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Isolation, characterisation and RP-HPLC estimation of berberine in homeopathic formulation

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ABSTRACT

Berberine is an iso-quinoline alkaloid and is a major constituent of the species Berberis aquifolium (Berberidaceae.). Analytical thin layer chromatography was carried out on silica gel GF_{254}TLC plates and the chromatoplate was developed with n-proponol: Formic acid: water (90:01:09v/v) as mobile phase. In this study isolation of berberine was achieved by preparative TLC and the compound thus isolated was characterised by Ultraviolet, Mass spectral analysis. Mass spectral data shows molecular ion peak of m/z=366. An isocratic RP-HPLC method was developed for the estimation of berberine from mother tincture of Berberis aquifolium. The chromatographic separations was achieved by RP-C_{18} Column (250 mm X 4.6 mm, 5µ), Shimadzu LC-20AT Prominence liquid chromatograph and a mobile phase composed of 0.1%tri fluoro acetic acid:acetonitrile (60:40v/v). The flow rate was 1.0 ml/min and the analyses of column effluents were performed using UV-Visible detector at 344 nm. Retention time of berberine was found as 5.133 min. This method has obeyed linearity over the concentration range of 2-12µg/ml and the regression coefficient obtained from linearity plot for berberine was found as 0.997. RP-HPLC method was validated in pursuance of ICH guidelines.

Key words: Berberine, Isocratic RP-HPLC, UV detection, Validation.

INTRODUCTION

Plants are used as a major source of drugs for the treatment of various health disorders. There are total of some 250,000 species of higher plants in the world, much less than the species of animals (5-10 million). However, plants contribute to our lives more than animals mainly due to their extraordinary array of diverse classes of biochemical with a variety of biological activities [1]. Plants with medicinal properties, the gift of Mother Nature to mankind, are in use for centuries in the traditional systems of Ayurveda, Siddha, and Unani etc. The plant kingdom has immensely contributed to the health needs of man when no concept of surgical management existed. Even today almost 25 % of all prescribed medicines in the developed world contain ingredients derived from medicinal plants [2]. Berberine is one of the major alkaloid constituent in C. fenestratum. Berberine has broad spectrum of pharmacological activities. The drug is useful in vitiated conditions of, inflammations, wounds, ulcers, jaundice, burns, skin diseases, abdominal disorders, diabetes, fever and general debility [3-12]. An infusion, tincture and concentrated liquor are also prepared to wash wounds and skin rashes. Stem pieces are boiled and one cup is given in case of a fresh, deep cut, being the most common use against tetanus[14-18]. It purifies the blood. Decoction of stem is given internally in cases of bites from monkeys, snakes, brahmin-lizards and geckos. The root bark is used for dressing wounds, ulcers and in cutaneous leishmaniasis [19]. A combination of the bark and honey is taken internally for the treatment of jaundice. Bark is also used in the treatment of leucorrhoea and other gynecological troubles [20]. According to them its aqueous extract is more useful but due to non-availability of fresh bark, a decoction by boiling the bark in water is usedby taking it in empty stomach daily morning. It has also been applied in a complex decoction after childbirth in Peninsular Malaysia. In Vietnam, tablets made from crude alcoholic C. fenestratum extracts are prescribed to cure dysentery [21]. The methods to analyse the alkaloid content of *B.*

aquifolium consisting formulation (tincture) by spectrometric method, but these methods lack sensitivity and specificity. The chromatographic methods of analysis include TLC, HPLC methods and MS methods. The existing HPLC methods [22-23] for determination of berberin either did not have sufficiently low level of detection or they required column-switching technique [13] or havea long run time or did not result in sufficient resolution. Thus, an attempt has been made to develop and validate HPLC method for the analysis of berberin, which would be highly sensitive, having good resolution, shorter retention time and reproducible.

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MATERIALS AND METHODS

Apparatus: Continuous Soxhlet apparatus (18cm length and i.d. of 4.8cm) Heating mantle, vacuum filtration assembly, sieves etc.

