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HPTLC Method Development and Validation for Standardization of Ayurvedic Formulation: Trikatu Churna

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ABSTRACT: Ayurveda medicineis concerned with healthy living along with curative measures that synchronize an individual physically, mentally and spiritually. In modern culture, it is getting accepted as a self-care system for individual well-being. About 50% of drugs are natural products, which play an important role in pharmaceutical industry. In Ayurveda, the drugs are selected for performing a type of work according to its *Gunas* (properties). In ayurveda, the *Dravyas* are multidimensional in nature. Trikatu is the sanskrit name indicates its meaning, "tri" in stands for three and "*katu"* stands for acrids. Trikatu acts mainly effect on stomach, liver, and pancreas. In stomach, trikatu increases production of digestive juices thereby stimulating digestion. In liver, it is used to increases production of bile salts by stimulating gallbladder functioning. Trikatu also used for the pancreatic functioning. In a nutshell, Trikatu affects overall digestive system along with its curative effects on respiratory, urinary, immunity, skin, and metabolic systems of our body.In the proposed work, attempt has been made for standardization of trikatu churna by developing chromatographic method. Piperine from piper longum and piper nigrum present in formulation were selected as marker compounds. A new, rapid, simple, precise, selective HPTLC method was developed for marketed preparation of Trikatu churna. The separation of piperine was performed on TLC aluminium plates precoated with silica gel 60 F_{254}, using toluene: ethyl acetate: glacial acetic acid (8:2:0.1 v/v/v) as mobile phase. The densitometric analysis was carried out at the detection wavelength of 360 nm. The R_f values of piperine was found to be 0.65.The developed method has been validated as per ICH guidelines.

KEYWORDS: Trikatu churna, Standardization, HPTLC, Piperine.

I. INTRODUCTION

Ayurveda is concerned with the healthy living along with curative measures that synchronize an individual physically, mentally and spiritually. In this modern era, it is getting accepted as a self-care system for individual well-being[1]. The Ayurvedic treatment is although highly effective; proper mode of action, pharmacology, pharmacokinetics, and pharmacovigilance of many important Ayurvedic drugs are still not fully explored.The comprehensive knowledge of the basic ideologies of Ayurveda is poorly acceptable scientifically due to lack of evidence[2]. About 50% of drugs are natural products, which play an important role in pharmaceutical industry.In Ayurveda, the drugs are selected for performing any type of work according to its *Gunas* (properties). The *Dravyas* in Ayurveda are multidimensional in nature[3].

Trikatu is a Sanskrit word it means, "tri" stands for three and "*katu"* stands for acrids. The three acrid herbs are *Maricha* (Black pepper), *Peepli* (Long Pepper), and *Sunthi* (Ginger), when combined in equal quantities, forms the miraculous formulation Trikatu. Ayurvedic system of medicine prescribes Trikatu for the management of tastelessness (*Arocaka*) disturbed digestion (*Agnimandya),* diseases of nose (*Pinasa*) and upper respiratory tract (*Gala*), excess and frequent urination (*Meha*), edema (*Gulma),* obesity (*Sthaulya*), Filariasis (*Slipada*), and skin diseases (*Tvakroga)*[4].

Literature review and market survey states that the above formulation available in market is product of numerous companies. Modern analytical methods are not yet reported for standardization of Trikatu churna. As it is difficult to estimate each and every ingredient for its chemical constituents, piperine **(Figure.1)** from *Piper longum* and *Piper nigrum* present in formulation were selected as marker compounds. Literature survey reveals that

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few HPTLC, RP-HPLC and UV methods are reported for estimation of piperine [5, 6, 7, 8] individually as well as in combination with other constituents. However, no analytical method has been reported for estimation of piperine which can be further applied for standardization of Trikatu churna.

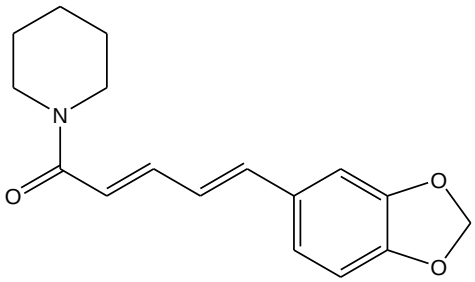

Figure.1: Structure of Piperine

The present research work deals with development of HPTLC method for standardization of Trikatu churna by detection and quantification of marker piperine simultaneously from in-house and marketed formulations. The proposed research method was validated on the basis of its linearity, accuracy, specificity, precision, limit of detection (LOD), limit of quantification (LOQ) and robustness according to ICH guidelines.

II. MATERIALS AND METHODS

2.1.1 MATERIALS

Raw materials used for the preparation of Trikatu churna and two diiferent marketed brands (M_1&M_2) of Trikatu churna were procured from Ayurvedic medical shop, Coimbatore and stored in air tight containers at room temperature. The stationary phase used was TLC plates precoated with silica gel 60 F254 (20×20 cm) of 0.2 mm thickness obtained from E. Merck Ltd. Mumbai, India.

