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Rapid validated HPTLC method for estimation of piperine and piperlongumine in root of *Piper longum* extract and its commercial formulation

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Abstract: Piperine and piperlongumine, alkaloids having diverse biological activities, commonly occur in roots of *Piper longum* L., Piperaceae, which have high commercial, economical and medicinal value. In present study, rapid, validated HPTLC method has been established for the determination of piperine and piperlongumine in methanolic root extract and its commercial formulation 'Mahasudarshan churna®' using ICH guidelines. The use of Accelerated Solvent Extraction (ASE) as an alternative to conventional techniques has been explored. The methanol extracts of root, its formulation and both standard solutions were applied on silica gel F_{254} HPTLC plates. The plates were developed in Twin chamber using mobile phase toluene: ethyl acetate (6:4, *v/v*) and scanned at 342 and 325 nm (λ_{max} of piperine and piperlongumine, respectively) using Camag TLC scanner 3 with CATS 4 software. A linear relationship was obtained between response (peak area) and amount of piperine and piperlongumine in the range of 20-100 and 30-150 ng/spot, respectively; the correlation coefficient was 0.9957 and 0.9941 respectively. Sharp, symmetrical and well resolved peaks of piperine and piperlongumine spots resolved at R_f 0.51 and 0.74, respectively from other components of the sample extracts. The HPTLC method showed good linearity, recovery and high precision of both markers. Extraction of plant using ASE and rapid HPTLC method provides a new and powerful approach to estimate piperine and piperlongumine as phytomarkers in the extract as well as its commercial formulations for routine quality control.

Article

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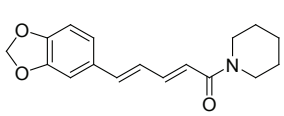
Introduction

Root of *Piper longum* L., Piperaceae, is used in home remedies as well as Indian System of Traditional Medicine against various disorders like pasly, gout, rheumatism, lumbago, bronchitis and abortifacient (Kirtikar & Basu, 1984). It is a major constituent of many common Ayurvedic and polyherbal formulations such as 'Mahasudarshan churna' which is used for complications associated with fever, typhoid, jwar etc. (Desai, 1975; Kirtikar & Basu, 1984; Deshpande et al., 1989). Root is reported as antiamoebic (Ghoshal & Lakshmi, 2002), analgesic (Vedhanayaki et al., 2003), hepatoprotective (Patel & Shah, 2009), antioxidant (Jagdale et al., 2009) and antimicrobial (Gupta et al., 2010) activity. It is a well known source of pharmacologically active alkaloids piperine (1) [CAS 94-62-2] and piperlongumine (**2**) [CAS 20069-09-4] (Dutta et al., 1977).

Piperine (**1**) is reported for diverse therapeutic actions like central nervous system depressant, analgesic (Singh et al., 1973), inhibition of hepatic drug metabolism (Bhat & Chandrasekhara, 1987), enhancing pentobarbitone induced hyponosis (Mujumdar et al., 1990a), bioavailability of oxyphenylbutazone (Mujumdar et al., 1999), hepatoprotective activity (Desai et al., 2008), anti-inflammatory activity (Mujumdar et al., 1990b), inhibition of lipid peroxidation during experimental inflammation (Dhuley et al., 1993), devoid of genotoxic effects (Karekar et al., 1996), antifertility (Daware et al., 2000) and antidiarrhoeal (Bajad et al., 2001). It also possesses radioprotective effects (Aggarwal & Kaul, 1992). Piperlongumine (**2**) is reported for killing of cancer cells by a small molecule targeting the stress response to ROS (Raj et al., 2011), antiplatelet (Lee et al., 2010), melanocyte stimulatory activity (Lin et al., 2007). It also acts as an antiaflatoxin agent (Lee et al., 2002). Due to diverse therapeutic activities, both compounds can be used as marker compounds for quality control and standardization of

root of *P. longum*. A perusal of literature showed no reports are available on analytical methods for analysis of piperine and piperlongumine in roots of this plant, its extracts or commercial formulations. Hence, the aim of this study was development and validation of a new, low-cost and high throughout analytical method for simultaneous quantitation of piperine and piperlongumine in methanolic root extract of *P. longum* as well as its commercial product 'Mahasudarshan churna' using ICH guidelines (ICH, 1996) and also assesses the possible application of Accelerated Solvent Extraction (ASE) in extracting both bioactive markers.



