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Novel Rp-Hplc Method for Simultaneous Estimation of Andrographolide and Berberinein Polyherbal Formulation

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Abstract
A novel, simple, rapid, isocratic reverse phase high-performance liquid chromatographic method was developed for simultaneous estimation of andrographolide and berberine. The analysis was carried on Prontosil C18 (250 ×4.6 mm,5µ)column, with mobile phase consisting of acetonitrile and potassium dihydrogen phosphate buffer (pH 2.2 adjusted with ortho phosphoric acid) in the ratio of 30:70. The flow rate was set at 1.0ml/minand effluents were monitored at 235nm.The retention time of andrographolide and berberine werefound to be 10.08±0.2 and 12.47±0.2 minrespectively.Linear responses were obtained in concentration ranges of 5-50µg/ml, with R^2 of 0.9989 and 0.9993 for andrographolide and berberine respectively. As per ICH guidelines, this method is accurate, simple, rapid and sensitive, hence can also be used in the evaluation of other formulations containing andrographolide and berberine.

Keywords: Reverse phase high performance liquid chromatography, andrographolide, berberine,ICH guidelines.

INTRODUCTION

Medicinal herbs are an integral part of human society to tackle diseases, from the dawn of civilization[1].Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities and medicinal properties, higher safety margins and lesser costs[2].To characterize herbal medicines, quality assurance also requires the control of starting materials, storage and processing, hence modern analytical techniques like highperformance thin-layer chromatography (HPTLC), gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS) are used[3]. The literature survey discloses that various analytical methods were developed for estimation of andrographolide and berberine alone or in combination with other markers[4-10],to the best of our knowledge no such HPLC analysis method for simultaneous estimation of andrographolide and berberine is reported yet.

This paper presents the development of a novel reverse phase HPLC method for simultaneous estimation of andrographolide and berberine. The selected marketed formulation supports healthy liver functions, helps control hepatocellular regeneration and fatty infiltration in the liver, it also promotes appetite andalso used for other conditions[11]. This tablet consists of Guduchi (*Tinosporacordifolia*), Kalmegh (*Andrographis paniculata*), Bhringraj (*Ecliptaalba*), Bhuiamla (*Phyllanthus niruri*),Tulsi (*Ocimumsanctum*), Punarnava (*Boerhaaviadiffusa*), Sharpunkha (*Tephrosiapurpurea*), Kutki (*Picrorhizakurroa*), Kasni (*Cichoriumintybuus*), Arjuna (*Terminalia arjuna*), Biranjasipha (*Tamarixgallica*), Jhavuka (*Tamarix gallica*).Andrographolide from *A. paniculata* possesses antibacterial, antiviral, antineoplastic, cardioprotective, digestive, hepatoprotective, hypoglycaemicand immune enhancementactivity[12]. It also possesses anti-inflammatory activity through different mechanisms[13].Berberine present in *T.*

Cordifolia possesses anti-diabetic,anti-diarrheal activity and it also exhibits antioxidant effect[14]. It also possesseshepatoprotective, antioxidant, anticancer, antimicrobial and antimalarialactivity[15].

MATERIALS AND METHOD

Marketed formulation Liv-first Liver support of Herbal Hills was procured from the local market of Mumbai, Maharashtra, India.

Standards and reagents

Standards of andrographolide and berberine were procured from Yucca Enterprises, Mumbai, Maharashtra, India. Acetonitrile and methanol of HPLC grade were procured from Thermo Fisher Scientific, IndiaPvt.Ltd., Powai, Mumbai. Potassium dihydrogen phosphate was purchased from Research-Lab Fine Chem Industries, Mumbai, India.

Preparation of Standard Stock Solutions

100mg of each andrographolide and berberine were accurately weighed and transferred into two separate volumetric flasks and the volume was made up to 100ml using methanol and the resulting solution was of 1000µg/ml. The stock solutions were further diluted before injections.

