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Development of standardization of trikatu churna: A spectrophotometric approach

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Abstract

Churnas are important group of formulations used by Ayurvedic and Siddha physicians to treat various types of diseases. Trikatu Churna is an important ayurvedic formulation, is official in ayurvedic formulary of India is combination of three reputed herbs, comprised of the fruits *Piper longum*, *Piper nigrum* and rhizomes of *Zingiber officinale* was estimated by High Performance Liquid Chromatography for its total Piperine content. This study was aimed to develop finger printing methods for well known Ayurvedic formulation. Three laboratory batch of Trikatu Churna were estimated for their Piperine content against standard Piperine solution by High Performance Liquid Chromatography at λ_{max} 342-343nm. The concentration of Piperine in raw material was found to be 1.4±0.010% w/w in *Piper longum* fruits and 3.8±0.088 in *Piper nigrum* fruits respectively. The content of Piperine in laboratory formulation and marketed formulation were found to be 0.0062±0.050%w/w, 0.0074±0.021%w/w and 0.0056±0.032% w/w respectively. In order to obtain accuracy and precision the recovery study was performed and result obtained with mean value 99.35±0.25%, which proves reproducibility of the result. This shows significant precision of methods at 95% confidence level. Results of statistical analysis show present HPLC method for determination of Piperine is simple, sensitive, precise and accurate and can be adopted for routine quality control of Trikatu Churna and Piperine can be used as a possible marker compound for fingerprinting of Trikatu churna.

Key-Words: Trikatu Churna, Piperine, High Performance Liquid Chromatography (HPLC)

Introduction

Most of the Ayurvedic formulation are lacked in their defined quality control parameter and method of evaluation.[1] World Health Organization has emphasized the need to ensure the quality of medicinal plant product by using modern controlled technique and applying suitable standards.[2] The present paper is afford to develop the quality control parameter of Trikatu Churna by High Performance Liquid Chromatography (HPLC) method using Piperine as a internal standard.

For standardization of natural product drugs, single chemical entities, "marker compounds," may be used as potency standards in high performance liquid chromatography (HPLC) analysis. Using well-characterized marker compounds, conventional pharmaceutical manufacturing criteria for assay and content uniformity may be applied.

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These marker compounds may be used to help identify herbal materials, set specification for raw materials, standardize botanical preparation during all aspects of manufacturing processes, and obtain stability profiles. HPLC analysis for marker compounds may provide additional information in the form of chromatographic fingerprints.[3]

The present study is undertaken to develop certain fingerprints for an Ayurvedic formulation Trikatu Churna.

Trikatu Churna is a well known Ayurvedic formulation comprised of the fruits of three medicinal plants *Piper longum*, *Piper nigrum* and rhizomes of *Zingiber officinale*. Trikatu Churna is the digestive tonic for the assimilation of the other food in the body. It is also used as a rejuvenator and stimulant. Trikatu plays an essential role in the treatment of wide variety of condition. It eliminates the aggravated Kapha in the respiratory tract and in the digestive channel. It also regulates the path for the Vata and helps in minimize gas formation in the abdomen being hot in nature.[4, 5]

The present study is an attempt to develop the finger print method for Trikatu Churna by High Performance

Liquid Chromatography (HPLC) method using Piperine as a internal standard is an important and major content in formulation. The HPLC analysis can be considered as one of the quality control method for routine analysis.

Material and Methods
All the chemical and solvent were used of AR grade.

Procurement of crude drug
Dried fruits of *Piper longum*, *Piper nigrum* and rhizomes of *Zingiber officinale* were procured from local market of Ujjain (MP, India) and identified on the basis of morphological and microscopical character and compared with standard pharmacopoeial monograph.[6-9]

Preparation of Trikatu Churna
Trikatu Churna three batch name TK-I, TK-II and TK-III were prepared in laboratory using method described in Ayurvedic Formulary. These three batches of Trikatu Churna and powdered *Piper longum* and *Piper nigrum* were estimated for their Piperine content against Piperine standard solution on HPLC. As *Zingiber officinale* does not contain Piperine is not include in present study.

Qualitative chemical examination
Ethanolic extracts were qualitatively evaluated by chemical tests for the presence of various phytoconstituents like alkaloids, glycosides, saponins, phenolic compounds tannins, and phytosterols.[10-12]

Preparation of Piperine extract of Trikatu Churna
Reflux the powdered Trikatu Churna (1g) with 60 mL ethanol for 1 hr. Filter the extract and reflux the marc left with 40 mL of ethanol for 1 hr. Filter and combine the filtrate. Concentrate the ethanol extract under the vacuum till the semi solid mass is obtained. Dissolve the residue in 75 mL of ethanol and filtered through the sintered glass funnel (G-2) by vacuum filtration assembly. Centrifuge the filtrate at 2000 rpm for 20min. the supernatant was collected in 100 mL volumetric flask and volume made with ethanol.

The same procedure was used for all batches and separately powdered *Piper nigrum* and *Piper longum* and solution (100 mL) of their Piperine extract were prepared.

