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DEVELOPMENT AND VALIDATION OF UV-SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF PICROSIDE-II IN *PICRORHIZA KURROA* RHIZHOME EXTRACTS

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ABSTRACT

Aim: *To develop and validate a simple, precise and cost-effective UV- visible spectrophotometric method for the estimation of Picroside II in Picrorhiza kurroa rhizome extracts according to the ICH Q2 (R1) guideline.* ***Methods:*** *Spiked Picroside-II solution was scanned over UV-visible range for its wavelength of maximum absorbance. Various calibration standards of Picroside II were prepared. Calibration curve of concentration vs. absorbance was plotted. Various analytical method validation parameters were calculated.* ***Results:*** *The maximum wavelength of Picroside II was found to be 266 nm. The correlation coefficient at concentration range of 20-140 µg/ml was found to be 0.9995. The validation of the developed UV method was carried out by conducting linearity, accuracy, precision, robustness, ruggedness, limit of detection and limit of quantitation studies. Developed UV method was found to be precise for the intra-day and inter-day study and shows percent relative standard deviation in the range of 0.1179 to 1.3001 & 0.241 to 0.8882 respectively. The total percent recovery of Picroside II was found to be 99.85 to 99.85 %.* ***Conclusion:*** *A simple, precise and cost-effective UV- visible spectrometry method for the estimation of Picroside II in standardized extract of rhizomes of Picrorhiza kurroa was developed. The said method was developed using solvent containing economical percentage of organic phase in aqueous media. Said validated UV-visible method can be efficiently used for the estimation of Picroside II in extracts of rhizomes of Picrorhiza kurroa.*

KEY WORDS

UV- visible spectrometry, Picroside II, Picrorhiza kurroa, Validation

INTRODUCTION

Picrorhiza kurroa commonly known as Kutki, belongs to family Scrophulariaceae. It is a perennial herb with elongated rhizomes. Rhizomes of kutki consist of variety of chemicals with wide range of activities [1-2]. Among all the chemicals in rhizome, Picroside-II (Figure-1) has gained the attention of researchers working in natural products. It is a derivative of glycoside and its chemical formula is $C_{23}H_{28}O_{15}$ [3-4]. It is used for the treatments of liver disorders, fever, asthma, gastrointestinal and urinary disorders, snake bite and leukoderma. It has several pharmacological effects viz. anti-inflammatory, antioxidant, hepato-protective, neuroprotective, anticarcinogenic and anticholestatic [5-6]. Picroside II occurs as colorless, odorless white powder. It is soluble in organic solvent like ethanol, methanol and chloroform. Abundant presence of Picroside-II imparts prominent hepatoprotective activity to *Picrorhiza kurroa* extracts therefore standardized *Picrorhiza kurroa* extracts are gaining commercial importance [7-8]. Till date, there is no single UV-visible spectrophotometric method available for estimation of Picroside-II in extracts of *Picrorhiza kurroa* rhizomes. Even, a precise UV-visible

spectrophotometric method capable of estimating Picroside-II in variety of dosage forms like powder and solutions is not available. Therefore, considering the commercial importance and the needs of herbal industries, a simple yet precise and economical UV-visible spectrophotometric method capable of estimating Picroside-II was developed and validated.

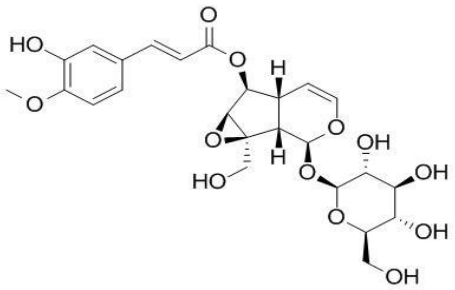

Fig. 1: Chemical structure of Picroside II

MATERIALS AND METHOD

Materials

Picroside II (purity 98% by HPLC) was obtained as gift sample from Natural Products Chemistry Division of Indian Institute of Integrative Medicine (CSIR), Jammu. Methanol and ethanol was purchase from Merck. All the chemicals of analytical grade were used for the proposed study.

Instruments Used

A double beam UV-visible spectrometer (UV-530, Jasco) with spectra manager software were used for the analysis. Quartz cells having 3 cm length with 1 cm path length were used for spectral measurement. Weighing balance (Essae, Vibra HT) with internal calibration mode was used for the accurate weighing purpose.

