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A GREEN APPROACH FOR SIMULTANEOUS QUANTITATIVE ANALYSIS OF BERBERINE & ANDROGRAPHOLIDE IN POLYHERBAL TABLET USING RP-HPLC-UV-DAD

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ABSTRACT

A selective, sensitive and precise reversed phase HPLC-UV-DAD method was developed for the simultaneous determination of two natural hepatoprotective drugs in Polyherbal Tablet, and validated as per the International Conference on Harmonization guidelines. The first component is berberine whereas second is andrographolide. The analysis was conducted using RP-18 column (250 mm x 4.6 mm x 5 µm id) and Potassium Di-hydrogen phosphate along with Acetonitrile as a mobile phase in a flow rate of 1.0 mL/min and detection at 226 nm at 30ºC. The method was evaluated for precision, selectivity, linearity, accuracy. The presence of other chemical constituents did not interfere with the quantification of berberine and andrographolide. The calibration curve was linear in the concentration range of 29 to 90 ppm and 56 to 158 ppm respectively. The recovery ranged from 98.0% to 102.0% with relative standard deviations not higher than 2%. Finally, this developed HPLC method is simple, reliable and successfully applied to identify and quantify the berberine and andrographolide in Polyherbal Tablet, which can be useful for the analysis of infusions that people consume in folk medicine.

INTRODUCTION

Ayurveda is also known as “Goddess of All Healing” and is considered as one of the most effective traditional system of medicine with many curing and healing properties. According to Ayurveda, individual herbs are insufficient to achieve a desired therapeutic effect. When it is optimized as multiple herbs composition in a particular ratio it will give a therapeutic effect in a better way with reduced toxicity[1]. About 31.4% population in industrialized societies, 42%–69% in United States, 71% Canadians and 90% British population are consuming dietary supplements or natural health products (vitamins, minerals, amino acids, essential fatty acids, herbal products, traditional Chinese medicines, Homeopathic medicines, and probiotics) for treatment, including elimination of disease causing agents, avoidance of side effects and for getting quality life [2]. The quality of herbal products is a major public health concern both in developed and resource-poor countries, where fakers sell adulterated herbal medicines. It is feasible that the introduction of scientific validation would control the production of impure or poor quality herbal products and would eventually ensure their rational use[3]. Physicochemical parameters, biochemical analysis, microbiological features, HPLC and HPTLC fingerprint profile may be usedas

marker parameters for quality evaluation and standardization of polyherbal formulations. HPLC is a rapid and precise technique for quantification of individual compounds. [4]. Polyherbal tablet is a classical ayurvedic polyherbal formulation for the treatment of Liver infections. That formulation contain two natural hepatoprotective drugs i.e. berberine and andrographolide. Berberine is an isoquinoline alkaloid of the protoberberine type, which could be found in the root, rhizome, and stem bark of many plant species traditionally used for treatment of hepatic disorders [5]. Extensive research within the past decade indicates that berberine possesses a wide range of pharmacological activities, including antioxidative, anti-inflammatory, and immunoregulative activities. Several studies demonstrated the inhibitory effects of berberine on chemically induced cytotoxicity, lipid peroxidation, and oxidative stress in the liver, including CCl4- induced liver damage[6]. Andrographolide is a diterpenoid lactone which is widely used as a bitter tonic, for snake bite and for the treatment of hepatitis. Experimental studies have shown that andrographolide may help in liver regeneration. This may stem from its cholinomimetic property and resultant stimulation of insulin secretion [7,8]. In the present study, we aimed to separate, identify, and quantify two natural hepatoprotective drugs i.e. berberine and andrographolide in polyherbal formulation using RP-HPLC-UV-DAD. The HPLC-DAD method for quantification of these drugs was also validated. The validation of chromatographic identification of berberine and andrographolide from polyherbal tablet may contribute to the standardization of polyherbal formulation and drug development process.

