mythological term that refers to the heavently elixer witch has saved celestial being from old age and kept them eternally young. Guduchi, the Sanskrit name means one which protects the entire body. The term Amrita meaning divine nectar is attributed to this drug in recognition of its capacity to impart youthfulness, vitality, and longevity to the consumer [5]. Drug consists of the dried stem with bark intact. It is widely used in folk and traditional systems of medicine for its general tonic, antipyretic properties [6].

Depending on the objective of the analytical procedure, the typical validation characteristics which can be considered through a statistical approach are accuracy, precision, specificity or selectivity, detection limit, quantification limit, linearity and ruggedness [7].

The concept of validation applied to densitometric determinations on HPTLC indeed varies according to the goal of the analysis and the steps required for a formal validation have been thoroughly investigated [8, 9] notably for purity testing [10], pharmaceutical dosage forms assay [11] and herbals fingerprinting [12]. The advances in chromatographic techniques made it possible to quantify the chemical constituents in a mixture with comparatively little clean-up using HPTLC [13]. Present study deals with development and validation of methods for quantification of some of the important marker compound of berberine in Habb-e-Bukhar. The proposed method was validated in compliance with ICH (International conference on harmonization) guidelines [14].

MATERIAL AND METHODS

Reference compounds and reagents

Reference Berberine was purchased from Sigma–Aldrich (Germany). All solvents used were of analytical grade and was procured from Merck (Mumbai, India).

Procurement of crude drug:

Crude drugs were procured from local market and identification was conformed by macroscopic and microscopic characters.

Apparatus and chromatographic conditions

Spotting device: Linomat V Automatic Sample Spotter; CAMAG (Muttenz, Switzerland)
Syringe: 100 µL Hamilton (Bonaduz, Switzerland)
TLC Chamber: Glass twin trough chamber (20 x 10 x 4 cm); CAMAG
Densitometer: TLC Scanner 3 linked to WinCats software V.4.06; CAMAG
HPTLC plates: 10 x 10 cm, 0.2 mm thickness precoated with silica gel 60 F_{254}; E. Merck KgaA, Cat. no. 1.05548; (Darmstadt, Germany)
Experimental conditions: Temperature 25 ± 2 ºC, relative humidity 40 %
Solvent system: Methanol: Acetic Acid: Water (8:1:1)
Detection Wavelength: 350 nm
Slit dimension: 6.00 x 0.20 mm, Micro
Scanning Speed: 20 mm/s

Preparation of the unani formulation:

Habb-e-Bukhar, two laboratory batches (Coded HLS-I and HLS-II) were prepared in laboratory according to reported method of Unani formulary of India [3]. The available commercially brand (HMS-A and HMS-B) of Habb-e-Bukhar was procured from their authorized retail shops in New Delhi, India.

Preparation of standard solutions

Accurately weighed Berberine (10 mg) was transferred in 100 ml volumetric flask and dissolved in and diluted to 100 ml with methanol. The final solution contained 100 µg of the Berberine per ml of the solution.

Calibration for HPTLC Method

Aliquots of standard solution (1mgmL^{-1}) equivalent to (100-500 ng of Berberine) was applied to HPTLC plate by Linomat V Automatic Sample Spotter with the help of micro syringe and developed as described under chromatographic conditions previously mentioned under 'chromatographic conditions.' The plates were visualized at 350 nm by densitometer. Calibration

curve was plotted representing the relationship between the average peak area and concentration and the regression equation was recorded.

Efficiency of different solvents system for separation of Berberine by TLC

The analytical separation of Berberine pigments by TLC was investigated using silica gel 60 F_{254} plates 10 cm ×10 cm developed with different solvent system. The method was selected according to the Rf values for each pigment. The different solvents were tested for TLC including the elution system toluene, ethyl acetate, chloroform, acetic acid and methanol (**Table 1)**.

