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Development and Validation of Stability Indicating Hptlc Method for Determination of Picroside-I and Picroside-II in *Picrorhiza kurroa*

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Abstract

Picrorhiza kurroa Royle commonly known as 'Kutki or Kutaki' is an important medicinal plant in Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract. The plant is the principle source of iridoid glycosides, picrosides I, II and kutkoside used in various herbal drug formulations mainly as strong hepatoprotective and immune-modulatory compound. An approach for the stress degradation was successfully applied for the picrosides I, II development of stability indicating HPTLC method for the determination of Separation was achieved on the plates pecoated with silica gel 60 F254. The mobile phase used was chloroform: methanol: formic acid (8:1.5:0.5 v/v/v) and quantification was carried out wavelength at 274 nm. Stress testing of picrosides I, II was carried out according to the International Conference of Harmonization (ICH) guideline Q1A(R2). The markers were subjected to acid, base, neutral hydrolysis, oxidation, thermal degradation and photolysis. The system showed a peak for picrosides I, II at Rf value of 0.47, 0.57 ± 0.02 resp. The method was successfully validated according to ICH guidelines Q2 (R1). The data of linear regression analysis indicated a good linear relationship over the range of 200–1000 ng/band concentrations with correlation coefficient value of 0.999. The accuracy of the method was established based on the recovery studies. The LOD of picrosides I, II were found 135,108 ng/ band. LOQ of picrosides I, II were found 410,327 ng/ band, respectively. Under various tested stressed conditions, picrosides I, II showed considerable degradation under alkali, acidic, oxidative and neutral hydrolytic condition.

Keywords: Picrosides; High performance thin layer chromatography; Stability indicating method

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Introduction

The important medicinal herb Kutki (*Picrorhiza kurroa* Royle ex Benth.) belongs to the family of Scrophulariaceae and the dried underground part (rhizomes and roots) of this plant has found to possess hepatoprotectice, antioxidant, anti-allergic, antiasthamatic, anticancerous and immunomodulatory properties [1]. This is one of the renowned plants in India, China, Tibet, Nepal and Sri Lanka from the prehistoric period having been used for the treatment of numerous immune-related diseases in Ayurvedic as well as other different traditional system of medicine [2]. It is traditionally applied in the treatment of disorders like liver and upper respiratory tract, fevers, dyspepsia, chronic diarrhea, and scorpion sting [3] *Picrorhiza kurrooa* (Family: Scrophulariaceae; local/ trade name: Kutki), is an important medicinal herb, endemic to alpine Himalaya, is distributed between 2800-4800 m altitude. The plant has been listed as an 'endangered' due to reckless collection and indiscriminate exploitationfrom its natural habitat. Over exploitation, consequent degradation from natural habitat, narrow distribution range, small population size and high economic value were major threats for its survival. Current research on *Picrorhiza kurroa* has focused on its hepatoprotective,

anticholestatic, antioxidant, and immunemodulating activity. P. kurroa is a prosperous source of hepatoprotective picrosides like picroside I, picroside II and other metabolites like picroside III, picroside IV, apocynin, androsin, catechol, kutkoside,etc. The roots of the plants have been used to treat disorders of the liver, chronic diarrhea as well as bitter tonic, antiperiodic, cholagouge, stomatic, laxative in small doses and cathartic in large doses [4]. Structure of the markers is shown in **Figure 1**. Literature survey reveals HPTLC, HPLC, RP-HPLC [4-11], Profile and methods reported for estimation of picrosides I, II. But there is no report on stability indicating method validation of picrosides I, II in *Picrorhiza kurroa* Hence, a *Picrorhiza kurroa* densitometric HPTLC method has been developed in the present work for quantitation of picrosides I, II from *Picrorhiza kurroa*

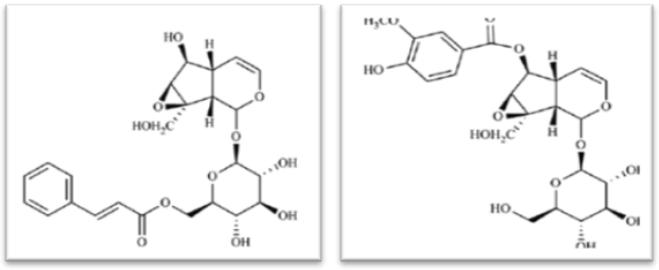

Figure 1 Structure of picrosides I, II.