MATERIALS AND METHODS:

Chemicals: HPLC-grade solvents such as acetonitrile, methanol, orthophosphoric acid and water were obtained from Merck Ltd. Bangalore India. Standards were purchased from Natural Remedies Ltd. Bangalore India [**Table 1**].

Preparation of standard solution: 10 mg Berberine as well as Andrographolide was accurately weighed and transferred into 25 mL volumetric flask. 20 mL of diluent was added and sonicated in ultrasonic water bath for 15 minutes. The solution was cooled and volume was made up to the mark with diluent.

Preparation of test solution: 20 tablets of formulation were weighed and powdered. 2000 mg of powdered tablet formulation was taken into 100 mL volumetric flask. 70 mL of diluent was added and sonicated in ultrasonic water bath for 30 minutes. The resulting solution was cooled and volume was made up to the mark with diluent. The content of volumetric flask was filtered through Whatman filter paper No. 41 and then 0.45 µ syringe filter. Resulting solution was used as test solution.

Chromatographic conditions for HPLC: HPLC was performed using a Waters 2695 Alliance system with a 2996 photodiode array detector (PDA) and 2489 UV/Visible detector (UV). The standards as Berberine as well as Andrographolide were resolved on a reverse-phase 250 × 4.6 mm, 5-µm, Sunfire C18 column (Mumbai, India). The mobile phase was prepared from 50 milimole of Potassium Di-hydrogen phosphate in water and adjusted the pH 3.0 with diluted OPA (solvent-A) and Acetonitrile (100%) (solvent-B). The gradient program used is given in **Table 2**. The mobile phase flow rate was kept at 1 ml/min. Before the first injection, the column was saturated for 30 min with the initial mobile phase. Temperature was maintained at 25°C. Injection volume was decided tomaintainat 10 µL. The PDA was set by optimizing wavelength to give best response for two peaks at 226 nm to acquire the chromatogram. The standard berberine and andrographolide were identified by comparing the retention time and spectra obtained from the sample and standard solutions. The present work was performed in an air-conditioned room maintained at 25°C [9-11].

Preparation of Calibration Graph: The linearity of peak area response for Berberine & Andrographolide was determined from 50 % to 150 % level of working concentration for Berberine & Andrographolide. The stock solutions of Berberine & Andrographolide were diluted in seven different known

concentrations. Graphs of concentration (as x-value) versus area (as y-value) were plotted.

VALIDATION OF HPLC METHOD

The proposed HPLC method was validated in terms of specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), standard solution stability, sample solution stability and robustness as per the International Conference on Harmonization (ICH) guidelines[12-15].

Specificity: The specificity of the method was studied by assessment of peak purity of berberine and andrographolide using the Waters empower software and diode array detector and represented in terms of purity angle, purity threshold, and purity flag.

Precision: Precision was studied in terms of system precision, method precision, and intermediate precision.

System precision: System precision was carried out by six replicate injections from the same vial of standard and was expressed in terms of percent relative standard deviation (% RSD) tailing, plate count, and resolution.

Method precision: The sample was analyzed for six times by mentioned procedure. The % assay for each analyte was expressed in terms of % RSD.

Intermediate precision: Intermediate precision was performed on different systems, one the Waters e2695 Alliance system with a 2996 PDA and the other a 2489 ultraviolet (UV) detector by different analysts by analyzing six different sample of extract and was expressed in terms of % RSD.

Recovery studies: The accuracy of the method was determined from recovery studies by adding a known amount of each standard at the 80%, 100%, and 120% level to the pre-analyzed sample followed by replicate quantitative analyses by the proposed method.

Robustness: Robustness of the method was determined by slight deviation in the method parameters. The parameters selected were deviation in column chemistry, wavelength, column temperature, flow rate, and mobile phase gradient. The retention time of berberine and andrographolide respectively, was determined and % RSD using system suitability parameters was observed. Polyherbal tablet formulation was analyzed to determine the contents of berberine and andrographolideas per method described under chromatographic conditions by HPLC. All analysis was repeated three times and results were expressed in mean ± SD.

