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HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF PIPERINE AND 6-GINGEROL IN TRIKATU CHURNA

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ABSTRACT

A sensitive high-performance thin layer chromatographic (HPTLC) method was developed for simultaneous determination of piperine and 6-gingerol from ayurvedic formulation. Chromatographic separation was achieved on aluminium plates pre-coated with silica gel G60 F254 as the stationary phase and hexane:ethyl acetate:toluene:diethyl ether (4.5:5.5:1.0:0.5) as the mobile phase. The densitometric evaluation was carried out at 282 nm. The developed method was validated as per the ICH guidelines. The Rf value of piperine and 6-gingerol was found to be 0.45±0.02 and 0.60±0.02, correspondingly. The response in terms of peak area was linear over the concentration range of 100-500 ng/spot for piperine and 6-gingerol, individually, with the regression coefficient values greater than 0.99 for both the drugs. The limits of detection were found to be 7.65 ng/spot and 8.83 ng/spot for piperine and 6-gingerol, respectively. The method can be applied for the simultaneous estimation of piperine and 6-gingerol in ayurvedic formulation.

Keywords: piperine, 6-gingerol, HPTLC, ayurvedic formulation.

INTRODUCTION

Trikatu churna is an Ayurvedic formulation composed of equal parts of Pipali (Black pepper, Piper longum), Maricha (Long pepper, Piper longum) and Sunthi (Ginger, Zingiber officinalis) [1]. Piperine is an active marker constituent of Pipali and Maricha whereas 6- Gingerol is of Sunthi. Piperine is an alkaloid and chemically it is (2E,4E)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one (Figure 1A). Gingerol is an pungent principle present in ginger. Chemically, it is (5S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)decan-3-one (Figure 1B). Several studies have shown that Trikatu and its constituents possessed bioavailability enhancing activity [1-3].

Spectrophotometric [4, 5], HPLC [6, 7] and HPTLC [8-10] methods are reported in the literature for determination of piperine in sample material and formulations. Several methods are also reported for simultaneous determination of piperine and other phytocompounds [11, 12]. The HPLC method with UV [13], MS [14] and electrochemical [15] detection and HPTLC method [16, 17] are reported for determination of 6-gingerol in the literature. Literature survey revealed that there is no HPTLC method reported for simultaneous estimation of piperine and 6-gingerol in ayurvedic formulation. Therefore, the aim of this present work was to develop rapid and sensitive HPTLC method for simultaneous estimation of piperine and 6-gingerol. The developed HPTLC method was validated and found to be simple, rapid, sensitive and robust and can be successfully applied for estimation of piperine and 6-gingerol in ayurvedic formulation.

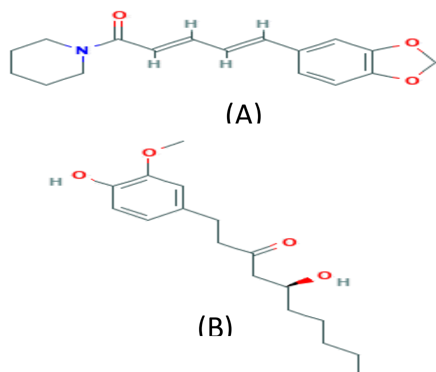

Figure 1. Chemical Structure of (A) Piperine and (B) 6-gingerol

METHODOLOGY

Instrumentation

The HPTLC system (Camag Sonnenmattstr, Mutenz, Switzerland) consisting of a Linomat V semi-automatic spotting device connected to a nitrogen cylinder, a glass twin-trough TLC chamber (20×10 cm), a TLC scanner-III, a data station with winCATS (V 1.4.7) software and an HPTLC syringe (100 µL capacity; Hamilton Company, NV, USA) was used for thin layer chromatographic studies.

Chemicals

Piperine (>95%) was procured from Sigma Aldrich, Mumbai, India. 6- gingerol (>95%) was purchased from Naturl Remedies, Bangalore, India. Methanol, ethyl acetate, toluene, diethyl ether, and n-hexane were purchased from Sisco Chem Pvt. Ltd., Mumbai, India. Analytical reagent grade solvents were used for HPTLC analysis. Marketed formulation containing piperine and 6-gingerol was purchased from local pharmacy.

