DEVELOPMENT OF FINGERPRINTS FOR SINGLE COMPONENT ANALYSIS OF AN AYURVEDIC FORMULATION (*PIPPLI CHURNA*) BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Pippli Churna (PC) is a most popular Ayurvedic formulations among the Ayurvedic medicines, Piperine is one of the major constituent of Pippli Churna. The process of development of HPLC fingerprints for Pippli Churna extract is introduced in detail. The three laboratory batches and three marketed batches were taken in this study to estimate the % of piperine in this indigenous formulation. The selection of a suitable chromatographic system, the screening for important parameters, and gradient optimization to method validation, and an integrated and universal HPLC fingerprint approach was performed. This improves the separation quality of the fingerprint. The detection wavelength of piperine was 343 nm. Thethree laboratory batches and three marketed batches were taken in this study to estimate the % of piperine in this indigenous formulation. The results of the method validation, based on the relative standard deviation of relative retention times and relative peak areas, were acceptable. Calibration curves showed good linearregression ($R^2 > 0.999$) within test range. The LODs and the LOQs for the piperine were 0.063 mg/ml and 0.071 mg/ml. This strategy is used for the estimation of piperine in Pippli Churna(PC) formulation and identifies and assessedits quality. Results of statistical analysis show present HPLC method for determination of Piperine is simple, precise, accurate and suitable for routine analysis of Piperine in PC. The developed fingerprints can be used as a standard and Piperine can be used as a possible marker compound for fingerprinting of PC.

Keywords: Fingerprints, Pippli churna (PC), Piper species, Piperine marker, HPLC.

INRODUCTION

India has a vast heritage of traditional system of medicine (Ayurveda, Siddha and Unani), due to the lack of precise quality control measures and finger printing methods, the benefits of these systems remains largely underutilized [1.] Keeping this thing in mind, from the last two decades, efforts have been made in developing quality control parameters for Ayurvedic formulations by means of chromatographic finger printing methods[2,12]. World Health Organization (WHO) has emphasized the need to ensure the quality of medicinal plant products by using modern controlled technique and applying suitable standards[8] . For standardization of natural product drugs,

single chemical entities, "marker compounds," may be used as potency standards in high performance liquid chromatography (HPLC) analysis. These marker compounds may be used to help identify herbal materials, set specifications for raw materials, HPLC analysis for marker compounds may provide additional information in the form of chromatographic fingerprints. The present study is undertaken to develop certain fingerprints for an Ayurvedic formulation. Pippli churna used for cough and cold, spleen disorders, fever, diabetes, piles, tuberculosis, abdominal disease ,thrust warms, leprosy, pain (colic-spasmodic) and digestive impairments. Pippli churna possesses bioavailability enhancing properties.,ie. antitubercular drugs & antilaprotic drug. The essential oils of pipali churna have antibacterial and antifungal activities.

Ayurvedic formulary of India has given the specification for the composition of PC, it should contain piper species as a major ingredient apart from different herbs[3,4].

AIMS AND OBJECTIVES

Pharmacopoeial standards for Ayurvedic formulations published by the Central Council for Research in Ayurveda and Siddha gives certain physical parameters as standards for churna, these standards are not based on modern analytical methods[5]. It is therefore essential to develop definite and accurate analytical tools to as certain consistency and quality of Ayurvedic preparation from batch to batch in pharmaceuticals which may results in acceptability world wide. In present study we tried to develop a method that serves as fingerprinting method for Pippli churna(PC).

EXPERIMENTAL

All the solvents for HPLC analysis were HPLC grade and purchased from E. Merck and S. D. Fine Chemicals, Mumbai. All solvents used for extraction were primarily distilled before use. SHIMADZU – LC10AT HPLC was used for piperine analysis. All the results are obtained by repetition of the each experiment six times (n= 6).

Procurement of drug

Crude drugs were procured from local market and identified by macroscopic[10,11] and microscopic characters[6,7,8,9].

Preparation of formulations

1. Three batches were prepared in laboratory (named as PC-I,PC-II and PC-III) according to strict methods of 'Ayurvedic formulary of India' and Sarangadhara-samhita.

2. Commercially available brands PC-A, PC-B, and PC-C, of Pippli churna were procured from local market.

Sample preparation for estimation of Piperine content

1.5 gm Pippli churna was refluxed for 1 hour with 100 ml of methanol. The volume were reduced under pressure and filtered by 0.2 μm membrane filters. The filtrate was diluted up to 100 ml with methanol. To the 20 ml of resulting solution, 2 ml of 0.5-mg/ml solution of p-dimethyl amino benzaldehyde (internal standard) was added, and made the final volume 25 ml with methanol .[7,12] [Fig. 1].