RESULTS AND DISCUSSION

The composition of the mobile phase in the HPLC method was optimized by testing different solvent compositions of varying polarity, column chemistry, column temperature, and pH of mobile phase, and the best results were obtained by using the present method, which produces highly symmetrical peaks showing good resolution between each standard and other peaks **[Figure 1]**. The scanning wavelength selected was 226 nm to provide comparable results and at this wavelength all analyte showed optimum response. Berberine and andrographolide were satisfactorily resolved with retention time about 11.5 and 13.0 min, respectively. The calibration graph was in 50 % to 150 % level of working concentration for berberine & andrographolide, with acceptable correlation coefficients 0.9990 and 0.9992 for berberine (28-90 µg/ml) and andrographolide AKBA (56-158 µg/ml) respectively **[Table 3]**.The graph for each standard is given in **Figure 2**. The values of system precision, method precision, and intermediate precision are given against sample application and scanning of peak area and are expressed in terms of % RSD. For system precision % RSD values were found to be 0.67 % and 0.42 % for Berberine and andrographolide respectively.Method precision was done and % RSD value were found to be 0.46 % and 0.64 % for Berberine and andrographoliderespectively. For intermediate precision % RSD values between the two analysts were found to be 1.99 % and 1.62 % for Berberine and andrographolide respectively. For the values of system precision, method precision, and intermediate precision, the %RSD values showed that the proposed method provides an acceptable level of system precision, method precision, and intermediate precision.

Table 1: Standard and Test used for validation studies

Sr. No	Name of Standard/Test	Batch No	Potency (%)
1	Berberine	T12H010	93.80
2	Andrographolide	T12G001	99.80
3	Polyherbal Tablet Placebo	NA	NA
4	Polyherbal Tablet	NA	NA

Table 2: Details of Gradient programme

Time (minute)	Flow (mL/minute)	% solvent A	% solvent B
0	1.0	80	20
25	1.0	20	80
30	1.0	50	50
40	1.0	50	50
45	1.0	80	20
50	1.0	80	20

