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RESEARCH ARTICLE

Development and Validation of HPTLC Method for Simultaneous Estimation of Piperine and Scopoletin in *Ajmodadi churna*

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Abstract

In this recent study, several trials were made to develop a HPTLC method for quantification of scopoletin and piperine in the Ajmodadi formulation. HPTLC was done on pre-coated silica gel 60 F254 plates with a mobile phase of toluene:ethyl acetate:methanol:formic acid (3.9:3.9:0.3:1.7 v/v/v/v). The retardation factors for scopoletin 0.75 and for piperine was 0.86 and found a good and defined resolution peak. Densitometer analysis of scopoletin and piperine was carried out at 335 wavelength (nm). The developed method was validated as reported in ICH guidelines (Q2R1). A linearity study shows that scopoletin and piperine was linear, and the recovery studies revealed a recovery in between 98-102 % as per guideline. This method was established to be rapid, delicate, accurate and specific, thus, these methods may be used in the quantification of the drug in any polyherbal formulation.

Keywords: *Ajmodadi Churna*, HPTLC, Piperine, Scopoletin

1. Introduction

Ayurveda is the most common and popular medicinal system, with a centuries-long history[1]. Polyherbal formulations are widely used as healing agents for a variety of persistent disorders such as immunodeficiency, diabetes mellitus, rheumatoid arthritis, cough and cold, memory loss, liver disorders, gastrointestinal disorders[2]. This formulation is an official Ayurvedic Formulary formulation that is used for back stiffness and painful diseases such as sciatica, as well as a carminative and anti-spasmodic[3]. *Ajmodadi churna* is made up of twelve active ingredients, which include: Ajmoda (*Trachyspermum ammi*), Vidanga (*Embelia ribes*), Saindhavalavana, Devdaru (*Cedrus deodara*), Chitraka (*Plumbago zeylanica*), Pipalimula (*Piper longum* (stems)), Satapuspa (*Anethum graveolens*), Pipali (*Piper longum (fruit)*), Marica (*Piper nigrum*), Pathya (*Terminalia chebula*), Vrddhadaruka (*Argyreia nervosa*), Nagara (*Zingiber officinale*)[4,5]. In this formulation, piperine has been noted in two herbs, *Piper longum* (pippali), *Piper nigrum* (marica). Piperine is an alkaloid (2*E*,4*E*)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one shown in Figure 1 belonging to family Piperaceae[5]. Current research explains that piperine is beneficial in relieving pain for better digestion, reducing inflammation and asthma[2]. It also increases the bioavailability of the other drugs. In formulation, scopoletin is present in the herb *Argyreia nervosa* (Vrddhadaruka). Scopoletin is a hydroxycouramin, 7-hydroxy-6-methoxychromen-2-one[6] shown in Figure 2 belonging to the family Umbelliferone[7]. Nowadays, HPTLC is a commonly used analytical method. It is cost-effective as compare to HPLC, simultaneously number of sample may be applied with a minor amount of samples and solvents. It reduces the time and cost of analysis[8]. One significant advantage of this method is that repeatedly scanning (detection) of chromatograph can be done with the same or different conditions repeatedly[9]. In the present study, several trials

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were done to evolve a sensitive, accurate and simple HPTLC method for the identification of scopoletin and piperine in Ajmodadi formulations. According to the literature review, there was individual quantification of scopoletin and piperine in plant extracts and in other ayurvedic formulations, but simultaneously there was no reported method for scopoletin and piperine in any plant extract neither in any ayurvedic formulation[10].

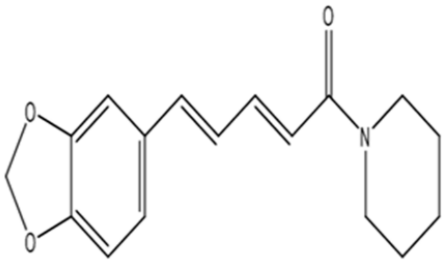

Figure 1. Structure of Piperine.