Preparation of working solutions

Working solutions were prepared using standard stock solutions. Combined solutions of markers having concentrations of100 µg/mlwere prepared from the stock solution(1000µg/ml). This was further diluted to get solutions of 5, 10, 20, 30,40,50µg/ml which were used for calibration curve construction. These solutions were filtered through a filter having a pore size of 0.45µm.

Preparation of Test solution

Twentytablets were triturated and accurately 2 g of powder was weighed. The powder was transferred to round bottom flask and it was subjected to extraction with 100 ml methanol for 20 min using reflux assembly. The solution was filtered through Whatman filter paper to get a clear solution and the volume was made up to 100 ml. The final solution obtained was used for analysis.

HPLC METHOD DEVELOPMENT

Chromatographic conditions:

Selection of wavelength

Standard solutions of andrographolide and berberine were prepared and scanned using a UV spectrophotometer. The detection range was kept from 200-400 nm and the overlay spectra of andrographolide and berberine obtained is shown in fig.1. The detection wavelength was selected as 235 nm for the analysis of andrographolide and berberine, as both the markers showed appreciable absorption at this wavelength.

Selection of mobile phase

The standard solution of andrographolide and berberine (20 µg/ml) were injected into the HPLC system and run in different solvent systems. Initially, trials were carried out using acetonitrile and KH_2PO_4(potassium dihydrogen phosphate having molarity 0.05) having pH 2.5in the ratio of 60:40, the next trial was done by changing mobile phase composition (30:70) and molarity of KH_2PO_4 was 0.02. Further changes were done in pH 2.3,2.1. Finally, acetonitrile and KH_2PO_4 (adjusted pH 2.2 with orthophosphoric acid having molarity 0.01) in the ratio of 30: 70 was selected as the mobile phase for analysis, which gave good resolution and proper peak shape.

Optimized Chromatographic conditions:

The mobile phase finalized was acetonitrile and potassium dihydrogen phosphate buffer (30:70) having pH 2.2. The molarity of phosphate buffer used was 0.01M.The flow rate was kept 1.0ml/min, column

temperature was set at 28°C. Run time was kept 15 min. The detection wavelength was 235 nm and injection volume was 10 µl. The retention time of andrographolide and berberine was found to be 10.08 ± 0.2 and 12.47 ± 0.2 min respectively.

HPLC METHOD VALIDATION

The developed method was validated as per ICH guidelines Q2 (R1) for parameters such as specificity, linearity, precision, accuracy, limit of detection, limit of quantification and robustness[16].

Specificity

It is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. It is performed in order to ensure the identification of marker compound from the ayurvedic formulation under analysis. Chromatogram of blank, standard solution, sample solution was used to compare the specificity of the method.Specificity was confirmed by comparing the retention times. HPLC chromatograms show that andrographolide and berberine were eluted at 10.08 ± 0.2 min and 12.47 ± 0.2 min respectively. The chromatogram of spiked standard, as well as sample solution showed more intense peak at the same retention time. The developed method was found to be specific as there was no interference of any other constituents at the retention times of both markers andrographolide and berberine as depicted in the chromatogram. (fig.2 and 3).

Linearity

Andrographolide and berberine (100 µg/ml) were accurately weighed and diluted with methanol to prepare stock solutions. The stock solutions were further diluted to get solutions of 5, 10, 20, 30, 40 ,50 µg/ml.Linearity was evaluated by analysing the plot area as a function of concentration of an analyte. Andrographolide and berberine showed a linear response in the concentration range of 5-50 µg/ml. Linear regression analysis was used to determine linearity. The linearity was validated by the high value of correlation coefficient(R^2)of 0.998 and 0.999 for andrographolide and berberine respectively. The results are depicted in table 1 and fig.4.

Quantification of markers

The amount of andrographolide and berberine present in the formulation was calculated using linear regression analysis. Quantification of the markers was done by performing HPLC analysis of test solutions. The results obtained were used for further recovery studies. The results are presented in table 2.