Instruments:
Chromatographic separations were achieved by using Shimadzu LC-20AT Prominence Liquid chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C18 column (4.6 mm i.d. X 250 mm, 5 micron particle size). 20 µL of sample was introduced into the HPLC system. The HPLC system data acquisition was performed with Spinchrom" software. Double beam UV-Visible Spectrophotometer (Systronics model 2203) with matched cuvettes was used in this study. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), and an ultrasonic bath sonicator (spectra lab, model UCB 40).

Chemicals and Reagents:
The berberine was procured from Sigma Aldrich. Methanol, acetonitrile etc, were purchased from Merck Pvt. Ltd. Mumbai, India. All the other chemicals used including the solvents were of analytical grade. Formic acid (Rankem Ltd., Mumbai, India,) while 0.1%trifluoro acetic acid, Acetonitrile and water (HPLC grade) from Pvt. Ltd. Mumbai, India**.**

Preparation of Reagents and Standards
Mobile phase (0.1%tri fluoro acetic acid: acetonitrile (60:40v/v)
To 60 parts of 0.1%tri fluoro acetic acid and 40parts of acetonitrile was mixed to get one litre of the mobile phase. The mobile phase was then filtered through 0.22 µm nylon membrane vacuum filtration and degassed by sonication.

Preparation of Standard Solutions
A standard stock solution was prepared by dissolving 100 mg of berberine in 100 ml volumetric flask containing 60 ml mobile phase, then sonicated for about 10 minutes and made upto 100 mL with mobile phase to get the primary standard stock solution containing 1000 µg/mL of berberine. Working standard solutions were prepared by further dilution with mobile phase.

Isolation and Purification of active compound
Analytical TLC:
Analytical TLC was carried out on preparative TLC plates (5 × 5 cm with 0.2mm thickness, silica gel GF_{254}, Merck, Darmstadt, Germany) cut from the commercially available sheets. An aliquot of standard solution of berberine and a sample solution of crude extract was spotted onto the silica gel plate and allowed to dry for a few minutes. Afterwards, the chromatoplate was developed with n-proponol: Formic acid: water (90:01:09v/v) as mobile phase in a previously saturated glass chamber with eluting solvents for some time at room temperature. The developed plate was dried under normal air and the spots were visualised by spraying with a solution of dragendorff and dried under oven. The Rf (retention factor) values of isolated compounds and standard were calculated and compared.

Preparative TLC for Purification.
A streak of crude extract was applied manually on a preparative TLC glass plate (20 cm × 20 cm; 1500 μm thickness) with inorganic fluorescent indicator binder (Analtech, Sigma-Aldrich, Steinheim, Germany). After air drying, the plate was developed, using the same mobile phase as used in the analytical TLC, in a pre-saturated glass chamber. In each experiment, two plates were used in parallel. One of the plates from each set of experiment was sprayed with as described above, and the bands were scraped off carefully from the plate. The scratched sample was dissolved in HPLC grade methanol and centrifuged at 12000 rpm for 15 min in order to remove silica. The supernatant was collected, filtered from 0.22 μm filter, and dried under reduced pressure. Further, all the dried samples were passed under nitrogen gas for 5min and then dissolved in methanol for further characterization and quantitative HPLC analysis. The entire purification process was carried out under dark or dim light conditions.

Characterization of Purified Compound

The UV-visible spectrum of the purified compound was recorded from 200 to 400 nm on a *ELICO* double beam spectrophotometer UV-visible spectrophotometer. ESI mass spectra was acquired from isolated compound and characterised. Methanol was used as solvent.

Recommended procedure for determination of berberin in *B. aquifolium* by RP-HPLC method

The chromatograph was stabilised for about 45minutes with mobile consisting of 0.1%tri fluoro acetic acid: acetonitrile (60:40v/v). The flow rate was 1.0 ml/min phase at the required flow rate to get a steady base line. Aliquots of standard solution containing berberine were transferred to a series of 10ml capacity volumetric flasks to get the concentrations ranging from 2-12 µg/ml. accurately injected about 20µl of each calibration standard into the chromatograph. Peak areas of each solution were recorded. A calibration curve was plotted between concentration and peak area response. 20 µl of sample solution prepared from berberis aquifolium was injected and the area of peak was recorded duly maintaining the ambient experimental conditions as followed for the standard drug solutions. The amount of berberine present in the sample was computed from its calibration graph.

