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Validated high-performance thin-layer chromatography method for determination of Piperine in herbal extracts and pharmaceutical dosage form

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Abstract
Herbal medicines widely used in health-care in both developed as well as developing nations. Herbals are usually considered harmless and ever supplementary being consumed by people devoid of prescription. Though, some of them leads to health troubles, some are not effective and some might be interact with supplementary drugs. Consequently, standardization of herbal formulations is essential in order to estimate the quality of drugs, based on the concentration of their active principles. In the present study, Shatavari based Galactogogue, herbal formulation manufactured by Fredun Healthcare Pvt. Ltd., Mumbai is used for standardization. The HPTLC method was validated according to ICH guidelines and shown to be specific, linear, repeatable and accurate, within the established ranges. Statistical analysis proves that the method is reproducible and selective. This method can be used for the quantitative determination of piperine in herbal extract and its formulations.

Keywords: HPTLC, standardization, piperine, herbal formulation

Introduction
Herbal remedy has been enjoying revitalization among the consumers all over the world. Aboriginal herbs are utilized as remedies against a variety of diseases in the traditional system of medicines well as used as ethno medicinal practices. From the past few decades active ingredient or compounds from natural sources have been gaining significance because of the enormous chemical assortment that they tender. This has led to extraordinary boost in the demand for the herbal medicine in the preceding few decades and a need has been felt for ensuring the safety, quality and efficacy of the herbal drugs. Phytochemical assessment is one of the tools for the quality appraisal, which includes preliminary phytochemical screening, chromatographic finger printing and marker compound analysis by means of recent analytical techniques. In the last two decades high-performance thin-layer chromatography (HPTLC) has emerged as an essential tool for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes development of TLC fingerprint profile of herbal drugs and estimation of chemical markers as well as biomarkers. The chief advantage of HPTLC is that a number of samples able to be analyzed simultaneously using a small extent of mobile phase [1, 2].

Herbal medicines extensively used in health-care in both developed as well as developing countries. They are complex chemical mixtures prepared from plants and are limited in their efficiency since they are inadequately absorbed when taken orally. Herbals are traditionally considered harmless and ever more being consumed by people devoid of prescription. Though, some can cause health troubles, some are not effective and some might be interact with supplementary drugs. Therefore, standardization of herbal formulations is necessary in order to evaluate the quality of drugs, based on the concentration of their active principles [3]. Standardization of Certain herbal extracts and polyherbal formulations containing active constituents performed by high-performance thin layer chromatography (HPTLC) method. This method was reported to be the most appropriate method for the estimation of active constituents of extracts, plant species (raw material) or polyherbal formulations [4, 5].

Shatavari based Galactogogue, herbal formulation manufactured by Fredun Healthcare Pvt. Ltd., Mumbai is used for standardization. It contain ten herbal medicines *Asparagus recemosus, Withannia somnifera, Tinospora cardifolia, Trigonella foenum, Dioscorea burbifera, Pueraria tuberosa, piper longum, Nardostachy's jatamanshi, Leptadenia reticulata, Piper nigrum*. In the present study, an attempt has been made to develop a simple,

rapid and accurate HPTLC method for estimation of piperine in marketed formulations of Galactogogue.

Materials and Methods [6-17]

Equipment and chromatographic condition

Chromatography was performed on 10 cm x 10 cm aluminum plate coated with 0.2 mm layer of silica gel 60 F254 (E. Merck, Germany). Samples were applied to the plate as bands width 6 mm by using Camag (Muttenz, Switzerland) Linomat 5 applicator fitted with 100 µl syringe (Hamilton, Switzerland). The rate of application was constant at 150 nl sec^{-1} and space between two bands was 14 mm. Linear ascending development of the plate was carried out in twin – trough glass chamber previously saturated with mobile phase for 20 min at room temp. (25^0C ± 2) and relative humidity 60 % ± 5. The length of chromatogram run was approximately 80 mm. After development the plate was removed and dried in current of air. Densitometric scanning was performed at their absorption maxima using Camag TLC scanner 3.

Chemicals

Standard Piperine (Sigma Aldrich), precoated silica gel G 60 F254 TLC aluminium plates (20 _ 20 cm, 0.2 mm thick) (Merck Ltd. Germany) and AR grade chemicals were used. The samples of formulations and extracts were procured from Fredun Healthcare Pvt. Ltd., Mumbai, Maharashtra.

HPTLC method for estimation of Phytoconstituent

Preparation of standard stock solution

A stock solution of 200 µg/ml was prepared by dissolving 2 mg of piperine in methanol and volume was made to 10 ml and different amounts were applied in triplicate on TLC plates, using a Camag Linomat IV sample applicator.

Preparation of Herbal Formulation solution

The sample was weighed (1 g); sonicated with methanol for 25 min, filtered through Whatmann filter paper and volume was made to 10 ml in volumetric flask. Samples were applied on plate in 4 mm band with the help of Linomat IV applicator and developed under the same conditions as described for the standards.