Accuracy (Recovery)

The accuracy of the proposed method was assessed by spiking the known number of standards in the sample solution. These sample solutions were filtered through a 0.45µmmembrane filter before analysis. Percent recovery and its standard deviation were calculated to determine the accuracy.Recovery of andrographolide and berberine from formulation was checked by spiking a known number of standards at three concentration levels (i.e. 80%, 100% and 120% of the quantified amount) to the test samples in triplicate using HPLC.In this way, accuracy was performed and calculated for nine determinations over a specified range and mean recovery was calculated.The mean percent recovery of andrographolide and berberine were found to be 99.97 and 99.91% respectively. They were in acceptance criteria in the range of 98-102 %. The percentage of recovery results are demonstrated in table 3. These results revealed that even a small change in the drug concentration of the solution could be determined by the developed method.

Precision

Repeatabilityof the proposed method was determined by injecting six replicates of standard and sample solutions for system and method precision respectively. The precision was presented as percentrelative standard deviation (% RSD) of the response. The results of repeatability showed % RSD of the peak area of standard solution were 0.53 and 0.34 while for sample solution were 0.59 and 0.83 for andrographolide and

berberine respectively. The standard analysis of the results proved that RSD of the peak areas obtained was less than 2%, hence, the proposed method was found to be precise. The data of precision is presented in tables 4 and 5.

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

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value.

The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

The LOD expresses as,LOD =3.3 σ/S. The LOQ is a parameter of quantitative assays for low levels of compounds (markers) in extracts. The LOQ is expressed as: LOQ =10 σ/S. The linear regression equation was used to determine LOD and LOQ. The LOD and LOQ of andrographolide were found to be 0.13 and 0.39µg/ml respectively and that of berberine was found to be 0.19 and 0.46 µg/ml respectively.

Robustness

The robustness of the analytical method was evaluated by making deliberate changes in the chromatographic conditions. The factors selected for this purpose were flow rate of mobile phase (±0.2 ml/min), wavelength (±1nm)and column temperature (±1°C). Each marker was analysed in triplicate to ensure that the method is robust. The %RSD of robustness testing under different conditions are depicted in table 6.

Solution stability

The solution of andrographolide and berberine were injected at different time intervals for evaluating the stability of solution. The solution stability of 24 h depicted that the sample solution can be used over 24 h. These solutions did not show any degradation up till 24 h. The % RSDwas calculated for indicating the stability of the solution. The results are shown in table 7.

DISCUSSION

The developed RP-HPLC method developed for the simultaneous estimation of andrographolide and berberine was found to benovel, simple, sensitive, accurate, and precise. Peaksof both the markerswere sharp and well resolved. Both the markers were quantified from formulation under study.This method was validated according to the ICH Q2(R1) guidelines in the terms of linearity, specificity, precision, limit of detection, limit of quantification, accuracy and robustness. The developed RP-HPLC method can be used to standardize the formulations containing andrographolide and berberine as marker.

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RESULTS

Table 1: Linear regression data obtained from the calibration curves of andrographolide and berberine.

Parameter	Andrographolide	Berberine
Linearity Range(µg/ml)	5-50	5-50
Equation of regression line	y=39806x-85354	y=60505x-30056
Coefficient of correlation(R^2)	0.9989	0.9993
Slope	39806	60505
Intercept	-85354	-30056

Table 2: Content of markers in marketed formulation

S.No.	Markers	%w/w content
1.	Andrographolide	0.0114
2.	Berberine	0.0102

Table 3: Results of accuracy studies for andrographolide and berberine

Marketed formulation	Level of recovery (%)	Theoretical content of marker (µg/ml)	Amount of marker Recovered (µg/ml)	%Recovery	Average % recovery
Andrographolide	80	41.04	41.00	99.90	99.97
	100	45.60	45.62	100.04	
	120	50.16	50.15	99.98	
Berberine	80	36.72	36.73	100.02	99.91
	100	40.80	40.80	100	
	120	44.48	44.75	99.71	