Figure 1: Chromatograms for Blank, Standard and Sample

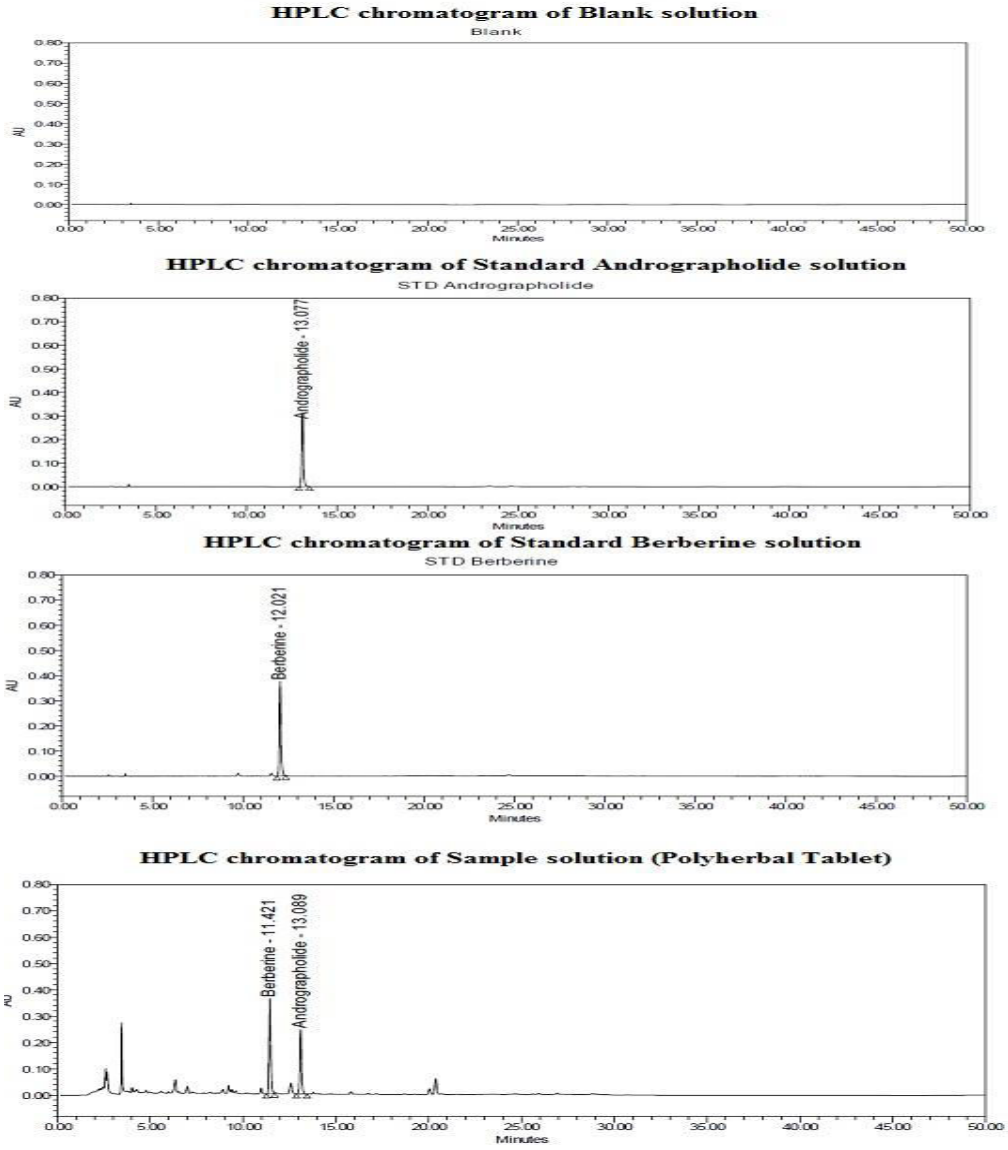

Table 3: Linearity of Berberine & Andrographolide

% Level	Conc. of Berberine (ppm)	Average Peak area of Berberine	Conc. of Andrographolide (ppm)	Average Peak area of Andrographolide
50	28.6	1074530	56.7	1153180
60	36.7	1307752	73.5	1551426
70	40.8	1488237	79.8	1689344
100	61.2	2233159	100.8	2205954
120	81.6	2929592	126.0	2896229
140	85.7	3093405	136.5	3156834
150	89.8	3304119	157.5	3630600
R^2	0.9990		0.9992	
Slope of Regression line	36169		25014	
y-intercept	11942		285218	

Figure 2: Linearity graphs for standard

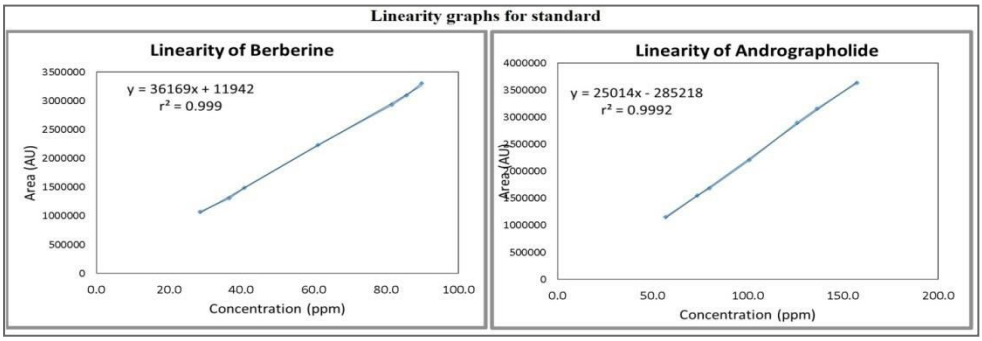

Table 4: Specificity of Berberine & Andrographolide

Sr. No.	Sample name	Analyte name	Purity flag	Specificity
1.	Polyherbal Tablet	Berberine & Andrographolide	No	Specific
2.	Standard	Berberine	No	Specific
3.	Standard	Andrographolide	No	Specific
4.	Blank	No Peak	-	-
5.	Polyherbal Tablet Placebo	No Peak	-	-