Validation of the developed method

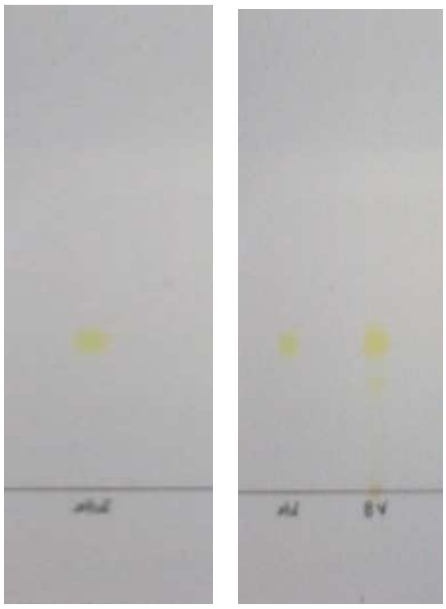
System suitability: The chromatograph was stabilised for about 45minutes with mobile phase at the required flow rate to get a steady base line. System suitability was ascertained by six replicate analyses of the drugs at concentrations of 10 µg/mL of berberine. The %RSD of the three injections of the same quantity of standard drug solutions of berberine in terms of their peak areas, retention time, efficiency or number of theoretical plates and asymmetry factor to ascertain its system suitability. The effect of wide range of other constituents and other additives usually present in the extract was investigated to know the specificity of the method. It shows no interference from other compounds. For linearity**,** Aliquots of primary working standard solutions containing berberine were diluted such a way that the final concentrations of berberine are in the range of 2-12 µg/mL. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curve was performed. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. Precision was determined by intra-day and inter-day study. Precision of the method was evaluated by carrying out the assay and analyzing corresponding responses 6 times on the same day and on different days for the sample solution. The percent relative standard deviation (% RSD) was calculated. Accuracy studies were performed for berberine at three different levels (25%, 50% and 75%) and the mixtures were analyzed in triplicate by the proposed method. Known amount of standard berberine at 25%, 50% and 75% of sample (which was previously analysed) was added and it was reanalysed by the proposed method. And the percentage recovery was evaluated. The robustness of the developed method was evaluated by small deliberate changes in flow rate (± 0.1 ml/min), detection wavelength (±5 nm) and mobile phase composition (±2%). The effect of these variables on the developed method was determined. Limit of Detection and Limit of Quantitation were calculated using following formula LOD= 3.3(σ)/S and LOQ= 10 (σ)/S, Where (σ) = standard deviation of response (peak area) and S= slope of the calibration curve.

RESULTS AND DISCUSSION

The berberine was achieved by marketed formulation of mother tincture. Several mobile phase combinations were tried and n-proponol: Formic acid: water (90:01:09v/v) was found optimum for separation of berberin from B. *aquifolium* and B. *vulgaris* and clear stone drops. The R_f values of standard and sample compound matches each other and the R_f value was found as 0.52. TLC profile of compound was represented in Figure1. Isolation of berberine from the extract was achieved by preparative thin layer chromatography using the same chromatographic conditions followed for identification of active constituent. Characterisation of isolated compound was done by studying ultraviolet, mass spectra. Berberine shows UV absorption at about at 424nm in methanol indicates the presence of conjugation and hydroxyl auxochrome which shifts the absorption maximum towards visible side of the spectrum and it was represented in Figure 2. Mass spectral data shows molecular ion peak m/z=366 which has a moderate abundance (Figure 3).