Calibration curve of Piperine

Calibration curve was constructed according to requirement of ICH guidelines (7). A stock solution of Piperine (200 µg/ml) was prepared in methanol. Different volumes of stock solution in the range 800-4000 ng of Piperine were spotted on the TLC plate. Estimation was done using linear regression analysis via peak areas and calibration curve was prepared by plotting peak area vs. concentration applied.

Method validation

The HPTLC method developed was validated for the following parameters.

Precision

The ICH guideline breaks precision into two parts:

Intraday precision: Repeatability expresses the precision of the method under the identical operating conditions over a short period of time.

Intermediate precision (Interday precision): Intermediate precision express the precision variation within laboratory in different days as well as different analysts or different equipments and is expressed as %R.S.D.

Accuracy

The accuracy of the analytical procedure was evaluated by using the recovery test. The analytical procedure for accuracy involved the addition of known quantities of the reference standard compound taken from stock solution. The known standards were diluted based on the percentage of present in the pre-analyzed sample. Three concentration levels were tested (80%, 100% & 120% i.e. low, middle and high). At each level, samples were prepared in triplicate and analyzed according to previously described procedure. Accuracy was expressed as percentage (observed concentration× 100/theoretical concentration).

Specificity

Specificity of the method was ascertained by analyzing the standard, extract and sample solutions. The bands of Piperine in the samples were confirmed by comparing their R_f values and overlaid spectra of the spotted bands with standards, extract and formulation.

Sensitivity

The sensitivity of the method was determined with respect to Limit of Detection (LOD) and Limit of Quantification (LOQ). The standard solutions were spotted in the range from 800 to 1600 ng/spot for Piperine (n = 3). The limit of detection and quantification were calculated based on calibration curve and experimentally verified as per the ICH guidelines.

Repeatability

Repeatability of sample application was assessed by spotting 8 µL contain 1600 ng/spot of standard Piperine on TLC plate in triplicate and experimentally verified as per the ICH guidelines.

Results and Discussion

Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the standard, herbal extract & formulation. Initially, different mobile phases were tried but dragging of spot, tailing of spots were observed. Finally, Toluene: Ethyl Acetate: Formic Acid, in varying ratios was tried; the mobile phase consisting of (4.8:4:0.5) (*v/v*) gave good resolution, sharp and symmetrical peak with 0.70 R_f value for Piperine.

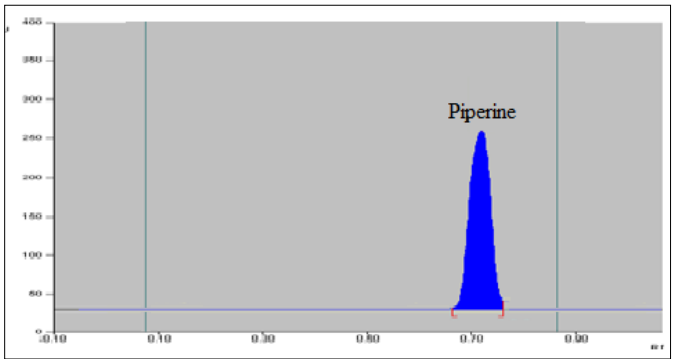

Fig 1: Chromatogram of standard piperine (1600 ng $spot^{-1}$), (Rf = 0.70±0.02), mobile phase: toluene: ethyl acetate: formic acid (4.8:4:0.5, v/v/v)

Calibration curves

The developed HPTLC method for estimation of piperine showed a good correlation coefficient (r2 = 0.9994±0.0002) in concentration range of 800–4000 ng spot−1 with respect to the peak area. The mean value (±S.D.) of slope and intercept

were 1.9229±0.031 and 187.21 ±1.1129, respectively. No significant difference was observed in the slopes of standard curves (ANOVA, $P > 0.05$).

Method Validation

Precision

Intraday precision

For Intraday precision, six samples of same concentration were prepared as per method and analyzed by proposed method to determine variation arising from method and expressed as % R.S.D. Percentage R.S.D. of method precision was in the range of 0.81-0.96%.

Inter day precision

Inter day precision or intermediate precision express within laboratory variations in different days. The % R.S.D. varies from 1.39–1.57%.

Table 1: Intra- and Inter-Day Precision of HPTLC Method

Concentration (ng/spot)	Intra-day precision (RSD, %, *n*=3)	Inter-day precision (RSD, %, *n=3*)
1600	0.96	1.39
2400	0.73	1.25
3200	0.81	1.57

Accuracy

The percentage mean recovery values for piperine were (100%, 100.4%, 102.3% & 100.4%) from lowest to highest level spiked, i.e.0%, 80%, 100%, 120%, respectively (Table).

Table 2: Accuracy studies for Piperine

Drug	Initial Amount [ng]	Amount added [ng]	Peak area ± S.D.	% Recovered	% R.S.D.
Piperine	1600	0	3283.3 ±57.25	100.6	1.72
	1600	1280	5751.3 ±95.12	100.4	1.67
	1600	1600	6485.4 ±62.27	102.3	0.95
	1600	1920	6985.3 ±127.04	100.4	1.83

Specificity

The bands of piperine in the extract & formulation were confirmed by comparing R_f values and overlaid spectra of the spotted bands with standards.