Preparation of standard solution

Piperine was purchased from Lancaster, England. Standard solution was prepared by the addition of 2 ml of solution a (1mg/ml) of Piperine and 2 ml of internal standard solution (0.5 mg/ml of p-dimethylamino benzaldehyde) in a 25 ml volumetric flask made the final volume to 25 ml with methanol.

HPLC studies[7]

Estimation of Piperine was carried from different batches (three marketed and laboratory batch) of PC with following conditions:

Column : C 18 (25cm X 4.6 mm i.d.) 10 μ,
Mobile phase: methanol: water (69:31),
Detection: at 343 nm (reference wavelength: 343 nm),
Injection volume: 20 μl and
Flow rate: 1.5ml/min.

Calibration

The Piperine content of PC was determined using a calibration curve established with seven dilutions, at concentrations ranging from 0.5-20 μg/ml. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentration of the Piperine injected. Peak identification was achieved by comparison of both the retention time and UV absorption spectrum with those obtained for standards.
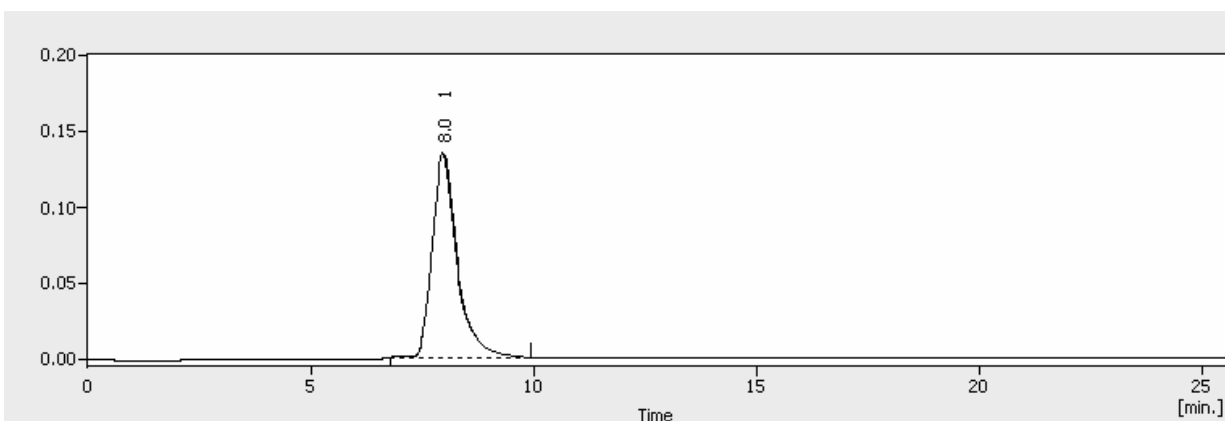
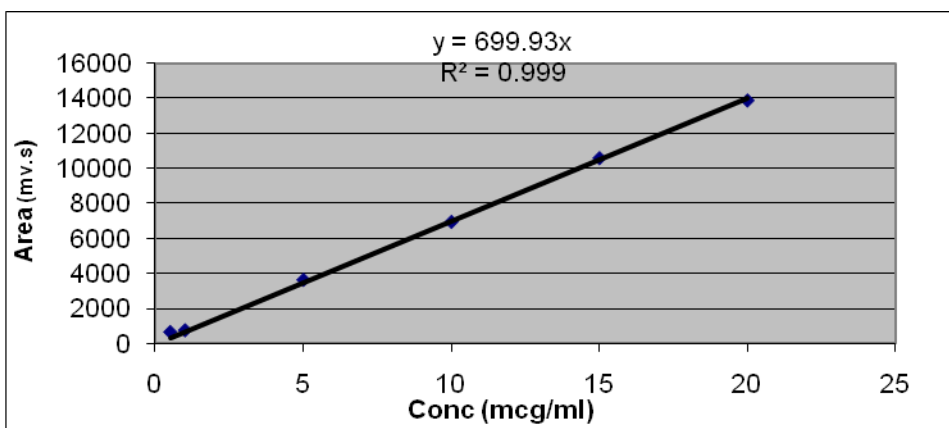**Figure:1. RP-HPLC Chromatogram of standard Piperine at 343 nm**

Figure 2: Calibration curve of standard Piperine

Validation parameters

Selectivity and peak purity

Selectivity was checked by using prepared solutions of PC and available standards optimizing separation and detection. The purity of the peaks was checked by multivariate analysis. The three spectra corresponding to up slope, apex and down slope of each peak were computer normalized and super imposed. Peaks were considered pure when there was a co-incidence between the three spectra (match factor was =98%).(Table-I)

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 PC prepared sample solutions. After chromatographic separation, the peak areas obtained were plotted against concentrations by linear regression. Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method.

