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Development and Validation of HPTLC Method for Determination of Betulinic Acid in *Helicteres isora* root Extract

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ABSTRACT: A new, simple, sensitive, selective, precise and robust high-performance thin-layer chromatographic (HPTLC) method for analysis of betulinic acid (BTA) has been developed and validated for the determination of BTA in *Helicteres isora* root extracts. The analyte was extracted with methanol and applied on TLC aluminium plates along with standard using Linomat IV spray on sample applicator (CAMAG). Analysis of BTA was performed on pre-coated TLC aluminium plates with silica gel as the stationary phase and prewashed with methanol. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of Toluene: Acetone: Formic acid (2.5: 0.5: 0.2 v/v/v). Spectrodensitometric scanning was performed by TLC scanner III (CAMAG) in absorbance mode at the wavelength of 540 nm. The system was found to give compact spots for BTA (Rf value of 0.43 ± 0.04). The linear regression analysis data for the calibration curve showed good linear relationship (r^2=0.9987) in the concentration range 10-50 µl, with respect to peak area. According to the International Conference on Harmonization (ICH) guidelines the method was validated for precision, recovery, stability in solution and robustness. The BTA content quantified from extracts was found well within limits. Statistical analysis of the data showed that the method is reproducible and selective for the estimation of BTA.

Keywords: *Helicteres isora* root extracts, Betulinic acid (BTA), HPTLC, Method development and validation

INTRODUCTION

Helicteres isora Linn (Sterculiaceae) is a plant growing gregariously throughout India. The plant is commonly called as 'Mrigashringa' in Sanskrit is an important medicinal plant described in indigenous system of medicine [1]. Its Roots are astringent, demulcent, diuretic, antigalactogogue. Moreover, the root juice or decoction is used in diarrhoea, dysentry, griping pain in the bowels, flatulence and in diabetes [2]. Major chemical constituents reported include Betulinic acid [3]. Betulinic acid is the triterpenoidal saponin and molecular formula $C_{30}H_{48}O_3$ (Mol.wt. 456.7 g/mol) (Figure-1). The present paper describes precise, accurate, sensitive HPTLC method for determination of Betulinic acid from the extract of *Helicteres isora* Linn root.

MATERIAL AND METHODS

MATERIALS

H. isora roots were identified and collected from Jamnagar in the month of November 2008. Authentication was done by the taxonomist at the Gujarat Ayurvedic University, Jamnagar. CAMAG HPTLC system (Switzerland) comprising of Hamilton 100 µl HPTLC syringe, Camag Linomat IV semiautomatic sample applicator, CAMAG twin trough chamber (20x20 cm), CAMAG TLC scanner III, Camag CATS IV integration software. Silica gel-G60F_{254}, 20 X 20cm TLC plate was used as stationary phase. Betulinic acid (BTA) procured from Sigma Chemicals Ltd, USA, was employed as reference standard. Methanol, ethyl acetate, toluene, acetone, formic acid, chloroform and *n*-butanol used were of analytical grade. The solvent was run for 80 mm, band

length 6 mm, slit dimension 6.00 x 0.30 mm and detection wavelength 560 nm were configured as standard parameters for the present study.

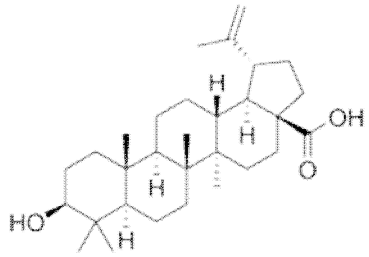

Figure 1. Chemical structure of Betulinic acid

METHODS

***H. isora* root extraction procedure:**

400g of dried powder (60#) was defatted with petroleum ether (60˚-80˚) (3 X 500ml) by refluxing on a boiling water bath for 1h; allowing to macerate for 24 h each time. The combined extracts upon concentrating in vacuo yielded yellow semisolid residue (0.24% w/w). Remaining marc was treated with ethyl acetate (500 ml x 3) and the combined extracts upon concentrating gives reddish brown residue (0.488%w/w), then the remaining marc after extraction with ethyl acetate was extracted with *n*-butanol (500mlx3).The combined *n*-butanol extract (0.408%w/w) was dissolved in methanol and concentrated. By adding excess of acetone and kept in refrigerator, precipitates were formed. Further precipitation done with diethyl ether and the final precipitates of saponin (0.302%w/w) were separated. This saponin precipitates were hydrolysed with 25 ml 2N Hydrochloric Acid and refluxed for 4 hours in waterbath. After hydrolysis sapogenin were separated by extraction with chloroform (25 ml x 3). Chloroform extract was evaporated in a porcelain dish. The dried extract (Ext A, 0.065%w/w) was subjected to HPTLC analysis.