Table 1: Different solvent systems on the separation of Berberine by TLC

TLC Mobile phase	***Rf* Values**
Toluene : Ethyl acetate (70: 30)	0.82
Chloroform : Methanol (9:1)	0.76
Methanol : Water (1:1)	0.78
Methanol: Acetic Acid: Water (8:1:1)	0.74
Acetonitrile : Water (1:9)	0.84
Acetonitrile : Methanol (1:1)	0.86

HPTLC procedure for identification of herbal samples

One gram powdered herbal sample was extracted with 10 mL methanol for 30 min by sonication. After centrifugation, the supernatant was filtered through a 0.45 µm filter for TLC analysis. HPTLC silica gel 60 F_{254} plates (10 cm ×10 cm, Merck) were heated for 15 min at 60^0C before use. The plates were then developed in a double-trough, 10 cm×10 cm chamber with a stainless steel lid. The chamber was pre-equilibrated with the mobile phase Methanol: Acetic Acid: Water (8:1:1). After developing over a path of 8 cm, the plate was air-dried, and the image was captured under UV light (350 nm).

Method validation

5.9.1. Precision

Precision of the method was determined with the product. An amount of the product powder equivalent to 100% of the label claim of Berberine was accurately weighed and assayed. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of relative standard deviation (R.S.D %) and standard error (S.E.). Method repeatability was obtained from R.S.D. value by repeating the assay six times in same day for intra-day precision. Intermediate precision was assessed by the assay of two, six sample sets on different days (inter-day precision). The intra- and inter-day variation for determination of Berberine was carried out at three different concentration levels 100, 300 and 500 ng/spot.

5.9.2. Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like Methanol: Acetic Acid: Water (8:1:1) and Methanol: Acetic Acid: Water (7:1:2) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ±5%. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 100, 300 and 500 ng/spot.

5.9.3. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method as explained in **Section 2.3.** The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of Berberine until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

5.9.4. Specificity

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

5.9.5. Recovery studies

The pre-analyzed samples were spiked with extra 50, 100 and 150 % of the standard Berberine and the mixtures were analyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the formulations.

5.10. Estimation of Berberine in the formulations

To determine the content Berberine in lab formulations, 500 mg was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to 100 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 15 min and supernatant was analyzed for drug content. The filtered solution was applied on the TLC plate followed by double development and scanning as described in **Section 2.3.** The same procedure was applied for the estimation of Berberine in the marketed formulations.

The analysis was repeated six times. The possibility of interference from other components of extract in the analysis was studied.

RESULTS AND DISCUSSION

6.1. HPTLC fingerprinting identification

The performance of TLC using a sorbent with a homogeneous particle size of 5 μm and with a narrow particle size distribution has been confirmed to be superior to that of the conventional TLC plate that was used in many pharmacopoeias for the identification of herbal materials. Therefore, in this study HPTLC plates were used to establish a TLC fingerprinting method. The chromatographic conditions, in particular the developing solvents (i.e., types of solvents and ratio), were carefully optimized before the crude drug and formulation samples were analysed. The results observed under UV light showed a good separation for all compounds. A reference marker compound of Berberine was separated on the same plates for the authentication of each sample. The samples were identified by matching the colors and Rf values of bands in their fingerprints with those of marker compounds. For positive identification, the sample must exhibit bands with chromatographic characteristics, including colors and Rf values (0.74 for Berberine), similar to those of reference marker compound. **(Fig.1)**

