WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Development and Validation of Spectrofluorimetric Method for the Estimation Berberine in *Argemone Mexicana* Extracts: Application in Standardization.

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ABSTRACT:

A rapid, simple, sensitive and cost effective spectrofluorimetric method was developed for the estimation of Berberine in pure pharmaceutical dosage form. The relative florescence intensity of Berberine was measured in an at excitation wavelength of 360 nm and emission wavelength 465 nm. This reaction product was measured spectrofluorimetrically at 465 nm after excitation at 360 nm under optimum conditions, linear relationship with best correlation coefficient 0.9999 and the linearity was detected in between the range of 100-4000 ng/mL. The limits of detection (LOD) and limit of quantification (LOQ) were found in the range of 15.76 and 47.78 ng/ml respectively. The validation of the developed spectrofluorimetric method was carried out by conducting linearity, accuracy, precision, and robustness and ruggedness, limit of detection and limit of quantitation studies. Developed spectrofluorimetric method was found to be precise for the intra-day and inter-day study and shows percent relative standard deviation in the range of 0.016 and 0.657 and 0.005 and 0.575 respectively. The total percent recovery of Berberine was found to be 99.76 to 99.99.

Keywords: Berberine, detection limit, fluorescence spectrophotometry, fluorimetry, linearity, quantitation limit, validation.

INTRODUCTION

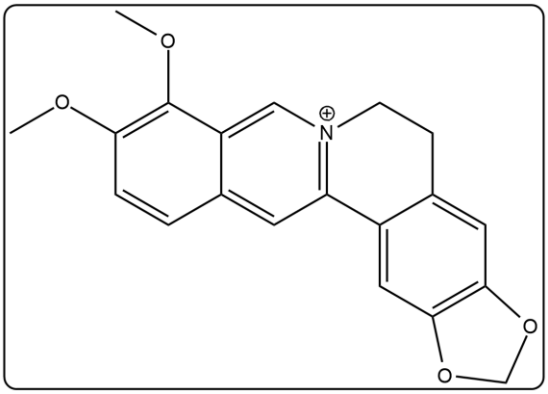

Figure No.1 Chemical structure of Berberine

The plant, Argemone mexicana Linn. belongs to the family Papaveraceae, is a widely distributed plant throughout the subtropical and tropical regions of the world. Argemone mexicana is considered as significant medicinal plant in India; the yellow juice, which exudes when the plant is injured, has long been used in India as traditional medicine for dropsy, jaundice, ophthalmia, scabies, and cutaneous affections.[1-3] Different parts of this plant are used in chronic skin diseases, and also as an emetic, expectorant, demulcent, and diuretic; the seeds and seed oil are employed as a remedy for dysentery, ulcers, asthma, and other intestinal affections. Berberine hydrochloride (Fig. 1), a kind of isoquinoline alkaloid, is a commonly used drug extracted from a variety of herbs, including Coptidis rhizoma, Phellodendron Chinese schneid, and Phellodendron amurense.[4-6] It exhibited a variety of biological and pharmacological actions, such as antidiabetic activity, antitumor properties, bactericidal properties, as well as antiatherosclerosis activity. It is soluble in an organic solvent like Ethanol and Methanol, Insoluble in water.[7-9] Till date, there is no single economical spectrofluorimetric method for estimation of Berberine in extracts Argemone mexicana Linn. The said method was a precise and economical spectrofluorimetric method capable of estimating Berberine in a variety of dosage forms like powder and solutions and the standardized extract is also unavailable. Therefore, considering the commercial

importance and the needs of herbal industries, a simple yet precise and economical spectrofluorimetric method capable of estimating Berberine was developed and validated.[10-11]

EXPREMENTAL

Materials and Method

Chemicals and reagents

Berberine was purchased from TCI Chemicals (India) Pvt. Ltd, Chennai. Ethanol was purchase from Merck. All the chemicals of analytical grade were used for the proposed study.

Apparatus and instruments

The spectrofluorimetric method development and its validation study were carried out using a Shimadzu RF-5301 Fluorimeter A xenon 150 w lamp was used as a light source. Quartz cells having 48mm height, 10 mm path length with 0.5mm slit width were used for fluorescence measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the weighing purpose.