Preparation of sample solutions:

10 mg residue of Extract of *H. isora* root was taken in 10 ml volumetric flask. The residue was dissolved in sufficient quantity of methanol and volume was adjusted to 10 ml with methanol to get (1 mg/ml) concentration which was further diluted with methanol to get necessary concentrations (100 µg/µl).

Preparation of Betulinic acid (BTA) solution

The standard solution was prepared by dissolving 10 mg BTA in 10 ml methanol solution which gives 1000µg/ml. The working standard of 200 µg/ml was prepared from standard solution by diluting with methanol. Different concentrations of 10, 20, 30, 40, 50 µg/ml were prepared from standard solutions.

Chromatographic Conditions

Analysis was performed on 20 cm × 20 cm HPTLC silica gel-G60F_{254} plates. The plate cleaned by predevelopment to the top with methanol, and dried in an oven 105^{0}C for 5 min. Sample and standard zones were applied to the layer as bands by means of a CAMAG. Linomat-4 semiautomatic sample applicator equipped with a 100 µl syringe and operated with the settings band length 6 mm, application rate 150 µl/sec, distance between bands 8 mm, distance from the plate side edge 6.5 mm, and distance from the bottom of the plate 2 cm.

Calibration curve of Betulinic acid

10, 20, 30, 40 and 50 µl standard solution of Betulinic acid (BTA) was applied onto TLC plate to generate Calibration curve. The plate was developed in the mobile phase Toluene: Acetone: Formic acid (2.5: 0.5: 0.2 v/v/v) and dried in an oven 105^{0}C for 5 min. The standard zones were quantified by linear scanning at 540 nm by use of a TLC Scanner III CAMAG. Data of peak height and peak area of each spot was recorded. The calibration curve was prepared by plotting concentration (µg/spot) versus peak area corresponding to each spot.

Identification of Betulinic acid in *H. isora* root extract

10 µl of test and 3 µl of standard solutions were spotted on precoated silica gel-G60 F_{254} plate. The plate was developed in the mobile phase and was sprayed with anisaldehyde-sulphuric acid reagent followed by heating for 5 min at 105^{0}C. The plate was observed under 560 nm.

METHOD VALIDATION [4]:

Precision

Repeatability:

Repeatability of sample application and measurement of peak area was carried out using the three replicates of same spot 30 µg/spot. Repeatability is also termed intra-day precision.

Intermediate precision:

The intra-day and inter-day variations for determination of BTA were carried out at three different concentration levels 10, 30, 50 µg/spot.

Specificity

The specificity of method was ascertained by standard BTA and samples (extracted from *H. isora* root). Spots of the diluent methanol, standard BTA, extracted samples were spotted on TLC plate in duplicate and

run. The spots for BTA that eluted were confirmed with Rf value of standard BTA.

Recovery Studies

Recovery Study was performed by spiking 10, 20 and 30 µg/spot of standard drug externally to the pre-spotted (10 µg/spot) samples. The experiment was conducted in triplicate and applied onto the plate in duplicate.

Robustness

The robustness of method can be performed by allowing variation in sample application time to scanning.

RESULT AND DISCUSSION

Mobile Phase Development

The mixtures of several mobile phases were tried to separate spot of BTA from other spots and get stable peak. The solvent system used was Toluene: Acetone: Formic acid (2.5: 0.5: 0.2 v/v/v) was selected for estimation of BTA, which gave good resolution. Good chromatogram in Figure-2 was attained with Rf value 0.43 ± 0.04 (Figure-2). The wavelength of 554 nm was used for quantification of sample.

