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Research Article

A NOVEL REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF ALOE-EMODIN AND PIPERINE IN AYURVEDIC FORMULATION

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ABSTRACT

Objective: The objective of this study was to develop and validate a novel, simple, rapid, accurate, and precise reverse-phase high-performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of aloe-emodin and piperine in the ayurvedic formulation as per the International Conference on Harmonization guidelines.

Methods: Chromatographic separation was achieved using a Prontosil C18 (250 × 4.6 mm, 5 µ), with a mobile phase consisting of 0.05% orthophosphoric acid and acetonitrile in the ratio of 50:50, at a flow rate of 1 ml/min and column temperature maintained at 28°C and ultraviolet (UV) detection at 225 nm.

Results: The retention time of aloe-emodin and piperine was found to be 9.38±0.2 min and 13.45±0.2 min, respectively. The linearity of aloe-emodin and piperine was tested in the range of 1–20 µg/ml. The correlation coefficient for aloe-emodin and piperine was found to be 0.998 and 0.997, respectively. The recovery values (98–102%) indicate a satisfactory accuracy. The percentage relative standard deviation for precision was found to be <2% which indicates that the method is precise.

Conclusion: A precise method for the simultaneous quantification of aloe-emodin and piperine was developed using high-performance liquid chromatography. Hence, the developed method can be used for quantitative and quality control analysis of formulations containing these phytoconstituents.

Keywords: Aloe-emodin, Piperine, Ayurvedic formulation, Reverse-phase high-performance liquid chromatographic, Validation.

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INTRODUCTION

Ayurveda, the ancient system of Indian medicine, is widely practiced in modern times due to its safety and efficacy [1]. Ayurvedic medicines are polyherbal formulations and each herb consists of a number of chemical constituents. Hence, each ayurvedic formulation is a source of many different phytochemicals, in which individuals may have different medicinal properties [2]. Standardization and analysis of chemical markers in ayurvedic or polyherbal formulations are important aspects for maintaining and assessing the quality and safety of the polyherbal formulation. Standardization assures safety, efficacy, and quality and minimizes batch-to-batch variation of the polyherbal formulations. An attempt for the standardization of polyherbal formulation has been carried out with respect to the active principles [3].

The present study focuses on the standardization of an ayurvedic formulation using high- performance liquid chromatography. Pylowin is a well-known marketed ayurvedic formulation that is indicated mainly against pile ailments such as piles and fistula which are characterized by pain, burning, swelling at the anus, bleeding from the rectum, constipation, or difficulty while passing stools.

The selected ayurvedic formulation consists of powder of Kumari ghanasar (*Aloe indica*), Marich (*Piper nigrum*), and other crude drugs. Two chemical markers, one from each medicinal herb, were selected for the present work, namely, aloe-emodin from *A. indica* and piperine from *P. nigrum*. These markers are mainly responsible for the bioactivity of the formulation. Aloe-emodin possesses antimicrobial, anticancer, antifungal, antitumor, and anti-aging [4]. Piperine, a principle pungent alkaloid, possesses central nervous system depressant, analgesic, anti-inflammatory, antimicrobial, antioxidant, and hepatoprotective properties [5].

The literature survey reveals that various analytical methods for the estimation of aloe-emodin and piperine were reported alone and in combination with other drugs [6-12]. However, no high-performance liquid chromatographic (HPLC) method has been reported for the simultaneous estimation of aloe-emodin and piperine. Therefore, an attempt was taken to develop a novel reverse-phase HPLC method for the simultaneous estimation of aloe-emodin and piperine in the ayurvedic formulation and validate the developed method in accordance with the International Council for Harmonization (ICH) guidelines [13].

METHODS

Instrument

HPLC chromatographic separation was performed on Shimadzu (LC 2030) model with Lab Solution software. Ultraviolet (UV)–visible spectrometer was used for obtaining the maximum wavelength of compounds.

Standards and reagents

HPLC grade aloe-emodin and piperine (purity 99%) were purchased from Sigma-Aldrich, Mumbai, India. Ayurvedic formulation of Pylowin tablet (Sharangdhar Pharmaceuticals Pvt. Ltd., Pune, India) used for analysis was purchased from the local market. HPLC grade solvents were purchased from Thermo Fisher Scientific India Pvt. Ltd.

Chromatographic conditions

The method was developed using RP, Prontosil C18 column (250×4.6 mm, 5 µ). The run time was 15 min. The mobile phase used was 0.05% orthophosphoric acid and acetonitrile in the ratio of 50:50 at a flow rate of 1 ml/min, column temperature maintained at 28°C and a detection wavelength of 225 nm using a UV–visible detector.