2.1.2 Standards and reagents:

The organic solvents and chemicals of analytical grade were procured from Technico laboratory, coimbatore, Tamilnadu, India. Standard piperine were procured from sulur,ciombatore India.

2.1.3 Instrumentation:

Camag Linomat 5 semiautomatic sample applicator with a 100µl Hamilton syringe (Camag, Switzerland) and winCATS software (CAMAG Ver.1.4.1), Camag TLC Scanner 3, Twin trough chamber.

2.2 METHOD:

2.2.1 Selection of mobile phase

Selection mobile phase was done by trial and error method by trying on different mobile phases. Based on R_f value of the drugs, the best mobile phase was selected. Mobile phase tried were as follows:

Toluene: Ethyl acetate (7:3)

Toluene: Ethyl acetate : Glacial acetic acid (8:2:0.1)

Fixed mobile phase

Toluene : Ethyl acetate : Glacial acetic acid (8:2:0.1)

2.2.3 Optimization of Mobile phase:

The standard stock solution containing 100µg/ml of piperine was spotted on to TLC plate and developed in different solvent systems. Many of the preliminary trials were carried out for selection of mobile phase. Mobile phase composition was optimized to provide accurate, precise and reproducible results for the determination of piperine.

2.2.4 Preparation of standard solution:

Stock solutions of piperine (1000µg/ml) were prepared separately by dissolving 10 mg of accurately weighed standard in 10 ml of methanol. From this stock solution, 1ml stock solution to 10 ml volumetric flask then volume adjusted with methanol.

2.2.5 Preparation of In-house formulation:

All the ingredients were collected, dried and powdered separately, passed through 100 # sieve and mixed together in specified proportions in a geometrical manner to get uniform mixture. This mixture was grounded well to form a homogenous blend. They were dried and packed in air tight containers for further analysis.

2.2.6 Extraction of piperine from marketed and in-house formulations:

Churnam equivalent to 5g were triturated and extracted with 25ml methanol, reflux for 30 min, filtered through Whatmann filter paper no. 41 and this procedure was repeated for three times using fresh 25ml of methanol. The final volume was make up to 100 ml with methanol. This solution was used for quantification of piperine.

2.2.7 Chromatographic conditions:

Chromatographic separation was achieved on HPTLC plates (10×10 cm) pre-coated with silica gel 60 F_{254} of 0.2 mm thickness with aluminium sheet support. Standard solutions of marker and extracts were applied to the plates as bands 6.0 mm wide, 10.0 mm from the bottom edge of the same chromatographic plate by using of a Camag (Muttenz, Switzerland) Linomat 5 sample applicator with a 100µl Hamilton syringe. The Ascending development to a distance of 80 mm was performed at room temperature (24 ± 2°C) with mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 30 min. After development, the plates were dried and then scanned at 360nm with a Camag TLC Scanner 3

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using the deuterium lamp with win CATS software.

2.2.8 Assay:

For purpose of this assay standard and sample (extract) solutions were applied on TLC plate in triplicates. Standard solutions of piperine 100μg/ml were applied. Calibration curve constructed from peak areas obtained from standard solutions of piperine. Sample (extract) was used for quantification of marker. The amount of piperine present per gram of formulation was calculated by comparison of the areas measured for the sample with the calibration curves.

2.3 METHOD VALIDATION: [9,10]

In accordance with ICH guidelines Q_2 (R_1) the optimized HPTLC method was validated with respect to following parameters.

2.3.1 Linearity:

The linearity is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. It is determined by plotting a graph of peak area v/s concentration of standards to obtain correlation coefficient (r_2) and equation of the line.

2.3.2 Specificity:

Specificity is defind as the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix. The specificity of the method was ascertained by comparing the R_f value and the peak purity was assessed by comparing the spectrum of standard piperine with sample.

2.3.3 Precision:

Precision is defind as the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) for a statistically significant number of samples. As per the ICH guidelines precision should be performed at three different levels like low quality control (LQC), medium quality control (MQC) and high quality control (HQC). The Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision. It was determined by using minimum of 9 determinations covering the specified range for the procedure. The intra-day assay precision was performed 3 times on same day, while the inter-assay precision was performed on 3 different days.

2.3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ):

Limit of detection (LOD) is measured by the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of Quantification (LOQ) is defined as the lowest amount analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. LOD and LOQ were measured by k x SD/s where k is a constant (3.3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal and s is the slope of the calibration curve.

2.3.5 Accuracy:

Accuracy is the percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Accuracy should be assessed by using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations 3 replicates each of the total analytical procedure). The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels via. 80%, 100%, 120% by adding known amount of standard mixture of piperine. These samples were analyzed and the results obtained were compared with expected results.