Table 4: System precision results

S.No.	Andrographolide (20 µg/ml)	Berberine (20 µg/ml)
	Peak area	Peak area
1	738365	1220153
2	729520	1221098
3	738618	1229723
4	731635	1222648
5	733940	1228970
6	738123	1221443
Mean±SD	735034 ± 3915.06	1224006 ± 4219.967
% RSD	0.53	0.34

Table 5: Method precision results

S.No.	Andrographolide	Berberine
	Peak area	Peak area
1	177849	368243
2	177619	363304
3	176012	364112
4	176622	360389
5	175001	359809
6	176620	362371
Mean±SD	176621 ± 1048.739	363038 ± 3039.894
% RSD	0.59	0.83

Table 6: Robustness results of andrographolide and berberine.

Time	% RSD	
	Andrographolide	Berberine
Initial	0.39	0.33
6 h	0.41	0.44
12h	0.46	0.52
24 h	0.55	0.58

Table7: Solution stability of andrographolide and berberine.

Parameter	Deviation	%RSD			
		Andrographolide		Berberine	
		Area	Retention time	Area	Retention time
Flow rate(ml/min)	0.8ml	0.40	0.58	0.68	0.32
	1.2ml	0.75	0.66	0.99	0.61
Column temperature	27°C	0.32	0.42	0.66	0.34
	29°C	0.36	0.31	0.51	0.25
Wavelength	234nm	0.35	0.33	0.50	0.18
	236nm	0.18	0.29	0.41	0.21

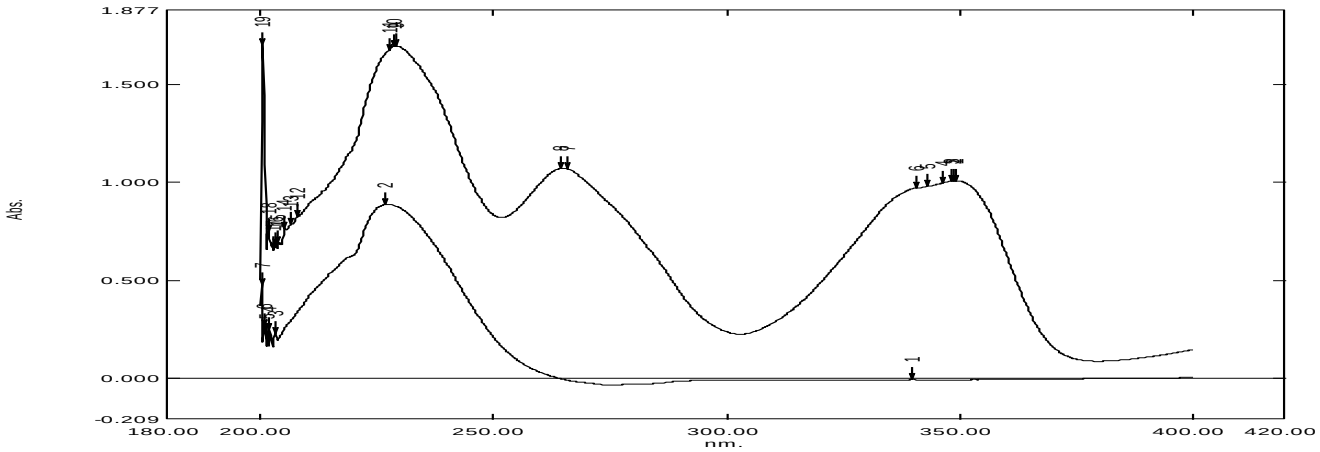

Fig.1: UV overlap spectrum of andrographolide and berberine at 235nm.