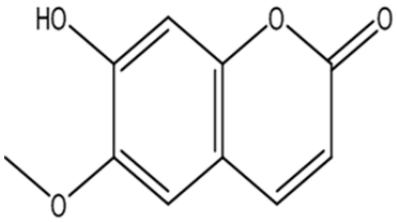

Figure 2. Structure of Scopoletin.

2. Materials and Methods

2.1 Materials and Reagents

Scopoletin and piperine standard markers were procured from Yucca Enterprises. The sample formulation (Patanjali Divya) was purchased from a local ayurvedic market. All other solvents used during the experimental work were methanol, ethyl acetate, toluene and formic acid of laboratory grade or analytical grade. Methanol was purchased from Astron Chemicals, toluene from the Central Drug House, ethyl acetate from Atur Laboratories and formic acid from Suvidhinath Laboratories.

2.2 Development of HPTLC Method

2.2.1 Chromatographic Conditions and Instrumentation

Spotting device: LINOMAT V automatic sample applicator assisted by continuous pressure of nitrogen gas. Syringe: Hamilton 100 µl

HPTLC chamber: Glass Twin Trough Chamber (TTC), dimensions: 10x10 cm and 20x10 cm;

Densitometer: HPTLC Scanner 4 linked to Win-CATS software

HPTLC plates: Pre-coated silica gel 60 F_{254}, 20x20 cm; Merck, Germany

Band size: Slit dimensions: 6x0.45mm. Scanning speed: 100mm/s Source of radiation: Deuterium and Tungsten.

2.2.2 Experimental Conditions

Solvent system; Toluene:ethyl acetate:methanol:formic acid (3.9:3.9:0.3:1.7 v/v/v/v); Saturation time: 20 minute; Drying time: 5 minute; Detection wavelength: 335 nm.

2.3 Preparation of Standard

Accurately, weigh 1 mg of scopoletin and 1 mg of piperine standard in 1 ml of methanol, separately. The final concentration is mg/ml (1000 µg/ml). Further, from 1000 µg/ml 1 ml of solution diluted upto 10 ml methanol to access (100 µg/ml) of solution was diluted upto 10 ml methanol to get (100 µg/ml) of stock solution.

2.4 Preparation of the Sample

Accurately, 1 gm of *Ajmodadi churna* was macerated with 10 ml of methanol. After filtering, the filtrate was completely dried. Then, from the dried extract, 1 mg of the dried extract was soluble in 1 ml of methanol (1000 µg/ml).

2.5 Selection of Wavelength

Isosbestic points were obtained by overlaying the spectra of scopoletin and piperine, scopoletin at 366 nm and piperine at 254 nm. The finalized wavelength was 335 nm as spectra of scopoletin and piperine shows the overlaying of the spectrum at this wavelength as shown Figure 5.

2.6 Calibration Curve for Scopoletin and Piperine

A standard solution of scopoletin and piperine was prepared of 1000 µg/ml by dissolving 1 mg of scopoletin and piperine in 1 ml of Laboratory Grade (LG) methanol. A distinct volume of standard was applied to the HPTLC plate in the range of 1 µl – 6 µl to get the concentration of 100 – 600 µg/band of scopoletin and piperine, respectively. By comparing the concentration of drug versus the data of the peak area, the calibration curve was represented as shown in Figures 4 and 5.

3. Method Validation

The developed procedures were validated according to the ICH guidelines (Q2R1). Specificity, accuracy, robustness, precision, LOD and LOQ were determined by the standard deviation of slope and response (Table 1).

3.1 Specificity

The ability to evaluate the analyte definitely depends on the existence of components that may be anticipated to be present. This method was analyzed by comparing standard drugs with the sample formulation and by collating the retardation factor and the spectrum of the band of standard drugs with the sample formulation.