Selection of wavelength

The suitable wavelength for the HPLC analysis was determined by UV spectrums in the range of 200–400 nm for individual drug solutions of aloe-emodin and piperine then overlapped. UV overlain spectra of these two markers showed that the drugs absorbed appreciably at 225 nm, and hence, 225 nm was taken as a detection wavelength for HPLC analysis (Fig. 1).

Preparation of stock solution

One hundred milligrams of each marker (aloe-emodin and piperine) were transferred separately in 100 ml volumetric flask and the volume was made up with methanol to obtain a solution of 1000 µg/ml.

Preparation of standard solution

A standard solution was prepared from the stock solution of aloe-emodin and piperine after suitable dilutions.

Preparation of sample solution

Ten tablets were triturated and 1 g of powder was accurately weighed. The powder was extracted with methanol for 30 min using the reflux assembly. The extract was made up to 100 ml with methanol. The solution was then filtered through Whatman filter paper to obtain a clear solution. The solution was injected after suitable dilutions.

RESULTS AND DISCUSSION

Method development

The developed optimized method resulted in the elution of aloe-emodin at 9.38±0.2 min and piperine at 13.45±0.2 min. The total run time was 15 min. Chromatograms of standard and sample of aloe-emodin and piperine are shown in Figs. 2 and 3. The optimized chromatographic conditions are tabulated in Table 1. The analysis of a sample, the amount of aloe-emodin and piperine present in the formulation was calculated using linear regression analysis. The results of the sample analysis are reported in Table 2.

Method validation

The developed method was validated as per the ICH guidelines Q2 (R1) for parameters such as specificity, precision, linearity, accuracy, robustness, limit of detection (LOD), and limit of quantitation (LOQ) [13].

Specificity

Figs. 2-3 for standard marker solutions and sample chromatograms reveal that the peaks obtained in the standard solutions and sample solution at working concentrations is just due to the drugs and blank has no peak at the retention time of aloe-emodin and piperine. Hence, the method is said to be specific.

Precision

System precision

System precision was carried out with six replicates (n=6) of standard solution at the working concentration of selected two markers. The repeatability was expressed in terms of percentage relative standard deviation (%RSD). The %RSD of the peak areas obtained was <2.0% which indicates the acceptable reproducibility; hence, the developed method was found to be precise. System precision results are tabulated in Table 3.