Chromatographic conditions

Separation was performed on pre-coated silica gel G60 F254 aluminum plates (20×10 cm) with 0.2 mm thickness (E. Merck, Darmstadt, Germany). Samples were spotted on the TLC plate in the form of band leaving 10 mm from the bottom edge using Linomat V semi-automatic spotter and analyzed using following parameters; bandwidth, 4 mm; track distance, 10 mm; migration distance, 40 mm; spraying rate, 150 nL/s; volume of mobile phase, 7.15 mL; temperature, 27±2 ◦C; chamber saturation time, 15 min; migration distance, 40 mm; slit dimension, 3.00×0.30 mm; scanning speed, 20 mm/s; detection wavelength, 232 nm. Mobile phase consisted of methanol: ethyl acetate: toluene: ammonia (3: 1: 3: 0.15 v/v/v/v).

Preparation of Standard Solutions

Accurately weighed 10 mg of Piperine drug powder was transferred to 10 ml volumetric flask, and was dissolved in 3 ml of Methanol. The volume was made up with Methanol to get a stock solution containing 1mg/ml of Piperine (1000 µg/ml of Piperine). Accurately weighed 10 mg of 6-Gingerol drug powder was transferred to 10 ml volumetric flask, and was dissolved in 3 ml of Methanol. The volume was made up with methanol to get a stock solution containing 6-gingerol (1000 µg/ml of 6-Gingerol). Stock solutions of standard piperine (0.5 ml) and standard 6-gingerol (0.5 ml) were transferred into the 10 ml volumetric flask and diluted using methanol up to the mark to get the concentration of 50 µg/ml of Piperine and 50 µg/ml of 6-Gingerol.

Preparation of Sample Solution

Ayurvedic lab formulation (3 g) or marketed ayurvedic formulation (2 g) was accurately weighed and transferred to 25 ml volumetric flask and methanol (10 ml) was added. The volume was made up to the mark with Methanol. The solution was sonicated for 45 min and filtered. This solution was used for HPTLC analysis.

Calibration curve of Piperine and 6-Gingerol

Combined working standard solution (2 µl, 4 µl, 6 µl , 8 µl, 10 µl), was spotted on the TLC plates which cover the range of 100-500 ng/spot for piperine and 100-500ng/spot for 6-gingerol to determine the linearity. Each concentration was spotted three times on TLC plate and mobile phase was run up to 70 mm and scanned by scanner. Calibration curve was constructed by plotting mean peak areas of piperine and 6-gingerol against respective concentration.

Validation of developed HPTLC method

The specificity of the method was ascertained by analyzing standard drug and sample solutions. The spot for piperine and 6-gingerol in sample solution prepared from marketed formulation was confirmed by comparing absorbance/reflectance spectrum with that of standard piperine and 6-gingerol. The peak purity of piperine and 6-gingerol was assessed by correlating the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) position of the spot. The linearity of piperine and 6-gingerol were determined in the range of 100-500 ng/spot. Five sets of such solutions were prepared and analyzed by plotting a calibration curve of mean peak area versus concentration. Standard deviation (SD), slope, intercept and correlation coefficient (r) of the calibration curves were calculated to ascertain linearity of the method.

The precision is measured by the degree of reproducibility and repeatability of analytical method. The precision of analytical method is expressed as a %RSD. Repeatability of measurement of peak area was carried out by repeated scan of the same spot (100 ng/spot of piperine and 6-gingerol) seven times without changing the plate position. The % RSD for peak area was calculated. Repeatability of sample application is based on seven-time application of combined standard solution. The % RSD for peak area was computed. Variations of results within same day (intra-day precision) and among days (inter-day precision) are called as reproducibility. The intra-day precision (% RSD) was determined by analyzing standard solution of piperine and 6-gingerol for three times on the same day. The inter-day precision (% RSD) was determined by analyzing standard solution of piperine and 6-gingerol for 3 days. The intra- and inter-day variation for determination of piperine and 6-gingerol was carried out at three different concentration levels 100, 300, 500 ng/spot of piperine and 6-gingerol.

Recovery study was performed by addition of known amounts of standard drugs to pre-analyzed formulation extract (standard addition method). To a fixed amount of sample, an increasing amount of standard drugs, piperine and 6-gingerol, was added at 3 levels and the amount of drugs recovered was calculated at each level. The average recoveries after the analysis were calculated. Robustness of the method was evaluated by altering parameters such as volume of mobile

phase, saturation time and solvent front position. The measurement of signal to noise ratio approach was used for determination of LOD and LOQ. Signal to noise ratio of 3:1 and 10:1 were considered acceptable for estimating the detection limit and quantification limit respectively.

Analysis of marketed formulations

Sample solution (10 µL) was spotted on the TLC plate and analyzed. The experiment was repeated 3 times. The peak areas of the spots were measured. The % content was calculated using straight line equation derived from calibration curves for piperine and 6-gingerol.

