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(RESEARCH ARTICLE)

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Validated HPTLC method development, comparative evaluation and stability study for berberine in herbal preparations

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Abstract

The study focusing on the development and validation of a high-performance thin-layer chromatography (HPTLC) method for quantifying berberine in pharmaceutical formulations. Various chromatographic parameters were optimized to ensure precise and reproducible results, including mobile phase composition, linearity range, detection wavelength, and spot characteristics. The method demonstrated high accuracy and linearity within the concentration range of 5 - 35 µg/mL, with a determined Rf value of 0.52±0.02 for berberine. Additionally, stability studies revealed that berberine underwent degradation under all stress conditions examined.

Keywords: HPTLC; Berberine; Stability Study; Quantitative Evaluation; Qualitative evaluation.

1. Introduction

Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium) is a nonbasic and quaternary benzylisoquinoline alkaloid, significant in pharmacology and medicinal chemistry. It serves as a crucial natural alkaloid for synthesizing various bioactive derivatives through condensation, modification, and substitution of functional groups at strategic positions, paving the way for designing new, selective, and potent drugs. In Ayurveda, Berberis species are traditionally employed for treating a spectrum of infections in the ear, eye, and mouth, facilitating wound healing, alleviating hemorrhoids, indigestion, and dysentery, and managing uterine and vaginal disorders. Moreover, it is used to combat obesity and serves as an antidote for scorpion stings or snakebites. Berberine extracts and decoctions are revered for their efficacy against diverse microorganisms, spanning bacteria, viruses, fungi, protozoa, and helminths, as per Ayurvedic, Chinese, and Middle-Eastern folk medicines. In Yunani medicine, Berberis asiatica finds utility in treating asthma, eye sores, jaundice, skin pigmentation, toothaches, inflammation, swelling, and ulcers. Decoctions from the roots and stem barks of Indian Berberis species like Berberis aristata, B. chitria, and B. lycium are used domestically for conjunctivitis, enlarged liver and spleen, hemorrhages, jaundice, and skin diseases. Additionally, a concoction of Indian barberry and Emblic myrobalan mixed with honey is employed to treat urinary disorders, specifically painful micturition[1-8]

1.1. Analytical Chemistry

Analytical chemistry encompasses the identification and quantification of substances within samples or mixtures. Its techniques involve processes for identifying, quantifying, and purifying substances, as well as separating components of solutions or mixtures, and determining the structure of chemical compounds. Analytical methods can be classified into two categories: qualitative, focusing on identification, and quantitative, centered on determining the amount of components present. [9-13]

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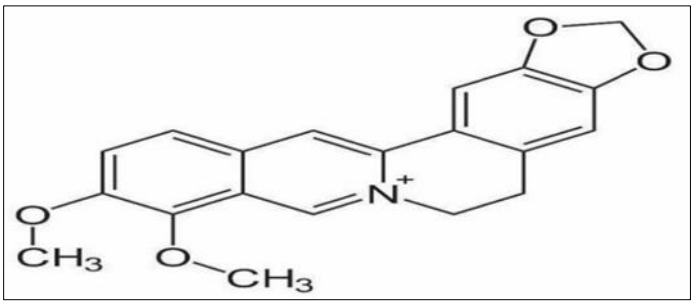


Figure 1 Chemical structure of Berberine

2. Materials and methods: materials and equipments

The drugs, chemicals, reagents, instruments and filters used during the experiment.

Berberine were purchased from Xyrex Pharmaceutical pvt. ltd.

Equipment: A CAMAG HPTLC system comprising of a Linomat V applicator and CAMAG HPTLC scanner and single pan balance of Shimadzu model was used, for the present study.

Chemicals: Analytical grade Toluene, Ethyl ether,Methanol, Formic acid, formic acid was obtained from Loba & CDH Chemical Pvt Ltd. Stationary phase used was silica gel G60F254, 20x10 cm TLC plate were obtained from E. Merk Ltd (Mumbai, India).

2.1. Instruments Used

2.1.1. Procrurement of drug sample

Table 1 Chemicals and Suppliers

Chemicals	Supplier
Berberine	Zyerx Chemical Pvt .Ltd
Methanol	Loba Chemical Pvt .Ltd
Hydrochloric acid	Loba Chemical Pvt. Ltd
Ethanol	CDH Chemical Pvt .Ltd
Toluene	Loba Chemical Pvt .Ltd
Ethyle-ether	CDH Chemical Pvt .Ltd
Formic acid	CDH Chemical Pvt .Ltd
Water	Fusion Pharma

Table 2 System and Instrument

Sr.no	Particulars	Details
1.	System	CAMAG
2.	Model no	CAMAG Linomat V Sample Applicator
3.	Detector	CAMAG UV Cabinet
4.	Pump	TLC Silica gel 60 F 254
5.	Column	CAMAG TLC Scanner 3
6.	Software	Wincat
7.	chamber	CAMAG Twin Plate Development Chamber

3. Experimental work [14]

Standardization parameters were established for qualitative evaluation:

3.1. Organoleptic Evaluation

Organoleptic evaluation involves assessing the drug based on its color, odor, taste, etc., utilizing our sensory organs.