An accurate isocratic RP-HPLC method was developed and validated by optimised chromatographic conditions. The conditions and system suitability was presented in Table 2. Chromatograms showed a peak of berberine at retention time of 5.133 min. The calibration curve was obtained and the data of regression analysis of the method is depicted in Table 3. The regression coefficient obtained from linearity plot for berberine was found as 0.997, which indicates this method had good linearity and the linearity data was given in Table 4. The representative chromatograms of this method were given in Figure 7. And Figure. 8. for calibration standard and sample. The calibration plot for berberine was shown in Figure 9. The developed method was applied to the determination of concentration and results are shown in Table 5. The amount of berberine was found as 0.0015mg/30ml extract. The method validation parameters were established in this work, LOD and LOQ of the berberine were found as 0.488µg/ml and 1.478

µg/ml and the proposed method was found to be precise for the determination. The %RSD for the proposed method was found to be less than 2.0 which indicate the method's precision. Results of the precision study are shown in the Table 5. Recovery studies of the method were found to be good and % recovery was represented in Table 6. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, λmax, mobile phase composition. The proposed method was found to be robust as there were no marked changes in the performance characteristics of the method.



(a) **(b)**

Figure 1: Figure 1.0. Identification of berberine on TLC plate: (a) chromatogram showing identification of berberine standard, (b) Separation of berberine from B. *aquifolium*

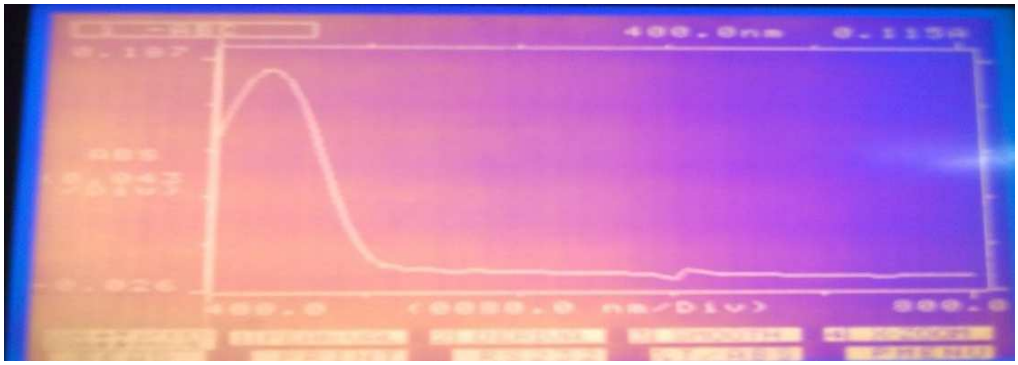


Figure 2: UV absorption spectrum of berberine in methanol

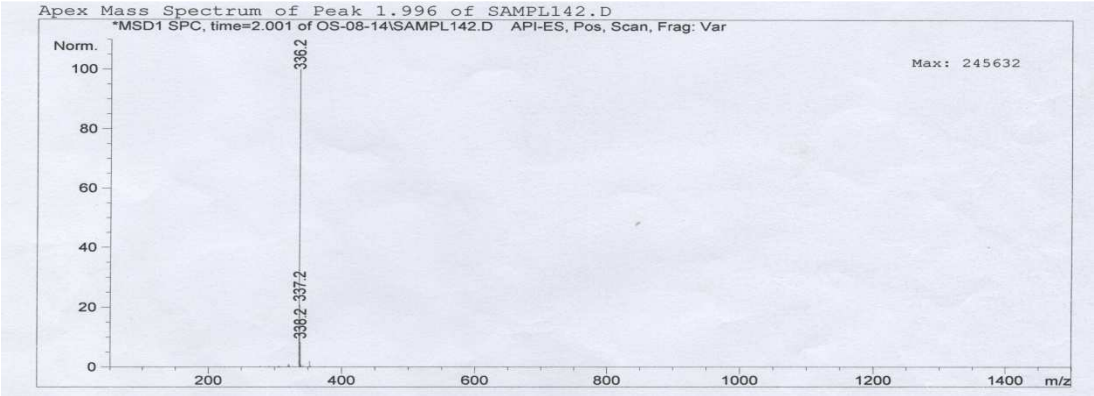


Figure 3: Mass spectra of berberine isolated from mother tincture of berberis aquifolium