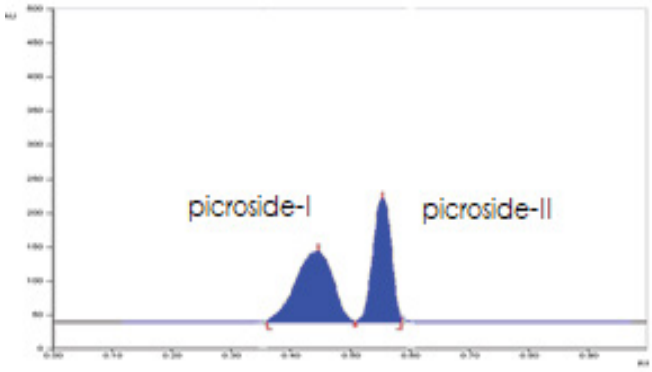

Figure 2 Chromatographic conditions and instrumentation.

Materials and Methods

Chemicals and reagents

Picrosides I, II purchased form Yucca Enterprises, Mumbai, were used as such, without any further purification. The roots of *Picrorhiza kurroa* were purchase from local area of Ahmednagar. These were authenticated from Agharker Research Institute, Pune. Aluminum sheets pre-coated with silica gel (60 F254, 20 cm × 20 cm with 250 µm layer thickness) were purchased from E-Merck, Darmstadt, Merck (Germany). Methanol (HPLC grade), Ethanol (AR grade), Hydrochloric acid (HCl), hydrogen peroxide (H_2O_2, 30% v/v) were purchased from S. D. fine chemical Laboratories, Mumbai.

Chromatographic conditions and instrumentation

Chromatographic separation of drug was performed on Aluminum plates precoated with silica gel 60 F254, (10 cm × 10 cm with 250 µm layer thickness). Samples were applied on the plate as a band with 6mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). The mobile phase was composed chloroform: methanol: formic acid (8:1.5:0.5 v/v/v) 10 cm × 10 cm CAMAG twin trough glass chamber was used for linear ascending development of TLC plate under 16 min saturation conditions and 13.2 ml of mobile phase was used per run, migration distance was 80 mm. Densitometric scanning was performed using Camag TLC scanner 3, operated by win CATS software (Version 1.4.3, Camag).

Preparation of standard solutions

Standard stock solution was prepared separately by dissolving 10 mg picrosides I, II in 10 ml of methanol to get concentration of 1000 µg/ml. From the standard stock solution, working standard mixture of picrosides I, II was prepared containing 100 µg/ml of picrosides I, II . Standard densitogram obtained is as shown **Figure 2.**

Stress degradation study of Picroside-I and Picroside-II

Stress degradation studies were carried under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolysis as per ICH Q1A (R2) guideline. For each study, samples were prepared as follows: Picrosides I, II working standard solution subjected to stress condition. Stress condition was optimized terms of strength of reapent and time of exposure to obtain 10-30% degradation [12].

Alkaline hydrolysis: To 1 ml of 1000 µg/ml solution of Picrosides I, II with 1 ml of 0.1 N NaOH (Methanolic) was added. The volume was made up to 10 ml with methanol. The above solution was kept 6hrs at room temperature in dark place.

Acidic hydrolysis: To 1 ml working standard solution of Picrosides I, II (1000 µg/ml) with 1 ml of 0.1 N HCl (Methanolic) was added. The volume made up to 10 ml with methanol. Solution was kept for 6 hrs in dark place.

Neutral hydrolysis: To 1 ml working standard solution of Picrosides I, II (1000 µg/ml) with 1 ml water and volume made up to 10 ml with methanol. The solution was kept for 6 hrs in dark place.

Oxidation: To 1 ml working standard solution of Picrosides I, II 1000 µg/ml) was mixed with 1 ml of 6% v/v solution of H_2O_2 and volume made up to 10 ml with methanol. Solution was kept for 6 hrs in dark place.

Degradation under dry heat: Dry heat studies were performed by keeping drug sample in oven (60°C) for 6 hrs.

Photo-degradation studies: Photolytic studies were carried out by exposure of drug to UV light up to 200 watt hours/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux hrs. Sample was weighed, dissolved in methanol and appropriate dilutions were made to get final concentration of 100 µg/ml.