2.3.6 Robustness:

The robustness is defined as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It was studied in triplicate at 300 ng/spot and 400 ng/spot by making small changes in mobile phase composition and the mobile phase saturation time. The final results were examined by calculation of %RSD of concentration.

III. RESULT & DISCUSSION

In situ HPTLC spectral of piperine taken and found at 360 nm it was selected as scanning wavelength **(Figure.2)**.

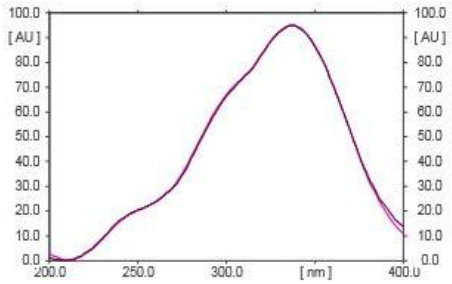

Figure.2: HPTLC Spectra of Standard Piperine

Good resolution and sharp peaks with minimum tailing were obtained with mobile phase consist of

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toluene: ethyl acetate: glacial acetic acid 8:2:0.1 (v/v/v). Piperine, were satisfactorily resolved with R_f value at 0.65**(Figure.3)**

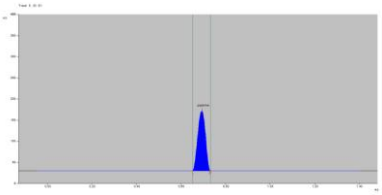

figure3: chromatogram of standard piperine

3.1 HPTLC Method Validation:

3.1.1 Linearity:

Linear relationship was observed by plotting drug concentration vs peak area for each compound. Piperine showed linear response in the concentration range of 200-800 ng/spot **(Figure.4)**.The linearity was validated by the high value of the correlation coefficients. The results are tabulated in **(Table.1**

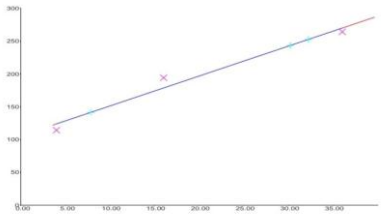

Figure.4: Linearity (200-800 ng/spot)

S. no	Parameters	Piperine
1	Linearity (ng/spot)	200-800
2	Equation Correlation Coefficient (r^2±SD)	y=19.71x+2775 0.9997 ± 0.003493
3	Slope ± SD Intercept ± SD	19.71 ± 0.3080 2775 ± 175.5055

Table.1: Linear Regression Data For Calibration Plot For Piperine

3.1.2 Specificity:

When the spectra of standard piperine were overlayed **(Figure.5)** or compared with extracts of it was observed that constituents present in the extract did not interfere with the peaks of piperine. Thus the proposed method was proved to be Specific.

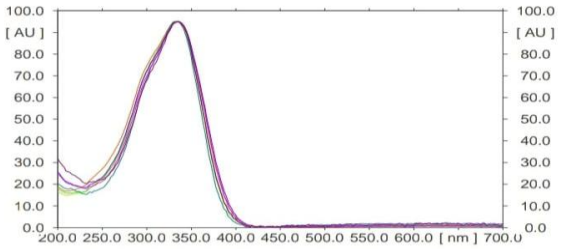

Figure.5: Overlay Spectra of Standard Piperine and Piperine From Extract.

3.1.3 Precision:

Intraday precision is used to describe the variation of the method, at three different concentration levels within the same day while interday precision is used for the variation between different days. The % RSD values for both intraday and interday precision were found within acceptable limit as shown in **(Table.2).**

Piperine	Concentration (ng/spot)	Interday			Intraday		
		Mean Area	S.D	%RSD	Mean Area	S.D	%RSD
	300	8651.3	61.89	0.33	8610.37	98.79	0.71
	500	13040.3	250.79	1.24	13033.9	117.6	1.92
	700	16082.2	31.79	1.86	16093.2	55.43	1.44

Table.2: Intra-day and Inter-day precision results of pipeline

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3.1.4 Limit of detection (LOD) and Limit of quantification (LOQ):

LOD and LOQ results of piperine are as shown in **Table.3.**

	LOD (ng/spot)	LOQ (ng/spot)
Piperine	19.97	60.52

Table.3: LOD and LOQ Results of Piperine

3.1.5 Accuracy:

Accuracy of the sample is reported as percent recovery of known amount of analyte was added in the sample. The percent recovery of the sample was calculated by performing recovery studies in triplicates of three concentration levels via. 80%, 100%, 120% by adding known amount of standard mixture of piperine. Results obtained were given in **Table.4.**