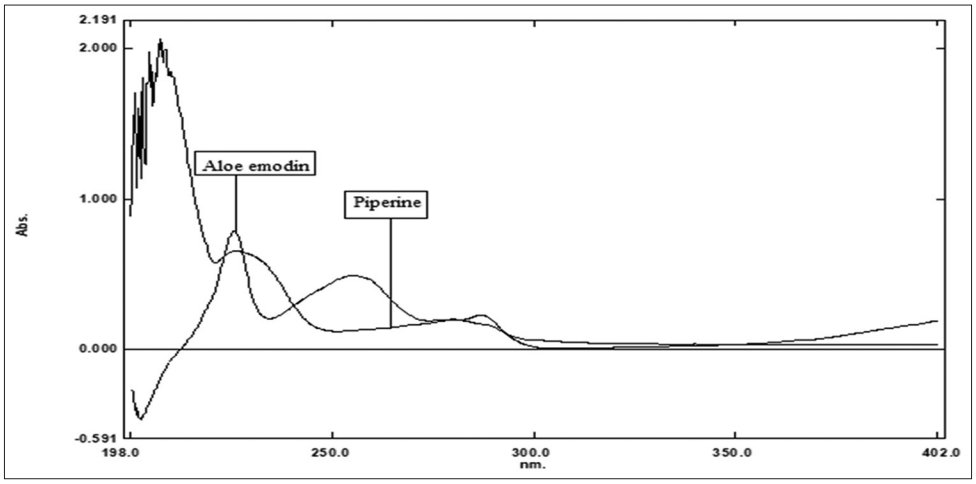

Fig. 1: Ultraviolet overlap spectrum of aloe-emodin and piperine

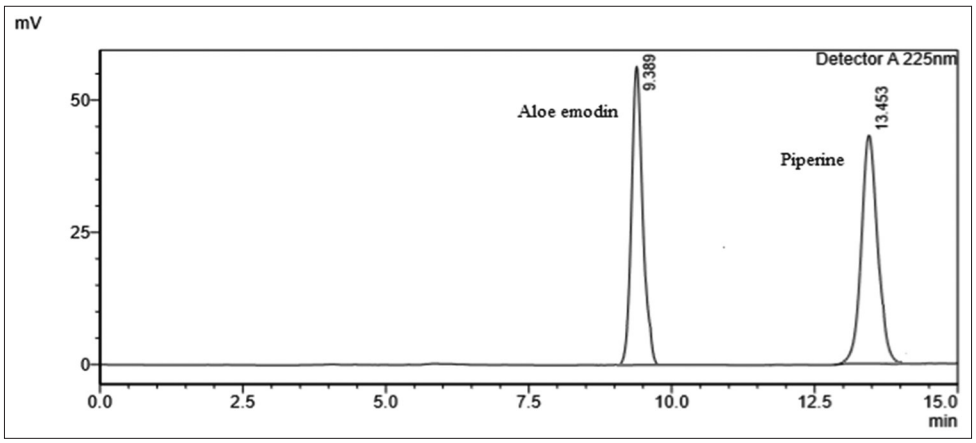

Fig. 2: High-performance liquid chromatographic chromatogram of standard mixture of aloe-emodin and piperine obtained using optimized conditions

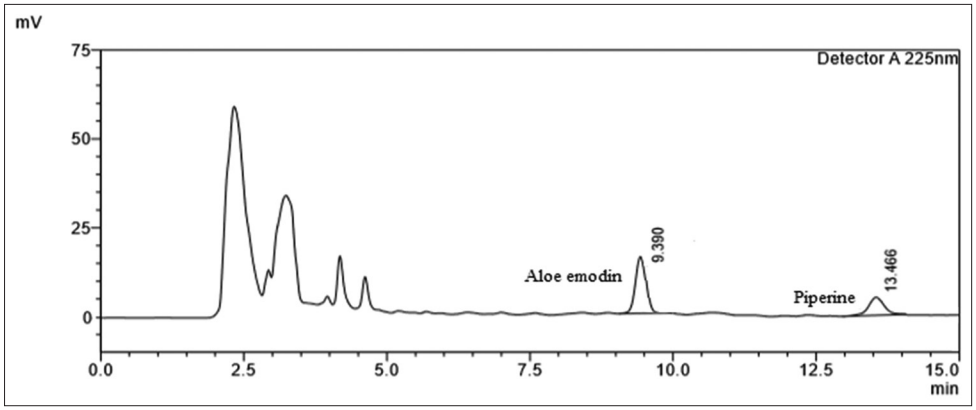

Fig. 3: High-performance liquid chromatographic chromatogram of sample mixture of aloe-emodin and piperine obtained using optimized conditions

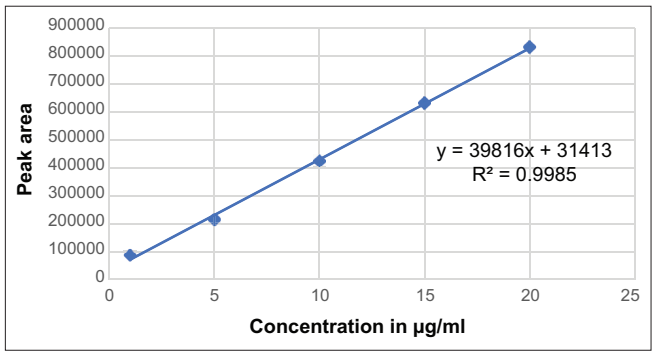

Fig. 4: Calibration curve of aloe-emodin

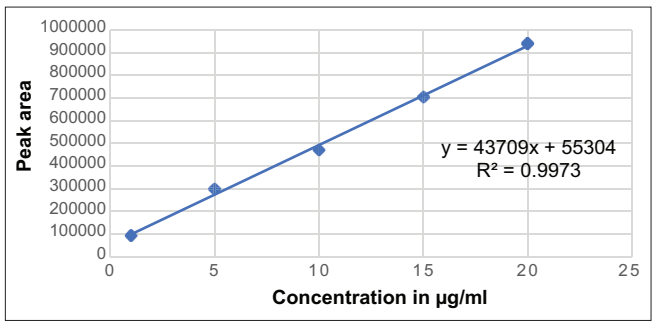

Fig. 5: Calibration curve of piperine

Method precision

Method precision was determined by injecting six replicates (n=6) of the sample under the test of repeatability at working concentration. The repeatability was expressed in terms of %RSD. The %RSD of the selected marker is <2.0% which indicates the acceptable reproducibility; hence, the developed method was found to be precise. Method precision results are tabulated in Table 4.

Linearity

The linearity between peak area and concentration was analyzed using calibration curves obtained with standard solutions of aloe-emodin and piperine with different concentrations of each standard. The proposed method was found to be linear over a wide range of concentrations 1–20 µg/ml for aloe-emodin and piperine with a good regression coefficient of 0.998 and 0.997, respectively. Hence, the method was found to be linear. The plots obtained from linear regression are given in Figs. 4 and 5.