Preparation of standard solution of Piperine
An accurately weighed Piperine (100 mg) was dissolved in ethanol and volume was made up to 100 mL with ethanol in volumetric flask. 2 mL of this solution was diluted with ethanol up to 100 mL in volumetric flask to give 20 mg/mL Piperine solution. Calibration curve from standard solution of Piperine was prepared and with the help of this curve the Piperine of Trikatu Churna was estimated. The method was validated for precision and accuracy.

Calibration curve of Piperine
A series of calibrated 10 mL volumetric flask were taken and appropriate aliquots of the working standard solution of Piperine were withdrawn and diluted up to 10 mL with ethanol. The absorbance was measured at absorption maxima 342.5nm, against the reagent blank prepared in similar without the Piperine. The absorption maxima and Beer's law were recorded and data that prove the linearity and obey Beer's law limit were noted.

The linear correlation between these concentrations (X-axis) and absorbance (Y-axis) were graphically presented and the slope (m), intercept (c) and correlation (r^2) were calculated for the linear equation ($Y = mx+c$) by regression analysis using the method of the least square, (Table- 1 and Fig. 1).

Validation parameters
Selectivity and peak purity
Selectivity was checked by using prepared solutions of Trikatu churna and available standards optimizing separation and detection. The purity of the peaks was checked by multivariate analysis. The three spectra corresponding to upslope, apex and down slope of each peak were computer normalized and superimposed. Peaks were considered pure when there was a coincidence between three spectra. (Match factor was = 98%)

Linearity, limits of detection and quantification
The linearity of the detector response for the prepared standards was assessed by means of linear regression regarding the amounts of each standard, measured in µg, and the area of the corresponding peak on the chromatogram. Linearity was also confirmed for Trikatu churna prepared sample solutions. After chromatographic separation, the peak areas obtained were plotted against concentration by linear regression. Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. signal-to-noise ratios approximately 3:8 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method.

Precision
The repeatability of the injection was determined for both standard Piperine and the content of Piperine in Trikatu churna. A standard solution containing reference compounds and prepared sample solution was injected. The mean amount and R.S.D. values were calculated. The precision was calculated at two different concentrations high and low tested in the concentration range. For standardization the sample

was injected at six different concentrations and linearity was noted.

Accuracy

The accuracy of the method was determined by analyzing the percentage of recovery of the Piperine in the Trikatu churna. The samples were spiked with two different amounts (8, 10µg) of standard compounds before sample preparation. He spiked samples were extracted by triplicate and analyzed under the previously established optimal conditions. The obtained average contents of the target compounds were used as the real values to calculate the spike recoveries.

Robustness

For the determination of the method's robustness a number of chromatographic parameters, such as column package and size, mobile phase composition and gradient ratio, flow rate and detection wavelength, were varied to determine their influence on the quantitative analysis.

Statistics

When applicable, one-way or two-way analyses of variance were used to assess the observed differences in the Piperine content. Differences were considered to be statistically significant when the P-value <0.05.

Estimation of Piperine

The appropriate aliquots from Piperine extract of each batch of Trikatu Churna and separately *Piper longum* (Pippali) and *Piper nigrum* (Marica) were withdrawn in 10 mL volumetric flask separately. The absorbance for aliquots of each was noted at 342.5 nm. The corresponding concentration of Piperine against respective absorbance value was determines using calibration curve. The statistical analysis for checking uniformity in batches is also performed.

Precision and accuracy

The method was validated for precision and accuracy, by performing the recovery studies at two levels by adding known amount of Piperine extract of Trikatu Churna, of which the Piperine content have been estimated previously. The data were obtained and recovery was calculated. (Table- 3).

Result and Discussion

In the present study phytochemical, spectral and chromatographic studies were performed. Phytochemical tests performed using standar test procedure with specific reagents and the result of phytochemical investigation indicates the presence of alkaloids, glycosides, phytosterols, tannins, carbohydrates and flavonoids which support significant addition of crude drugs in the formulation.

Piperine obeys Beer Lambert's law in concentration range 1-6 µg/mL at λ_{max} 343 nm. The correlation coefficient (r^2) was calculated where the r^2 value 0.9989 indicates the good linearity between the concentration and absorbance.

The estimation of Piperine content of Trikatu Churna (three identical laboratory batch) and powdered *Piper nigrum* (Pippali) and *Piper longum* (Marica) was carried out separately. The concentration of Piperine present in raw material was found to be 3.8±0.088 (w/w) in Marica and 1.4±0.010 (w/w) in pippali, respectively and in three identical laboratory batches of Trikatu name TK-I, TK- II and TK- III was 0.0062±0.050, 0.0074±0.021, and 0.0056±0.032 (w/w) (Table-2).

The HPLC method was validated by defining th linearity, peak purity, limit of quantification and detection, precision, accuracy, specificity and robustness. For the qualitative purposes, the method was evaluated by taking into account the precision in the retention time, peak purity, and selectivity of Piperine elutes. A high repeatability in the retention time was obtained with (R.S.D.) value lower than 1.5% for both standard and samples even at higher concentration. The peak purity was studied in the major peaks.