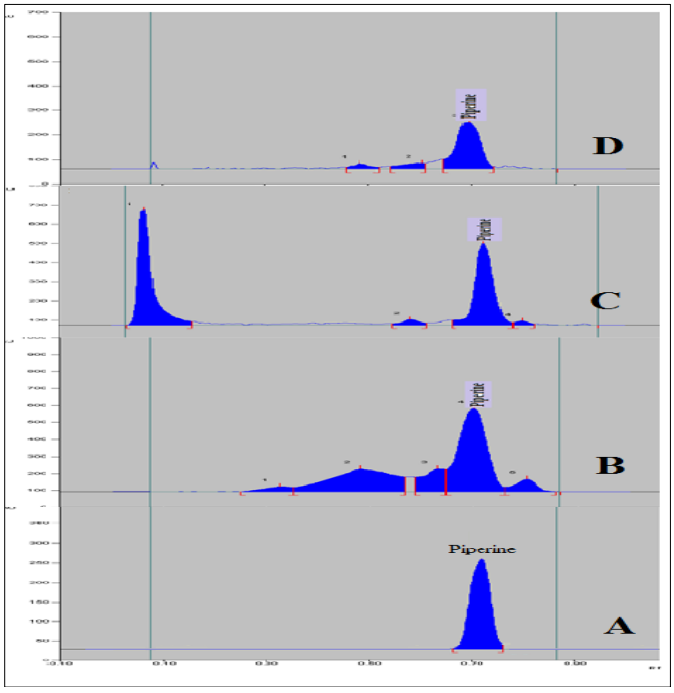

Fig 2: Overlay chromatogram of standard and sample for specificity

A-Standard showing piperine, B-Piper Extract showing piperine, C-Piplamool Extract showing piperine, D- Ossitone Plus formulation showing piperine.

Sensitivity

Under the proposed experimental conditions, the lowest amount of compounds which could be detected were found to be 81.03 ng /spot and the lowest amount of compound which could be quantified was found to be 245.55 ng /spot for piperine. The calibration curve was found to be linear in the range of 800-1600 ng/spot for piperine with good correlation coefficient 0.999.

Repeatability

Repeatability of sample application was assessed by spotting 8 µL contain 1600 ng/spot of standard Piperine on TLC plate in triplicate; results were shown in Table.

Table 3: Repeatability studies for Piperine

Sr. no.	Application volume [µL]	Area of Piperine	Mean	S.D.	%R.S.D.
1	8	3295.4	3271.73	34.14	1.16
2	8	3226.5			
3	8	3285.2			
4	8	3298.6			
5	8	3295.1			
6	8	3229.6			

Table 4: Summary of Validation Parameters for Piperine

Parameter	Data
Linearity range (ng/spot)	800-4000
Regression equation	Y = 1.9229X + 187.21
Limit of detection (ng/spot)	81.03
Limit of quantitation (ng/spot)	245.55
% Recovery	100.4 -102.3
Precision (%R.S.D.)	
Intra-day (n = 3)	1.25-1.57
Inter-day (n = 3)	1.13-1.69
Repeatability of application (n = 6)	1.04
Specificity	Specific

Application of the Method

The proposed method was applied for the estimation of piperine content in extract of *Piper longum and Piper nigrum* extract and its formulations. The obtained results are depicted in Table.

Table 5: Assay of piperine in ossitone plus formulation, *Piper longum* and *Piper nigrum* extract

Component	Amount taken [ng/spot]	Peak area of Piperine in formulation	Amount found [ng/spot]	Amount found [%]
Ossitone Plus formulation	2500	1135.3	493.0522	0.091
	2500	1169.8	510.9938	0.020
	2500	1142.2	496.6405	0.019
	2500	1149.4	500.3848	0.020
	2500	1160.5	506.1574	0.020
	Mean±S.D.	1151.44±13.86	501.44±7.21	0.020±0.0002
	% R.S.D.	1.20	1.43	1.44
Piper nigrum Extract	100	4019.5	1992.9	1.992
	100	3925.6	1944.1	1.944
	100	4015.6	1990.9	1.990
	100	3916.4	1939.3	1.939
	100	4012.6	1989.3	1.989
	Mean±S.D.	3977.94±52.13	1971.3±27.11	1.971±0.027
	% R.S.D.	1.31	1.37	1.38
Piper longum Extract	100	2556.6	1232.1	1.232
	100	2551.6	1229.5	1.229
	100	2556.5	1232.1	1.232
	100	2545.6	1226.4	1.226
	100	2494.6	1199.9	1.199
	Mean±S.D.	2540.9±26.31	1224.07±13.68	1.224±0.013
	% R.S.D.	1.03	1.11	1.12

Conclusions

The HPTLC method was validated according to ICH guidelines and shown to be specific, linear, repeatable and accurate, within the established ranges. Statistical analysis proves that the method is reproducible and selective. This method can be used for the quantitative determination of piperine in herbal extract and its formulations.

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