Precision

The repeatability of the injection integration was determined for both standard piperine and the content of piperine in Pippli churna. A standard solution containing reference compounds and prepared sample solutions was injected. Pippli churna samples were also prepared 2 times to evaluate the repeatability of the process. 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 eight different concentrations and linearity was noted [Table-1].

Accuracy

The accuracy of the method was determined by analyzing the percentage of recovery of the piperine in the Pippli churna. The samples were spiked with two different amounts (100, 150 μg) of standard compounds before sample preparation. The 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 [Table-1].

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. Interday and intraday variability was studied for the sample, by injecting the same concentration of the sample on three different days and the standard error mean was calculated.

RESULTS AND DISCUSSION

In the present study, spectral and chromatographic studies were performed. Results of the The RP-HPLC analyses of PC were performed, samples were injected at seven different concentrations and the linearity was observed with in the concentration range of 0.5-20 μg/ml [Fig. 2].Both Piperine was well separated at retention time 8.020 respectively. The concentration of Piperine present in raw material was found to be 1.41±0.62 w/w in Piper longum fruits (pippli). The content of

Piperine in laboratory formulations (PC-I, II, III) were found to be 0.16±0.002 ,0.19±0.006 ,and 0.18±0.004 respectively and in different marketed formulations of PC were, for PC-A (0.15 ±0.002 %), PC-B (0.16±0.004%), PC-C (0.17±0.008 %) w/w respectively [Table-2, Fig-1]. The HPLC method was validated by defining the 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. (Table-3).The peak purity was studied in the major peaks. Linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated for quantitative purposes [Table-1].Thus LOD and LOQ found to be 0.063, 0.071mg/ml respectively which suggest full capacity for quantification of piperine content in different laboratory and marketed batches of PC. R^2 value for the regression equation of the Piperine was higher than 0.9988 thus confirm the linearity of the method. The recovery study was performed at two levels by adding known amount (100, 150 μg/ml) of piperine with reanalyzed sample of PC found to be close to 99.48(mean)% and a higher repeatability indicate a satisfactory accuracy in the proposed methods [Table-3].Finally the robustness of the method was also assessed. Minor modification of the initial mobile phase gradient (from 25 to 30% solvent instead of 31%) had no effect on the peak resolution of the compound. Therefore, this HPLC method for fingerprinting of PC can be regarded as selective, accurate, precise, and robust. The method is very adaptable because of the precision and repeatability for the traditional Ayurvedic formulation like PC and suitable for routine analysis of Piperine in PC. There was not much variation in the interday and intraday injections performed with the R.S.D. value was found to be 0.080%with the mean standard error 0.03% respectively. Piperine estimation can be utilized as a possible analytical marker for fingerprinting of Pippli churna.

CONCLUSION

The developed high performance liquid chromatographic method for estimation of Piperine from Pippli 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.

Table-I Validation parameter (Mean% ± SD,n=3)

S. no.	parameter	value
1	Absorption maxima	343
2	Bee's law limit	0.5-20ug/ml
3	Regression equation(y=bx+a)	699.9x
4	Intercept(a)	0
5	Slope(b)	699.9
6	Correlation coefficients(r^2)	0.999
7	LOD mg/ml	0.063
8	LOQ mg/ml	0.071
9	Precision (n=6, % RSD)	0.080
10	Accuracy (%)	99.48

Table-2 Estimation of piperine (Mean% ± SD,n=3)

S.no.	name		Piperine content%w/w
01	piper longum(pippli)		1.41±0.62
02		PC-I	0.16±0.002
03		PC-II	0.19±0.006
04	PIPPLI CHURNA	PC-III	0.18±0.004
05		PC-A	0.15±0.002
06		PC-B	0.16±0.004
07		PC-C	0.17±0.008

Table-3 Recovery study (Mean% ± SD,n=3)

S.no.	Amount of piperine(microgm/ml)			RSD%	SE	Recovery%
	In sample	added	estimated			
01	100	100	198.4±0.14	0.070	0.038	99.2
02	200	150	349.2±0.32	0.091	0.022	99.77
mean				0.080	0.03	99.48

Mean ± SD of six determinations,   RSD= Relative standard deviation
SE=Standard error

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