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Stability indicating HPTLC studies of piperine through method development and analysis

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Abstract

Objective: To develop simple, selective, precise and stability indicating high-performance thin-layer chromatographic method of analysis of piperine and validation as per ICH guidelines.

Methods: The method employed TLC plates precoated with silica gel 60F-254 as the stationary phase. Ideal solvent system was developed after several trials. The method was validated as per ICH guidelines for linearity, accuracy, precision, specificity and limits of detection and quantification. Standard piperine was subjected to different ICH prescribed stress conditions (acid and base hydrolysis, oxidation, dry and wet heat degradation and photo-degradation) and establishment of stability indicating HPTLC assay.

Results: The solvent system consists of toluene: ethyl acetate: diethyl ether (12:6:1) was found to give well separated spots of piperine at R_f 0.26. The linear regression analysis data for the calibration plots showed good linear relationship with r 0.997 respects to peak height and peak area, respectively, in the concentration range 200-700 ng per spot. The limits of detection and quantification were 5.7 ng and 17.27 ng per spot, respectively. The degraded product peaks were well resolved from the standard piperine with significant difference in their R_f values.

Conclusion: The stability studies indicate that standard piperine was susceptible to alkali hydrolysis, acid hydrolysis, oxidative stress degradation, photolytic degradation and dry heat degradation. The developed novel HPTLC method could effectively separate piperine from its degradation products thus it can be employed as a stability indicating one.

Keywords: Piperine, *Piper longum*, HPTLC, validation, stability indicating studies, forced degradation studies

1. Introduction

Piperine is an alkaloid found naturally in plants belonging to the Piperaceae family, such as *Piper nigrum* L [1], commonly known as black pepper and *Piper longum* L, commonly known as long pepper. Piperine is reported to have diverse pharmacological activities, for example central nervous system depressant, analgesic [2], inhibition of hepatic drug metabolism [3], enhancing pentobarbitone induced hypnosis [4], bioavailability of oxyphenylbutazone [5], hepatoprotective activity [6], anti-inflammatory activity [7], inhibition of lipid peroxidation during experimental inflammation [8], antifertility [9], and antidiarrheal [10]. It is also radioprotective [11], and devoid of genotoxic effects [12]. Literature survey reveals that, various chromatographic methods such as HPTLC [13, 14], HPLC [15, 16] have been reported for the quantification of piperine. However these methods suffer from drawbacks such as poor resolution, lack of sensitivity and reproducibility. Moreover, none of them is stability indicating method by HPTLC and till date no stability studies have been carried out so far on piperine. On the basis of that, the present study was planned to study the stability profile of important bioactive compound piperine. The International Conference on Harmonization (ICH) guideline entitled 'stability testing of new drug substances and products' requires the stress testing to be carried out to explore intrinsic stability parameters of the active drug substances [17]. Stability indicating method can be defined that the method which determines the drug along with its degradation and reaction products. Acid, alkali, oxidation and light are very common stress conditions employed for stability studies using titrimetric, spectrophotometric and chromatographic methods.

Among chromatographic methods, nowadays, HPTLC is becoming a very common analytical technique where number of samples can be analyzed at a time for fast, reliable quantitative determination of drugs [18, 20]. Hence in the present work it was decided to develop an accurate, specific, repeatable and stability indicating assay method for the determination of piperine in the presence of its degradation products and related impurities as per ICH guidelines.

2. Materials and Methods

2.1 Chemicals

The reagents used in this wok were methanol, toluene, ethyl acetate, diethyl ether, distilled water, HCl from Merck (AR), NaOH from Merck, hydrogen peroxide (3% w/v) from Merck, India. Marker piperine was procured from Sigma Aldrich.

2.1.1. Equipments

HPTLC instrument consists of CAMAG (Muttenz, Switzerland) Linomat V sample applicator with 100µL applicator syringe (Hamilton, Bonadauz, Switzerland). Chromatography was performed on 10 cm × 10 cm aluminum TLC plates precoated with silica gel 60-F254 (E. Merck, Darmstadt, Germany; supplied by Anchrom Technologists, Mumbai, India). CAMAG TLC scanner III was used for the densitometric scanning of the developed chromatogram. All drugs and chemicals were weighed on Shimadzu electronic balance (AX 200, Shimadzu Corp., Japan).

