Quantification of Piperine by TLC densitometer method and forced degradation study in a Classical Ayurvedic formulation-Trikatu Churna

ABSTRACT:

Developed a thin layer chromatography (TLC) method for the quantification of piperine in Trikatu Churna formulation it is a major and important ingredient in the formulation. TLC methods for the determination of piperine from the *Trikatu churna* along with its raw materials have been developed,as per the ICH guideline. The developed method has been validated, experimented with parameters like linearity, accuracy, the limit of detection, the limit of quantification, inter-day and intra-day assay precision, repeatability of measurement, and repeatability of a sample application. Performed a degradation study in different conditions. Prepared a calibration curve in the concentration range (100-800ng/spot) with correlation coefficients r2 (0.997) with Rf value 0.46 ± 0.03. The Limit of detection (LOD) and Limit of quantification (LOQ) value has 100 ng and 329 ng, respectively. The maximum degradation found in the benchtop (98%) follow with an acidic condition (86%), almost similar in the range of condition basic, oxidation and wet (75-77%), and a minimum in dry heat condition (49%). The freeze-thaw stability study, the accelerated stability study, the real-time stability study result of these conditions almost is the same range (92-99%).

Keywords: TLC, Validation, ICH guideline, LOD, LOQ, Trikatu Churna

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INTRODUCTION

The fruits of black and long peppers, *P. Nigrum* and *P. Longum*, belonging to the family Piperaceae have piperine as a major constituent [1]. Adulteration in aromatic plants directly affects their medicinal and economic importance. Authentication of the medicinal in quality, as well as the quantity of the drugs can be done by the different analytical methods. Conventionally medicines are obtained from medicinal plants, minerals, and organic matter, but the herbal drugs are produced from medicinal plants only. Globally, a plant as a source of medicines has been used from ancient times in the health care system and it growing very rapidly [2]. Unsound food habits and lifestyles produce so many diseases like cancer, diabetes, heart problems, depression, and many others. Allopathic medicines have so many disadvantages most prominent is its side effects, in that situations require alternative therapy to good health for all. Herbal drugs are the best choice to overcome the problems that produce in the same [3]. The development of herbal drugs requires guidelines for its preparation; globally International Regulatory Cooperation for Herbal Medicines (IRCH) is an authority that comes under the WHO guideline. The objective of this authority is to encourage the use of safe herbal medicines by sharing valuable information. Countries like Armenia, Australia, Argentina, Darussalam, Canada, Brazil, Brunei, Chile, China, Cuba, Ghana, Hungary, Japan, Malaysia, India, Indonesia, Italy, Pakistan, Peru, Portugal, Republic of Korea, Saudi Arabia, Singapore, UAE, UK, United Republic of Tanzania, and USA are members of this authority.

In quality control, standardization is a process by which prepare a set of standards, constant parameters, definitive quality and quantitative values that assure the quality, efficacy, safety, and reproducibility. Different analytical techniques are available, but modern chromatography

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methods of identification, separation, and quantification of such active components are very valuable for the quality control of herbal drugs [4]. Testing of new drug substances performed under the ICH guideline entitled stability testing, formulation requires studying force degradation to elucidate the inherent stability characteristics of the active substance [5]. In developed as well as developing countries, the quality drug is a major problem, where adulterated medicines spread in the market rapidly, which harms the health of the people. Validation is very important step to check the quality of the medicines, it is very necessary to follow the ICH guideline when a new method developed in the laboratory for the estimation of drugs in the formulation and herbal extract, It is way by which the production of quality drugs can be checked and confirm their rational use [6, 7].

To cure the disease like asthma, cough and cold, tuberculosis, fever, indigestion, chronic rhinitis/sinusitis and other inflammatory and respiratory disorders from ancient times by Ayurveda formulation, available different types of powder (churna) such as *Trikatu churna, Sitopaladi churna, Hingavastaka churna, Avipattikara churna, Sringyadi churna, and Talisadya churna* [8]. From research it has been confirmed, piperine an alkaloid has IUPAC name (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-petadienyl] piperidine) and molecular formula $C_{17}H_{19}NO_3$ is an active constituent in the ayurvedic formulation. This herbal constituent's origin from the *Piperaceae* family, of plants *Piper longum* (pipli), *Piper nigrum* (peppercorn), *Piper chaba* (Pipli), etc. [9, 10, 11, 12]. The role of the piperine is to increase the bioavailability of other nutritive substances including b-carotene, curcumin, selenium, pyridoxine, glucose, and amino acids [13, 14]. The purpose of this research was to find out the quantity of piperine in the formulation and its stability.