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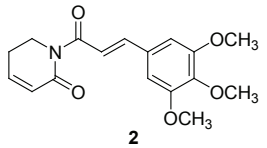

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Materials and Methods

Plant material and chemicals

Root of *Piper longum* L., Piperaceae, was collected from Amboli, Maharashtra, India in January 2009. The sample was authenticated by Dr. A. S. Upadhhye and deposited in the Agharkar Herbarium at Maharashtra Association of Cultivation Sciences, Agharkar Research Institute, Pune, vide voucher specimen number AHMA-0025384.

'Mahasudarshan churna®' (Shree Baidyanath Ayurved Bhawan Pvt. Ltd., Napur, India) was procured from local market, Pune, India. Silica gel F_{254} HPTLC plates were purchased from Merck, Darmstadt, Germany. Other analytical grade solvents and reagents were obtained from sd fine chemicals, Mumbai, India.

Isolation of piperine and piperlongumine

Piperine (**1**) and piperlongumine (**2**) were isolated from roots of *P. longum* using the method reported by Chatterjee & Dutta (1967). The isolated compounds showed single spot on TLC. The crystallized pure compounds (≥97%) were identified based on melting point, UV and co-TLC with reference piperine and piperlongumine (Sigma Aldrich, Germany).

Sample extraction procedure for HPTLC analysis

Soxhlet extraction

Accurately weighed powder of root sample (5 g) and 'Mahasudarshan churna®' sample were extracted exhaustively with methanol (100 mL) using Soxhlet apparatus for 360-600 min (6-10 h). The optimum yield was obtained in methanol with extraction time of 480 min (8 h). The extract was then filtered through Whatman no. 41 (E. Merck, Mumbai, India) and concentrated under reduced temperature and pressure using rotary evaporator. The extractions were performed in triplicate. Respective yields of methanol extract of root and formulation sample were 1.602±3.56 and 0.901±3.78 g.

Accelerated solvent extraction

Accurately weighed powder of root sample (5 g) and 'Mahasudarshan churna®' sample were extracted with methanol placed in the stainless-steel cell of a Dionex ASE™ 100 system (Sunnyvale, CA, USA). Extraction was performed at 100 bar and three temperatures, 40, 60 and 80 ºC, for 20 min (1 cycle). The optimum yield was obtained at 60 ºC. In the experiment at 60 ºC, five replicate cycles were performed. The extracts were concentrated under reduced temperature and pressure using rotary evaporator. The extractions were performed in triplicate. Respective yields of methanol extract of root and formulation sample were 1.718±2.89 and 1.052±4.78 g.

Chromatographic experiments

HPTLC analysis was performed on aluminium backed HPTLC plates 10×10 cm coated with 0.2 mm layers of silica gel 60 F_{254} (E. Merck, Germany). Samples (4 µL) were applied on the plate with band width 6 mm employing Linomat IV sample applicator (Camag, Switzerland) fitted with a microlitre syringe. Linear ascending development of the plate to a distance of 80 mm was performed with mobile phase toluene:ethyl acetate (6:4, *v/v*) in a twin-trough glass chamber previously saturated with mobile phase vapour for 10 min at 25 °C. The dried plate was scanned at wavelength of 342 and 325 nm (λ_{max} of piperine and piperlongumine, respectively) using a Camag TLC scanner 3 with CATS 4 software. A variety of mobile phases were tried for analysis of piperine and piperlongumine in methanolic extracts of root and commercial formulation. These included toluene :ethyl acetate :diethyl ether (6:3:1, *v/v/v*), toluene:ethyl acetate:diethyl ether (5:4:1, *v/v/v*), toluene:ethyl acetate (7:3 *v/v*), toluene:ethyl acetate (5:5, *v/v*).

Calibration plot of piperine and piperlongumine

Stock solutions of piperine and piperlongumine were individually prepared by dissolving pure substances at concentrations of 1.0 mg/mL in methanol. Standard solutions were prepared by diluting the stock

solutions with methanol to obtain concentrations of 1, 2, 3, 4 and 5 µg/mL for different concentrations of piperine (20, 40, 60, 80 and 100 ng, respectively) and 1.5, 3, 4.5, 6 and 7.5 µg/mL for different concentrations of piperlongumine (30, 60, 90, 120 and 150 ng, respectively).

