Development and Validation of a Method for Densitometric Analysis of Berberine in Herbal Extract and Polyherbal Formulation

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Key Words

High-performance thin-layer chromatography, validation · Berberine · *Berberis aristata*

Abstract

Objective: To develop a new high-performance thin-layer chromatography (HPTLC) method for the quantification of berberine in herbal extract and pharmaceutical dosage form. **Materials and Methods:** The HPTLC was performed on aluminium foil plates coated with 200 µm silica gel 60F₂₅₄. Linear ascending development with toluene:ethyl acetate:formic acid:methanol 9:9:3:1 (v/v/v/v) was performed at room temperature (25 ± 2°C) in a twin-trough glass chamber saturated with mobile-phase vapour. Compact bands (R_F 0.58 ± 0.02) were obtained for berberine. Spectrodensitometric scanning was performed in fluorescence mode at 350 nm. The method was validated for precision, recovery, robustness, specificity, and detection and quantification limits, in accordance with International Conference on Harmonization guidelines. **Results:** Linear regression analysis of the calibration plots showed a good linear relationship ($r^2 = 0.9996 \pm 0.0001$) between peak area and concentration in the range 10–100 ng/band, respectively. The limits of detection and quantification were 2.8 and 9.3

ng/band. The recovery of the method was 98.5–100.6%. **Conclusion:** The above method was a rapid and cost-effective quality-control tool for routine analysis of berberine in herbal extracts and in pharmaceutical dosage form.

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Introduction

Berberis aristata (Berberidaceae), commonly known as daruharidra, is commonly used in the Indian traditional system of medicine for the treatment of skin, ophthalmic and hepatic disorders [1, 2]. Daruharidra is an official herbal drug in the treatment of skin disorders [3] and the plant is reported to possess tonic, stomachic, astringent, antiperiodic, diaphoretic, antipyretic and purgative components [4]. *B. aristata* root is mainly recognized for its isoquinoline alkaloids like berberine, barbamine, aromoline, karachine, palmatine, oxyacanthine, and oxyberberine [5]. Berberine is pharmacologically most active among the other phytoconstituents and exhibits antimicrobial, antidiabetic, hepatoprotective and anticancer activities [6–9].

A limited number of high-performance thin-layer chromatography (HPTLC) methods have been used for

the quantitative determination of berberine in *B. aristata* [10] and in other plant extracts [11–13]. The berberine concentrations were also determined by UV spectrophotometry [14], ^1H NMR [15] and liquid chromatography coupled with different types of detectors [16–22]. Even though these analytical procedures are suitable for the detection of berberine in samples originating from plants, they have limitations with respect to their applications in the determination of berberine in plant samples. The reported colorimetric method lacks sensitivity and is tedious and time-consuming. Even though high-performance liquid chromatography (HPLC) is a method of choice, it is limited by extensive sample clean-up and requires expensive solvents and longer periods of column stabilization. In comparison to HPLC, HPTLC is a versatile analytical technique that requires less expensive instrumentation and expertise.

An earlier developed method based on HPTLC wherein berberine was detected in *B. aristata* and other plant extracts by using butanol:acetic acid:water as a developing solvent system was unsatisfactory because flow rate was slow, and it was reported to take about 4 h to run 10 cm [23]. The present study was therefore carried out to develop a rapid, sensitive and accurate analytical method for estimation of berberine in *B. aristata* and its pharmaceutical dosage form for the routine analysis of a large number of plant extract samples and their formulations.

Materials and Methods

Chemicals and Standards

The berberine standard was obtained from Yucca Enterprises, Mumbai, India. The daruharidra extract was purchased from Herbal Extract Supplier (Bangalore, India). Different marketed formulations containing daruharidra extract were obtained from a local market in the form of tablets and capsules. All the chemicals and reagents used were of analytical grade and were purchased from Merck Ltd, Mumbai, India.

Preparation of Standard and Test Solution

A stock solution of berberine was prepared in methanol at a concentration of 1 mg/ml. The standard solutions were prepared from the stock solution by appropriate dilution with the same solvent. 20 tablets or capsules were weighed accurately and finely powdered. Powder equivalent to 50 mg of daruharidra was accurately weighed and transferred to a 25 ml volumetric flask and about 15 ml of methanol was added. The flask was sonicated for 20 min in an ultrasonic water bath and diluted up to the mark with methanol. Then 1 ml of this solution was transferred to a 10 ml volumetric flask and 9 ml volume with methanol was added, then filtered through Whatman No. 41 filter paper and used for further chromatographic analysis.

Chromatography

The HPTLC was performed on 20×10 cm aluminium foil plates coated with a $200 \mu\text{m}$ layer of silica gel 60F₂₅₄ (E. Merck, Germany). Samples were applied as 6-mm bands, by means of a Camag (Switzerland) Linomat 5 applicator fitted with a 100 μl syringe. A constant application rate of 150 nl/s was used. Linear ascending development with toluene:ethyl acetate:formic acid:methanol 9:9:3:1 (v/v/v/v) as a mobile phase was performed in a glass twin-trough chamber (Camag) previously saturated with solvent for 5 min (optimized chamber saturation time) at room temperature and relative humidity $55 \pm 5\%$. The development distance was 80 mm. After development the plates were air-dried. Densitometric scanning was then performed at 350 nm with a Camag TLC Scanner 3 in fluorescence mode operated by winCATS software (v. 1.4.3.6336). The slit dimension was 5×0.45 mm with a scanning speed of 20 mm/s. The proposed method was validated as per International Conference on Harmonization guidelines [24].