2. MATERIALS AND METHODS

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2.1 Plants

the vegetative parts used as the fruits of~~The crude drugs(fruits) comprising~~ Piper longum (pippali), fruits of Piper nigrum (black paper), and rhizome of Zingiber Officinale (shunt). The nuts were purchased in~~Crude drug purchased~~ from the local market, Kharibaoli, old Delhi, ~~The crude drugs~~ the fruits were dried in the shade, powdered to coarse and were kept in air-tight containers individually, away from moisture. The powder~~seddrugs~~ were standardized for the following parameters (microscopy, foreign organic matter, organoleptic properties).

2.2 CHEMICAL, SOLVENTS, AND REAGENTS:

Piperine was procured in analytical grade, LR grade solvents from "chemical labs" were used,**and**for another laboratory, either LR grade or its equivalent grade solvents were used. For thin-layer chromatography, precoated silica gel plates were used. Solvent distilled water is used for the solution and also, for another purpose.

2.3 CHROMATOGRAPHIC METHOD

2.3.1 Thin Layer Chromatography (TLC)

In the laboratory made TLC plates prepared, used silica gel G (0.3 mm thickness), activating them for 30 min~~s~~ at 110ºC. The quantitative analysis by thin-layer chromatography and fingerprint profiling were done using pre-coated silica gel TLC plates (E. Merck) of 200 µm~~micrometre~~ thickness after developing an appropriate solvent system, the number and position of bands in the plate were observed under ultraviolet light. Various compositions of the solvent system were tried for developing the fingerprint profiles. CAMAG TLC scanner was used for analytical studies. The data was altered and processed through software CAMAG Win cats 3.0. The samples were applied using CAMAG automatic TLC was sampled by TLC sampler four and were developed in CAMAG twin through the chamber using appropriate solvents. The plates were dried, and images of TLC chromatography were taken under 284 nm UV light using

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CAMAG Reporter scanner. Peaks were recorded. The amount of the desired component was calculated from the standard of the respective marker.

2.3.2Method Development:

TLC fingerprint profile- The standard solution prepared by dissolving 1mg of piperine in ethanol and making volume up to 1ml.Sample application - A uniform volume of 1, 2, 3, 4, 5, 6, 7, 8µl of standard and 4µl test solution were applied separately using CAMAG automatic TLC sampler in the form of a 6 mm band on pre-coated silica gel 60F254 TLC plates of uniform thickness of 200 µm.

Development of the plate and visualization: The plate was developed in the appropriate solvent system to a distance of 80% of the plate in a twin trough chamber with a pre-saturation time of 10 min. The plate was air-dried and image of TLC chromatogram was taken under 254 nm ultraviolet light using CAMAG Reporter.

2.3.3 Quantitative estimation of Piperine in *Trikatu Churna* formulation:

TLC plates – Pre-coated plates of silica gel 60F254with a uniform thickness of 200µm. Solvent system–Toluene: Ethyl acetate: Formic acid (7: 2: 1).Scanning wavelength –350 nm. Preparation of sample – prepared as described under section.

2.3.4 Method Validation: After the development of new method further it was validated as per ICH guidelines carried out an experiment [N=3]for linearity range, precision, accuracy as recovery, limits of detection (LOD) and limits of quantification (LOQ)[15,16,17.]

2.3.5 Stress degradation studies:

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The stability of the pharmaceutical ~~drug~~ substance or pharmaceutical~~drug~~ product defined as its extent to be remaining within the established specifications to maintain its identity, strength, quality, quantity, and purity throughout the retest or expiration period. In the drug development process, its role is significant. The purpose of the stability study is to give the authentication for the quality and purity of drug ~~drug~~ substance or pharmaceutical~~drug~~ product. Condition like temperature, humidity, oxidation, light, pH and moisture, etc. drug substance or pharmaceutical~~drug~~ product must be stable. The pharmaceutical formulation must have experimented with stability profile at accelerated temperature. The result of the study beneficial to forecast the authentic shelf life at room temperature by adopting certain assumptions [18.]