Preliminary analysis

A preliminary analysis was carried out to determine the excitation and emission wavelength of berberine. Various solvents like distilled water, ethanol, methanol, acetonitrile, isopropyl alcohol and their combinations were used to determine appropriate media for berberine. Berberine showed maximum fluorescence intensity in ethanol as a media. Initially, berberine solution of 4 µg/ml strength was prepared in ethanol. Prepared solution was scanned spectrofluorimetrically to obtain the excitation and emission wavelengths. The scanning was performed over 220 nm to 800 nm range and excitation and emission wavelength were found to be 360 nm and 465 nm (figure.2) respectively.

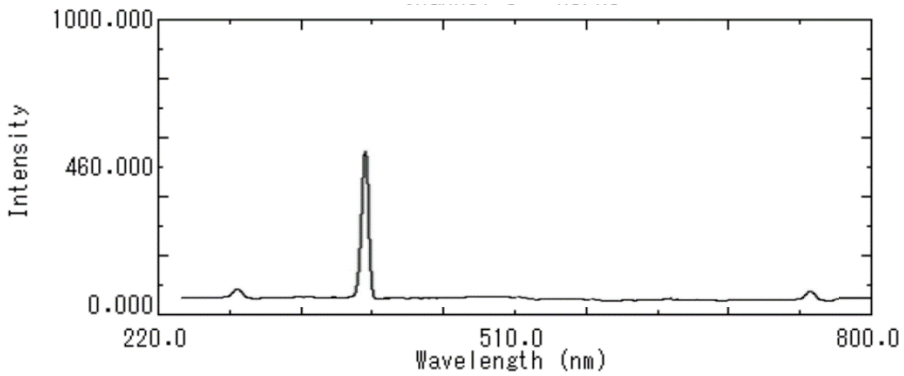

Fig. 2: Excitation Spectra of Berberine

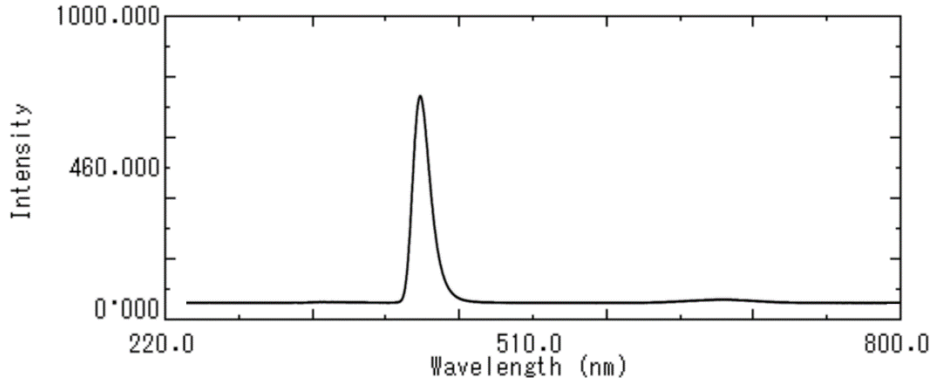

Fig. 3: Emission Spectra of Berberine

Preparation of standard stock solution

A 1 mg of Berberine was accurately weighed, poured into 5 ml volumetric flask and dissolved in 1 ml Ethanol. It was sonicated for 5 min and the resultant solution was diluted with ethanol to achieve a stock solution-1 of 1 mg/mL strength. Stock solution-1 diluted further with ethanol to get stock solution-II and III of 10 µg/mL and 1 µg/mL strength respectively.

Preparation of calibration curve

Calibration curve was prepared by diluting the stock-I, stock-II and stock-III solution to achieve the seven different calibration standards representing 100, 200, 400, 800, 1000, 2000, 4000 ng/ml strength. Fluorescence Intensity of each calibration standard was measured at pre-identified $\lambda_{Excitation}$ 360 nm and $\lambda_{Emission}$ 465 nm. The calibration curve representing concentration vs. fluorescence intensity was plotted. Above mentioned procedure was repeated five times so that reproducible results can be obtained.