Linearity

The stock solution (10 $\mu\text{g/ml}$) of berberine was prepared in methanol. Different volumes of the stock solution (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μl) were located to a plate to provide 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ng berberine/band, respectively. Each volume was applied three times on the TLC plate. Peak area data and the corresponding amounts were treated with linear least-square regression analysis.

Method Validation

Precision. The precision of the system was determined by measuring repeatability of sample application and measurement of peak areas for seven replicates of the same band (50 ng berberine/band). To evaluate intra-day precision, seven samples at three different concentrations (30, 50 and 80 ng/band) were prepared and analyzed on the same day. The inter-day precision was studied by comparing the assay performed on the three different days. Precision of the method was determined with the product against standard berberine. An amount of the product powder equivalent to 100% of the label claim of berberine was accurately weighed and assayed. The precision of the method and system were expressed as relative standard deviation (RSD, %) and the standard error (SE) of peak area.

Robustness. Following the introduction of small changes in the mobile-phase composition ($\pm 5\%$ for each component), the effect on the result was examined. Mobile phases having different compositions, e.g. toluene:ethyl acetate:formic acid:methanol 8.5:9.5:3:1 v/v/v/v, 8.5:9:3.5:1 v/v/v/v, 8.5:9:3:1.5 v/v/v/v, 9.5:8.5:3:1 v/v/v/v, 9.5:9:2.5:1 v/v/v/v, and 9.5:8.5:3:0.5 v/v/v/v were tried. The volume of mobile phase and duration of saturation investigated were 20 ± 2 ml (18, 20 and 22 ml) and 5 ± 1 min (4, 5 and 6 min), respectively. The robustness of the method was measured for three different amounts of berberine: 30, 50, and 80 ng/band.

Limits of Detection and Quantification. Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ. The LOD and LOQ were determined by measuring the magnitude of the analytical background by spotting a blank and calculating the signal-to-noise ratio for berberine by spotting a series of solutions until the signal-to-noise ratio 3 for LOD and 10 for LOQ were obtained. To determine the LOD and

LOQ, serial dilutions of standard solutions of berberine were made from the standard stock solution in the range of 1–100 ng/spot. The diluted standard solutions were applied to a TLC plate and the chromatogram was run and the measured signal from the standard was compared to those of blank samples.

Recovery Study. Recovery study was performed to confirm accuracy and suitability of the method. The daruharidra placebo (identical formulation without active ingredient) was prepared in-house. The placebo samples were spiked at 80, 100 and 120% level of working concentration with standard berberine, then 5 µl of each solution was applied on a TLC plate. The mixtures were analyzed (n = 6) by the proposed method.

Specificity. The specificity of the method was ascertained by analyzing a standard drug sample of equivalent concentration (50 ng/band) and placebo. The band for the berberine in the sample was confirmed by comparing the R_F value and spectra of the band from the samples and from the standard as well as absence of any band in placebo in the same R_F value also help in the confirmation of band for the standard. The peak purity of berberine was assessed by comparing the spectra at three levels, i.e. peak start, peak apex, and peak end.

Analysis of Herbal Extract and Pharmaceutical Dosage Form. Five microlitres of standard, daruharidra extract, tablet dosage form (*B. aristata*, *Embllica officinalis*, *Rubia cordifolia* and *Acacia catechu*) and placebo were spotted in the same plate (20 × 10 cm), then developed and scanned by the proposed chromatographic conditions above. The analysis was repeated in triplicate. The possibility of interference of other phytoconstituents or excipients with the analysis was studied.

Results

A variety of mobile phases that included toluene:ethyl acetate, toluene:ethyl acetate:acetic acid, or toluene:ethyl acetate:formic acid were investigated for separation of the berberine spot from other components in herbal extract and excipients present in different commercial polyherbal formulations. The mixture toluene:ethyl acetate:formic acid:methanol 9:9:3:1 (v/v/v/v) yielded the most satisfactory resolution of berberine with good peak shape. The R_F value for berberine was 0.58 ± 0.02 , with resolution of 2.14. Pre-saturation of the TLC chamber with a mobile phase for 5 min ensured good migration reproducibility of berberine and better resolution.

Linearity

Calibration plots of peak area against concentration were linear in the range 10–100 ng for berberine. The calibration lines were represented by linear equation: $y = 21.310 + 1,149.3x$, where y is response and x is amount (table 1). For this equation the correlation coefficient, r^2 , was 0.9980 ± 0.0003 .