2.1.2 Chromatography conditions

Chromatography was performed on a 10x10 cm HPTLC Silica gel 60 F254 plates (Merck, Darmstadt, Germany). Aliquots of each of the extracts were separately applied (Samples and standard) to the plate as 6 mm wide band with an automatic TLC applicator Linomat-V with N2 flow (CAMAG, Switzerland), 8

mm from the bottom. Densitometric scanning was performed on CAMAG scanner III. Linear ascending development was carried out in 10x10 cm twin glass chamber saturated with the mobile phase.

2.2 Method Development

2.2.1 Preparation of standard sample

10mg of piperine was taken in 10ml volumetric flask. The residue was dissolved in sufficient quantity of methanol and volume was adjusted to 10ml with methanol to get (1mg/ml) concentration which was further diluted with methanol to get necessary concentration 100 µg/µl.

2.2.2 Development of solvent system

Varieties of mobile phases were tried for analysis of piperine and *Piper longum* extract. According to different solvent polarity HPTLC method was developed by hit and trials methods using various solvent systems. Best solvent system selected that contained good resolution, good chromatogram with sharp, stable peak.

2.3 Method Validation

A simple, rapid and selective high performance thin layer chromatography (HPTLC) method was developed for piperine. The developed method was validated according to the International Conference on Harmonization (ICH) guidelines regarding; linearity, ranges, accuracy, precision, specificity and limits of detection and quantification [21].

2.3.1 Linearity

The content of piperine compound was determined by using a calibration curve established with a standard concentration range from 200 to 700 ng/spot. A stock solution of standard piperine (10µg/ml) was prepared in methanol. The different volumes of stock solution 2, 3, 4, 5, 6 and 7 µl/spot were spotted on HPTLC plate to obtained concentration 200, 300, 400, 500, 600 and 700 ng/spot, respectively (band width 6 mm, distance between tracks 12 mm) using automatic sample spotter. Each concentration peak area was plotted against the concentration of piperine spotted or injected.

2.3.2 Precision

Instrumental precision, intra-day precision and interday precision of the method were determined. Instrumental precision was measured by replicate (n-6) applications of same piperine solution. Intra-day assay precision was evaluated by analysis of replicate (n-6) applications of freshly prepared standard solution of same concentration (200 ng/spot), on the same day. Intermediate precision was evaluated by analysis of replicate (n-6) applications of standard solution of the same concentration (200 ng/spot) on six different days. The repeatability of sample application and measurement of peak area have been expressed in terms of % RSD.

2.3.3 Limit of detection and limit of quantification

For the evaluation of limit of detection and limit of quantification different concentrations (200-700ng/spot) of the standard solutions of piperine were applied along with methanol as blank and determined on the basis of signal to noise ratio.

LOD = $3.3 \times \sigma/S$

LOQ = $10 \times \sigma/S$, Where, σ = standard deviation of the response, S = slope of the calibration

2.3.4 Specificity

The specificity of the method was ascertained by analyzing standard piperine and extracts. The spot for piperine in the sample was confirmed by comparing the R_f and spectra of the spot with that of sample. The peak purity of piperine was assessed by comparing the spectra at three different levels, i.e., peak start, peak middle and peak end positions of the spot/ bands.

2.4 Forced degradation studies

The specificity of the method can be demonstrated through forced degradation studies conducted

on the sample using acid, alkaline, oxidative, thermal, photolytic, and UV degradations. The sample was exposed to these conditions and the main peak along with peaks of the degraded products were studied to check whether the method could effectively separate the degradation products from the pure active ingredient [22].

2.4.1 Alkali Hydrolysis

To perform alkali degradation study, appropriate aliquots of stock solutions of piperine and *piper longum* extract were taken in two different 10 ml volumetric flasks and 2 ml of 0.1 N NaOH was added. All the mixtures were heated in a water bath at 80^0 C for 2 h. and allowed to cool to room temperature. Solutions were neutralized with 0.1 N HCl and diluted up to the mark with diluted up to the mark with methanol.