Validation of Spectrofluorimetric Method

Developed spectrofluorimetric method for the estimation of Berberine was validated as per the ICH guideline. Said method was validated using different parameters like linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ). [12-16]

Linearity and Range

Linearity of the proposed spectrofluorimetric method was established using seven different calibration standards. Based on analysis of calibration standards, calibration curves in terms of florescence intensity vs. concentration plots were developed and subjected to linear least square regression analysis. R square value was considered to be important factor for establishing linearity of the proposed method. The interval between lower and upper concentration limit with acceptable linearity was reported to be the range of the proposed spectrofluorimetric method.

Accuracy

The accuracy of the proposed spectrofluorimetric method was evaluated using recovery studies after standard addition of analyte of interest. Three different solutions of Berberine were prepared in triplicate at level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of Berberine were added (standard addition method) and the accuracy was calculated on the basis of percent recovery. For calculating the percent recovery following formula was used.

$$\% RC = (SPS-S/SP) \times 100$$

Where,

SPS = Amount found in the spiked sample

S = Amount found in the sample

SP = Amount added to the sample

% RC = Percent recovery

Precision

The precision of the proposed spectrofluorimetric method was established by performing intra- and inter-day spectrofluorimetric analysis of predefined samples. The study was performed at three concentration levels. Intra-day precision study was carried out by preparing three different concentrations of Berberine solutions of 150, 2000 and 3900 ng/ml strength (3 solutions of each concentration) and analysing the same at morning, afternoon and evening time of the same day. Deviation in the results was calculated in terms of % relative standard deviation (% RSD). Similarly, inter-day precision study was carried out by analysing the above-mentioned solutions at three consecutive days.

Ruggedness

Ruggedness study of the method by preparing the middle level sample (2000 ng/ml) by the three different analyst and analysed at 360 nm excitation and 465 nm emission wavelength of Berberine. The results were represented in term of %RSD.

Robustness

Robustness of the method was determined by changing the solvents. Three different solvents viz. 0.01 M NaOH, methanol and distilled water were used for dissolving Berberine and the fluorescence intensity of each was determined at preidentified excitation and emission wavelengths. Results were represented in terms of % RSD

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ of the developed spectrofluorimetric method was calculated by using following formula

$$LOD=3.3\times SD/S$$

$$LOQ= 10\times SD/S$$

Where, SD= Standard deviation of lower most concentration of calibration curve

S= Slope of calibration curve

Estimation of Berberine in *Argemone Mexicana* extract

A. mexicana powder was dried at 500 C using a Micro tray drier (S.B. Paschal and company, Mumbai, India) and powdered using a twin-blade mixer (Bajaj electrical ltd, Mumbai, India). To select uniform particle size, the powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, India) with sieves of different sizes (12, 24, 45, 85, and 120 mesh, Swastika electric and scientific works, Ambala, India) for 15 min. Powder passed through 120 mesh sieve was collected and used for further extraction. Soxhlet assisted extraction (SAE) technique was used for the extraction.10gm of powdered A. mexicana Powder was placed in a thimble (Borosil, Mumbai, India) which was inserted into a Soxhlet apparatus. The material was exhaustively extracted with ethanol. SAE was performed for 2 h. After a predefined extraction period, the solvent was distilled off under reduced pressure using a rotary vacuum evaporator (Heidolph Instruments GmbH & co. Germany) to obtain the dry extract. Accurately weighed 1 mg of dry extract of A. Mexicana Powder was transferred into the calibrated volumetric flask and dissolved using 1 ml of ethanol to achieve a stock solution of 1000 µg/ml (Stock-III). Stock- III solution was suitably diluted with a co-solvent system and analyzed for the Berberine content using the proposed spectrofluorimetric method.