Quantification of piperine and piperlongumine

Each test extract (2.5 mg) was dissolved in methanol (2 mL) to serve as test solution for HPTLC analysis. Test sample solutions 4 µL (5000 ng) were applied in duplicate on a HPTLC plate along with standards. The plate was developed under predetermined conditions described above and scanned at 342 and 325 nm (λ_{max} of piperine and piperlongumine, respectively). Peak areas were recorded and piperine and piperlongumine content in the samples were calculated using the calibration plot.

Validation

The method was validated according to the ICH guidelines (ICH, 1996) by determining peak purity, limit of detection (LOD), limit of quantitation (LOQ), precision and recovery of piperine and piperlongumine from test samples.

Instrument precision was checked by repeated scanning of piperine and piperlongumine band (80 and 120 ng, respectively) six times and expressed as relative standard deviation (% *RSD*). Precision was studied by analyzing six bands of sample solutions per plate on three plates (intra-day precision) and by analyzing six bands of sample solution per plate on three consecutive days (inter-days precision) at three different quantities (40, 60, 80 ng/spot for piperine; 60, 90, 120 ng/spot for piperlongumine) and calculated % *RSD*. Accuracy of the method was tested by determination of recovery at three levels. Pre-analyzed samples were spiked with extra individual standards (50, 100 and 150 %) and the mixtures were reanalyzed. Robustness of the method was studied at three different concentrations - 40, 60, 80 ng/spot for piperine; 60, 90, 120 ng/spot for piperlongumine by introducing small deliberate changes in mobile phase composition (toluene:ethyl acetate, 6.2:3.8, 5.8:4.2, *v/v*). Repeatability of the method was assessed by analysis of 60 and 90 ng/spot of standard solutions of piperine and piperlongumine (n=6), respectively and expressed as *RSD* (%) of peak areas. Percentage recovery and standard deviation (SD) were calculated for each concentration level. LOD and LOQ were determined by standard deviation (SD) method from the slope (*S*) of calibration plot and the SD of a blank sample (blank methanol was spotted three times), by use of the equations LOD=3.3×SD/S and LOQ=10×SD/*S*.

Results and Discussion

Accelerated solvent extraction (ASE) was studied along with Soxhlet extraction for the development of rapid sample preparation method. ASE was being applied for the first time for extraction of piperine and piperlongumine in *Piper longum* L., Piperaceae, root extract. The mobile phase toluene:ethyl acetate (6:4, *v/v*) gave optimized result with sharp, symmetrical and well resolved peaks of piperine and piperlongumine at R_f 0.51 and 0.74, respectively from other components of the sample extracts (Figure 1). A linear relationship was obtained between response (peak area) and amount of piperine and piperlongumine in the range of 20-100 and 30-150 ng/spot, respectively; the correlation coefficient was 0.9957 and 0.9941, respectively. Piperine (**1**) and piperlongumine (**2**) in the methanolic extract of *P. longum* roots were quantitated using the developed HPTLC method and the results for each extraction technique employed are summarized in Table 1. Both the marker compounds were observed in chromatograms of all sample extracts with variation in their contents. As evident, ASE at 60 ºC for 20 min gave almost the same results as in Soxhlet extraction for 480 min (Table 1). However, taking into consideration the time of extraction and solvent consumption, ASE proves to be a promising alternative to Soxhlet extraction.

The method was validated in terms of peak purity, precision, LOD, LOQ and accuracy (Tables 2-4). Method was specific for analysis of both active principles piperine and piperlongumine because it resolved the compounds at R_f 0.51 and 0.74, respectively in the presence of other components. Purity of piperine and piperlongumine peak was checked from the samples by recording UV spectra. The method selectivity was demonstrated by the absence of overlapping spots. The identified spots of piperine and piperlongumine were confirmed from sample extracts by overlaying UV absorption spectrum of samples with standards at 342 and 325 nm, respectively.