- Appearance: Visual examination was conducted to assess the appearance.
- Color: The dried sample was placed in a test tube, and its color was observed in sunlight.
- Odour: Evaluation of odour was performed using a freshly prepared sample.
- Taste: The taste was evaluated using a freshly prepared sample.

Table 3 Organoleptic Properties of Berberine Drug

Sr no.	Sample	Appearance	Colour	Taste	Odour
1	Berberine	Powder	Yellow-brown	Bitter	Woody

3.2. Microscopical Evaluation: [14]

3.2.1. Phytochemical Investigation

- Detection of Alkaloids

(Hager's Test) A small amount of the drug was taken in a test tube, Hager's reagent was added, shaken well, and filtered. The formation of a yellowish color indicated the presence of alkaloids.

- Detection of Carbohydrates

(Seliwanoff's Test) The drug was heated with a solution of phenylhydrazine hydrochloride, sodium acetate, and acetic acid. The formation of yellow crystals indicated the presence of carbohydrates.

- Detection of Proteins

(Xanthoproteic Test) A small quantity of the test solution was taken, and 1 mL of nitric acid was added. The mixture was boiled, resulting in the formation of a yellow precipitate. Upon cooling, 40% sodium hydroxide solution was added, resulting in the formation of an orange color.

- Detection of Flavonoids

(Alkaline Reagent Test) A small quantity of the test solution was taken, and a few drops of sodium hydroxide solution were added. The appearance of an intense yellow color, which turned colorless upon addition of dilute acid, indicated the presence of flavonoids.

- Detection of Tannins

(Ferric Chloride Test) The extract was treated with a ferric chloride solution. The appearance of a blue color indicated the presence of hydrolysable tannins, while a green color indicated the presence of condensed tannins.

- Detection of Cardiac Glycosides

(Baljet's Test) The test solution was treated with picric acid and shaken well. The formation of a yellowish-brown color indicated the presence of glycosides.

Table 4 Observations and Results of Phytochemical Investigation

Sr no.	Test	Observation	Result
1.	Alkaloids	Yellowish coloured was observed	Present
2.	Glycosides	No Yellowish brown coloured was observed	Absent
3.	Carbohydrates	No Yellow coloured was observed	Absent
4.	Proteins	No Yellow coloured was observed	Absent
5	Flavonoids	No Yellow coloured was observed	Absent
6.	Tannis	No Blue coloured was observed	Absent

3.2.2. Determination of Physical Characteristics of Powder

Bulk Density and Tap Density

- Bulk Density

The term bulk density refers to a measure used to describe the packing of particles or granules. It is calculated using the formula: Bulk Density (Db) = M/Vb, where M is the mass of the particles and Vb is the total volume of the packing.

- Tap Density

Tap density refers to the density of a powder after it has been tapped or compacted. It is typically determined using an apparatus consisting of a graduated cylinder mounted on a mechanical tapping device (Jolting Volumeter) with specially cut rotating. 100 grams of the weighed formulation powder was carefully added to the cylinder with the aid of a funnel. The initial volume was noted, and then the sample was tapped until no further reduction in volume was observed. The initial volume provided the bulk density value, and after tapping, the volume decreased, providing the value of tapped density.

Table 5 Observations and results bulk density of berberine

Sr.no	Sample	Mass of powder (gm)	Bulk volume of powder (ml)	Bulk density (g/cc)
	Berberine	20	36	0.555

Table 6 Observations and results tapped density of berberine

Sr. no	Sample	Mass of powder(gm)	Tapped volume of powder (ml)	Tapped density (g/cc)
	Berberine	20	27	0.740

3.2.3. Angle of Repose

The angle of repose is defined as the internal angle between the surface of a pile of powder and the horizontal surface. To measure it, the powder is passed through a funnel fixed to a burette at a height of 4 cm. Graph paper is placed below the funnel to collect the powder. The height (H) and radius (r) of the resulting pile are measured.

The angle of repose of the powder is then calculated using the formula: Angle of

Repose = tan^(-1)(H/r).