RESULTS AND DISCUSSION

Construction of calibration curve

Quantification of unknown samples by any instrumental method of analysis needs a reproducible calibration curve and a mathematical equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. To establish linearity of the proposed method, seven different calibration standards were prepared from the stock solution and analyzed at excitation wavelength 360 nm and emission wavelength 465 nm (Fig. 2) by Spectrofluorometer. Least square linear regression analysis was performed for the obtained spectrofluorimetric data using MS-Excel 2016. Calibration curve was repeated five times for reproducibility. Various concentrations and their fluorescence intensities with mean ± standard deviation were reported (Table 1)

Table No 1: Calibration standard data for Berberine

Sr No.	Concentration	Fluorescence intensity
1	100	20.863±0.402
2	200	41.820±0.408
3	400	82.236±0.394
4	800	167.880±0.414
5	1000	202.096±0.400
6	2000	403.216±0.401
7	4000	810.973±0.401

Linearity and Range

Linearity and range are the key parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven point calibration curve of Berberine covering a range of 100-4000ng/ml was plotted. Details of concentrations and the respective mean absorbance values are depicted in Table 1. Calibration curve when subjected to least square regression analysis yielded an equation; y = 0.2024x + 0.9965 with correlation coefficient 0.999 as shown in Figure 3. From the linearity study, it was revealed that, developed spectrofluorimetric method was linear in the pre-defined concentration range of calibration standards.

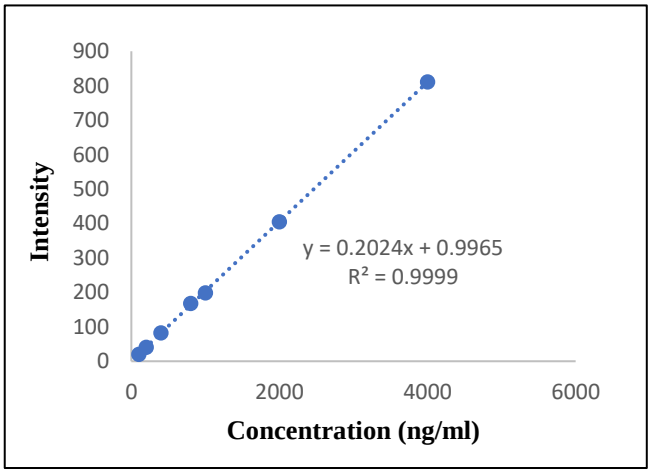

Figure No. 4 Calibration curve for Berberine

Accuracy

Accuracy is a measure of the closeness of the experimental value to the actual amount of the substance in the matrix. Accuracy is to be established over the entire calibration range of the analytical method so that at any point of determination, results obtained would be reliable. In case of spectrofluorimetric method for Berberine, accuracy was established using recovery studies. At 80 % standard addition, mean recovery of Berberine was found to be 99.76% whereas at 100 and 120 % standard addition, it was found to be 99.99 and 99.83% respectively. % RSD was found to be less than 2 for the Berberine recovery studies as shown in Table 2. From the results of accuracy studies, it was observed that developed spectrofluorimetric method is highly accurate as the percent recovery was in between 99.51 to 100% and the % RSD was well below 2%.

Table No 2: Accuracy data of Spectrofluorimetric method for Berberine

Sr No.	Concentration (%)	Origin level (ng/ml)	Amount added (ng/ml)	% Recovery	Mean % Recovery	% RSD
1	80	150	120	99.77	99.76	1.250
2	80	150	120	100.00		
3	80	150	120	99.51		
4	100	2000	2000	99.72	99.99	0.408
5	100	2000	2000	100.00		
6	100	2000	2000	99.98		
7	120	3900	4680	99.87	99.83	0.352
8	120	3900	4680	99.63		
9	120	3900	4680	100.00		

Precision

Precision is a measure of degree of scatter. It expresses the reproducibility of the measurements. It is expected that an analytical method should generate outcomes that are reproducible. Precise analytical method leads to accurate results. Considering the importance of reproducible yet accurate results, intra- and inter-day precision of developed spectrofluorimetric method was established at 150, 2000 and 3900 ng/ml levels of Berberine. The results in terms of mean florescence intensity values, percent assay and % RSD for the intra- and inter-day precision study are demonstrated in Table 3 and Table 4 respectively. % RSD values of intra-day precision study were found to be in between 0.016 and 0.657 whereas those of inter-day precision study were in between 0.005 and 0.575. Overall, % RSD values of less than 2 demonstrated the precision of developed spectrofluorimetric method.