Preparation of standard stock solution

Accurately weighed 5 mg of Picroside II was transferred in to the calibrated volumetric flask and dissolved using 5 ml co-solvent system consisting of ethanol and water (50:50 v/v) to achieve a stock solution of 1000 µg/ml (Stock-I). Stock- I solution was suitably diluted with co-solvent system to achieve solution of 100 µg/ml (Stock-II).

Determination of wavelength of maximum absorbance (λ_{max})

Stock-II solution was scanned using full scan mode for the entire range of UV and visible i.e. 800 to 200 nm with co-solvent system as a blank. After obtaining the spectrum, λ_{max} was identified with the help of software. In order to achieve reproducible results, above method was repeated five times.

Preparation of calibration curve

Calibration curve was prepared by diluting the stock-I solution to achieve the seven different calibration standards representing 20, 40, 60, 80, 100, 120, 140 µg/ml strength. Absorbance of each calibration standard was measured at pre-identified λ_{max}; 266 nm using fixed wavelength measurement mode. The calibration curve representing concentration vs. absorbance was plotted. Above mentioned procedure was repeated five times so that reproducible results can be obtained.

Method Validation

Developed UV method for the estimation of Picroside-II was validated as per the ICH guideline. Different parameters like linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated [9-10].

Linearity and Range

Linearity of the proposed UV method was established using seven different calibration standards. Based on analysis of calibration standards, calibration curves in terms of absorbance 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 upper and lower concentration limit with acceptable linearity was reported to be the range of the proposed UV method.

Accuracy

The accuracy of the proposed UV method was evaluated using recovery studies after standard addition of analyte of interest. Three different solutions of Picroside-II were prepared in triplicate at level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of Picroside-II 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 UV method was established by performing intra- and inter-day UV

analysis of predefined samples. The study was performed at three concentration levels. Intra-day precision study was carried out by preparing nine different Picroside-II solutions of 20, 80 and 140 µg/ml strength (3 solutions of each concentration) and analyzing the same at morning, afternoon and evening time of same day. Deviation in the results was calculated in terms of % relative standard deviation (% RSD). Similarly, inter-day precision study was carried out by analyzing the above-mentioned solutions at three consecutive days.

Robustness

Robustness of the developed UV method was established using different percentage of ethanol in co-solvent system. Ethanol percentage in co-solvent system was kept at 45 and 55 % and Picroside II was dissolved in said co-solvent system separately. Triplicate samples were analyzed at 266 nm for absorbance. Levels of Picroside II in each sample were estimated using respective calibration curve. The results were calculated in terms of % RSD.

Ruggedness

Ruggedness study of the method was carried out by analyzing triplicate samples of Picroside II solution (80 µg/ml) at three different (25°C, 35°C, and 45°C) temperatures and absorbance were noted in terms of % RSD.

Limit of Detection (LOD)

The LOD of the developed UV method was calculated by using following formula

LOD=3.3×SD/S

Where, SD= Standard deviation of Y-intercepts

S= Slope

Limit of Quantitation (LOQ)

The LOQ of the developed UV method was calculated by using following formula

LOQ= 10×SD/S

Where, SD= Standard deviation of Y-intercepts

S= Slope

Estimation of Picroside-II in *Picrorhiza kurroa* rhizome extracts

Picrorhiza kurroa rhizomes were dried at 50°C using a Microtray drier (S.B. Panchal and company, Mumbai, India) and powdered using twin blade mixer (Bajaj electrical ltd., Mumbai, India). To select uniform particle size, 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 a period of 15 min. Powder passed through 120 mesh sieves was collected and used for further extraction.

Soxhlet assisted extraction (SAE) technique was used for the extraction of *Picrorhiza kurroa* rhizomes. Fifty g of powdered *Picrorhiza kurroa* rhizomes was placed in a thimble (Borosil, Mumbai, India) which was inserted into a Soxhlet apparatus. The material was exhaustively extracted with 95% ethanol. SAE was performed for 8 h. After predefined extraction period, solvent was distilled off under reduced pressure using rotary vaccum evaporator (Heidolph instruments GmbH & co. Germany) to obtain the dry extract.