Table 7 Observation and Result Angle of repose of Bereberine.

Sr. no	Sample	Height of piles (cm)	Radius of circle(cm)	Angle of repose $\tan^{1}(h/r)$	Flow
	Berberine	2.8	4.7	30.75	Excellent

3.2.4. Carr's Index

Carr's Index is a measure of the propensity of a powder to be compressed. It is determined based on the apparent bulk density and tapped density of the powder. The percentage compressibility of the powder can be calculated using the following formula:

% compressibility = [(tapped density - bulk density) / tapped density] × 100

Table 8 Observation and Result Carr's Index of Berberine

Sr. no	Sample	Tapped density	Bulk density	Carrs index
1.	Berberine	0.740	0.555	25.0

3.2.5. Physical Evaluation

Physical standards are determined for drugs, although they may not be constant for crude drugs. These standards aid in evaluation, particularly regarding moisture content, drug gravity, density, optical rotation, refractive index, melting point, and solubility in different solvents.

- Solubility

Solubility studies help indicate the presence of adulterants in a drug. Berberine is soluble in organic solvents such as ethanol, methanol, and dimethylformamide and sparingly soluble in hot water.

- Melting Point

Determining the melting point is crucial for identifying a sample's purity and thermal stability. The method involves placing the sample in a capillary tube and heating it until it reaches its melting point. The melting point of Berberine is between 143-145°C.

- Moisture Content (Loss on Drying)

The drying oven method, a thermogravimetric technique, is employed to determine moisture content (Loss on Drying). In this method, the sample is dried for a specified period at a constant temperature. The moisture content is then calculated by weighing the sample before and after drying and determining the difference.

Table 9 Observations and results of loss on drying

Sr. no	sample	Weight of crucible of sample	Weight of crucible after incineration	Percentage (%)	Mean $\pm$ SD
1.	Berberine	5	4,2	0.84%	
2.		5	4.0	0.8%	0.8$\pm$ 0.04
3.		5	3.8	0.76%	

- Determination of Total Ash

About 2 to 3 grams of the sample are accurately weighed in a tarred silica dish and incinerated at a temperature not exceeding 450°C until it is free from carbon. The dish is then cooled and weighed again. The percentage of total ash is calculated with reference to the weight of the air-dried sample.

Total ash (%) = [(Weight of ash - Weight of dish) / Weight of sample taken] * 100

Table 10 Observations and results of total ash

Sr no	Sample	Wt.of crucible with sample (g)	Wt.of crucible after incineration	Wt. of ash (z)	Percentage of ash	Mean+SD
1	Berberine	34	33.86	0.14	7	
2		34	33.89	0.11	5.5	5.6+1.25
3		34	33.91	0.09	4.5	

3.3. Quantitative Evaluation

HPTLC Method Development ,Validation and Stability Study [15-25]

3.3.1. Preparation of Standard Solutions

Solution A: Approximately 25 mg of berberine was accurately weighed and dissolved in a 25.0 mL volumetric flask using methanol. The volume was adjusted to the mark with the same solvent, resulting in a concentration of 1 mg/mL.

Solution B: 5.0 mL of Solution A was accurately measured and further diluted in a 50.0 mL volumetric flask with methanol, resulting in a concentration of 100 µg/mL.

Solution C: 10.0 mL of Solution A was accurately measured and further diluted in a 50.0 mLvolumetric flask with methanol, resulting in a concentration of 200 µg/mL.

3.3.2. Selection of Mobile Phase

Solution B of Berberine was applied to TLC plates, and various solvents of different polarities were tested individually and in combination to optimize plate development for sharp, stable, and distinct peaks. The mobile phase consisting of toluene:ethyl acetate:methanol:formic acid (4:4:2:5 drop v/v) was found to be the most satisfactory and suitable for further experimentation.

3.3.3. Selection of Detection Wavelength for Densitometric Evaluation of Sample Spots:

After chromatographic development and plate drying, bands were scanned over a wavelength range of 350-450 nm. The wavelength selected for detection was 366 nm.