The purpose of the stability is to know about the stability of the drug substance in the dosage form. Establish degradation pathways of the active compound to interpret the structure of the degradation product, to decide the intrinsic stability of a drug substance in the dosage form. The exact mechanism of the degradation by hydrolysis, oxidation, and photolysis shall be beneficial to know the chemical properties of drug formulation further it possible to prepare a more stable formulation, minimize the unstability problems in the formulation. From stability data, the expiry date, retesting period and storage conditions of the active drug established. Many guidelines are exits for the stability testing [N=3] of the pharmaceutical formulation [19, 20.]

- **Acid stability**-0.1N HCl added to the mixture of 2gm of the powdered drug was extracted with a hydroalcoholic solution for 2h~~rs.~~ and filtered. The filtrate is concentrate to dryness and weighed. The sample made by taking 0.1mg of *Trikatu Churna* extract in 1ml ethanol. After 18h~~rs.~~of sample preparation analysis done.
- **Basic stability-** 4N NaOH added to the mixture of 2gm of the powdered drug (Ph-11) which was further extracted with a hydroalcoholic solution for 2h~~rs.~~ and filtered. The

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filtrate is concentrate to dryness and weighed. The sample made by taking 0.1mg of *Trikatu Churna* extract in 1ml ethanol. After 18h~~rs.~~ of sample preparation analysis done.

- **Oxidation stability-**1ml (3% v/v) hydrogen peroxide added to the mixture of 2gm of powdered drug which further extracted with a 70% hydroalcoholic solution for 2 h~~rs.~~ Filtered, concentrate to dryness and weighed. The analysis performed after 18h~~rs.~~ of sample preparation.
- **Wet heat stability**-2gm of the powdered drug refluxed with a hydroalcoholic solution on a water bath for 3h~~rs.~~ at 70ºC. The solution was filtered, concentrates to dryness and weighed. After 18 h~~rs.~~ of sample preparation analysis done.
- **Dry heat stability-**2gm of the powdered drug was kept in a hot air oven at 80ºC for 4h~~rs.~~ and then refluxed on a water bath for 2 h~~rs~~. The solutions was filtered, concentrate to dryness and weighed. After 18 h~~rs.~~ of sample preparation analysis done.
- **Benchtop stability**-2gm powdered drug extracted with a hydroalcoholic solution for 2h~~rs.~~ at 70ºC. The extract is filtered, concentrates to dryness and weighed. The residue reconstituted with ethanol (10ml). The prepared sample kept at room temperature for at least 24 hrs. After 18hrs of sample preparation analysis done
- **Stock solution stability**-2gm of the powdered drug extracted with a 100ml hydroalcoholic solution for 2h~~rs.~~ at 70ºC. The extract was filtered, concentrated to dryness and weighed. The residue reconstituted with ethanol and volume was made up to 10ml. The stock solution is then frozen at -20ºC for 7days and subsequently kept for 6h~~rs.~~ at room temperature. After 18h~~rs.~~ of sample preparation analysis done.

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Freeze-Thaw Stability: 2gm of the powdered drug was refluxed with a 100ml hydro alcoholic solution for 2 h~~rs~~. The extract was filtered, concentrates to dryness and weighed. The residue is reconstituted with ethanol (10ml). From these three concentrations of the high, medium, and low is prepared. The volume is made up to 5ml. These samples are frozen at -20 ºC for 24h~~rs.~~ and thawed unassisted for the next 24 h~~rs~~. This cycle was repeated 3 times before analysis. The analysis is done after 18 h~~rs.~~ of sample preparation.

Accelerated Stability Study:The study of the drug substance in the formulation of its chemical degradation or physical change using overstress storage conditions as a part of the formal stability studies. The conditions provided are temperature 40 ºC and relative humidity provided is 75% RH [21.]

Real-Time Stability Testing: This test was performed to know about product degradation under recommended storage conditions for a longer duration of time. The temperature in this varies between 15-30 ºC. [22.]

