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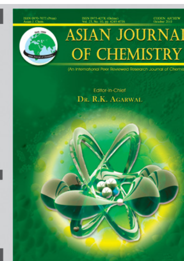

Simultaneoous Estimation of Piperne and 6-Gingerol in an Ayurvedic Formulation by HPLC

VISHAKHA S. KULKARNI* and SANJAY J. SURANA

R.C. Patel Institute of Pharmaceutical Education and Research, Near Karwand Naka, Shirpur-425405, India

*Corresponding author: Fax: +91 2563 251808; Tel: +91 2563 255189; E-mail: kulkarnivishakha@rediffmail.com

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Trikatu churna is an ayurvedic formulation used extensively by ayurvedic practitioners and contains equal amount of fine powders of rhizomes of *Zingiber officinale* (Zingiberaceae), fruits of *Piper longum* and *Piper nigrum* (Piperaceae). In general 6-gingerol from *Z. officinale* and piperine from *P. longum* and *P. nigrum* were analyzed by HPLC or by HPTLC. No analytical method is reported for the estimation of both the markers simultaneously. Trikatu churna needs simultaneous estimation of piperine and 6-gingerol. Attempt has been made to develop simple, precise, rapid and reproducible method for simultaneous estimation of piperine and 6-gingerol as a routine quality control tool of Trikatu churna. The analysis was carried out using toluene:ethyl acetate:formic acid (8:2:1, v/v/v) at room temperature. The R_f value of piperine and 6-gingerol were found to be 0.53 ± 0.04 and 0.67 ± 0.02 respectively. The method was validated by specificity, precision, recovery and robustness. Trikatu churna contains 4.38 % and 0.78 % of piperine and 6-gingerol, which were further compared with HPLC analysis.

Key Words: HPTLC, HPLC, Simultaneous estimation, Trikatu churna, Piperine, 6-Gingerol, Validation.

INTRODUCTION

Trikatu is Sanskrit word, which means three spices. Trikatu contains equal amounts of fine powders of rhizomes of *Zingiber officinale* (Zingiberaceae), fruits of *Piper longum* and *Piper nigrum* (Piperaceae)[1]. It is prescribed extensively alone or in combination of other formulations by ayurvedic practitioners in India. Trikatu has reported to possess an analgesic, antimicrobial, anthelmintic, antiinflammatory and hypolipidemic activity[2-5]. Hepatoprotective potential of Trikatu has also been reported[6]. Out of the 370 compound formulations listed in the Handbook of Domestic medicine and common ayurvedic remedies, 210 contain either Trikatu or its individual components[7].

The ingredients of Trikatu churna were standardized based on the major chemical constituent mainly piperine ad 6-gingerol. Literatures reveals that simultaneous estimation of piperine with other biologically important markers from the ayurvedic formulations was carried out *viz*. piperine, curcumin and thymol[8], piperine and withaferin A[9], piperine and vasicine[10] and piperine, cinnamaldehyde and eugenol[11]. Identification and quantification of 6-gingerol by HPTLC has been reported [12-14]. Three types of gingerols were also separated simultaneously by TLC[15]. All the reported methods in literatures are not suitable for the simultaneous analysis of piperine and 6-gingerol. So attempt has been made to analyze piperine and 6-gingerol simultaneously as a tool of standardization of widely used Trikatu churna.

EXPERIMENTAL

Preparation of formulation: The plant materials required for Trikatu churna were collected from local market, authen-ti-cated and voucher specimens were deposited at Agharkar Research Institute, Pune, Maharashtra India. The deposited voucher specimen numbers are *Zingiber officinale* (Zingiberaceae)-R-106, *Piper longum* (Piperaceae)-F-145, *Piper nigrum* (Piperaceae)-F-144. The formulation was prepared as per the Ayurvedic Formulary of India part-I. Rhizomes of *Zingiber officinale* (Zingiberaceae), fruits of *Piper longum* and *Piper nigrum* (Piperaceae) were taken in equal parts, powdered and then mixed uniformly.

The standard marker compound piperine (98.0 % purity) and 6-gingerol (97.0 % purity) were purchased from Sigma Aldrich Pvt. Ltd. (USA). All chemicals and reagents used were of analytical grade.