3.2 Linearity

The capacity of an analytical technique to produce results in the sample that are directly proportional to the concentration of an analyte (in a given range) is known as linearity. Standard stock of scopoletin and piperine was prepared of 1000 µg/ml by dissolving 1 mg of scopoletin and piperine in 1 ml of methanol. A distinct volume of standard was applied on HPTLC plate in the range of 1 µl – 6 µl to get the concentration of 100 – 600 µg/band, respectively. The calibration curve was represented by comparing the drug concentration versus the data from the peak area.

3.3 Accuracy

The preanalyzed sample solution was spiked with the biomarkers and then reanalyzed in order to calculate its average percent recovery and % RSD. Spiking standard in placebo and sample with known quantity it was carried out in triplicate at three different levels (80, 100, and 120%).

3.4 Precision

The degree of closeness of agreement that is obtained from numerous samples of an equivalent, consistent sample under the given conditions. It was carried out by the repeatability of the sample application and measurement of peak area was done by taking any one concentration six times (n = 6).

3.5 Robustness

It is defined as the ability to remain unchanged by a compressed change with minor variations in system parameters, indicating its dependability in regular use. This method was determined by deliberately changing the wavelength and the saturation time.

3.6 Limit of Detection

The Limit of Detection (LOD) is the lowest amount of analyte sample that can be detected but not necessarily quantified as an exact value. This method was calculated based on the standard deviation of the slope and response procedures.

3.7 Limit of Quantification

The Limit of Quantification (LOQ) is an individual procedure that is defined as the lowest amount of analyte in a sample that may be quantitatively determined with suitable precision and accuracy. It was calculated based on the standard deviation of the slope and response procedures.

Table 1. Summary for validation parameter

Parameters	Scopoletin	Piperine
Retardation factor (Rf)	0.75	0.86
Linearity range (µg)	100-600	100-600
Equation	y = 0.002x + 0.007	y = 0.003x + 0.017
R^2 value	0.995	0.996
Slope	0.007	0.0017
LOD(µg)	2 µg/band	2 µg/band
LOQ (µg)	7 µg/band	7 µg/band

4. Results and Discussion

4.1 Method Development

To identify the phytoconstituents in the Ajmodadi formulation, different compositions of mobile phases were optimized depending on the different polarities of solvents. Standard scopoletin and piperine with the Retardation factor (Rf) values of 0.75 and 0.86, respectively. It was found that the toluene: ethyl acetate: methanol: formic acid as a mobile phase with the ratio (3.9:3.9:0.3:1.7) shows a good and sharp peak when the complete chamber saturation was done for 20 mins with the mobile phase. It was found that a good and well-defined peak was shown in Figures 3 and 4.

4.2 Validation Parameters

The developed method was validated by parameters like linearity, precision, specificity, accuracy and robustness according to the ICH Q2R1 guidelines. Specificity was established by comparing standard drugs with the sample formulation and by collating the retardation factor and the spectrum of the band of standard with the sample formulation. The developed method was found to be specific, as shown in Table 2. The linearity of scopoletin and piperine was established in the concentration of 1 µl- 6 µl in the range of 100 – 600 µg/band as shown in Figures 8 and 9, and it was found to be linear in the range with a regression coefficient (R^2) 0.995 for scopoletin and 0.996 for piperine as shown in Table 3. The calibration curves for scopoletin and piperine are shown in Figures 6 and 7. A recovery study for accuracy was performed at three different levels 80%, 100% and 120% by spiking the standards and the percent recovery and percent relative standard deviation was calculated. The results are shown in Table 4. A precision study was performed by analyzing intraday and interday variations. It was carried out at one concentration in the six replicates. The procedures were found to be precise. Intraday was performed by repeating one concentration six times in the interval of time calculation shown in Table 5, and inter-day was performed by repeating one concentration in a day in six replicates as shown in Table 6. Robustness was performed by the minor changes in the wavelength 335 nm to 333 and 337 nm and the saturation time of 20 mins to 18 and 22 mins as shown in Table 7.