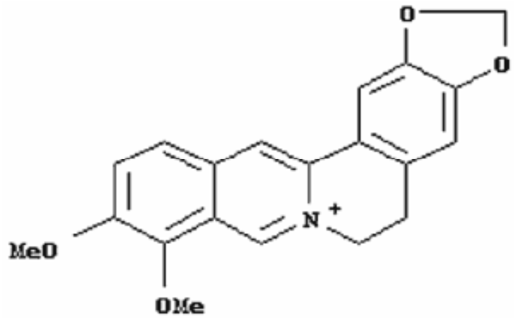


Figure 6: Chemical structure of berberine

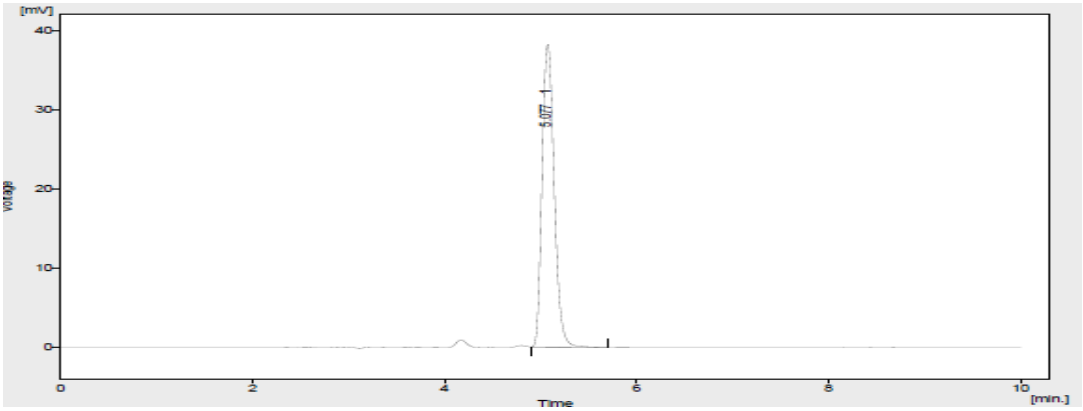


Figure 7: Chromatogram of berberine

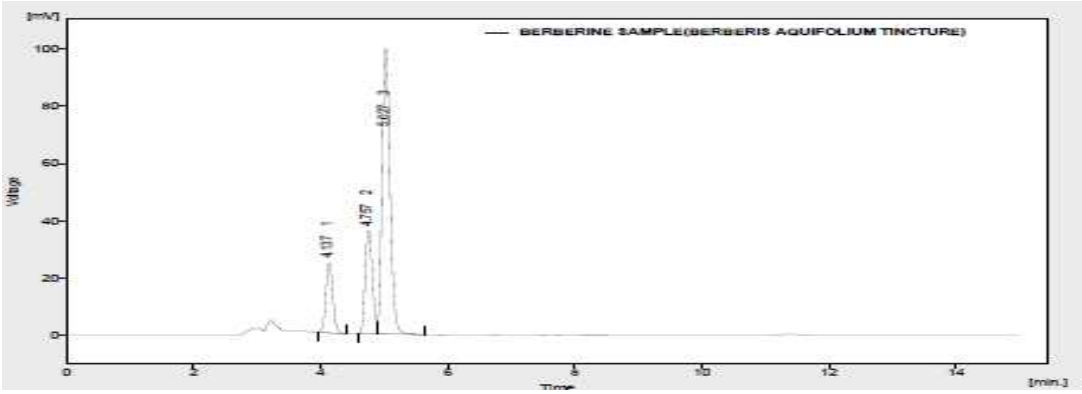


Figure 8: Chromatogram of mother tincture of B. *aquifolium*

Table 2: Optimised chromatographic conditions and system suitability

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT Prominence liquid chromatograph
Column	WELCHROM C_{18} Column (250mm X 4.6, 5µm)
Detector	SHIMADZU SPD-20A Prominence UV-Vis detector
Diluents	Mobile phase
Mobile phase	0.1%trifluro acetic acid:acetonitrile (60 :40 v/v)
Flow rate	1mL/min.
Detection wave length	UV at 344 nm.
Run time	10 minutes
Column back pressure	102 kgf
Temperature	Ambient temperature(25°C)
Injection Volume	20µL
System suitability parameters	
Retention time (min.)	5.077
Theoretical plates[th.pl] (Efficiency)	7448
Tailing factor (asymmetry)	1.344