Instrumentation and chromatographic conditions: The samples were spotted in the form of 6 mm bands with Camag microlitre syringe on aluminium plate pre-coated with silica gel 60 F_{254} (10 × 10 cm with 0.2 mm thickness) prewashed with methanol, using Camag Linomat V applicator fitted with a 100 μL syringe. A constant application rate of 150 nL/s was employed. The linear development was carried out in solvent system (11 mL) toluene:ethyl acetate:formic acid (8:2:1, v/v/v) in a 10 cm × 10 cm twin trough glass chamber previously saturated with solvent for 0.5 h at room temperature (25 ºC ± 2)

and relative humidity of 60 ± 5 %. The length of chromatogram run was approximately 80 mm from the point of application. Subsequent to the development, plates were dried in current of air with the help of an air dryer. Densitometric scanning was performed using Camag TLC scanner III in the reflectance-absorbance mode at 285 nm and operated by winCATS software (V 1.4.3.6336). The slit dimension was 5 × 0.45 mm with the scanning speed of 20 mm/s. Photo documentation of the chromatoplate was carried out with the help of Camag Reprostar 3 with cabinet cover and mounted digital camera.

Preparation of standard stock solutions: Piperine and 6-gingerol were weighed individually and dissolved in methanol to get a solution of 100 μg/mL of each.

Preparation of working standard stock solution: The stock solutions of piperine and 6-gingerol were further diluted to get working standard solution of 10 μg/mL of each and used as working standard solution for further chromatographic analysis.

Preparation of sample solution: An accurately weighed 100 mg of powder from Trikatu churna and 100 mg of its ingredients were extracted by sonication in ultrasonic water bath for 0.5 h with 80 mL methanol. Then the solutions were filtered through the Whatman filter paper No. 41. The extraction by sonication was performed three times successively. The samples for extraction efficiency study was withdrawn and successive extracts were combined and concentrated up to 100 mL of each and used for further chromatographic analysis. Extraction efficiency study was performed by proposed method and again confirmed by HPLC method. Filter paper interference and glass absorption study was performed by immersing the used filter paper and volumetric flask in methanol, sonicated and analyzed for the presence of any maker compound.

Calibration curve for piperine and 6-gingerol: The stock solution of piperine and 6-gingerol was applied in the concentration range of 10-90 ng/spot on the plate. The plate was developed by proposed method, dried and scanned at 285 nm. The concentrations were subjected to least square regression analysis to calculate calibration curve equation and correlation coefficient (r^2) (acceptance criterion: correlation coefficient should be not less than 0.995).

Method validation: The proposed method was validated as per ICH guidelines with respect to specificity, precision, limit of detection and quantitation, accuracy, robustness and stability[16].

Specificity: Specificity of the method was studied by analyzing standards, extracts, formulation and individual placeboes by simultaneously applying on the same plate. The spots of piperine and 6-gingerol in sample were confirmed by comparing R_f and spectra with respective standards.

Precision

Repeatability of measurement of peak area: For scanning precision the standard stock solution was spotted on the plate (5 μL). The separated spots were scanned seven times without changing the plate position at 285 nm. The average, standard deviation (SD) and percentage relative standard deviation (% RSD) of peak area was calculated.

Repeatability of sample application: For application precision seven spots of the standard stock solution were applied on the plate (5 μL). The plate was developed, dried and each track was scanned at 285 nm. The peak area of each standard was measured and the % RSD of peak area was calculated.

Intra-day precision: The intraday precision was determined by analyzing the sample at three different concentration levels (4, 5 and 6 μL) in triplicate on the same day for two times. The % RSD of peak areas was calculated.

Inter-day precision: The interday precision was determined by analyzing the sample at three different concentration levels (4, 5 and 6 μL) in triplicate on the two different days. The % RSD of peak areas was calculated.

Limit of detection (LOD) and limit of quantitation (LOQ): Determination of signal-to-noise ratio is performed by comparing measured signals from the sample with known low concentrations of analytes with blank solution (methanol) and establishing the minimum concentration at which the analytes can be reliably detected. LOD was considered as 3:1 and LOQ as 10:1 (signal to noise ratio).

Accuracy: The accuracy of the method was determined from recovery studies. A known amount of each standard was spiked to individual placebo at three different concentration levels (80, 100 and 120 %). 6-Gingerol was spiked with *Z. officinale* placebo and piperine in *P. longum* and *P. nigrum* placebo (only in *Z. officinale* extract). The analysis was done by the proposed method.

Robustness of the method: By small changes in the proposed chromatographic conditions, the effect on the results was examined. The saturation time of development was varied by ± 5 min and the distance of chromatogram run was varied by ± 5 mm. The robustness of the method was done at three different concentration levels 4, 5 and 6 μL of working standard solution in triplicate. The results were expressed in the terms of % RSD.

HPLC analysis: The chromatographic system used was Agilent liquid chromatography system series 1200 with quaternary pump, Rheodyne injector with 20 μL fixed loop and photodiode array detector. The separation was achieved on Waters symmetry C_{18} column (250 × 4.6 mm, particle size 5 μ) preceded by an ODS guard column (10 μm, 10 mm × 5 mm ID) at an ambient temperature. The analysis was isocratic with mixture of acetonitrile: potassium hydrogen phosphate buffer (10 mM, pH 7.5): methanol (65:20:15) with flow rate of 1 mL /min. The mobile phase was prepared freshly every day and filtered through filtered through a 0.2 μm Ultipor Nylon 66 membrane filter prior to use.