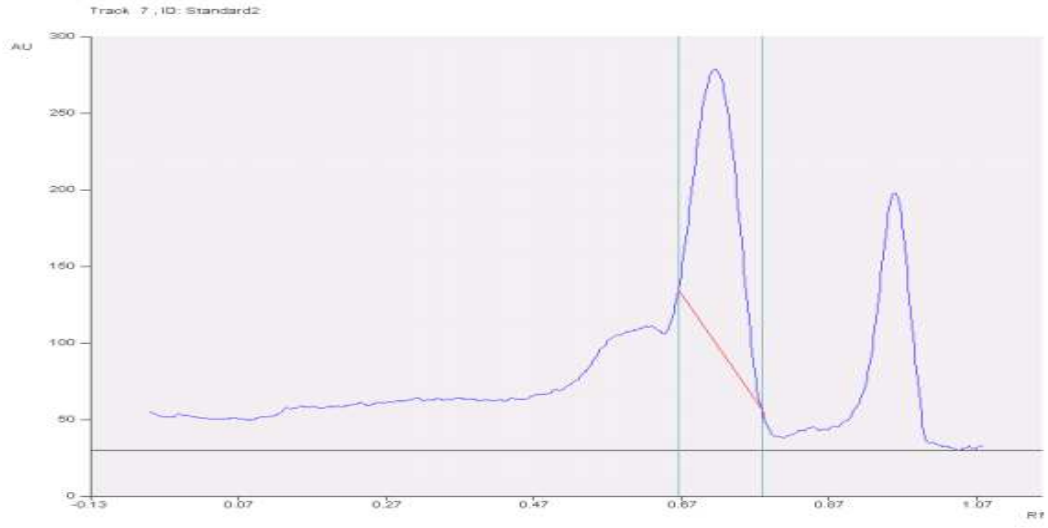


Fig. 1. Scan chromatogram of Berberine standards at 350 nm.

6.2. Peak purity test

Peak purity test of Berberine was done by comparing UV–visual spectra of Berberine in standard and sample tracks. Peak purity results (obtained by scanning at 350 nm) were satisfactory. Correlations of the peak start spectrum with the peak centre spectrum were 0.9999, 0.9989, 0.9998, 0.9997, 0.9996, 0.9997 for standard and sample tracks, respectively. Correlation of the peak centre spectrum with the peak end spectrum were also 0.9999, 0.9989, 0.9998, 0.9997, 0.9996, 0.9997 for standard and sample tracks, respectively.

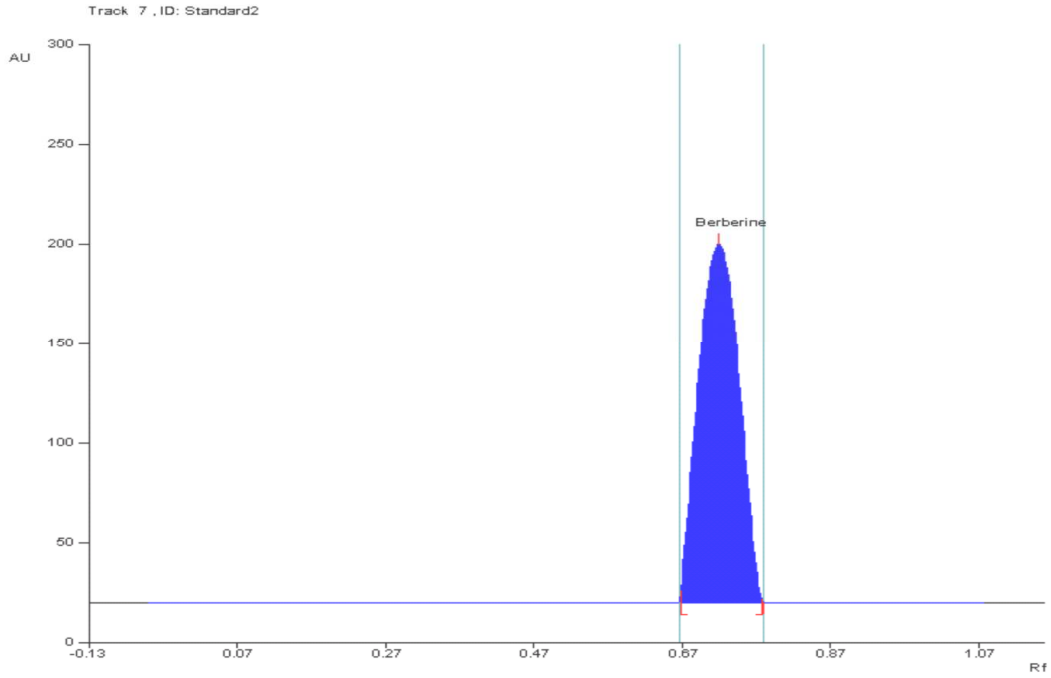


Fig. 2. HPTLC chromatogram of standard Berberine

6.3. Validation of Berberine

6. 3.1. Development of the optimum mobile phase

The TLC procedure was optimized with a view to develop a stability indicating assay method. Both the standard Berberine and the degraded products were spotted on the TLC plates and run

in different solvent systems. Initially Methanol: Acetic Acid: Water in varying ratios was tried. Finally, the mobile phase consisting Methanol: Acetic Acid: Water (8:1:1v/v) gave sharp and symmetrical peaks and improved spots characteristics of Berberine was obtained. Well-defined spots were obtained when the chamber was saturated with the mobile phase for 40 min at room temperature. The spot at Rf 0.74 was identified as Berberine with the help of the chromatograms **(Fig. 2).**