Table 5: Robustness for Berberine & Andrographolide

Robustness parameter		% RSD	Peak tailing	Theoretical plates	Remark
Berberine					
Wavelength (nm)	221	0.56	1.24	51927	Pass
	226	0.16	1.20	51908	Pass
	231	0.58	1.22	51611	Pass
Temperature (°C)	25	0.53	1.21	48926	Pass
	30	0.30	1.19	51970	Pass
	35	0.76	1.20	46453	Pass
Flow (mL/min)	0.9	0.56	1.22	51839	Pass
	1.0	0.19	1.20	51869	Pass
	1.1	0.44	1.21	45205	Pass

Andrographolide					
Wavelength (nm)	221	1.23	1.10	72036	Pass
	226	0.84	1.10	72340	Pass
	231	1.16	1.10	72171	Pass
Temperature (°C)	25	0.78	1.12	72722	Pass
	30	2.00	1.09	66045	Pass
	35	0.72	1.13	80339	Pass
Flow (mL/min)	0.9	1.11	1.12	77229	Pass
	1.0	0.80	1.10	70821	Pass
	1.1	1.45	1.05	57708	Pass

Table 6: Recovery for Berberine & Andrographolide

Analyte	Recovery level	% Recovery	Average % Recovery
Berberine	80% - 1	101.73	100.63
	80% - 2	101.24	
	80% - 3	98.93	
	100% - 1	100.32	100.18
	100% - 2	99.67	
	100% - 3	100.54	
	120% - 1	101.70	101.32
	120% - 2	101.04	
	120% - 3	101.22	
Andrographolide	80% - 1	100.45	100.57
	80% - 2	101.93	
	80% - 3	99.34	
	100% - 1	99.50	100.06
	100% - 2	101.86	
	100% - 3	98.83	
	120% - 1	101.48	100.72
	120% - 2	100.58	
	120% - 3	100.10	

The peak purity of for each analyte was assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the spot from standard and extracts. The purity angle and purity threshold values are given in table **[Table 4].** The given method was optimized by doing robustness. The peak area for each analyte was calculated for each parameter and % RSD was found to be less than 2%. The values of % RSD as shown in Table 5 indicate better robustness of the method. The recovery study was carried out by spiking known amount of standards into placebo solution at 80%, 100% and 120% of working concentration. The overall recovery percent were found to be for Berberine 100.71% and for Andrographolide 100.45% respectively. [Table 6]

CONCLUSION:

The present investigation resulted in the development of an RP-HPLC-UV-DAD analysis method for berberine and andrographolide that was validated in terms of linearity, precision, accuracy, specificity, system suitability and robustness. The presented method in addition to its novelty for determination of two ingredients i.e. berberine and andrographolide at single wavelength is sufficiently rapid, simple and sensitive as well as precise and accurate that complies with ICH guidelines. The assay of the two active ingredients was not interfered

by the excipients in the Polyherbal Tablet. Therefore, the proposed analytical method is recommended for the routine analysis of berberine and andrographolide as such, or in various dosage forms. In addition, the method can be applied in many developing countries or field stations where advanced analytical equipments are not available.

Authors' contribution: Gayatri Ganu and Rakesh Shivatare contribution included collecting samples, designing and performing laboratory work, analyzing the results, and preparing the paper. Gayatri Ganu, Rakesh Shivatare, Dr. Dheeraj Nagore and Dr. Tushar Deshmukh contribution included data interpretation and identification of the compounds. All the authors have read the final manuscript and approved the submission.

Conflicts of interest: The authors declare no conflicts of interest.

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