Table 2. Specificity of scopoletin and piperine

Sr. No	Standard Markers	Rf	
		Standard Formulation	
1	Scopoletin	0.75	0.76
2	Piperine	0.86	0.86

Table 3. Linearity of scopoletin and piperine

Sr. No	Standard	R2 value	Equation
1	Scopoletin	0.995	y = 0.002x+0.007
2	Piperine	0.996	y = 0.003x+0.017

Table 4. Recovery studies of scopoletin and piperine

Standard	Spiked Level	% Recovered (mean)	SD	% RSD
Scopoletin	80 %	101	1.509	1.49
	100%	102.08	1.151	1.12
	120%	100.8	0.987	0.97
Piperine	80 %	101.5	1.32	1.30
	100 %	100.1	1.41	1.40
	120 %	101.4	1.46	1.44

Table 5. Intra-day precision

Sr. No	Standard Markers	Mean	SD	% RSD
1	Scopoletin	0.015931	0.000212	1.332504
2	Piperine	0.033367	0.000349	1.047187

Table 6. Inter-day precision

Sr. no	Standard markers	Mean	SD	% RSD
1	Scopoletin	0.01601	0.000238	1.48578
2	Piperine	0.033181	0.00034	1.025299

Table 7. Robustness of scopoletin and piperine

Sr. No	Parameters	Changes	%RSD	
			Scopoletin	Piperine
1	Wavelength	335 ± 2 nm	1.67	1.60
2	Saturation time	20 ± 2 mins	1.77	0.87

4.3 Quantification of Standards

Quantification of standard in the formulation was done by the regression coefficient equation by the area of the standard in formulation as shown in Table 8.

Table 8. Quantification of standards

Standards	Amount present in sample
Scopoletin	130 µg/band
Piperine	550/band

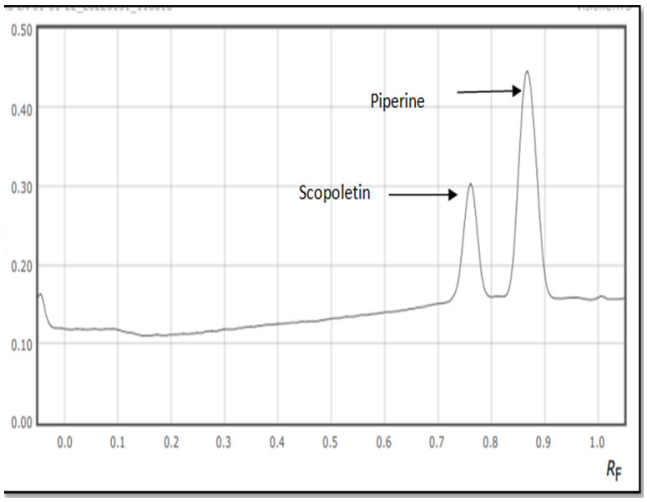

Figure 3. HPTLC Chromatograph of standard scopoletin and piperine.

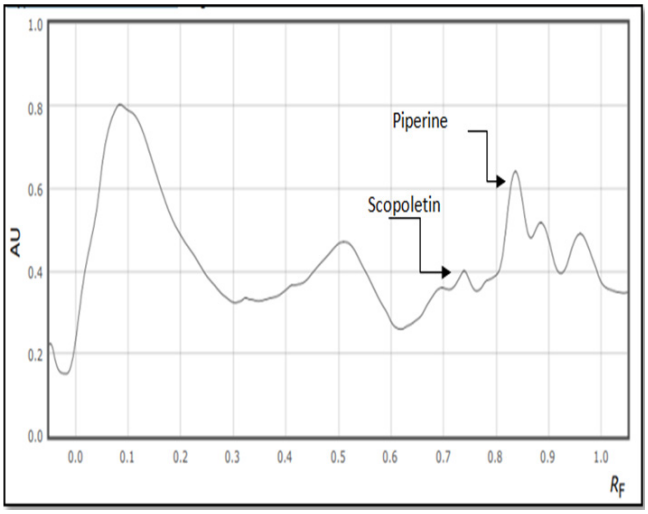

Figure 4. HPTLC chromatograph of Ajmodadi Formulation.