6. 3.2. Calibration curves of standard Berberine

Calibration curve obtained was linear which is adherence of the system to Kubelka Munk theory which relies on the idea that light is travelling in all directions simultaneously within the precoated TLC plate. This is approximated as a flux of light travelling upwards and a flux travelling downwards at any depth in the plate. When this flux passes through a thin layer of material, some of it passes through, some of it is scattered backwards and some of it is absorbed. Linearity was found over the concentration range 100–500 ng/spot for Berberine with r^2 ± S.D. = 0.9996 ± 0.0003. Linearity was evaluated by determining three standard working solutions containing 100, 300 and 500 ng/spot of Berberine in triplicate. Peak area and concentration was subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the range of 100–500 ng/spot. Three dimensional image of the calibration curve at 350 nm.The linearity of calibration graphs and adherence of the system to Beer's law was validated by high value of correlation coefficient and the S.D. for intercept value was less than 2%. No significant difference was observed in the slopes of standard curves (ANOVA; P < 0.05) **(Table 2, Fig. 3)**.

Table 2: Linear regression data for the calibration curve (n=6)

Linearity range (ng/spot)	r ± S.D.	Slope ± S.D.	Confidence limit of slope[a]	Intercept ± S.D.	Confidence limit of Intercept[a]
100 - 500	0.9996 ± 0.00035	0.4193 ± 0.00062	0.4186-0.4200	29.69 ± 0.02449	29.664 - 29.716

[a] **95% Confidence level**

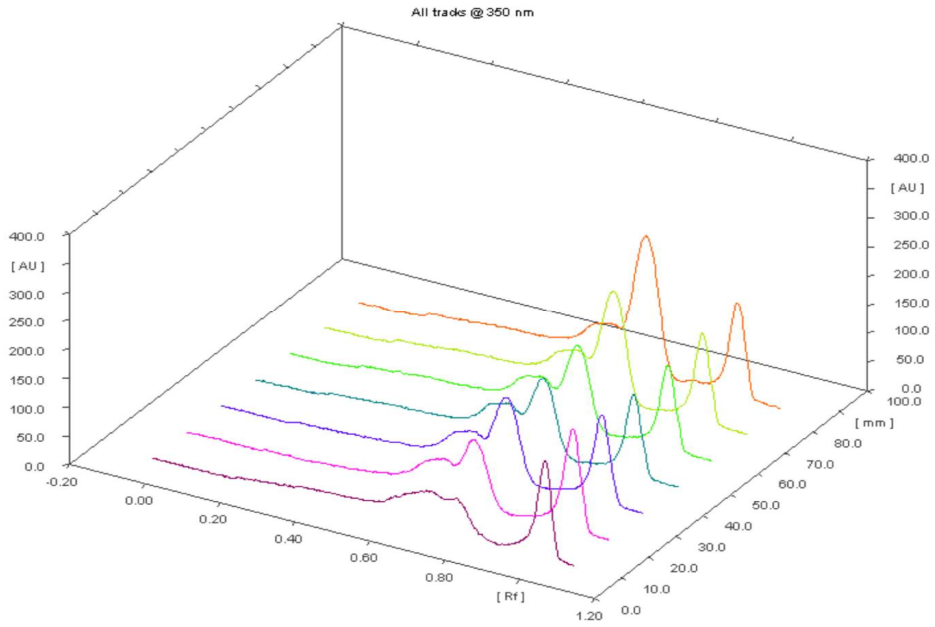


Fig. 3. Three dimensional image of the calibration spots for Berberine

6. 3.3. Validation Parameters

6. 3.3.1. Precision

The repeatability of sample application and measurement of peak area were expressed in terms of R.S.D. % and found to be 0.035 for Berberine. The results depicted in **Table 3** showed that no significant intra- and inter-day variation was observed in the analysis of Berberine at three different concentration levels 100, 300 and 500 ng/spot. The R.S.D.% for intra- and inter-day analysis was found to be less than 2% in all the cases.