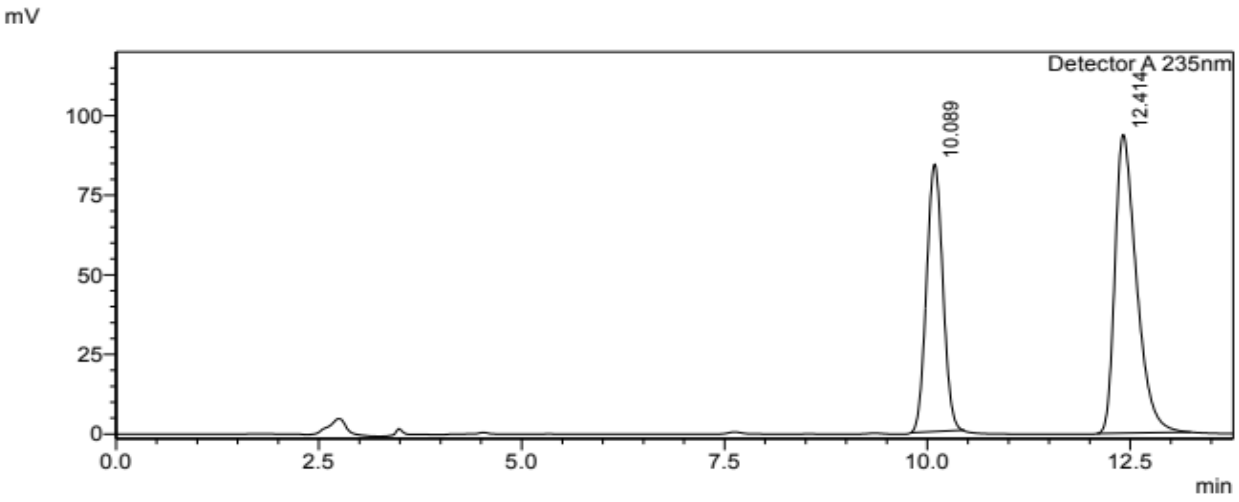

Fig.2: HPLC chromatogram of a standard mixture of andrographolide and berberine obtained using optimized conditions.

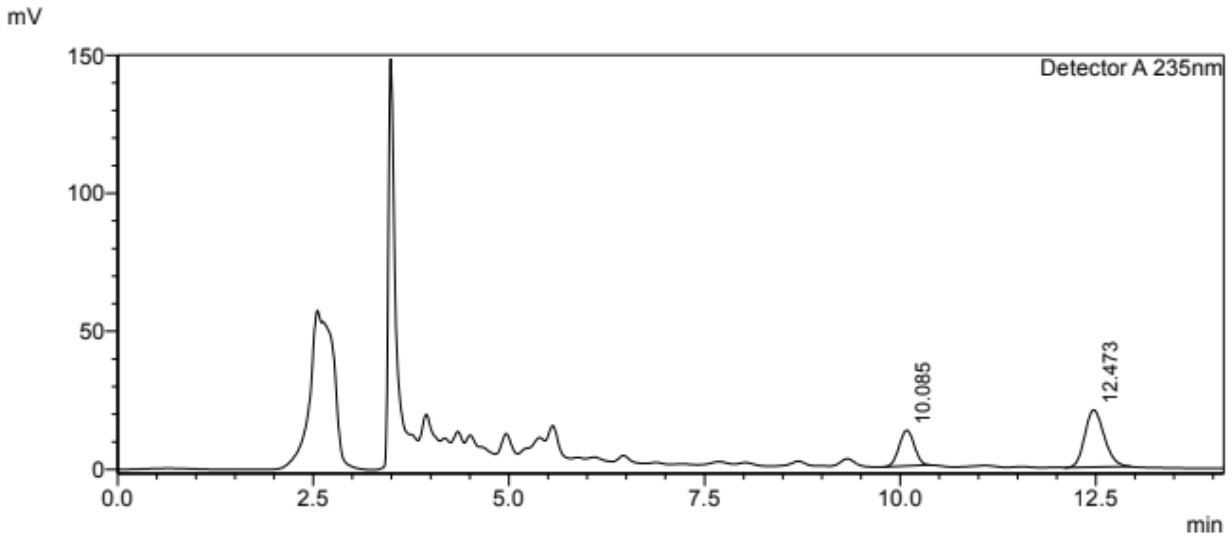

Fig.3: Chromatogram of extract of marketed formulation.