Compound	Level of % Recovery	Recovery (%)	RSD	Mean Recovery%
Marketed Formulation (M1)	80 100 120	98.916 103.6 99.47	1.26 0.94 1.39	99.40
Marketed Formulation (M2)	80 100 120	94.80 98.89 99.12	0.27 0.25 0.38	99.16
In house Formulation	80 100 120	99.15 98.63 98.74	0.61 0.52 0.68	98.84

Table.4: Accuracy data for Piperine

3.1.6 Robustness:

The % RSD of the peak area was calculated in triplicate for changes in mobile phase composition and duration of saturation time for 300 and 400 ng/spot. The values of % RSD were less than 2% which indicated that the developed method is robust as shown in **Table.5.**

Parameters	Piperine	
	300ng/spot % RSD	400ng/spot %RSD
Mobile phase composition		
Toluene: Ethyl Acetate: Glacial Acid (8:2:0.1) v/v/v	0.89	0.22
Toluene: Ethyl Acetate: Glacial Acid (7:3:0.5) v/v/v	0.92	0.24
Saturation time		
+5 min -5 min	0.33 0.46	1.33 0.72

Table.5: Robustness results of Piperine

3.1.7 Estimation of piperine in marketed and In-house formulations:

The developed method was applied for the detection and quantification of piperine, from marketed and in-house formulations of Trikatu churna . The peaks for piperine are observed at Rf0.65 ±0.02. in the densitogram of extracts. The test samples of marketed formulations and inhouse formulation were compared with the ingredients **Figure.6.**

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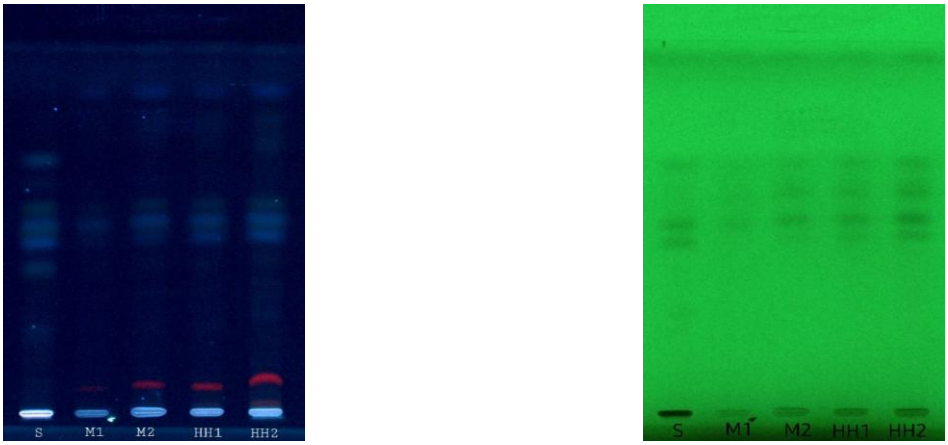

Figure.6: HPTLC Fingerprinting Profile of Extract of Trikatu churna and its Ingredients at 360nm respectively. S-Standard Piperine, M1-Marketed Formulation (M1); M2-Marketed Formulation (M2); HH1-Inhouse Formulation; HH2-Inhouse Formulation.

There was no interference from other compounds present in the Trikatu churna. The total content of piperine in marketed formulations M1, M2 and in-house formulations HH1, HH2 is as shown in **Table.6.**

Formulations	Piperine content (%w/w)
Marketed formulation(M1)	0.20
Marketed formulation(M2)	0.15
In House Formulation(HH1)	0.18
In House Formulation(HH2)	0.19

Table.6: **PIPERINE CONTENT IN POLYHERBAL FORMULATIONS**

IV. CONCLUSION

Herbal medicines are the high demand in the developed as well as in developing countries for primary health care because of their wide biological activities, higher safety margins and lower costs. In India, the herbal drug market is about $ one billion and the export of plant based crude drugs is about $ 80 million. Interest of the global resurgence of interest in herbal medicines has led to an increase in the demand for them. But the most important challenges are faced by these formulations arises because of their lack of complete standardization.

The main problem with polyherbal formulation is that it consists of a large number of phytoconstituents and each constituent has to be determined separately that is not an easy task. In presence of large number of ingredients in a formulation may cause interference in determination of each other. Based on these criteria the HPTLC method was developed for standardization of Trikatu churna using piperine as marker constituents. The HPTLC method was found to be simple, precise, accurate, specific and reproducible for standardization of Trikatu churna. The method based on estimation of piperine could be applied for both marketed and in house formulation as well as for routine quality control to check quality and batch-batch variations.

The separation was performed on TLC aluminium plates precoated with silica gel 60 F254, using toluene: ethyl acetate: glacial acetic acid (8:2:0.1 v/v/v) as mobile phase. The densitometric analysis was carried out at the detection wavelength of 360 nm. The R_f values of piperine was found to be 0.65.The developed method has been validated as per ICH guidelines.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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