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DEVELOPMENT AND VALIDATION OF SPECTROFLUORIMETRIC METHOD FOR THE ESTIMATION PICROSIDE II IN *PICRORHIZA KURROA* EXTRACTS: APPLICATION IN STANDARDIZATION

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ABSTRACT

A rapid, simple, sensitive and cost effective spectrofluorimetric method was developed for the estimation of Picroside II in pure pharmaceutical dosage form. The relative florescence intensity of Picroside II was measured in an at excitation wavelength of 269nm and emission wavelength 416nm. This reaction product was measured spectrofluorimetrically at 416nm after excitation at 269 nm under optimum conditions, linear relationship with best correlation coefficient 0.9999 and the linearity was detected in between the range of 25-10000ng/ml. The limits of detection (LOD) and limit of quantification (LOQ) were found in the range of 10.39 and 31.51ng/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 0.066 and 1.98 and 0.055 to 1.94 respectively. The total percent recovery of Picroside II was found to be 99.00 to 100.4.

KEYWORDS

Picroside-II, Detection limit, Fluorescence spectrophotometry, Fluorimetry, Linearity, Quantitation limit and Validation.

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INTRODUCTON

Picrorhiza kurroa (Kutki), belongs to family Scrophulariaceae. Rhizomes of Kutki are found at an altitudes of 3000-5000 m in north western Himalaya[1]. The plant is a small perennial herb, stem is small, weak creeping, erect at flowering, leafy, slightly hairy and is traditionally well known in India Ayurvedic system for the liver and respiratory disorder[2]. It is also used in chronic fevers, treatment

of upper respiratory tract infection, dyspepsia, and diarrhoea and scorpion sting. It is improve appetite and stimulate gastric secretions[3]. It is a well-known hepatoprotective plant and used widely in various herbal formulations[4]. Rhizomes of Kutki consist of various types of chemicals with wide range of activities[5,6]. Picroside II (PK-II) is one of the active constituents of Kutki rhizomes. It is a glycoside derivative and its chemical formula is $C_{23}H_{28}O_{15}$. PK-II (Figure No.1) occurs as colourless, odourless white powder. It is soluble in organic solvent like ethanol, methanol and chloroform[7,8]. Abundant presence of PK-II imparts prominent hepatoprotective activity to *Picrorhiza kurroa* extracts therefore standardized *Picrorhiza kurroa* extracts are gaining commercial importance.

Till today, number of analytical methods belonging to UV and HPLC are available for the estimation of PK-II either alone or in combination with PK-I but none of the above mentioned analytical methods are sensitive enough to estimate the PK-II in its ng level. Moreover, there are no reports of development of simple, sensitive, highly accurate and precise spectrofluorimetric method for the estimation of PK-II which can be used for the standardization of Picrorrhiza kurroa extracts. Considering the future prospects of standardized *Picrorrhiza kurroa* extracts in therapeutics, it was envisaged that development of spectrofluorimetric method for the estimation of PK-II will be worth.

EXPREMENTAL

MATERIAL AND METHODS

Chemicals and reagents

PK-II (purity 98% by HPLC) was obtained as gift sample from Natural Products Chemistry Division of Indian Institute of Integrative Medicine (CSIR), Jammu. Methanol 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 widthwere used for fluorescence measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the weighing purpose.

Preparation of standard stock solution

A 5 mg of PK-II was accurately weighed, poured into 5mL volumetric flask and dissolved in 5mL Methanol. It was sonicated for 5 min and the resultant solution was diluted with Methanol to achieve a stock solution-1 of 1 mg/mL strength. Stock solution-1 diluted further with Methanol to get stock solution-II and III of 100µg/mL and 10µg/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 25, 100, 200, 500, 1000, 5000, 10000ng/ml strength. Florescence Intensity of each calibration standard was measured at pre-identified $\lambda_{Excitation}$ 269nm and $\lambda_{Emission}$ 416nm. 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[9,10]

Developed spectrofluorimetric method for the estimation of PK-II 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).

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 PK-II were prepared in triplicate at level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of PK-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) × 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 concentration of PK-II solutions of 30, 4900 and 5900ng/ml strength (3 solutions of each concentration) and analyzing 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 analyzing the above mentioned solutions at three consecutive days.

Ruggedness

Ruggedness study of the method by preparing the middle level sample (4900 ng/ml) by the three different analyst and analyzed at 269nm excitation and 416nm emission wavelength of PK-II. 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 PK-II 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×SD/S

LOQ= 10×SD/S

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

S= Slope of calibration curve

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

Proposed spectrofluorimetric method was used for the estimation of PK-II in Soxhlet assisted extraction (SAE) of *Picrorhiza kurroa* rhizomes. Briefly, 30 g of powdered *Picrorhiza kurroa* rhizomes was put in a thimble (Borosil, Mumbai, India) which was placed into a Soxhlet apparatus. The material was exhaustively extracted with 95% ethanol. SAE was performed for 7 h. After predefined extraction period, solvent was distilled off under reduced pressure using rotary vaccum evaporator (Heidolph instruments GmbH and co. Germany) to obtain the dry extract. Accurately weighed 1 mg of dry extract of *Picrorhiza kurroa rhizomes* was transferred in to the eppendorf and dissolved using 1 ml of methanol to achieve a stock solution of 1000µg/ml (Stock-I and then to prepared solution 100 µg/ml stock-II). Stock- II solution was suitably diluted with methanol and analyzed for the Picroside-II content using 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 269 nm and emission wavelength 416 nm (Figure No.2) by Spectrofluorimeter. Least square linear regression analysis was performed for the obtained spectrofluorimetric data using MS-Excel 2013. Calibration curve was repeated five times for reproducibility. Various concentrations and their fluorescence intensities with mean ± standard deviation were reported (Table No.1).