Calibration Curve, Linearity and Range

Linear regression data showed a good linear relationship over concentration range (Table-1, Figure-3). The correlation coefficient r^2 was 0.9987 (Table-2).

Precision: The repeatability and intermediate precision were studied (Table-3 and -4 respectively).

Repeatability

It showed very low % RSD of peak area of drug (Table no. 3).

Specificity

The mobile phase was optimized and it showed good result. BTA was found to be well separated from other constituents present in extracted sample. There was no interference of diluents and other constituent's peaks from extracted sample found at the Rf value of BTA peak, indicates specificity of the method.

Robustness of method

The time from sample application to scanning varied from 0, 20, 30, 40 mins. The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 3 %.

Recovery studies

The proposed method when used for extraction and subsequent estimation of BTA from root extract after spiking with 20, 80, 120% standard BTA (Table no.5).

Estimation of BTA in *H. isora* root extract

The optimized solvent system was used for the estimation of the BTA in *H. isora* root extract. There was no interference in analysis from other components present in extract. The resolution was good and components were observed at different Rf value. The total BTA present in extract was found to be 0.02 % w/w.

CONCLUSION

The developed HPTLC method is fast, simple, precise, specific and accurate. Statistical analysis proved that method is repeatable and selective for determination of Betulinic acid.

Table no.1. Comparative data of standard and Extract of *H. isora* root

Track	Concentration of BTA (µg/spot)	Rf	Mean Peak Area± SD (n=3)	% RSD
1	10	0.43	5656.6±90.93	1.60
2	20	0.43	6625.8±150.83	2.27
3	30	0.43	7428.0±178.35	2.40
4	40	0.43	8225.5±174.92	2.12
5	50	0.43	9162.3±165.27	2.89
6	Extract of *H. isora* root	0.43	7233.5±169.05	2.56

Table 2. Linearity regression data for calibration curve

Parameters	Value
Linearity Range	10-50 µg/ml
Correlation of Coefficient (r)	0.9993
Slope	86.111

Table 3. Repeatability study

Sr. No.	Sample applied (µg)	Peak Area AU ± SD	%RSD
1	30	7428±45.25	0.609
2	30	7425±50.91	0.686
3	30	7424±45.96	0.619

Table 4. Intraday and Interday Precision (Intermediate precision)

Sr. No.	Sample applied (µg) (n=3)	Intraday %RSD	Interday %RSD
1	10	2.69	2.24
2	30	1.94	1.57
3	50	2.23	2.58

Table 5: Recovery studies

Concentration of BTA (µg/spot)		Total Conc. of BTA (µg/spot)	Conc. of BTA recovered (µg/spot) Mean±SD	%RSD	% Recovery
Conc. Taken	Conc. Added				
10	0	10	9.99±0.22	2.20	99.90
10	10	20	19.79±0.36	1.82	98.95
10	20	30	29.52±0.49	1.66	98.40
10	30	40	39.66±1.10	2.77	99.15