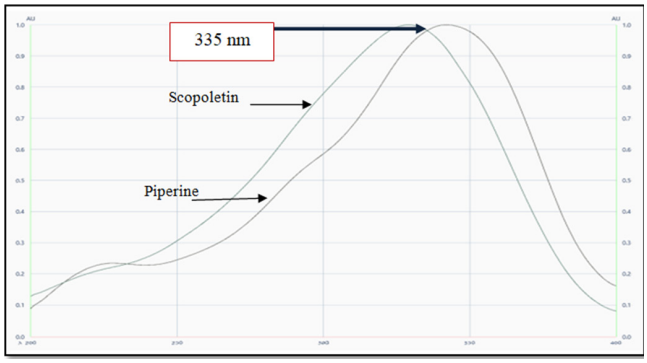

Figure 5. Spectrum of scopoletin and piperine.

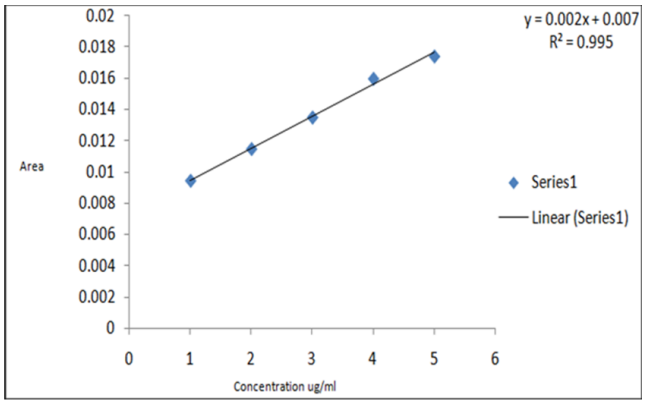

Figure 6. Calibration curve of scopoletin.

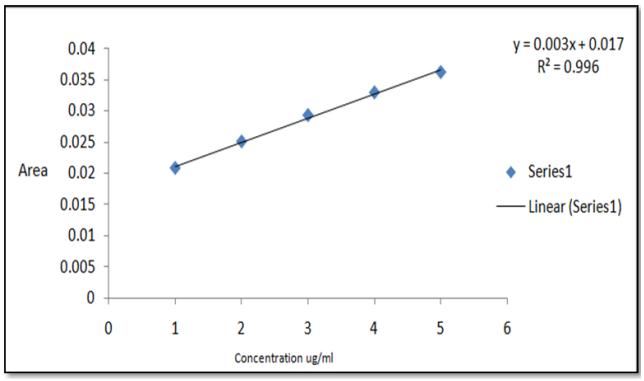

Figure 7. Calibration curve of Piperine.

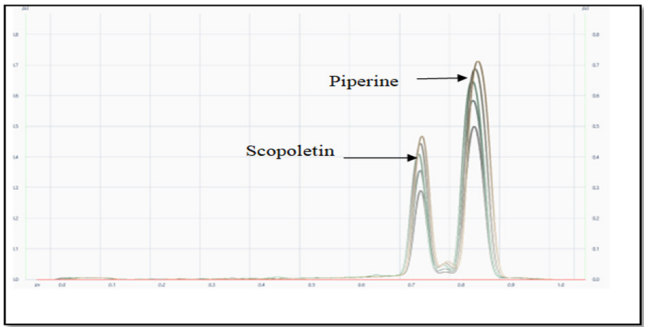

Figure 8. Linearity of standard scopoletin and piperine.