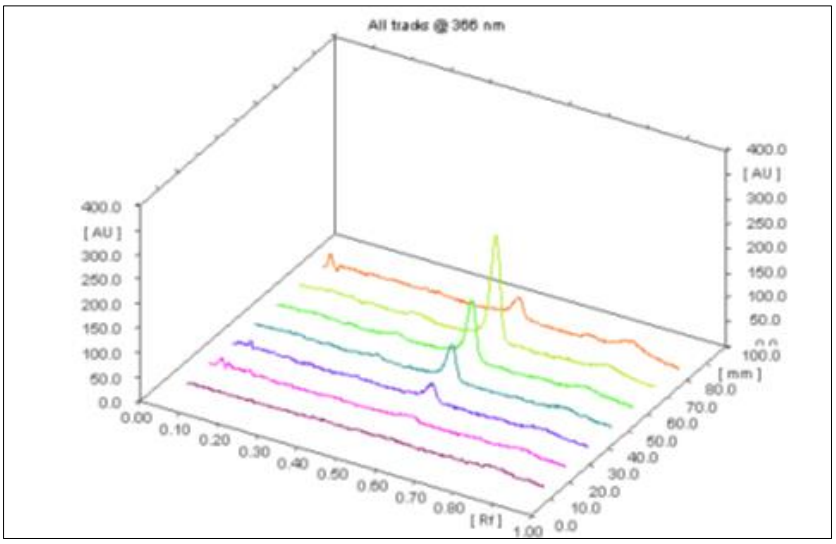


Figure 2 UV Spectra 366 nm.

3.3.4. Finalized Chromatographic Conditions

The chromatographic conditions were optimized by evaluating various parameters and were kept constant for further experimentation:

- Samples were applied as 6 mm wide bands with a spacing of 6 mm using a micro syringe (Hamilton, Switzerland) on pre-coated silica gel aluminum TLC plates 60F_{24} (20 x 10 cm, Merck) with a thickness of 250 µm using a CAMAG LINOMAT V automatic sample applicator.

- Slit dimensions of 3.0 x 45 mm and a scanning speed of 20 mm/s were employed in the analysis.

- Linear ascending development was conducted in a twin-trough glass chamber (10 x 10 cm, Camag, Switzerland) using toluene: ethyl acetate: methanol: formic acid (4:4:2:5 drop v/v) as the mobile phase.

- Chamber saturation time was 30 minutes, migration time was 15 minutes, and migrating distance was 75 mm. After application, TLC plates were dried using a dryer with a current of air.

- Densitometric scanning was conducted using the CAMAG TLC SCANNER 3 at 254 nm, employing WINCATS software. The scanner utilized a deuterium lamp as a radiation source emitting a continuous UV spectrum ranging from 350 to 450 nm.

3.4. Construction and Study of Calibration Curve

Following the outlined procedure, calibration curves were constructed by spotting 5-35µL of solution B on TLC plates, achieving a concentration range of 50 to 350 ng/band. The plates were developed and scanned under the optimized chromatographic conditions. This process was repeated three times, and the mean peak height and peak area were recorded for different drug concentrations. Calibration curves were then constructed by plotting concentration versus peak height and peak area. The resulting calibration curves are depicted below.