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 PK II covering a range of 25-1000ng/ml was plotted. Details of concentrations and the respective mean absorbance values are depicted in Table No.1. Calibration curve when subjected to least square regression analysis yielded an equation; $y = 0.0628x + 4.27622$ with correlation coefficient 0.999 as shown in Figure No.3. From the linearity study, it was revealed that, developed spectrofluorimetric method was linear in the pre-defined concentration range of calibration standards.

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 PK-II, accuracy was established using recovery studies. At 80 % standard addition, mean recovery of PK-II was found to be 101.5% whereas at 100 and 120 % standard addition, it was found to be 100.5 and 99.6 % respectively. % RSD was found to be less than 2 for the PK-II recovery studies as shown in Table No.2. From the results of accuracy studies, it was observed that developed spectrofluorimetric method is highly accurate as the percent recovery was in between 97 to 100% and the % RSD was well below 2%.

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 25, 4900 and 9900 ng/ml levels of PK II. 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 No.3 and Table No.4 respectively. %RSD values of intra-day precision study were found to be in between 0.066 and 1.98 whereas those of inter-day precision study were in between 0.055 and 1.94. Overall, % RSD values of less than 2 demonstrated the precision of developed spectrofluorimetric method.

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 prefered. Proposed spectrofluorimetric method was found to be robust as the % RSD values were found to be in between 1.31 and 1.57 as shown in Table No.5.

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, PK II solution was analyzed at two different analyst. Sample analysis and data processing resulted into % RSD values between

0.68 and 1.27. Results revealed that proposed spectrofluorimetric method was found to be rugged as it showed % RSD values less than 2 as shown in Table No.6.

Limit of Quantification (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 Spectrofluorimetric method was found to be 10.39 and 31.51 ng/ml respectively as shown in Table No.7. Lower LOQ value indicated that proposed method would be suitable for analyzing the samples containing even small quantities of PK-II.

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

Developed spectrofluorimetric method was successfully performed for estimation of Picroside-II content in *Picrorhiza kurroa* rhizome extracts. By proposed spectrofluorimetric method, Picroside-II content in Soxhlet extracts of *Picrorhiza kurroas* rhizomes was found to be 1.14±0.49mg/g feed.

Table No.1: Calibration standard data for PK II

S.No	Concentration	Fluorescence intensity
1	25	1.249±0.4026
2	100	6.895±0.4082
3	200	12.589±0.3940
4	500	41.428±0.4140
5	1000	75.380±0.4001
6	5000	317.467±0.4013
7	10000	632.209±0.4019

Table No.2: Accuracy data of Spectrofluorimetric method for PK II

S.No	Concentration (%)	Origin level (ng/ml)	Amount added (ng/ml)	% Recovery	Mean % Recovery	% RSD
1	80	30	24	102.50	101.102	1.25
2	80	30	24	100.08		
3	80	30	24	100.71		
4	100	4900	4900	100.96	100.54	0.414
5	100	4900	4900	100.54		
6	100	4900	4900	100.31		
7	120	9900	11880	99.87	99.63	0.526
8	120	9900	11880	99.03		
9	120	9900	1180	100.00		

Table No.3: Intra-day precision data of spectrofluorimetric method for PK-II

S.No	Concentration Range (ng/ml)	Morning			Afternoon			Evening		
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	30ng/ml	1.929	100.47	1.15	1.935	100.81	1.63	1.922	100.1	1.98
2	4900ng/ml	310.82	99.06	0.348	310.61	99.047	1.169	310.57	99.035	1.94
3	9900ng/ml	627.75	99.07	0.117	627.72	99.07	0.077	628.32	99.16	0.066

Table No.4: Inter-day precision data of spectrofluorimetric method for PK-II

S.No	Concentration Range (ng/ml)	1 Day			2 Day			3 Day		
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	30ng/ml	1.927	100.366	0.767	1.927	100.36	1.244	1.919	99.992	1.94
2	4900ng/ml	310.878	99.050	0.152	312.995	99.80	0.247	312.37	99.60	0.182
3	9900ng/ml	627.935	99.106	0.254	627.875	99.09	0.055	627.86	99.09	0.075

Table No.5: Robustness data of spectrofluorimetric method for PK-II

S.No	Concentration (ng/ml)	Solvent Ratio	Mean	% RSD
1	4900 ng/ml	Water	46.403	1.577
2	4900 ng/ml	MeOH	244.866	1.317
3	4900 ng/ml	0.1 N NaOH	6.543	1.552