The % RSD of instrument precision for peak area of piperine and piperlongumine was found to be 0.61 and 0.72, respectively. Intra-day and inter-days precision were studied by triplicate assay at three different quantities (40, 60, 80 ng/spot for piperine; 60, 90, 120 ng/spot for piperlongumine). Low RSD values (Table 2) indicated the method was precise. Small changes in mobile phase composition had no significant effect on the chromatographic profile. Low RSD values of peak areas calculated indicate robustness of the method (Table 4). Accuracy of the method was determined at three levels (50, 100, and 150%) by

adding known amounts of standards to samples extract. The result is represented in Table 3. High recovery indicated that the proposed method was reliable and reproducible. LOD was found to be 6.66 and 10 ng/spot for piperine and piperlongumine, respectively. LOQ was found to be 20 and 30 ng/spot for piperine and piperlongumine, respectively.

Herbal drug, singularly or in combinations is a multifarious mixture of phytoconstitutes in which no single constituent is responsible for the overall efficacy. It leads to create a challenge in establishing quality control standards for raw materials and the standardization of finished herbal drugs. Selecting one or more markers is a common practice in natural product analysis for purposes of identification and quality assessment. Estimation of the content of marker in herbal medicine is important in evaluating the phytochemical entity of the herb. Roots of *P. longum* are commonly used in various polyherbal formulations. In present study, a new, simple, accurate, precise, reproducible and sensitive HPTLC method was developed for simultaneous determination of piperine and piperlongumine in root of *P. longum* and its commercial product 'Mahasudarshan churna®'. Comparison of the extraction yields of both markers reveals ASE is a simple and efficient method. The method will be helpful for standardization and routine quality control of raw materials and herbal products containing root of *P. longum* as an ingredient. It provides significant advantages in terms of greater specificity and rapid analysis.

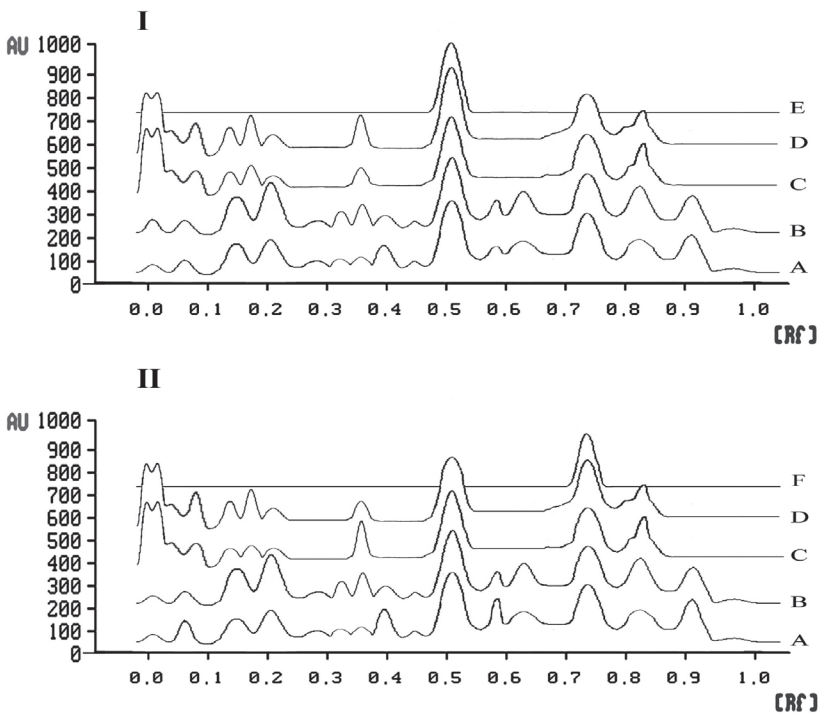

Figure 1. HPTLC chromatograms of methanolic extracts of root of *Piper longum*, its formulation along with standards piperine and piperlongumine. (I) For piperine standard scanned λ 342 nm; (II) for piperlongumine scanned at λ 325 nm. A. methanolic Soxhlet extract of root; B. methanolic ASE 60 ºC extract of root; C. methanolic Soxhlet extract of formulation; D. methanolic ASE 60 ºC extract of formulation; E. standard piperine; F. standard piperlongumine.

Table 1. Comparative content of piperine and piperlongumine in extracts of *P. longum* root and its formulation.

Sample	Piperine mg/g	Piperlongumine mg/g
Soxhlet extraction - methanol extract of root	9.56±0.83	4.67±1.23
ASE 60 ºC extraction - methanol extract of root	9.91±0.98	5.09±0.97
Soxhlet extraction - methanol extract of formulation	0.23±0.05	0.11±0.03
ASE 60 ºC extraction - methanol extract of formulation	0.27±0.09	0.17±0.04

Values are mean±SD of three experiments.