The mobile phase is mixed and degassed by sonication prior to use and found stable with no precipitation with time or change in temperature. The absorbance of both the markers piperine and 6-gingerol was good at 280 nm in this mobile phase. The developed RP-HPLC method was validated and the quantities of the markers present in the formulation were compared.

Determination of 6 -gingerol and piperine from Trikatu churna: Trikatu churna was analyzed by the proposed HPTLC and HPLC method. The peak area of respective marker was compared with peak area obtained in formulation and the % amount was calculated and compared.

RESULTS AND DISCUSSION

The various methods developed by the different researchers for the analysis of 6-gingerol and piperine were not suitable for simultaneous estimation of these markers. The reported methods were useful when the single marker has to be analyzed. When the compound formulation containing these two markers has to standardized, two different methods required, which is time consuming and expensive. To overcome this problem, attempt has been made to develop simple, rapid, precise and reproducible HPTLC method for simultaneous estimation piperine and 6-gingerol in compound formulations.

HPTLC analysis: The mobile phase for HPTLC was optimized by analyzing the samples in mixtures of solvents of varying polarity. Finally a mixture of toluene:ethyl acetate: formic acid (8:2:1, v/v/v) mobile phase gave a good resolution of the standards piperine and 6-gingerol at R_f 0.53 ± 0.04 and 0.67 ± 0.02 respectively in a binary mixture and formulation showing the specificity of the proposed method for formulation analysis (Fig. 1a-b). The scanning wavelength selected was 285 nm as absorption maxima for both the markers is in the range of 280-335 nm. The chromatogram recorded is shown in Fig. 2.

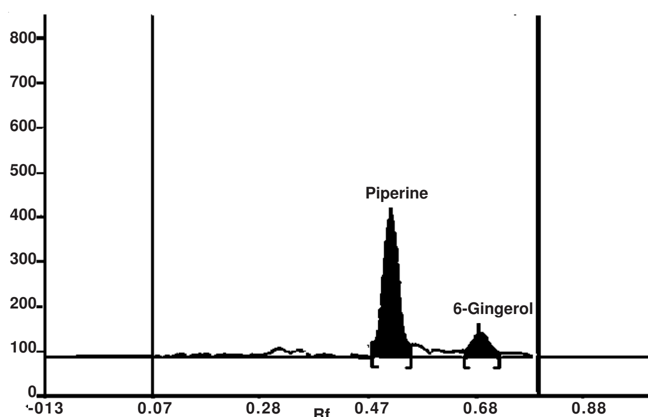

Fig. 1a Chromatogram of standard 6-gingerol and piperine in a binary mixture at 254 nm

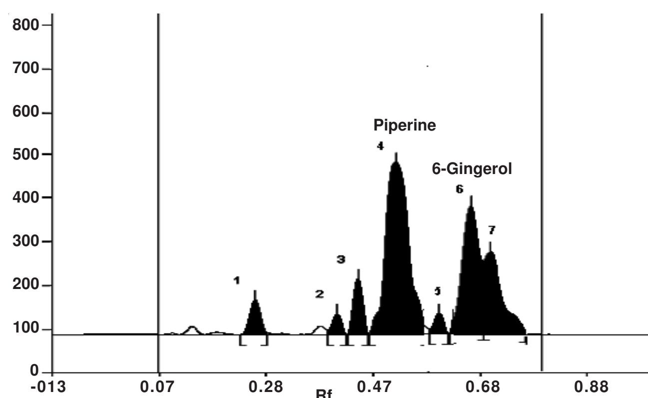

Fig. 1b Chromatogram of formulation at 254 nm; Peak 4→piperine and peak 6→6-gingerol

The extraction efficiency study showed that more than 90 % of piperine and 6-gingerol were extracted in first cycle of extraction, which suggests the complete exhaustive extraction of the plant material (Table-1). There is no glass absorption and filter paper interference was observed.

TABLE-1
EXTRACTION EFFICIENCY STUDY BY HPTLC AND HPLC ANALYSIS (n = 3)

Marker	HPTLC		HPLC	
	Content (%)	RSD (%)	Content (%)	RSD (%)
Piperine				
1st cycle	4.30	1.43	4.42	1.09
2nd cycle	0.11	1.67	0.03	1.21
3rd cycle	0.03	1.05	0.01	1.08
6-Gingerol				
1st cycle	0.69	1.14	0.71	1.12
2nd cycle	0.04	1.05	0.04	0.98
3rd cycle	0.04	1.45	0.02	1.54

Method validation

Calibration study: Calibration curve for both the markers were linear over the concentration range 10-90 ng/spot showing the correlation coefficient (R^2) and calibration curve equation were 0.9974, y = 437.17x - 92.676 and 0.9981, y = 230.6x - 23.926 for piperine and 6-gingerol (Table-2).