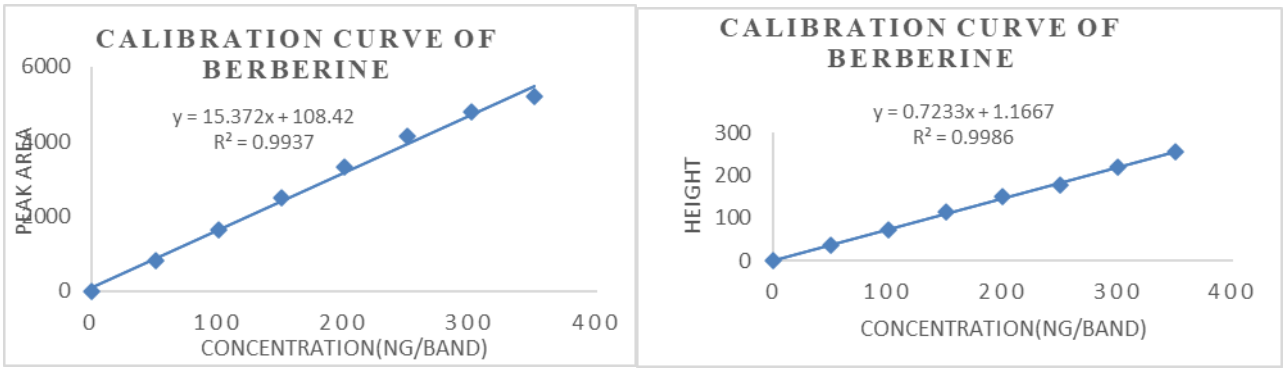


Figure 3 Calibration curve for Berberine Conc. Vs AUC and Conc. Vs Height

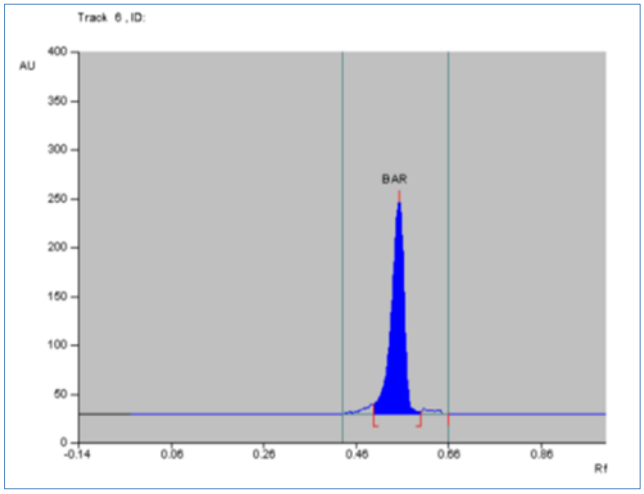


Figure 4 Typical densitogram of standard is shown in Figure 5.2 (Rf = 0.52)

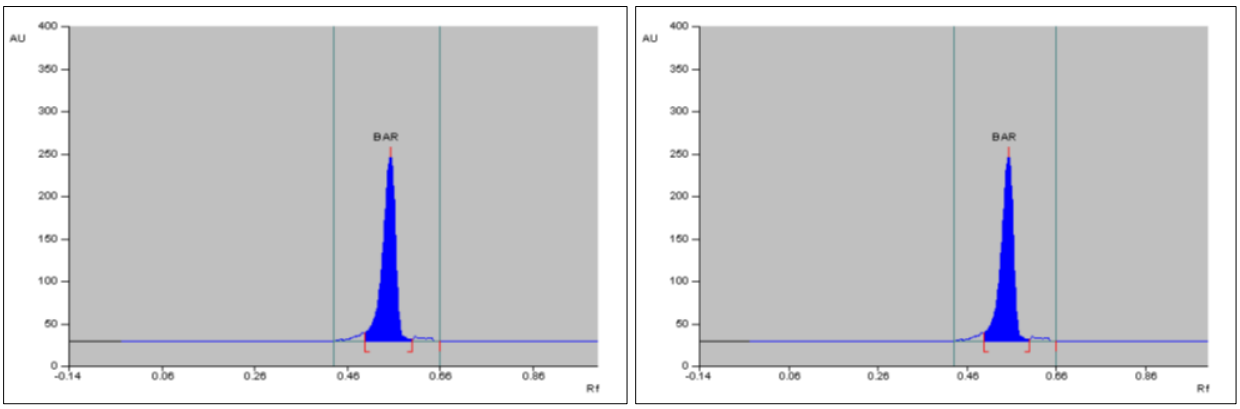


Figure 5 Typical densitograms of sample 1 (Rf = 0.52) and 2 is shown in Figure 5.3(Rf = 0.52)

3.5. Observations and results of marketed formulation analysis

Table 11 Observations and results of marketed formulation analysis sample 1

Trade Name: Nutrija Lifesciences				**Average weight: 820**			
Sr.No	Wt of Capsule taken (mg)	CA Estimated in (µg)		Amount estimated in average wt of capsule		% Labeled Claim*	
		Height	Area	Height	Area	Height	Area
1	8.37	218.24	4808	217.29	4806	100.43	100.04
2	8.39	220.40	4892	219.74	4872	100.30	100.41
3	8.375	219.90	4898	219.10	4876	100.36	100.45
4	8.395	224.60	4925	224.30	4910	100.13	100.30
5	8.310	239.90	4984	239.10	4974	100.33	100.20
					Mean	100.31	100.28
					S.D	0.111579	0.165981
					C.V	0.0010%	0.0015%
					R.S.D	0.111%	0.166 %
					S.E	0.049899	0.07422

Table 12 Observations and results of marketed formulation analysis

Trade Name: Himalaya				**Average weight: 620 mg**			
Sr.No	Wt of Capsule taken (mg)	CA Estimated in (µg)		Amount estimated in average wt of capsule		% Labeled Claim*	
		Height	Area	Height	Area	Height	Area
1	6.37	245.68	5104.2	244.68	5101.0	100.40	100.06
2	6.39	246.55	5125.4	246.10	5110.4	100.18	100.29
3	6.352	245.98	5114.6	244.23	5109.9	100.71	100.09
4	6037	246.59	5129.8	244.90	5112.4	100.69	100.34
5	6.32	247.10	5139.9	246.10	5111.9	100.40	100.547
					Mean	100.476	100.262
					S.D	0.1998	0.1780
					C.V	0.139%	0.175%
					R.S.D	0.222 %	0.153 %
					S.E	0.9999	0.0890

3.6. Validation parameters

Validation of the proposed method was carried out as per the USP guidelines.