Table 3: Intra- and inter-day precision of HPTLC method (n=6)

Amount (ng/spot)	Intra -day precision			Inter-day precision		
	S.D. of area	R.S.D. %	S.E.	S.D. of area	R.S.D. %	S.E.
100	0.263944	0.123156	0.1078	0.263944	0.122546	0.1078
300	0.242212	0.006253	0.09888	0.531664	0.01372	0.2171
500	0.360093	0.005056	0.1470	0.427395	0.005999	0.1745

6. 3.3.2. Robustness of the method

The standard deviation of peak areas was calculated for each parameter and R.S.D. % was found to be less than 2%. The low values of R.S.D.% as shown in **Table 4** indicated robustness of the method.

Table 4: Robustness testing (n=6)

Parameter	S. D.[a] of Peak area	R.S.D. %
Mobile phase composition	0.958263	0.170308
Amount of mobile phase	0.723484	0.127232
Temperature	1.089151	0.202707
Time from spotting to chromatography	0.410997	0.060242
Time from chromatography to scanning	0.459119	0.089389

a* Average of three concentrations 100, 300, 500 ng/spot

6. 3.3.3. LOD and LOQ

The LOD with signal/noise ratio of 3:1 was found to be 3.86 ng/spot for Berberine. The LOQ with signal/noise ratio of 10:1 was found to be 11.83 ng/spot for Berberine.

6. 3.3.4. Specificity

The peak purity of Berberine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., r (start, middle) = 0.9996 and r (middle, end) = 0.9994. Good correlation (r = 0.9996) was also obtained between standard and sample overlain spectra of Berberine **(Fig. 4)**.

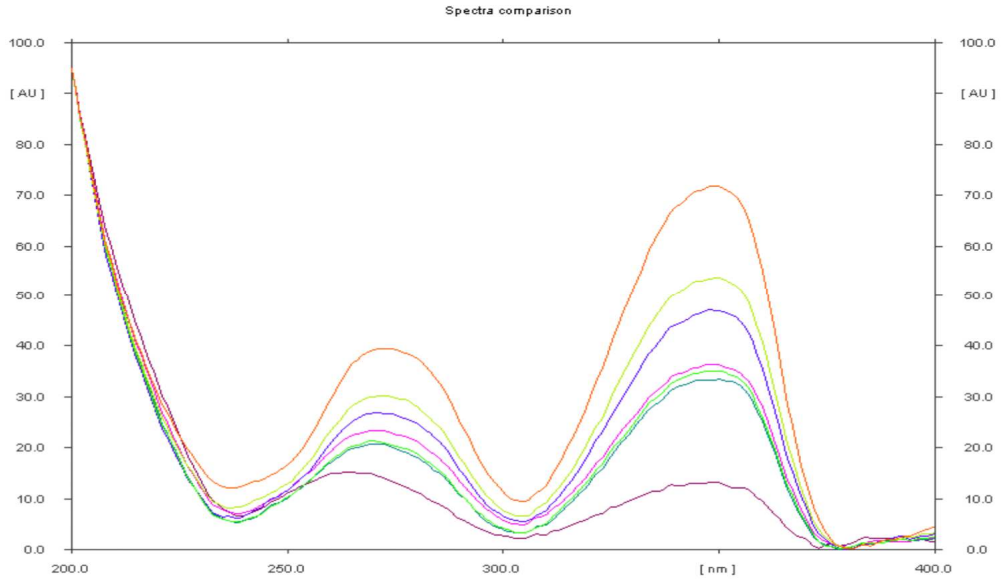


Fig. 4 Overlay spectra of Berberine Standard in AU mode, taken on the CAMAG TLC Scanner 3.