RESULTS AND DISCUSSION

Method development and optimization

To optimize the chromatographic conditions for the separation of piperine and 6-gingerol, mobile phase composition, effect of saturation time, and detection wavelength were investigated. Initially, trials for mobile phase optimization were carried out using experimental conditions: stationary phase, pre-coated silica gel G60 F254 aluminum sheets; standard solution, piperine 200 ng/spot and 6-gingerol 200 ng/spot; detection wavelength, 254 nm; saturation time, 30 min. Solvent system consisting of hexane:ethyl acetate:toluene:diethyl ether (4.5:5.5:1.0:0.5) resulted in separation of piperine and 6-gingerol spots at Rf values of 0.45±0.02 and 0.60±0.02, respectively (Figure 2A). Chromatographic plate was developed up to 70 mm migration distance. Pre-saturation of TLC chamber with mobile phase for 15 min produced good reproducibility and peak shape. Photometric evaluation was performed at 282 nm. Quantitative determinations of piperine and 6-gingerol were made by considering the peak areas from chromatograms and regression line equation using optimized conditions.

Method validation

Comparison of chromatograms of standard solution and sample solution from formulation showed identical Rf values i.e. 0.45±0.02 for piperine and 0.60±0.02 for 6-gingerol (Figure 2). Comparison of the spectra scanned at peak start (S), middle (M) and end (E) showed high degree of correlation (above 0.990). This confirmed the purity of the corresponding spots. Also, the spectrum of individual drug was compared with the spectrum of standard piperine and 6-gingerol. The correlation obtained was 0.9991 for piperine and 0.9997 for 6-gingerol; this confirmed the identity of spots. The excipients and other components present in the churna did not interfere in the resolution of piperine and 6-gingerol.

The calibration curves for piperine and 6-gingerol were found to be linear in the concentration range of 100-500 ng/spot for both the analytes with correlation coefficients greater than 0.99. The linear regression equations were found to be $y = 21.75x+1087$ for piperine and $y = 4.76x-159$ for 6-gingerol, where, y – peak area and x – concentration in ng/spot.

The repeatability (% RSD) of sample application was found to be 1.16 and 1.71 for piperine and 6-gingerol, respectively. The scanner precision (% RSD) for measurement of peak area was found to be 0.29 and 0.98 for piperine and 6-gingerol, respectively. The repeatability studies ensured precision of scanner and spotting devices. The % RSD for

intra-day precision was found to be 0.03-0.38 and 0.22-1.29 for piperine and 6-gingerol, respectively (Table 1). The % RSD for inter-day precision was found to be 1.00-1.61 and 1.43-1.76 for piperine and 6-gingerol, respectively (Table 2).

Accuracy of the developed method was calculated by performing recovery studies. Results of recovery studies are shown in Table 3. The % recoveries were found out to be 97.32–103.80 % for piperine and 97.91–102.20 % for 6-gingerol. The LODs and LOQs were found to be 7.65 ng/spot and 23.18 ng/spot for piperine and 8.83 ng/spot, 26.76 ng/spot for 6-gingerol, respectively. Results of robustness studies indicated that the selected chromatographic factors (Rf Value and peak purity) remained unaffected by small variation of these parameters, which demonstrates that the developed method is robust.

Analysis of ayurvedic formulations

The spots at Rf value 0.45 (for piperine) and 0.60 (for 6-gingerol) was observed in the densitogram of the drug samples extracted from ayurvedic formulations. Amounts of piperine and 6-gingerol were calculated using linear regression equation derived. The % contents of piperine and 6-gingerol are presented in Table 4.

CONCLUSION

The proposed HPTLC method provides precise, accurate and reproducible quantitative analysis for the simultaneous estimation of piperine and 6-gingerol in ayurvedic formulations. The method was validated as per the ICH guidelines. It can be concluded that the developed method is simple, accurate, sensitive and precise. The method is suitable for routine analysis of piperine and 6-gingerol in marketed ayurvedic formulations.