Table 2. Validation data of HPTLC method for estimation of piperine and piperlongumine.

Parameters	piperine	piperlongumine
Instrumental precision (% RSD, n=6)	0.61	0.72
Calibration range (ng/spot)	20-100	30-150
Regression equation	y=309.78x+801.85	y=3.0345x+1380.9
Correlation coefficient	0.9957	0.9941
Repeatability of standards (% RSD , n=6)	0.57	0.56
Repeatability of samples (% RSD, n=6)	0.91	0.94
Limit of detection (LOD) (ng/spot)	6.66	10
Limit of quantitation (LOQ) (ng/spot)	20	30
Intra-day precision (% RSD, n=6)	0.97	0.98
Inter-day precision (% RSD, n=6)	0.78	1.12

Table 3. Recovery study of the method for standards piperine and piperlongumine.

Compound	Amount of standard in sample [ng][a]	Amount of standard added [ng][a]	Amount of standard found in mixture [ng][a]	Recovery [%][a]	Average recovery [%][a]
Soxhlet extraction - methanol extract of root					
piperine	316±8.33	158	456±4.33	96.20±2.22	94.97±3.43
	316±8.33	316	600±5.87	94.93±3.45	
	316±8.33	474	741±9.03	93.79±3.85	
ASE 60 ºC - methanol extract of root					
piperine	316±8.33	158	451±3.84	95.55±4.25	95.94±3.67
	316±8.33	316	608±4.23	96.20±2.45	
	316±8.33	474	759±3.08	96.07±1.56	
Soxhlet extraction - methanol extract of root					
piperlongumine	234±5.67	117	329±1.56	93.23±2.35	94.38±1.89
	234±5.67	234	438±2.95	93.58±3.54	
	234±5.67	351	567±3.98	96.92±1.89	
ASE 60 ºC - methanol extract of root					
piperlongumine	234±5.67	117	321±2.34	91.45±3.34	93.72±2.78
	234±5.67	234	441±4.14	94.23±2.53	
	234±5.67	351	559±3.67	95.48±5.89	
Soxhlet extraction - methanol extract of formulation					
piperine	105±3.78	52.5	146±1.98	92.71±2.09	95.09±2.56
	105±3.78	105	199±2.07	95.22±3.55	
	105±3.78	157.5	255±2.35	97.36±1.01	
ASE 60 ºC extraction - methanol extract of formulation					
piperine	105±3.78	52.5	149±3.16	94.63±2.90	94.80±1.03
	105±3.78	105	199±2.67	94.77±3.63	
	105±3.78	157.5	250±2.39	95.01±4.91	
Soxhlet extraction - methanol extract of formulation					
piperlongumine	78±6.89	39	115±2.21	98.61±1.67	96.67±2.34
	78±6.89	78	151±3.67	97.32±1.66	
	78±6.89	117	185±3.09	95.01±3.04	
ASE 60 ºC - methanol extract of formulation					
piperlongumine	78±6.89	39	109±1.44	93.85±2.34	95.24±2.98
	78±6.89	78	147±2.39	94.55±3.48	
	78±6.89	117	189±2.11	97.33±1.02	

[a]Mean±SD (n=3).

Table 4. Robustness of the method for standards piperine and piperlongumine.

Condition	Amount of standard added (ng/spot)[a]	Amount of standard detected (ng/spot)[a]	% RSD
Mobile phase composition - toluene:ethyl acetate, 6.2:3.8, *v/v*			
piperine	40	39.78±1.34	1.09
	60	60.01±1.56	0.87
	80	79.01±1.7	0.76
piperlongumine	60	59.33±1.34	0.78
	90	88.45±1.23	1.1
	120	118.99±1.97	0.98
Mobile phase composition - toluene:ethyl acetate, 5.8 : 4.2, *v/v*			
piperine	40	38.88±1.45	1.45
	60	59.05±2.01	0.81
	80	79.44±1.34	0.89
piperlongumine	60	58.91±1.56	0.99
	90	88.97±1.73	1.45
	120	118.01±1.72	1.01

[a]Mean±SD (n=3).

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