Linearity, limit of detection (LOD), and limit of quantification (LOQ) were evaluated for quantitative purposes. LOD and LOQ found to be 0.6 and 2 mg/mL respectively which suggest full capacity for quantification of Piperine content in different laboratory and marketed batches.

In order to obtain precision and accuracy, the recovery study was performed at two levels by adding known amount of Piperine in Trikatu Churna. The experiment was repeated six times at both levels (Table- 3) and result shows 98.75 and 99.85% recovery of Piperine, which prove reproducibility of the result. This shows significant precision of method at 95% confidence level. The relative standard deviation (RSD%) value was found to be 0.409 and 0.292 with mean 0.351 at both level while the standard error was 0.25 and 0.237 with mean 0.244 respectively. From the data, it is obvious that the present method of determination of Piperine is simple, precise, accurate and suitable for routine analysis of Piperine in Trikatu Churna.

Finally robustness of the method was also assessed. Minor modification of the initial mobile phase gradient had no effect on the peak resolution.

The method is very adaptable because of the precision and repeatability for the traditional Ayurvedic formulation.

As Trikatu Churna is a good source of Piperine, these findings can be taken as one of the parameter, along with other parameters, for quality control of Trikatu Churna.

Conclusion

The developed high performance liquid chromatography method for estimation of Piperine from Trikatu churna could be used as a valuable analytical tool in the routine analysis, to check the batch to batch variation. Estimation of Piperine can be used as one of the appropriate analytical markers for the finger printing.

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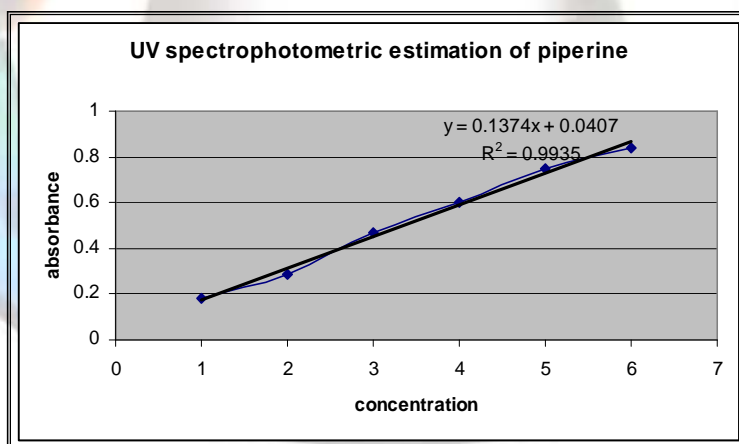12. Wagner H., Plant Drug Analysis, a thin layer chromatography atlas, II edition, Springer 2002; 298.

Fig. 1: Calibration curve of Piperine

Table 1: Optical Characteristics, Statistical Regression data and validation Parameter of Piperine

S/No.	Parameters	Value
1.	Absorption maxima	343 nm
2.	Beer's law limit	1-6 µg/ml
3.	Regression equation (y=mx + c)	y = 0.014x + 0.028
4.	Intercept (c)	0.028
5.	Slope (m)	0.014
6.	Correlation coefficient (r^2)	0.997
7.	LOD	0.6
8.	LOQ	2
9.	Precision(n=6,% RSD)	0.39
10.	Accuracy (%)	99.54%

Table 2: Estimation of Piperine Content in Trikatu Chunra

S/No.	Name	Piperine content (%w/w)	Confidence level (95%)
1.	*Piper longum*	1.4±0.010	±0.020
2.	*Piper nigrum*	3.8±0.088	±0.090
3.	TK-I	0.0062±0.050	±0.031
4.	TL-II	0.0074±0.021	±0.010
5.	TK-III	0.0056±0.032	±0.051

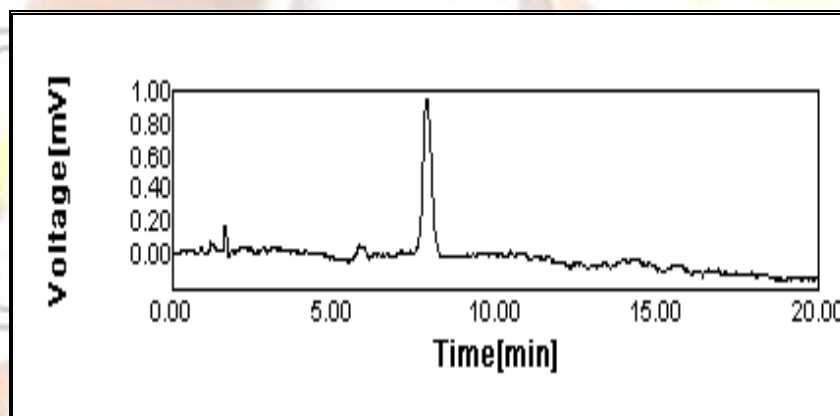**Fig. 2: Chromatogram of TK-L by HPLC**

Table 3: Compilation of Data of Recovery Study

S/ No.	Amount of Piperine (µg/ml)			Recovery %
	In sample	Added	Estimated	
1.	10	8	7.9	98.75
2.	10	10	9.9	99.85