Table 3: Regression analysis of the proposed method

Parameter	Berberine
Detection wavelength(nm)	UV at 344nm
Linearity range (µg/mL)	2-12 µg/mL
Regression equation	y=57.86x+0.407
(Y = aX + b)	R^2 = 0.997
Slope(a)	57.863
Intercept(b)	0.4077
Standard error of slope (S_a)	1.18661
Standard error of intercept (S_b)	8.55676
Standard error of estimation (S_y)	12.5579
Regression coefficient (R^2)	0.997
Limit of detection(µg/mL)	0.488
Percentage range of errors (Confidence limits)	
1.049 significance level	0.346
1.646 significance level	0.543

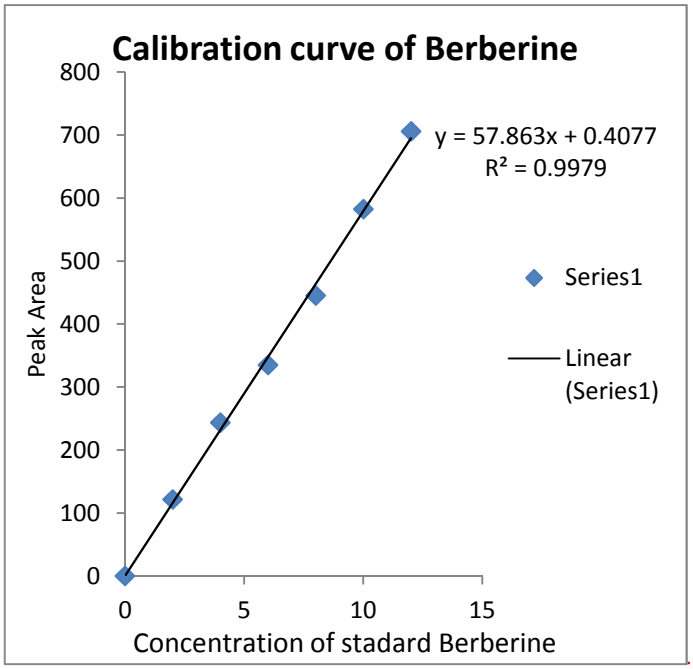


Figure 9: Linearity plot for berberin

Table 4: Linearity analysis of HPLC method

S. No.	Conc.(µg/ml)	Area of Berberine
1	0	0
2	2	121.559
3	4	243.534
4	6	334.810
5	8	445.206
6	10	582.214
7	12	705.786

Table 5: Determination of concentration of Berberine in B. *aquifolium* from its Precision Study

trial no	area of sample berberine	concentration	amount (µg/ml)
1	871.822	15.28756	1.52875
2	870.832	15.27012	1.52701
3	868.676	15.23214	1.52321
4	866.536	15.19444	1.51944
5	872.818	15.30511	1.53051
6	865.816	15.18175	1.51817
Mean	869.416	15.24519	1.52451
SD	2.8695	0.050553	0.00505
%RSD	0.3300	0.331603	0.00331

Table 6: Recovery Study berberin in mother tincture of B. *aquifolium* by HPLC method

S. No.	Level of spiking of standard	Amount recovered (µg/ml)	Amount found* (µg/ml)	% recovery*±S.D (N=3)
1	25%	1.8	1.7	94.68
2	50%	2.2	2.3	97.41
3	75%	3.0	3.01	99.66

**= Mean of three determinations*

CONCLUSION

Isolation, identification and characterisation of berberine were achieved successfully which will be helpful for the standardisation of homeopathic formulations containing this active constituent. The proposed HPLC method is linear, sensitive, accurate and precise and can be adopted for the determination of concentration of berberine in various formulations with shorter run time and good efficiency.

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