- Accuracy

Accuracy of the proposed method was ascertained on the basis of recovery studies. Recovery studies were performed by standard addition method.

Table 13 Accuracy

Label claim(mg in capsule)	**Initial amount in µg**	**Amount of standard drud added (µg)**	**Total amount recoverd**	**% Recovery**
	300	0	299	99.66
	300	200	501	100.22
300	300	250	551	100.1
	300	300	599	99.83
			Mean	99.94
			S.D	0.2594
			C.V	0.0026
			R.S.D	0.26%
			S.E	0.12971

- Precision

Precision of an analytical method is expressed as S.D. or R.S.D. of series of measurements. It was ascertained by replicate estimation of the drugs by proposed method. The results of estimation are shown in Table 5.1

- Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot of Quercetin in sample was confirmed by comparing the Re and spectra of spot with that of standard. The peak purity was assessed by comparing the spectra at three different levels peak start (S), peak apex (M) and peak end (E).

- Robustness and ruggedness

Robustness is the measure of capacity of a method to remain unaffected by small but deliberate variations in the method conditions and is an indication of the reliability of the method. To study the robustness of the method, small but deliberate variations in mobile phase composition (±2%) chamber saturation period (±10%), development distance (±10%), time from application to development (0, 10, 15 20 min), time from development to scanning (0, 30, 60, 90 min) were carried out. Results of robustness studies are given in Table 5.4

Table 14 Results of robustness studies

i) Chromatographic changes (% toluene in mobile phase)

% in mobile phase	**Rf ***
-2%	0.51
0%	0.53
2%	0.54
Mean+-S.D	0.5233 +- 0.0208

ii) Chromatographic changes (chamber saturation)

Chamber saturation (time in min)	Rf *
23	0.54
20	0.53
17	0.52
Mean+-S.D	0.523 +- 0.0208

iii) Chromatographic changes (development distance)

Development distance (mm)	Rf *
75	0.54
70	0.53
65	0.50
Mean+-S.D	0.523 +- 0.0206

iv) Chromatographic changes (time from application to development)

Time from application to development	Rf *
10	0.57
20	0.52
30	0.53
Mean+-S.D	0.52 +- 0.01

v) Chromatographic changes (time from development to scanning)

Time from development to scanning	Rf *
10 min	0.50
20 min	0.52
30 min	0.53
Mean+-S.D	0.5766 +- 0.0152

4. Results of ruggedness studies

The rugedness of the method was studied under three different parameters.

4.1. Intraday variation

The samples were analysed on different times on same day by proposed method. The percent labeled claim was calculated and results of estimation proposed method.

Table 14 Results and Statistical data for intraday study

Time	**%Labeled claim***	
	Height	Area
Time – 1	100.40	100.06
Time – 2	100.18	100.29
Time – 3	100.71	100.09
Mean	100.43	100.1466
S.D	0.2663	0.1250
C.V	0.002165	0.001019
R.S.D	0.265%	0.125%
S.E	0.1537	0.0721

ii) Interday variation

The samples were analysed by proposed method on three different days (1st 3rd & 5th day). The percent labeled claim was calculated and results of estimation are shown

Table 15 Results and Statistical data for interday study

Days	**%Labeled claim***	
	Height	Area
Day – 1	100.71	100.09
Day- 2	100.69	100.34
Day – 3	100.40	100.547
Mean	100.6	100.325
S.D	0.17349	0.2289
C.V	0.001408	0.001862
R.S.D	0.172%	0.228%
S.E	0.1002	0.1321

iii) Different analysts

The samples were analysed by three different analysts as per the proposed method. The percent labeled claim was calculated and results of estimation are shown