Results and Discussion

Under optimized chromatographic conditions retention factor of Picrosides I, II was found to be 0.47,0.57 ± 0.2 resp. The % assay was found to be 102% ± 1.64. Degradation was observed for Picrosides-I, II during stress conditions like hydrolysis, oxidation, dry heat and photolysis but the peak of degradation product of Picrosides-II was observed only under acid, alkali hydrolysis. Picrosides-II showed one degradation product (D1) in alkaline hydrolysis at Rf 0.61, one degradation product (D2) in acid catalysed hydrolysis at Rf 0.62 **(Figure 3).** The Rf of degradation product (D2) interfear with Rf of Picrosides I. Picrosides I does not show any degradation peak under hydrolysis, oxidation, dry heat and photolysis. Summary of stress degradation results is given in **Table 1.** Peak purity results greater than 0.999 indicate that Picrosides I peaks are homogeneous in all stress conditions tested indicating non-interference of product of degradation. The unaffected assay Picrosides I, II confirms the stability indicating power of the method.

Method validation

For the developed method, validation parameters checked were specificity, Linearity, Accuracy, Precision, Sensitivity i.e., LOD and LOQ, Robustness as per ICH Q2A (R1) guideline [13].

Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 0.997, indicating the non-interference of any other peak.

Linearity

Linearity studies were performed in the concentration range of 200-1000 ng/band an accurate correlation was found between peak area and amount spotted. Results are tabulated in **Table 2 and Figure 4.**

Precision

Interday precision: Precision of the system was evaluated by analyzing three independent standard preparations on three

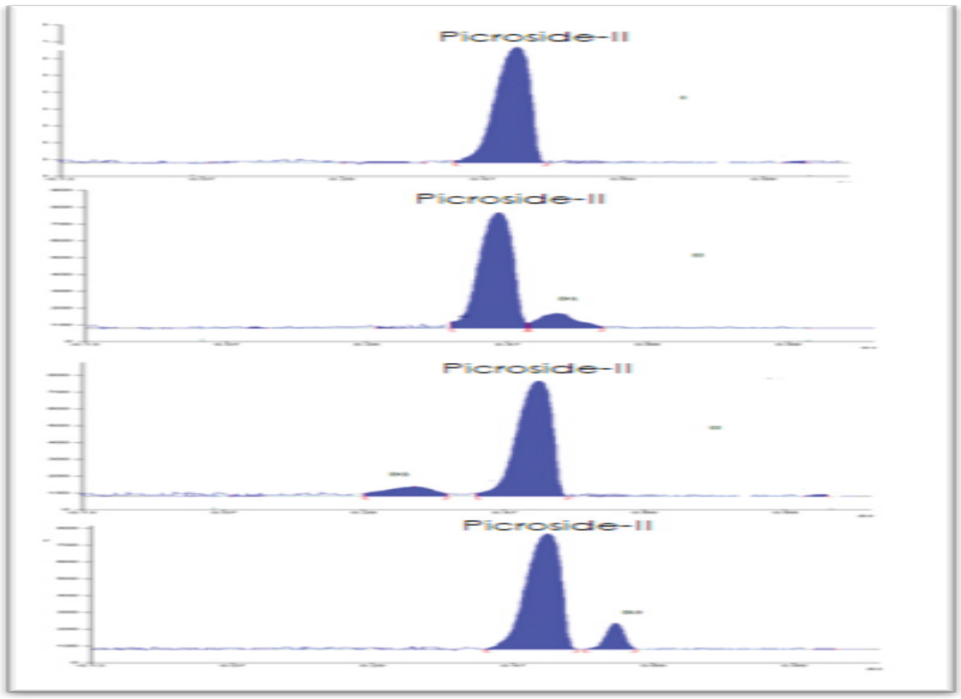

Figure 3 I- Densitogram of Picroside-II Standard, II- Alkali treated Picroside-II, III- oxidation treated Picroside-II, IV- Acid treated Picroside-II.

Table 1 Summary of stress degradation of P-I, P-II.

Stress Degradation Condition	Peak Area		% Recovery		R_f degradation product		Peak purity			
							R (s, m)		R (m, e)	
	P-I	P-II	P-I	P-II	P-I	P-II	P-I	P-II	P-I	P-II
Base (0.1 N NaOH, kept for 6hrs)	3100	3340	85.7	80.1	-	0.6	0.999	1	1	1
Acid (0.1 N HCl, kept for 6 hrs)	2812	1121	89	75.8	-	0.6	1	0.999	1	0.999
Neutral (kept for 6hr)	3012	1665	93.8	91.6	-	-	1	0.999	1	0.999
H_2O_2 6(kept for 6 hrs)	2890	1989	83.4	81.4	-	0.5	0.999	1	0.999	1
Heat dry (60^0C,6 hrs)	2970	2105	84	73.2	-	-	0.999	0.999	1	0.999
Photo stability	2570	1502	85.9	84.6	-	-	1	1	0.999	1

Table 2 Linearity of P-I and P-II.