TABLE-2
SUMMARY OF METHOD VALIDATION PARAMETERS FOR ANALYSIS OF PIPERINE AND 6- GINGEROL

Parameters	Piperine	6-Gingerol
Specificity	Specific	Specific
Correlation coefficient	0.997	0.998
Range (ng/spot)	10-90	10-90
Precision (%) RSD (n = 7)		
Repeatability of measurement of peak area	1.30	1.62
Repeatability of sample application	1.02	1.26
Intra-day precision	1.41	1.67
Inter-day precision	1.53	1.56
LOD (ng/spot)	10	10
LOQ (ng/spot)	1.78	1.53

Precision

Repeatability of measurement of peak area: The separated spots were scanned seven times and the % RSD of peak area for piperine and 6-gingerol was found to be 1.30 and 1.62, respectively. These low values of % RSD showed the precision of the method.

Repeatability of sample application: After scanning each track at 285 nm the % RSD of peak area for each standard was determined. The % RSD of piperine and 6-gingerol were found to be 1.02 and 1.26 respectively.

Intra-day and inter-day precision: The results of intra- and inter-day precision showed that no significant variation in % RSD of peak area was observed for each standard. The values of % RSD of intra-day precision were 1.41 and 1.67 while of inter-day the corresponding values were 1.53 and 1.56 for piperine and 6-gingerol respectively.

Limit of detection and limit of quantitation: The limit of detection was found to be 10 ng/spot for piperine and 6-gingerol. The limit of quantification was found to be 1.78 ng/spot and 1.53 ng/spot for piperine and 6-gingerol respectively.

Accuracy: The accuracy of the method was determined from recovery studies. The results of recovery studies are listed in Table-3. The recovery values for piperine and 6-gingerol were in the range of 99.04 to 101 and 99.59 to 100.28 respectively.

TABLE-3
RECOVERY STUDIES (n=3)

Standard (%)	Amount added (ng)	Amount recovered (ng)	Recovery (%)	RSD (%)
Piperine 80	0.699	0.687	98.236	0.301
100	0.871	0.862	98.966	0.528
120	1.048	1.024	97.679	0.584
6-Gingerol 80	0.123	0.122	99.186	0.382
100	0.155	0.153	99.138	0.305
120	0.186	0.183	98.565	0.678

All values are average of three readings; RSD-relative standard deviation

Robustness of the method: The % RSD of peak area for each parameter of robustness was determined. The % RSD of saturation time parameter was found to be 1.22 and 1.68 for piperine and 6-gingerol respectively. For the development distance parameter the values were 0.91 and 1.22 respectively. The low values of % RSD indicate the robustness of the method (Table-4).

TABLE-4
ROBUSTNESS OF THE DEVELOPED METHOD

Standard	Saturation time RSD[a] (%)	Development distance RSD[a] (%)
Piperine	1.22	1.68
6-Gingerol	0.91	1.22

[a]Average peak areas at 4, 5 and 6 μL of working standard

Determination of piperine and 6-gingerol from Trikatu churna: The content of piperine and 6-gingerol in formulation was found to be 0.78 % and 4.38 % with a % RSD of 1.19 and 1.78 respectively. The result of the HPTLC analysis was further compared by HPLC analysis. The HPLC analysis showed 4.41 % and 0.76 % of 6-gingerol and Piperine with retention time 5.3 ± 0.09 and 6.7 ± 0.08 min respectively. The developed HPLC method found to be linear with correlation coefficient 0.998; concentration range 10-50 mg/mL for both the markers with regression equation, y = 18115x - 18070 and y = 196793x - 194496 for 6-gingerol and piperine respectively. The results of the HPTLC analysis are comparable with those obtained by HPLC (Table-5).

TABLE-5
COMPARISON OF RESULTS OBTAINED BY HPTLC AND HPLC

Marker	HPTLC		HPLC	
	Content (%)	RSD (%)	Content (%)	RSD (%)
Piperine	4.38	1.78	4.41	1.25
6-Gingerol	0.78	1.19	0.76	1.92

The proposed method is simple and accurate. The method validation suggests that the method is reproducible, specific and rapid for the simultaneous estimation of piperine and 6-gingerol and can be used for the routine quality control tool for Trikatu churna.

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