Table 16 Results and statistical data for different analysts

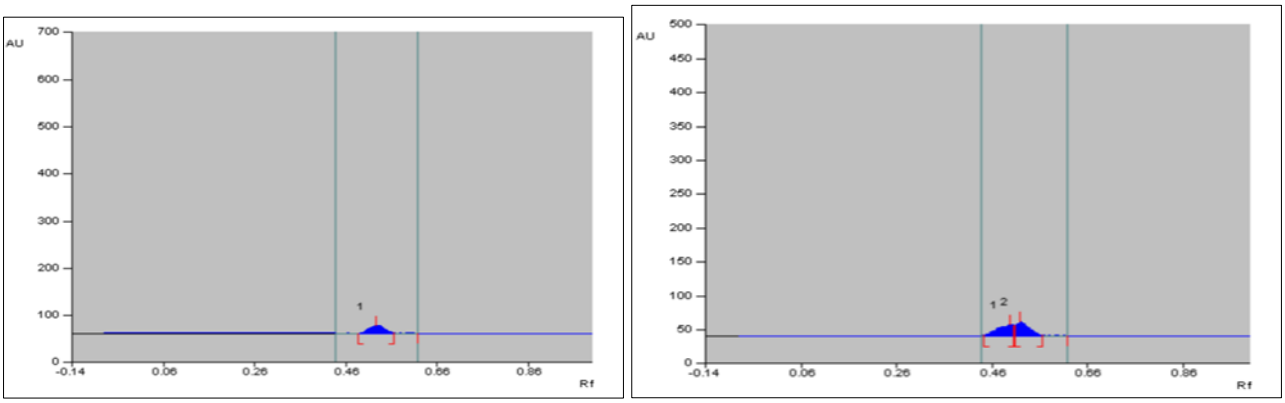
Analysts	**%Labeled claim***	
	Height	Area
Analyst - 1	100.71	100.09
Analyst - 2	99.12	99.78
Analyst – 3	100.05	100.02
Mean	99.96	99.96
S.D	0.7988	0.1626

C.V	0.006525	0.001328
R.S.D	0.799%	0.163%
S.E	0.4611	0.0939

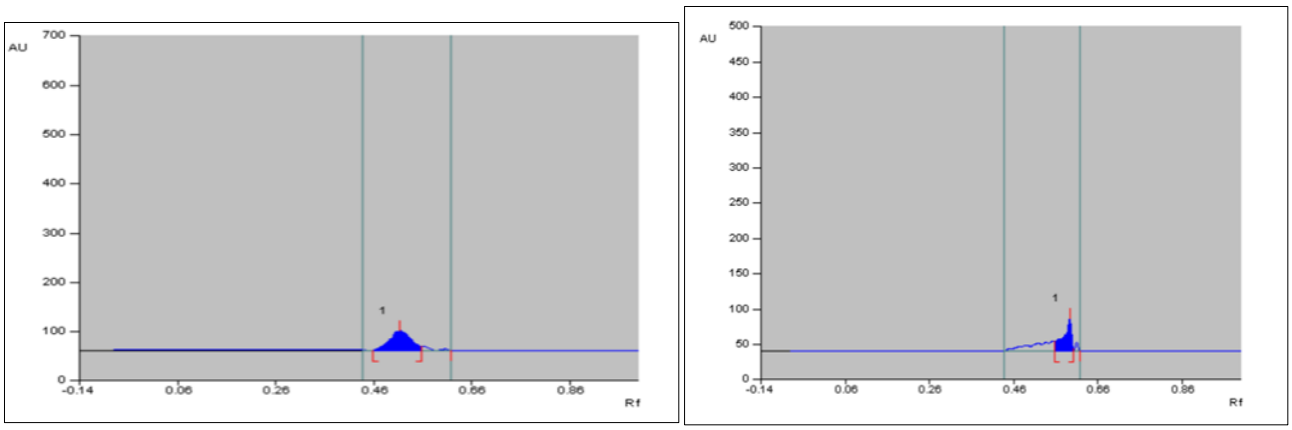
4.2. Forced degradation of berberine [265-34]

In order to ensure the stability indicating property and specificity of the proposed method stress studies were performed. In all following degradation studies, the average peak area Berberine of was determined by applying 200 ng/band .

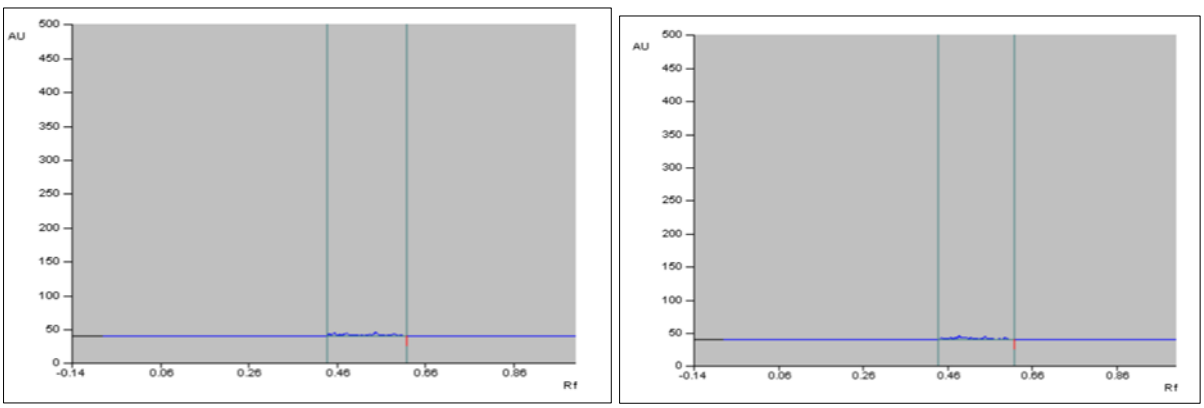
4.2.1. Acid and base induced degradation