3. RESULT AND DISCUSSION

3.1 Standard solution – Prepared a stock solution of piperine (1mg/ml) dissolving accurately weighed 1mg in 1ml of ethanol and making the volume up to 1 ml. CAMAG automatic sampler was then commanded to place the spots of 1,~~µl~~ 2,~~µl~~ 3,~~µl~~ 4,~~µl~~ 5,~~µl~~ 6,~~µl~~ 7,~~µl~~ and 8µl from the stock solution of piperine containing 1~~µg~~, 2~~µg~~, 3~~µg~~, 4~~µg~~, ~~and~~ 5,~~µg~~ 6,~~ µg~~ 7,~~ µg~~ and 8 µg of piperine respectively.

3.2 Calibration curve – Applied 6µl of the standard solutions using CAMAG automatic sampler, on a pre-washed, silica gel 60F254 plates. Development of the plate in the solvent system to a distance of 80% of the plate in a CAMAG twin trough chamber with a saturation time of 10 min.

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The plate was then scanned at 350 nm in the CAMAG TLC scanner. The peak areas for different concentrations were recorded. The curve of piperine was prepared by plotting mean AUC vs the concentration of piperine (Range-100-800 ng/spot, y= 0.028x+28.72 & r2= 0.997) and Rf value - 0.46. The calibration curve **(Figure-1).** Picture showing spots of Trikatu Churna extract (track no.9) in comparison with piperine track no. 1-8 **(Figure-2).** Denstiogram showing various peaks of standard piperine and extract of Trikatu Churna formulation along with a peak treated with base **(Figure 3)**& Standard Piperine (Rf= 0.46) **(Figure 4).**

3.3 Validation:

First in laboratory estimate the value for a limit of detection (LOD) and limit of quantification (LOQ) of piperine, found 100 ng and 329 ng / ml respectively. It is a good value and further performed the other validation parameter. Calculate the precision value as Intraday and inter-day precision. Analysis of standard drugs done on the same day for the determination of Intra-day precision and carried out analysis at three different days for the determination of Inter-day precision value. The % RSD was found to be ≤ 2 for both inter-day and intra-day precision. Performed experiment for the determination repeatability of sample application and repeatability of measurement. Spotting 10 ml of drug solution, six times for determining the value of Repeatability of the sample application, analysis of peak area done the %RSD (0.968) and determined Repeatability of measurement found % RSD (0.422). The complete validation parameters shown in**[Table -1].**

3.4 Stability Studies:

Estimation of piperine in dried fruit in Trikatu Churnaby densitometry

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For stability study, the extract of Trikatu Churna was prepared with coarse powder in 100ml of ethanol at 70ºC for 2 h~~rs,~~ and the sample was analyzed by TLC densitometry. The percentage content of piperine was calculated from the standard curve by considering the area under the curve. The content of piperine in the coarse powder of Trikatu Churna was found to be an indifferent condition.

- Stress Degradation Studies
- Freeze-Thaw Stability Study
- Accelerated Stability Study
- Real-Time Stability Study

Carried out experiments [N=3] in a different condition to the stability of the piperine, 0.566 % piperine present in the formulation, in the different conditions the percentage reduced mention in the table. Reduction in the percentage with respect to the extract of Trikatu Churna formulation of a different condition like acidic, basic, oxidation, wet heat, dry heat, and bench-top stability study result in **[Table -2]**& Chromatogram-Peak of different stress degradation study**(Figure–5).** The result of the Freeze-Thaw Stability Accelerated Stability Study and Real-Time Stability Study in **[Table-3]**and Chromatogram (Figure-6).

4. CONCLUSION:

Stability acts as a symbol of quality, purity, and efficacy in herbal drugs and pharmaceutical~~drug~~ products. Stability in itself is a very important criterion, without which it is difficult to ensure a drug product's safety and efficacy. The attempt was made to carry out stability studies on Trikatu Churna formulation herbal drug industry both in India as well as other countries.