Table No 3: Intra-day precision data of spectrofluorimetric method for Berberine

		Morning			Afternoon			Evening		
S. NO	Concentration Range (ng/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	150ng/ml	149.6	99.72	0.657	149.8	99.84	0.522	150	99.99	0.546
2	2000ng/ml	1999	99.97	0.102	2000	100	0.031	2000	99.99	0.042
3	3900ng/ml	3901	100	0.098	3900	100	0.016	3901	100	0.091

Table No 4: Inter-day precision data of spectrofluorimetric method for Berberine

		1 Day			2 Day			3 Day		
S. NO	Concentration Range (ng/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	150ng/ml	149.77	99.84	0.575	150.05	100.03	0.381	149.97	99.98	0.481
2	2000ng/ml	1999.85	99.99	0.058	2000.01	100.00	0.021	1999.9	99.99	0.009
3	3900ng/ml	3900.76	100.02	0.068	3899.91	99.99	0.015	3899.9	99.99	0.005

Robustness

Robustness of analytical method is the ability of a method to resist the change in its performance in spite of small, deliberate change in method parameters. It is most important parameter of analytical method as a small, un-intentional change in method parameters like solvent composition; pH etc. may occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the analytical method. Therefore, robust analytical method is preferred. Proposed spectrofluorimetric method was found to be robust as the % RSD values were found to be in between 0.0385 and 0.1259 as shown in Table 5.

Table 5: Robustness data of spectrofluorimetric method for Berberine

Sr. NO	Concentration (ng/ml)	Solvent Ratio	Mean	% RSD
1	2000 ng/ml	Water	405.54	0.1259
2	2000 ng/ml	MeOH	405.82	0.0396
3	2000 ng/ml	0.1 N NaOH	405.81	0.0385

Ruggedness

Ruggedness of analytical method is the ability of a method to resist the change in its performance in spite of influential environmental factors like temperature. Rugged analytical methods are preferred as these methods are free from impact of environmental/external factors. In order to establish the ruggedness of proposed spectrofluorimetric method, Berberine solution was analysed by three different analysts. Sample analysis and data processing resulted into % RSD values between 0.0073 and 0.0252 Results revealed that proposed spectrofluorimetric method was found to be rugged as it showed % RSD values less than 2 as shown in Table 6.

Table 6: Ruggedness data of spectrofluorimetric method for Berberine

Sr No.	Concentration (ng/mL)	Instrument	Mean	% RSD
1	2000ng/ml	Analyst 1	405.88	0.0252
2	2000ng/ml	Analyst 2	405.72	0.0065
3	2000ng/ml	Analyst 3	405.81	0.0073

Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOQ represents the lowermost concentration that can be analysed with acceptable accuracy and precision. Generally, LOQ is the first calibration standard. LOD and LOQ of proposed Spectrofluorimetric method was found to be 15.76 and 47.78 ng/ml respectively as shown in Table 7. Lower LOQ value indicated that proposed method would be suitable for analysing the samples containing even small quantities of Berberine.

Table No 7: LOD & LOQ data for spectrofluorimetric method for Berberine

1	LOD	15.76
2	LOQ	47.78

Estimation of Berberine in *Argemone Mexicana* extracts

Developed spectrofluorimetric method was successfully performed for estimation of Berberine content in *Argemone Mexicana* extracts. By proposed spectrofluorimetric method, Berberine content in Soxhlet extracts of *Argemone Mexicana* was found to be 2.44 ± 0.49 mg/g feed.

CONCLUSION

A simple, rapid, sensitive and reliable spectrofluorimetric method was developed for the estimation of Berberine in *Argemone Mexicana* extracts, said method was found to be accurate, precise, and easy to execute as compared to other reported methods. On the basis of Berberine content, proposed spectrofluorimetric method was found to be suitable for the standardization of *Argemone Mexicana* extract.

ACKNOWLEDGEMENT

The extra-mural grant support of DST-DPRP, Govt. of India (Ref:-VI-D&P/626/2018-19/TDT) dated 03/06/2019 sanctioned to P.I. Dr. Sachin S. Bhusari for the proposed research work is highly acknowledged.

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