Table 1. Summary of validation data for estimation of berberine in the herbal extract and its polyherbal formulations

Parameters			Berberine
Linear range, ng/band			10–100
Correlation coefficient, r^2			0.9996
<i>Precision</i>			
Repeatability of sample application, % (n = 7)			0.20
Repeatability of measurement of peak area, % (n = 7)			0.18
Method precision, % RSD (n = 6)			0.22
Limit of detection			2.8 ng
Limit of quantification			9.3 ng
Specificity			specific
<i>Recovery study</i>			
Level	Theoretical yield, mg	Actual yield, mg	% recovery $\pm$ SD
80%	2.0	1.988	99.46 $\pm$ 0.27
100%	2.5	2.515	100.64 $\pm$ 0.17
120%	3.0	2.955	98.52 $\pm$ 0.15

Precision

The RSD (%) for the repeatability of the assay method, sample application (50 ng/band) and measurement of peak areas were 0.22, 0.20 and 0.18%, respectively. Measurement of the peak area at three different concentrations resulted in low values of SE and RSD (<1%) for inter- and intra-day variation, which suggested the precision of the method was excellent (table 2).

Robustness

The SD, RSD (%) and SE of the peak area were calculated for changes in mobile-phase composition, mobile-phase volume and duration of saturation at three concentration levels (30, 50 and 80 ng/band). The low values of SD, RSD (%) and SE obtained after introducing small deliberate changes in the method indicated the robustness of the method (table 3).

LOD and LOQ

LOD represented the lowest concentrations of berberine that was detected, whereas the LOQ represented the lowest concentrations of berberine that was determined with acceptable precision and accuracy. The signal-to-noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were 2.8 and 9.3 ng/band for berberine.

Fig. 1. a Comparison of spectra of berberine standard and berberine from extract and pharmaceutical dosage form. **b** Three-dimensional overlay chromatograms showing the specificity of the method in presence of standard berberine, daruharidra extract, polyherbal formulation and daruharidra placebo (x-axis: retention factor in the plate).

Table 2. Intra- and inter-day precision of the method (n = 7)

Amount, ng/band	Intra-day precision				Inter-day precision			
	mean area	SD	% RSD	SE	mean area	SD	% RSD	SE
30	1,787.57	1.16	0.06	0.44	1,778.62	1.61	0.09	0.61
50	2,245.01	1.63	0.07	0.62	2,235.76	1.75	0.08	0.66
80	2,786.71	1.79	0.06	0.68	2,781.38	1.57	0.06	0.59

Table 3. Robustness of the method (n = 3; 30, 50 and 80 ng/band)

Condition	SD of peak area	% RSD	SE
Mobile phase (toluene:ethyl acetate:formic acid:methanol)	1.79	0.082	1.03
Mobile-phase volume (18, 20 and 22 ml)	1.22	0.055	0.71
Duration of saturation (4, 5 and 6 min)	1.28	0.058	0.74

Recovery

The percentage yield of daruharidra ranged from 98.5 to 100.6% as shown in table 1. The obtained recovery values were within the limit recommended for chemical analysis, which indicate the reliability and reproducibility of the present method.

Specificity

The peak purity of berberine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spots, i.e. r (start, middle) = 0.9959 and

r (middle, end) = 0.9916 and the overlain spectra are shown in figure 1a. This was further supported by good correlation ($r = 0.9979$) between the spectrum of standard berberine and the spectrum of berberine in polyherbal formulation.

Analysis of Herbal Extract and Pharmaceutical

Dosage Form

The band of berberine was observed at R_F 0.58 in the chromatogram obtained from herbal extract and commercial polyherbal formulations. There was no interference from excipients or other components (fig. 1b). The developed HPTLC method was used to estimate berberine in five different commercial polyherbal formulations and the drug content was found to be $3.96 \pm 1.21\%$ w/w.

Discussion

HPTLC is gradually being used routinely as an analytical tool because of low operating cost, high sample throughput and minimum sample clean-up. The most important advantage of HPTLC is that more than one sample can be run at a time using a small quantity of mobile phase unlike HPLC. While the previously reported HPTLC methods [10, 12] that used a mobile phase composition of butanol:acetic acid:water on silica plate took about 2–3 h for development up to 6–8 cm, the present method required only 30–45 min without compromising

the resolution and peak shape. Equally important, the previously mentioned methods did not study the different variables in the HPTLC method which could affect reproducibility and sensitivity as we did. The low values of SE (<1) in our method for all the variables confirmed that the method was robust. The LOD determination of berberine in both absorbance and fluorescence mode was helpful in deciding to add formic acid to the mobile phase because it drastically reduced the signal-to-noise ratio. Hence the low values of LOD and LOQ (2.8 and 9.3 ng/band, respectively) obtained in this study were lower than those of previous methods [10, 12]. The low LOD and LOQ values of our method were probably due to a baseline drift of the chromatogram.

The HPTLC method validation data suggest that the method had very good accuracy, precision and specificity with low LOD and a high level recovery value as compared to conventional HPTLC methods.

Conclusion

The newly developed HPTLC method for the quantification of berberine in *B. aristata* extract and its formulations was simple, rapid and easily adaptable for screening and quantitative determination. Hence we recommend the present method for rapid screening of a large number of plant extracts and routine quality control of formulations containing this compound.

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