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The evaluation of the stability studies has been carried out by both comparative fingerprints chromatograms and quantitative analysis of different markers evaluated by TLC densitometry. In a comparative fingerprint profile, the comparison was established after different stability studies and compared with reference extract. The condition leading to degradation wasnoted forthe drug. The change in contents of piperine for Trikatu Churna was further analyzed by TLC densitometry. The percentage reduction in the contents of the respective marker was calculated after the various stability studies and compared with the initial values.

Various stress degradation studies, the Benchtop conditions caused maximum (98%) degradation and minimum in dry heat (49%) condition. In accelerated stability study real-time stability studytesting showed almost the same level of degradation during 3 months with no further significant change during the next 3 month (6-month study)

The study is likely to be immense value for the herbal drug industry with respect to usage of crude drug and formulation development based on any of the three plants worked in this document.

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Figure Caption:

Fig.1 Calibration curve

Fig.2 Picture showing spots of Trikatu Churna extract (track no.9) in comparison with piperine track no. 1-8.

Fig.3 Denstiogram showing various peaks of standard piperine and extract of *Trikatu Churna* formulation along with a peak treated with base.

Fig. 4 Standard piperine (Rf= 0.46)

Fig.5 Peaks of different stress degradation study (1-Acidic,2-Basic,3-Oxidatio, 4-Wet heat, 5-Dry heat and 6-Bench Top.

Fig.6 Freeze-Thaw Peak (1&2), Accelerated Stability Peak (3, 4 & 5) and Real-Time Peak (6, 7 & 8).

Table Caption:

Table-1: Validation Parameter

Table-2: Degradation Study

Table 3: Freeze-Thaw, Accelerated Stability & Real-Time Stability Study

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Figure-1 Calibration curve

Please put the numerals of the samples in the image

Figure -2: Picture showing spots of Trikatu Churna extract (track no.9) in comparison with piperine track no. 1-8.

Figure 3: Denstiogram showing various peaks of standard piperine and extract of *Trikatu Churna* formulation along with a peak treated with base.

Figure 4: Standard piperine (R_f= 0.46)

Figure – 5: Peaks of different stress degradation study (1-Acidic,2-Basic,3-Oxidatio, 4-Wet heat, 5-Dry heat and 6-Bench Top)

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Figure-6: Freeze-Thaw Peak (1&2), Accelerated Stability Peak (3, 4 & 5) and Real-Time Peak (6,7 & 8)

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Table-1: Validation Parameter

Parameter	Value
Rf	0.46±0.03
Linearity (ng/spot)	100-800 ng
Correlation coefficients r^2	0.997
LOQ (ng /spot)	329 ng
LOD (ng /spot)	100ng
Precision (%RSD)	
Inter day	0.69
Intra day	0.57
Recovery Studies	
Accuracy(%RSD)	0.405
SE	0.46
Recovery%	99.18
Repeatability of sample application	0.968
Repeatability of measurements	0.422

Table-2: Degradation Study

Sample	AUC	Estimated content of piperine in spotted volume (ng)	Piperine content in the dried residue (mg)	% Piperine found in the coarse powder.	% degraded (% 0.566 Piperine in formulation extract)
Acidic	12188.8	9.2	1.50	0.075	86.74
Basic	21972.1	16.89	2.75	0.137	75.79
Oxidation	23024.3	17.72	2.894	0.144	75.26
Wet heat	20298.5	15.57	2.5431	0.127	77.03

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Dry heat	4974.1	3.513	5.76	0.288	49.11
Bench top	2304	1.43	5.23	0.011	98.23

Sample	AUC	Estimated content of piperine in spotted volume (ng)	Piperine content in the dried residue (mg)	% Piperine found in the coarse powder.	% degraded (% 0.566 Piperine in formulation extract)
FREEZE-THAW STABILITY					
Freeze-thaw first dilution	6769	4.92	0.8	0.04	92.93
Freeze-thaw second dilution	5334	3.81	0.62	0.031	94.52
ACCELERATED STABILITY STUDY					
3 month	6412.7	4.662	0.76	0.038	93.28
6 month	4762.7	3.365	0.54	0.027	95.22
REAL-TIME STABILITY STUDY					
3 month	2411.7	1.517	0.24	0.012	97.87
6 month	1201.8	0.565	0.092	0.005	99.11

Table 3:Freeze-Thaw, Accelerated Stability&Real-Time Stability Study

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