Accurately weighed 5 mg of dry extract of *Picrorhiza kurroa rhizomes* was transferred in to the calibrated volumetric flask and dissolved using 5 ml of ethanol to achieve a stock solution of 1000 µg/ml (Stock-III). Stock-III solution was suitably diluted with co-solvent system and analyzed for the Picroside-II content using proposed UV method.

RESULTS AND DISCUSSION

Determination of wavelength of maximum absorbance

Identification of wavelength of maximum absorbance is prerequisite for quantitative UV analysis. Solution representing absorbance value less than 1 is generally considered to be suitable for the determination of wavelength of maximum absorbance. Considering the prerequisite and the suitability, determination of maximum wavelength for Picroside-II solution was carried out using full scan mode of UV-Visible spectrophotometer (Figure 2). Full scan was processed using UV software and the λ_{max} was identified with the help of software. It was found to be 266 nm for Picroside-II.

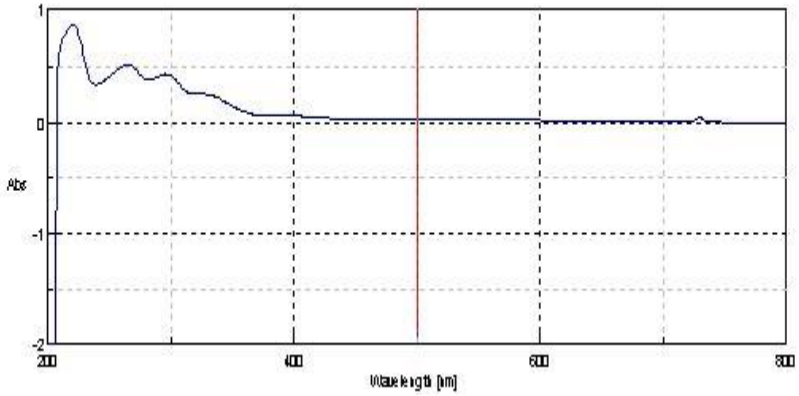

Fig. 2: UV-visible spectra of Picroside II

Preparation of calibration curve

Quantification of unknown samples by UV-Visible spectrophotometer or any other instrumental method of analysis needs a reproducible calibration curve and an equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. Considering the utility of quantitative analysis of Picroside-II, calibration curve for Picroside II was developed using seven different calibration standards. The absorbance of different calibration standards at 266 nm was recorded using fixed wavelength mode of UV-Visible spectrophotometer. Calibration curve was repeated five times and the mean values ± deviation was reported as shown in Table 1.

Table 1: Calibration standard data for Picroside-II

Concentration (µg/ml)	Absorbance
20	0.1951±0.0029
40	0.2907±0.0041
60	0.4037±0.0052
80	0.4926±0.0059
100	0.6001±0.0066
120	0.7112±0.0065
140	0.8101±0.0066

Method validation

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 Picroside II covering a range of 20-140 µg/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 = 0.0051x+0.0887 with correlation coefficient 0.9995 as shown in Figure 3. From the linearity study, it was revealed that, developed UV method was linear in the pre-defined concentration range of calibration standards.

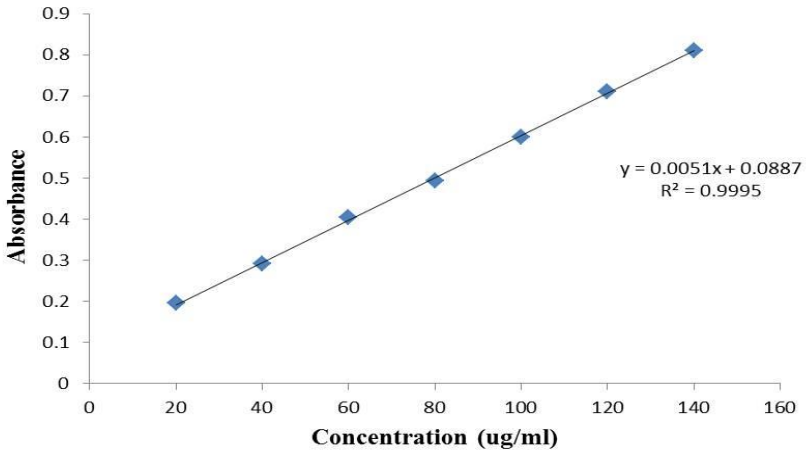

Fig. 3: Calibration curve for Picroside II

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 UV method for Picroside-II, accuracy was established using recovery studies. At 80 % standard addition, mean recovery of Picroside-II was found to be 99.88% whereas at 100 and 120 % standard addition, it was found to be 100.82 and 99.85% respectively. % RSD was found to be less than 2 for the Picroside-II recovery studies as shown in Table 2. From the results of accuracy studies, it was observed that developed UV method is highly accurate as the percent recovery was in between 98 to 102% and the % RSD was well below 2%.