6. 3.3.5. Recovery studies

The proposed method when used for extraction and subsequent estimation of Berberine from unani formulations form after spiking with 50, 100 and 150% of additional standard Berberine afforded recovery between 98.03-100.37% **(Table 5).** The data of validation parameters are listed in **Table 6**.

Table 5: Recovery Studies (n=6)

Excess drug added to the analyte (%)	Theoretical content (ng/spot)	Content found (ng/spot)	Recovery (%)	S.D.	R.S.D. (%)	S.E.
0	100	98.03	98.03	0.2945	0.3003	0.1202
50	150	150.55	100.37	1.3619	0.9044	0.5560
100	200	197.47	98.73	0.7005	0.3547	0.2860
150	250	247.87	99.15	0.6654	0.2684	0.2716

Table 6: Summary of Validation Parameters

Parameters	**Data of Berberine**
Linearity range	100-500 ng/spot
Correlation coefficient	0.9996 ± 0.00035
Limit of detection	3.865085
Limit of quantitation	11.8295
Recovery (n=6)	99.07 %
Precision (R.S.D. %)	
Repeatability of application (n=6)	0.034967
Inter-day (n=6)	0.047422
Intra-day (n=6)	0.044822
Robustness	Robust
Specificity	Specific

6. 4. Estimation of Berberine in formulations

The spot at Rf 0.74 was observed in the chromatogram of the Berberine isolated from extract along with other components. There was no interference in analysis of Berberine from the other components present in the extract. These components appear in the chromatogram at significantly different Rf values. The total Berberine content was found to be 11.75, 12.46 (mg/gm) in lab formulations, 10.05, 11.56 (mg/gm) in marketed formulations and 16.78 (mg/gm) in crude drug **(Table 7, Fig. 5).**

Table 7: Content of Berberine in the formulations

Sample	**Berberine content [Mean** ± SD**; n=3] (mg/g)**
HLS-I	11.75 ± 0.0208
HLS-II	12.46 ± 0.0264
HMS-A	10. 05 ± 0.0015
HMS-B	11.56 ± 0.0152
Tinospora cardifolia	16.78 ± 0.0305

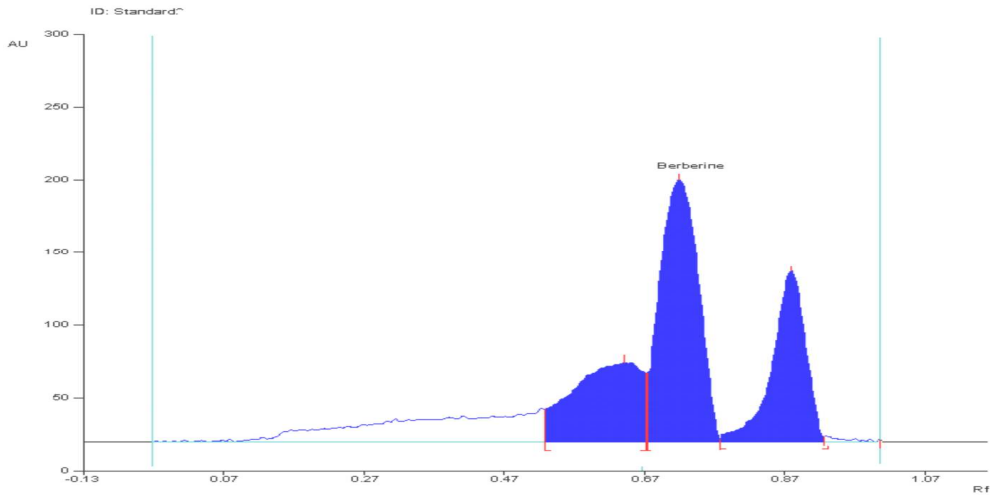


Fig. 5. HPTLC Chromatogram of Berberine extract of formulation

CONCLUSION

The developed HPTLC technique is a precise, specific, accurate and robust for the determination of Berberine. Statistical analysis proves that the method is reproducible and selective for the analysis of Berberine. Since the proposed mobile phase effectively resolves Berberine, the method can be used for quantitative analysis of Berberine in the extract of lab and marketed formulations.

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