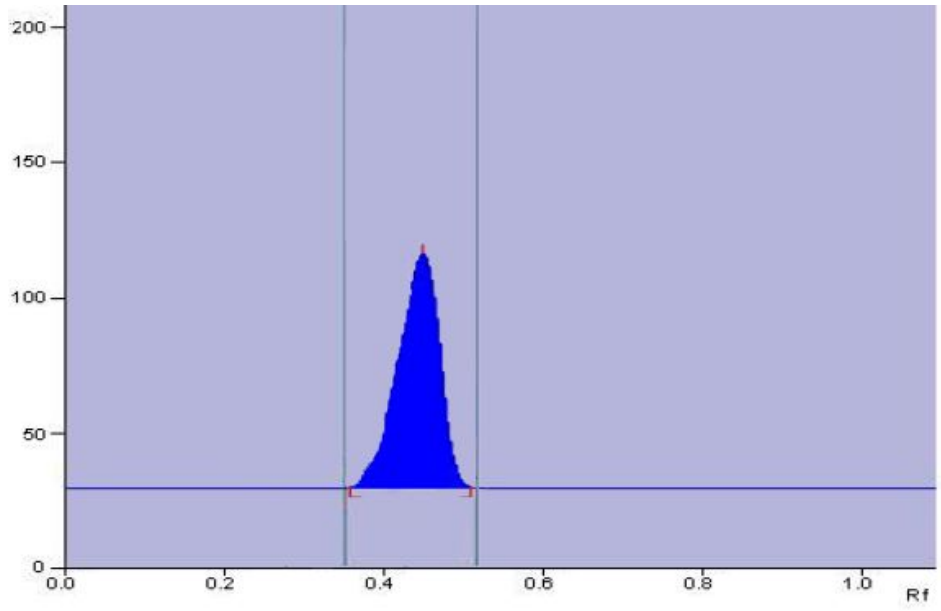

Figure 2. HPTLC Chromatogram of representative sample

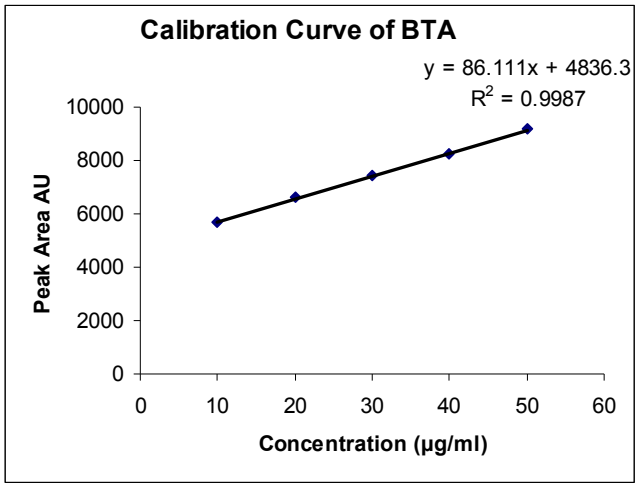

Figure 3 Calibration Curve of BTA

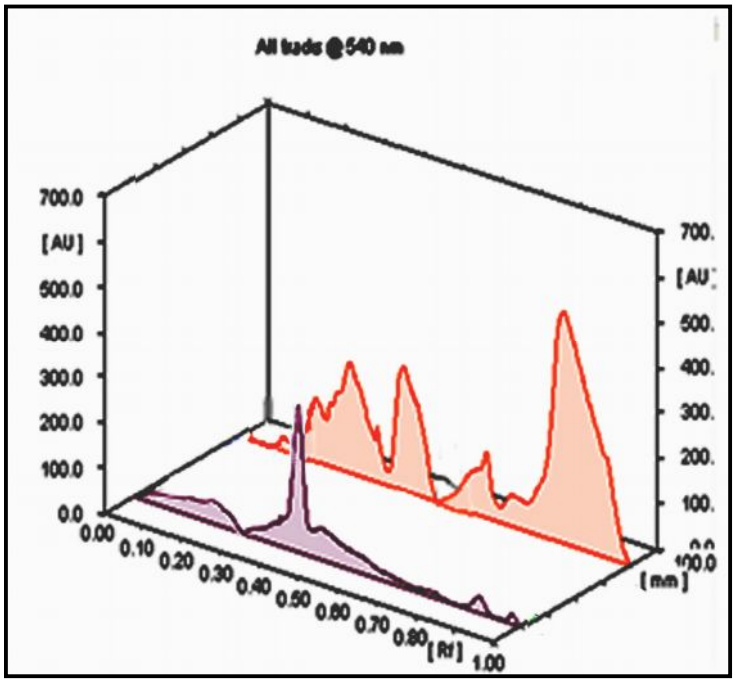

Figure 4. Densitometric chromatogram scanned at 540 nm

REFERENCES

[1] *Wealth of India*, Raw Materials, (Council of Industrial and Scientific Research, New Delhi, 1969) 5, p.27.

[2] Kirtikar K.R., Basu B.D., *Indian Medicinal Plants,* (Lalit Mohan Basu publication, 1975) 2nd edn Vol.1, p.371.

[3] Wenhao Qu, Jingrong Li, Mingshi W. Chemical studies on *Helicteres isora L. Zhongguo Yaoke Daxue Xuebao,* 22(4), 203-206, 1991.

[4] Validation of analytical Procedures, Methodology, ICH harmonized triapartite guidelines, 1996.

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