S. No	Concentration (ng/band)	Peak area	
		P-I	P-II
1	200	2073	1887
2	400	3510	3872
3	600	5517	5647
4	800	7202	7474
5	1000	8890	9293

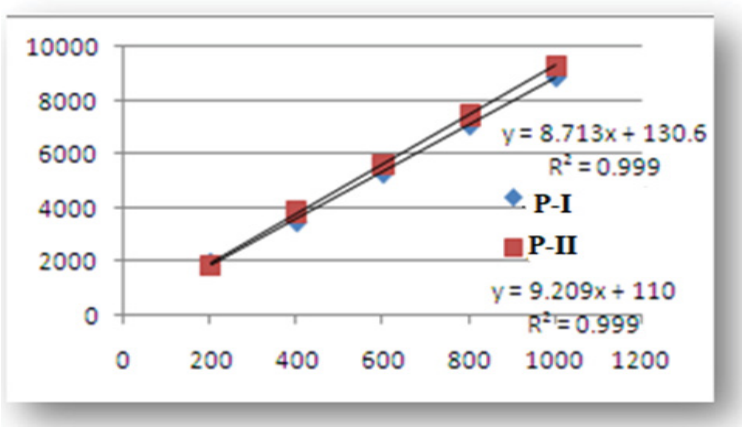

Figure 4 Calibration curve of P-I and P-II.

Table 3 Inter-day precision of P-I and P-II.

Conc. (ng/band)	Mean Area		SD		% RSD	
	P-I	P-II	P-I	P-II	P-I	P-II
200	2306	2064	30	30	1.3	1.3
400	4351	4231	37	31	1.5	1.4
600	6071	5231	40	34	1.7	1.6

Table 4 Intraday precision of P-I and P-II.

Replicates	Intraday	
	P-I	P-II
1	2306	2133
2	2311	2097
3	2323	2143
4	2347	2156
5	2359	2200
6	2383	2177
Mean Area	2383	2151
% RSD	1.28	1.66

Table 5 % Recovery.

Level %	Sample	Standard	Mean Area		% Recovery	
			P-I	P-II	P-I	P-II
80	400	320	20570	10649	97.020	98.68
100	400	400	22694	12446	94.583	100.24
120	400	480	24708	14122	99.81	101.26

Table 6 Results of robustness.

S. No	Parameters	% RSD	
		P-I	P-II
1	Chamber Saturation time (16) ± 2 min	1.55	1.74
2	Mobile phase composition-	1.81	1.57
3	Time from spotting to development (immediate& after 2 hrs)	0.45	1.3
4	Time from development to scanning	0.66	1.02

different days and % RSD value obtained was calculated to determine system precision. Results are tabulated in **Table 3.**

Intraday precision: Precision of the system was evaluated by analyzing six independent standard preparations in a day and % RSD value obtained was calculated to determine system precision. Result are tabulated in **Table 4.**

Accuracy

Accuracy studies were performed by adding 80%, 100%, and 120% solutions with respect to target assay concentration (400 ng/band). The amount of P-I and P-II was calculated and % recovery is tabulated in **Table 5.**

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of P-I and P-II were found 135,108ng/ band. LOQ of P-I and P-II were found 410,327ng/ band, respectively.

Robustness

Robustness was determined by carrying out the analysis under conditions during which mobile phase ratio, time form application to development and time form development to scanning, chamber saturation time were altered. It was found that method is robust. Results are tabulated in **Table 6.**

Conclusion

The accuracy of the method was established based on the recovery studies. The LOD of picrosides I, II were found 135,108 ng/ band. LOQ of picrosides I, II were found 410,327 ng/ band, respectively. Under various tested stressed conditions, picrosides I, II showed considerable degradation under alkali, acidic, oxidative and neutral hydrolytic condition. The developed method was found to be simple, time saving, economic, accurate and precise. This method can be used for quantitative analysis of Picrosides I. Picrosides-II.

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