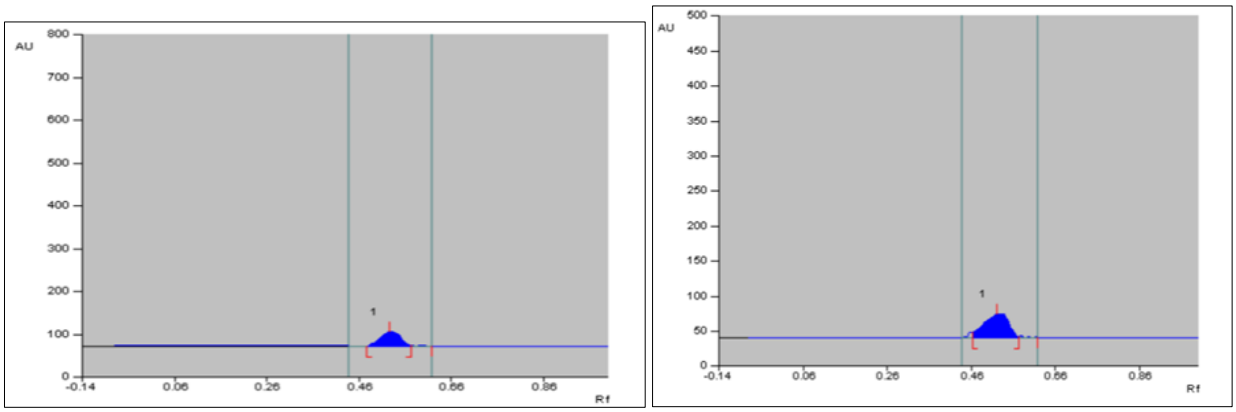
Base induced degradation



Hydrogen peroxide-induced degradation



Neutral degradation



Neutral induced degradation

Figure 5 Densitogram of Berberine stress condition medium marketed 1 and 2

Table 17 Results of forced degradation of marketed formulation

Sr. No	Degradation condition	concentration used(µg/ml)	Sample1 (% Recovery)	Sample 2 (% Recovery)
1	Acid Hydrolysis	30	9.7631 %	9.912 %
2	Alkali Hydrolysis	30	29.35 %	14.27 %
3	Oxidative Hydrolysis	30	0 %	0 %
4	Neutral Hydrolysis	30	33.23 %	24.30 %

5. Conclusion

In this study, we developed and applied a validated HPTLC (High-Performance Thin-Layer Chromatography) method for the quantitative estimation of Berberine in pharmaceutical marketed formulations. The chromatographic conditions were meticulously optimized, considering various parameters such as mobile phase composition, linearity range, detection wavelength, band size of the spots applied, chamber saturation time, solvent front migration, and slit width. These optimizations were crucial to ensure the accuracy and reproducibility of the results.

The Rf value obtained for Berberine was found to be 0.52 ± 0.02, indicating its characteristic migration behavior under the specific chromatographic conditions employed. The linearity of the method was established over a concentration range of 5 - 35 µg/mL, with high correlation coefficient values of 0.9993 and 0.9969 obtained for height and area, respectively. These high correlation coefficient values signify the excellent linear relationship between the concentration of Berberine and its corresponding response in the chromatogram.

The method exhibited high accuracy, with the results showing an accuracy of 99.94 ± 0.2594%. This high level of accuracy indicates the reliability of the method in quantifying Berberine content in the tested formulations.

Furthermore, degradation studies were conducted to assess the stability of Berberine under various stress conditions. It was observed that Berberine underwent degradation in all stress conditions tested, highlighting the importance of monitoring its stability in pharmaceutical formulations.

Overall, the developed HPTLC method proved to be robust, sensitive, and suitable for the quantitative analysis of Berberine in pharmaceutical formulations. The optimized chromatographic conditions, along with the observed accuracy and reproducibility, validate the applicability of this method for routine quality control analysis in the pharmaceutical industry.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Reference

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