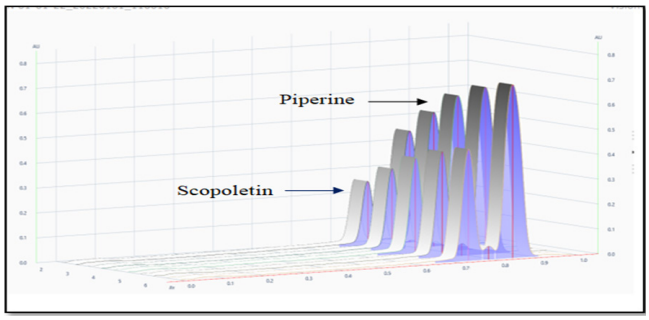

Figure 9. Overlay of scopoletin and piperine.

5. Conclusion

The chromatographic conditions like mobile phase, stationary phase, detection wavelength, and solubility were optimised for the development of the HPTLC method. Various trials of mobile phases were carried out to obtain the best results for the separation of phytoconstituents in the formulation. The analytical method was developed and optimised and found to be accurate, precise, linear, robust, and specific. Scopoletin and piperine were quantified simultaneously in *Ajmodadi churna* for the first time. This method is simple and is being used to investigate Scopoletin and Piperine in other herbal formulations. The validation was performed as reported in the ICH guideline. All the results were found to be within suitable criteria as per the guideline.

6. Acknowledgement

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7. List of Abbreviations

HPTLC: High Performance Thin Layer Chromatography
UV: Ultraviolet
ICH: International Conference for Harmonisation
R_f: Retardation factor
%RSD: Percent Relative Standard Deviation
LOD: Limit Of Detection
LOQ: Limit Of Quantification
TLC: Thin Layer Chromatography
cm: centimetre
µl: microlitre
mg: milligram
v: volume
nm: nanometer
TTC: Twin Trough Chamber

8. References

1. Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of ayurveda. Pharmacogn Rev. 2014; 8:73. https://doi.org/10.4103/0973-7847.134229
2. Verma S, Singh SP. Current and future status of herbal medicines. Vet. World. 2008; 1:347. https://doi.org/10.5455/vetworld.2008.347-350
3. Hazra AK, Chakraborty B, Mitra A, Sur TK. A rapid HPTLC method to estimate piperine in Ayurvedic formulations containing plant ingredients of Piperaceae family. J Ayurveda Integr Med. 2019; 10:248-254. https://doi.org/10.1016/j.jaim.2017.07.006
4. Sriwastava NK, Shreedhara CS, Ram HA. Standardization of *Ajmodadi churna*, a polyherbal formulation. Pharmacogn Res. 2019; 2:98. https://doi.org/10.4103/0974-8490.62957
5. Namjoshi AM. Ayurvedic formulary of India. Controller of Publications. 1976. Delhi, 85, page no. 106.
6. https://www.bimbima.com/ayurveda/ajmodadi-churna-for-arthritis-knee-and-joint-pain/1728/. Accessed 2 March 2022
7. https://pubchem.ncbi.nlm.nih.gov/compound/Piperine. Accessed 2 March 2022
8. https://pubchem.ncbi.nlm.nih.gov/compound/Scopoletin. Accessed 2 March 2022
9. Vyas J, Itankar P, Tauqeer M, Kelkar A, Agrawal M. Development of HPTLC method for estimation of piperine, guggulsterone E and Z in polyherbal formulation. Pharmacogn J. 2013; 5:259-64. https://doi.org/10.1016/j.phcgj.2013.10.003
10. Prabhu C, Subramanian GS, Karthik A, Kini S, Rajan MS, Udupa. Determination of telmisartan by HPTLC–a stability indicating assay. JPC- J Planar Chromat.2007; 20:477-81. https://doi.org/10.1556/JPC.20.2007.6.14
11. Patra KC, Kumar KJ. A validated HPTLC method for simultaneous analysis of eugenol and piperine in a siddha formulation. JPC–J Planar Chromat. 2010; 23:293-7. https://doi.org/10.1556/JPC.23.2010.4.11
12. ICH Q2R1 Validation of analytical procedures: methodology. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (Q2R1).