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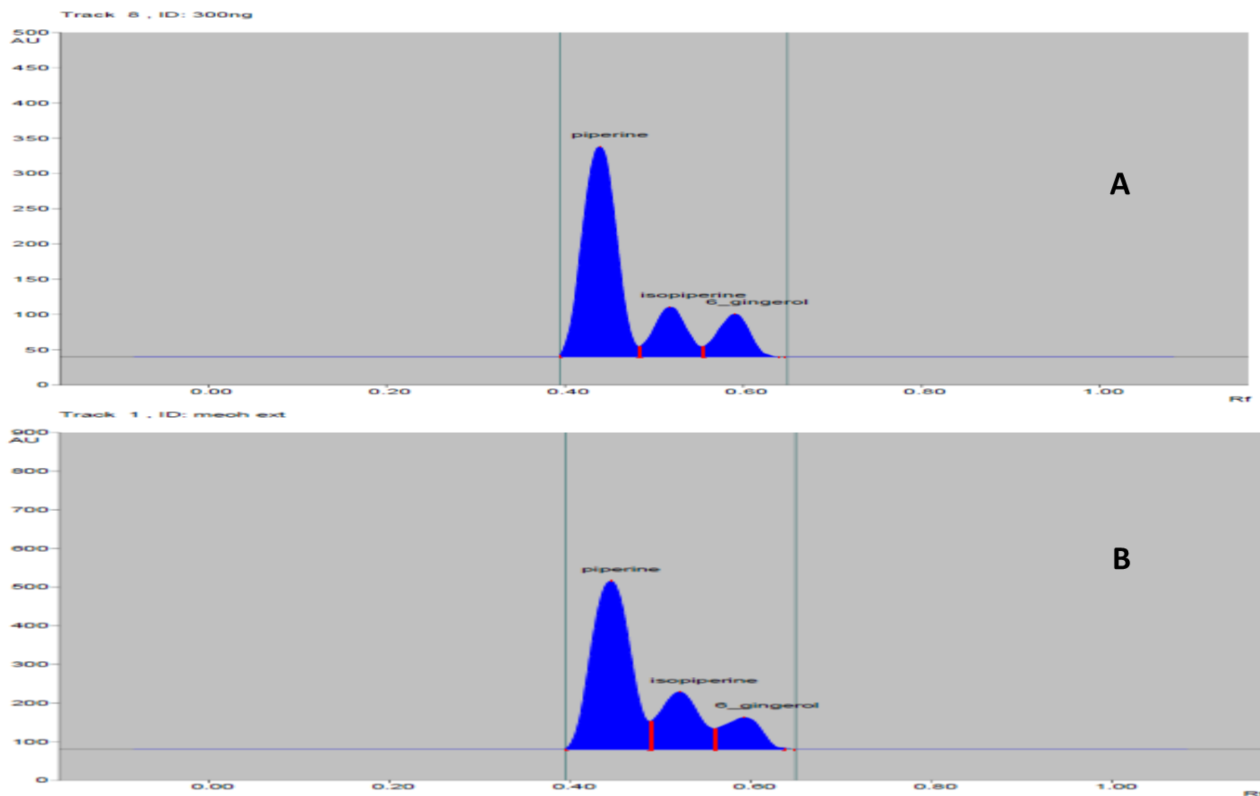

Figure 2 HPTLC chromatogram showing separation of piperine and 6-gingerol in (A) standard mixture solution and (B) ayurvedic formulation

Table-1 : Intra-day Precisions of piperine and 6-gingerol (*n*=3)

Name of Drug	Amount (ng/spot)	Intra-day Precision		
		Peak area (Mean±SD)	RSD	SE
Piperine	100	3135.18 ± 11.82	0.38	6.83
	300	7656.09 ± 11.79	0.15	6.81
	500	11251.94 ± 3.31	0.03	1.91
6-Gingerol	100	644.41 ± 8.31	1.29	4.79
	300	2157.90 ± 4.64	0.22	2.68
	500	2931.71 ± 14.43	0.49	8.33

Table-2 : Inter-day Precisions of piperine and 6-gingerol (*n*=3)

Name of Drug	Amount (ng/spot)	Inter-day Precision		
		Peak area (Mean±SD)	RSD	SE
Piperine	100	2858.93 ± 46.12	1.61	26.63
	300	7769.4 ± 77.89	1.00	44.97
	500	11517.27 ± 168.49	1.46	97.28
6-Gingerol	100	345.83 ± 6.07	1.76	3.50
	300	1180.5 ± 16.87	1.43	9.74
	500	1950.2 ± 33.34	1.71	19.25

Table-3 : Recovery studies for piperine and 6-gingerol

Name of the drug	Amount of standard spiked (ng)	Average of amount Recovered (ng)	Recovery (%) ± S.D	%RSD
Piperine	200	207.66	103.8 ± 1.55	1.50
	300	291.97	97.32 ± 1.67	1.71
	400	396.75	99.19 ± 1.81	1.83
6-Gingerol	200	195.83	97.91 ± 1.68	1.71
	300	306.58	102.2 ± 1.81	1.77
	400	401.17	100.3 ± 1.81	1.81

Table-4 : Analysis of ayurvedic formulation

Name of the drug		Ayurvedic lab formulation	Marketed formulation
Average content (%w/w)	Piperine	0.05	0.087
	6-Gingerol	0.06	0.07