Table 2: Accuracy data of UV method for Picroside-II

Concentration (%)	Origin level (µg/ml)	Amount added (µg/ml)	% Recovery	Mean % Recovery	% RSD
80	20	16	100.29		
80	20	16	99.27	99.88	0.785
80	20	16	100.10		
100	80	80	101.34		
100	80	80	99.28	100.82	1.41
100	80	80	101.84		
120	140	168	98.56		
120	140	168	100.17	99.85	0.961
120	140	168	100.84		

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 UV method was established at 20, 80 and 140 µg/ml levels of Picroside-II. The results in terms of mean absorbance 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 0.11 and 1.30 whereas those of inter-day precision study were in between 0.02 and 0.88. Overall, % RSD values of less than 2 showed the precision of developed UV method.

Table 3: Intra-day precision data of UV method for Picroside-II

	Morning			Afternoon			Evening		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
20	0.1962	100.67	0.4087	0.1951	100.20	0.6243	0.1934	98.86	1.3001
80	0.4907	101.12	0.3774	0.4894	99.86	0.9816	0.4962	100.71	0.4953
140	0.8111	100.72	0.1176	0.8067	100.45	0.6207	0.8104	101.02	0.3878

Table 4: Inter-day precision data of UV method for Picroside-II

	Day 1			Day 2			Day 3		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
20	0.1951	99.67	0.2071	0.1960	100.27	0.8554	0.1970	100.50	0.8882
80	0.4929	101.41	0.0652	0.4932	98.95	0.2186	0.4923	99.78	0.5230
140	0.8105	99.52	0.0241	0.8148	99.40	0.6503	0.8062	100.11	0.6741

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 an 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. Robustness of proposed UV method was established by modifying the composition of co-solvent system. Change in ethanol percentage (45 to 55 %) in co-solvent system did not affect the method performance. % RSD values were found to be in between 0.36 and 0.97 as shown in Table 5. % RSD values below 2 showed that proposed UV method is robust in nature.

Table 5: Robustness data of UV method for Picroside-II

Concentration (µg/ml)	% Ethanol	Absorbance	% RSD
80	45	0.4920	0.368
80	55	0.4886	0.978

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 UV method, Picroside-II solution was analyzed at three different temperature conditions. Sample analysis and data processing resulted into % RSD values between 0.63 and 1.48. Results revealed that proposed UV method was rugged as it showed % RSD values less than 2 as shown in Table 6.

Table 6: Ruggedness data of UV method for Picroside-II

Concentration (µg/ml)	Temperature (°C)	Absorbance	% RSD
80	25	0.4980	0.638
80	35	0.4935	1.283
80	45	0.4909	1.487

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

LOQ represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. Generally, LOQ is the first calibration standard. LOD and LOQ of proposed UV method was found to be 6.6 and 20 µg/ml respectively as shown in Table 7. Lower LOQ value indicated that proposed method would be suitable for analyzing the samples containing even small quantities of Picroside-II.

Table 7: LOD & LOQ data for UV method for Picroside-II

LOD	**6.60 µg/ml**
LOQ	**20.00 µg/ml**

Estimation of Picroside-II in *Picrorhiza kurroa* rhizome extracts

Developed UV method was successfully applied for estimation of Picroside-II content in *Picrorhiza kurroa* rhizome extracts. By proposed UV method, Picroside-II content in Soxhlet extracts of *Picrorhiza kurroa* rhizomes was found to be 10.927 ± 0.41 mg/g feed.

CONCLUSION

A simple, accurate and precise UV-Visible spectrophotometric method for the estimation of Picroside II in *Picrorhiza kurroa* rhizome extracts was developed and validated. Proposed method was found to be robust and rugged in nature and was successfully used for the estimation of Picroside-II present in *Picrorhiza kurroa* rhizome extracts.

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