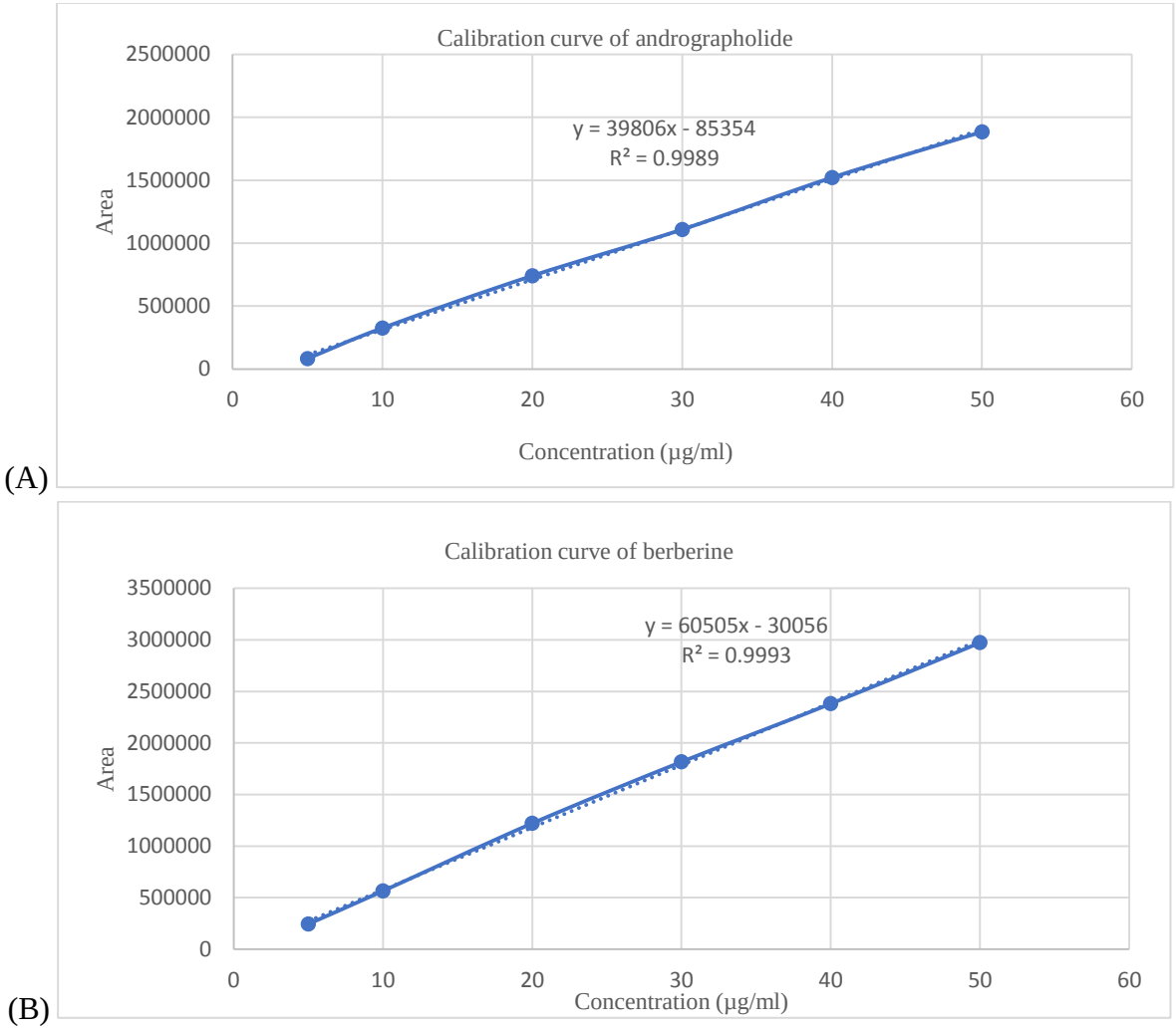

Fig. 4: Calibration curve for linearity of andrographolide (A) and berberine(B)

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