Table 1: Optimized chromatographic conditions for aloe-emodin and piperine

Parameters	Optimized conditions
Column	Prontosil C18 (250×4.6 mm, 5 µ)
Mobile phase	0.05% Orthophosphoric acid: acetonitrile (50:50)
Flow rate	1.0 ml/min
Run time	15 min
Column temperature	28°C
Injection volume	10 µl
Detection wavelength	225 nm
Retention time	9.38 and 13.45 min

Table 2: Analysis of ayurvedic formulation

Markers	%w/w content
Aloe-emodin	0.0087
Piperine	0.0023

Table 3: System precision result

S. No.	Peak area of aloe-emodin (10 µg/ml)	Peak area of piperine (10 µg/ml)
1.	426,897	472,263
2.	423,895	471,596
3.	425,369	473,892
4.	426,993	475,863
5.	423,686	469,986
6.	424,769	475,036
Mean±SD	425,268±1434	473,106±2221
%RSD	0.34	0.47

*SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 4: Method precision result

S. No.	Peak area of aloe-emodin	Peak area of piperine
1.	172,796	96,136
2.	173,588	96,125
3.	172,185	96,786
4.	172,659	96,640
5.	173,368	97,916
6.	171,173	96,087
Mean±SD	172,628±873	96,615±703
%RSD	0.51	0.73

*SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 5: Percentage recovery results for aloe-emodin

Level (%)	Sample (µg/ml)	Standard added (µg/ml)	Total amount	Recovery	% recovery
80	17.5	14	31.5	30.89	98.06
100	17.5	17.5	35	34.59	98.82
120	17.5	21	38.5	38.29	99.45

Table 6: Percentage recovery results for piperine

Level (%)	Sample (µg/ml)	Standard added (µg/ml)	Total amount	Recovery	% recovery
80	4.65	3.72	8.37	8.39	100.2
100	4.65	4.65	9.3	9.19	98.81
120	4.65	5.58	10.23	10.17	99.41

Table 7: Robustness results of aloe-emodin and piperine

Parameter	Deviation (n=3)	% RSD	
		Aloe-emodin	Piperine
Flow rate	0.8 ml/min	0.49	0.48
	1.2 ml/min	0.45	0.50
Column temperature	26°C	0.80	0.39
	30°C	0.68	0.30
Wavelength	224 nm	0.66	0.53
	226 nm	0.94	0.24

*n=Number of injections. % RSD: Percentage relative standard deviation

Accuracy

The accuracy of the method was confirmed by studying recovery at three different concentrations of 80, 100, and 120% in accordance with the ICH guidelines. Standard drug solutions were added to a pre-analyzed sample solution and the percentage of drug content was measured. The acceptance limit for recovery ranges from 98 to 102%. The mean percentage recovery was found to be within the range, which indicates good accuracy. The percentage recovery results are tabulated in Tables 5 and 6.

Robustness

Robustness was performed by small variations in the chromatographic conditions. The solution was prepared as per the test method described earlier and injected at different variable conditions such as column temperature (26°C and 30°C), flow rate (0.8 ml/min and 1.2 ml/min), and detection wavelength (224 nm and 226 nm). Robustness data clearly show that the proposed method is robust at small but deliberate change. Robustness data are given in Table 7.

Limit of detection and limit of quantitation

LOD and LOQ are expressed as follows:

$$LOD = 3\ \sigma/S$$

$$LOQ=10\ \sigma/S$$

Where, σ is the standard deviation of the responses and S is the slope of the calibration curve.

LOD and LOQ of aloe-emodin were found to be 0.13 and 0.39 µg/ml, respectively, and that of piperine were found to be 0.17 and 0.51 µg/ml, respectively.

A low LOD and LOQ indicate that the method is sensitive.

CONCLUSION

A novel HPLC method has been developed and validated for the simultaneous determination of aloe-emodin and piperine in the ayurvedic formulation. This developed method was validated as per the ICH guidelines and results found to be linear, accurate, precise, repeatable, specific, and selective for the detection and quantification of both drugs. Hence, the proposed method was found to be satisfactory and can be applied for routine qualitative and quantitative analysis of aloe-emodin and piperine in an ayurvedic formulation containing these markers as one of the ingredients.

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AUTHORS' CONTRIBUTIONS

All authors have equally contributed toward the preparation of the manuscript.

CONFLICTS OF INTEREST

Authors declare that no conflicts of interest exist in this research work.

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