Table No.6: Ruggedness data of spectrofluorimetric method for PK-II

S.No	Concentration (ng/mL)	Instrument	Mean	% RSD
1	4900ng/ml	Analyst 1	244.634	0.6892
2	4900ng/ml	Analyst 2	246.148	1.2737

Table No.7: LOD and LOQ data for spectrofluorimetric method for PK-I

1	LOD	10.398
2	LOQ	31.510

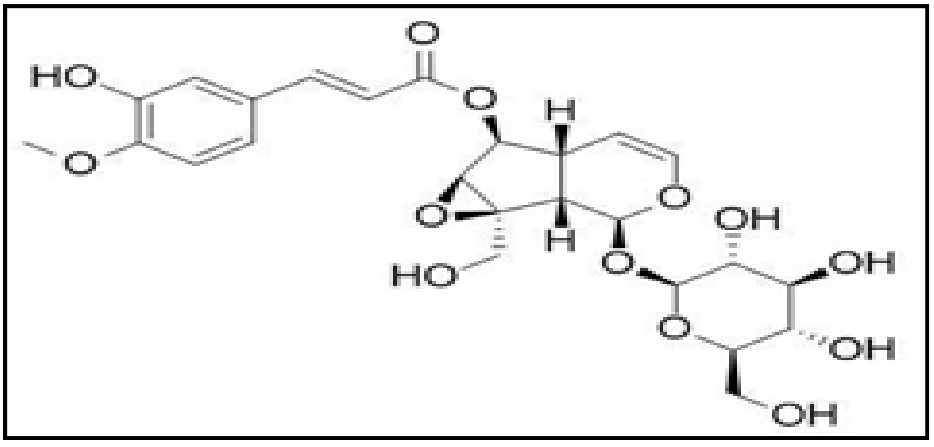

Figure No.1: Chemical structure of Picroside II

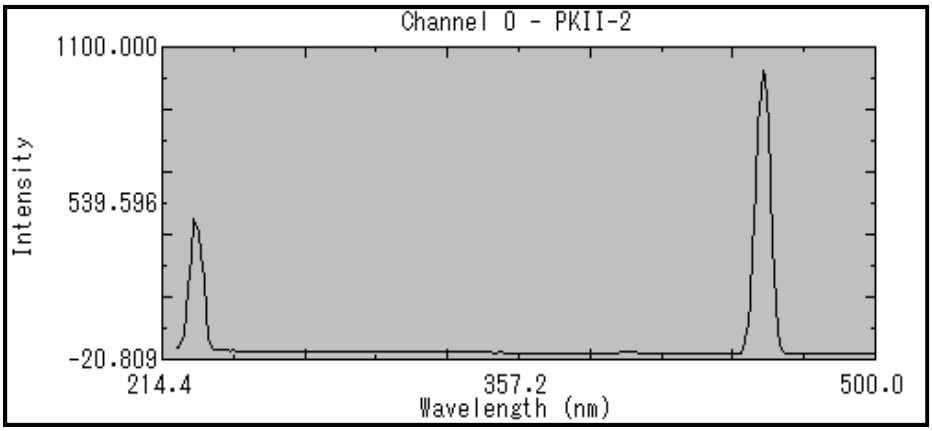

Figure No.2 Excitation scan of PK II at the Emission wavelength of 419 nm

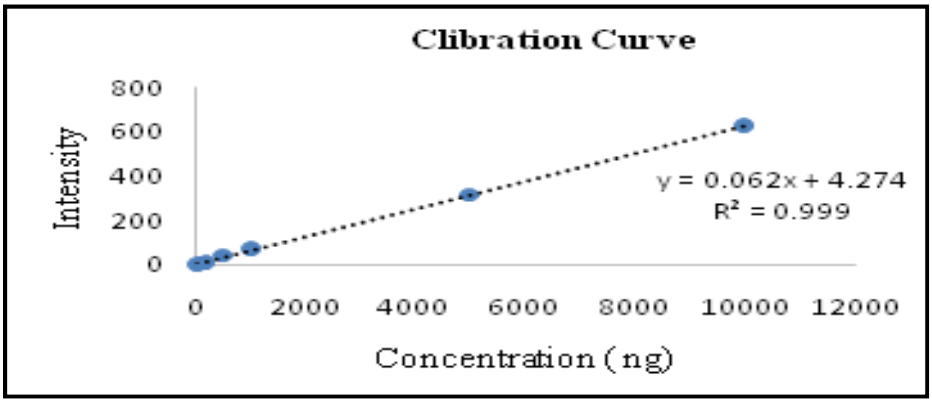

Figure No.3: Calibration curve for PK II

CONCLUSION

A simple, rapid, sensitive and reliable spectrofluorimetric method was developed for the estimation of PK II in *Picrorhiza kurroa* extracts. Said method was found to be accurate, precise, and easy to execute as compared to other reported methods. On the basis of PK-II content, proposed spectrofluorimetric method was found to be suitable for the standardization of *Picrorhiza kurroa* extract.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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