2.4.2 Acid Hydrolysis

To perform acid degradation study, appropriate aliquots of stock solutions of piperine and *piper longum* extract were taken in two different 10 ml volumetric flasks and 2 ml of 0.1 N HCl was added. All the mixtures were heated in a water bath at 80^0 C for 2 h. and allowed to cool to room temperature. Solutions were neutralized with 0.1 N NaOH and diluted up to the mark diluted up to the mark with methanol.

2.4.3 Oxidative Stress Degradation

To perform oxidative stress degradation, appropriate aliquots of stock solutions of piperine and *piper longum* extract were taken in two different 10 ml volumetric flasks and 2 ml of 3% hydrogen peroxide was added. All the mixtures were heated in a water bath at 80 ^{0}C for 2 h. and allowed to cool to room temperature and diluted up to the mark with methanol.

2.4.4 Dry Heat Degradation

Analytically pure samples of piperine and *piper longum* extract were exposed in oven at 80 ^{0}C for 2 h. The solids were allowed to cool and 10 mg each of piperine and *piper longum* extract were weighed, transferred to two separate volumetric flasks (10 ml) and dissolved in few ml of methanol. Volumes were made up to the mark with the methanol. Aliquots from the stock solutions of piperine and *piper longum* extract were appropriately diluted with methanol to obtain working standards of 100 µg/ml of both the drug.

2.4.5 Photo Degradation

Analytically pure samples of piperine and *piper longum* extract were exposed to UV light for 6 h. The solids were allowed to cool and 10 mg each of piperine and *piper longum* extract were weighed, transferred to two separate volumetric flasks (10ml) and dissolved in few ml of methanol. Volumes were made up to

the mark with the methanol. Aliquots from the stock solutions of piperine and *piper longum* extract were appropriately diluted with methanol to obtain working standards of 100 µg/ml of both the drug.
All the reaction solutions were applied on TLC plate and chromatograms were recorded using HPTLC method. Chromatography was performed on a 10x10 cm preactivated HPTLC Silica gel 60 F254 plates (Merck, Darmstadt, Germany). Aliquots of each of the degradated samples of standard piperine and extract were separately applied to the plate with an automatic TLC applicator Linomat-V with N2 flow (CAMAG, Switzerland), 8 mm from the bottom. Densitometric scanning was performed on CAMAG scanner III. Linear ascending development was carried out in 10x10 cm twin glass chamber saturated with the mobile phase.

3. Results and Discussion

3.1 Development of the optimum mobile phase

Best solvent system selected that contained good resolution, good chromatogram with sharp, stable peak. The mobile phase Toluene: ethyl acetate: Diethyl ether (12:6:2) and 20 min time of chamber saturation at room temperature gave good resolution with R_f value of 0.26 for piperine.

3.2 HPTLC Studies

Quantitative estimation of piperine was carried out using silica gel F254 HPTLC pre-coated plates with mobile phase Toluene: ethyl acetate: diethyl ether (12:6:2), the R_f value was about 0.26. (Figure 1) shows HPTLC chromatogram at 254 nm.

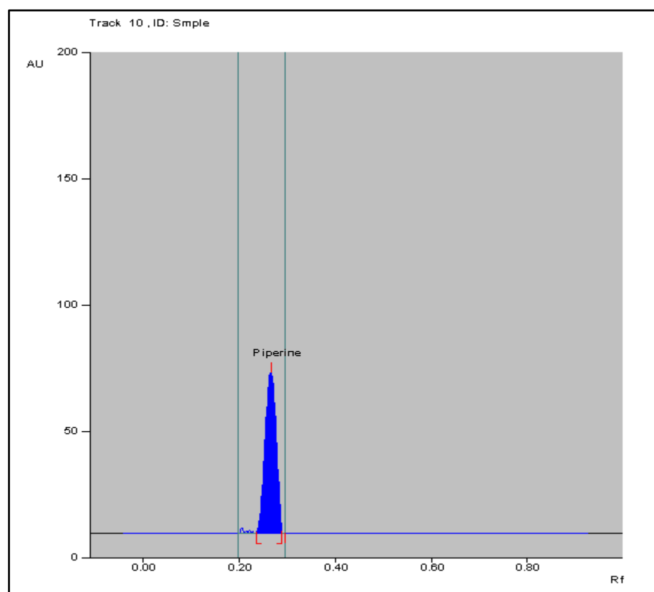


Fig 1: Chromatogram of standard piperine

3.4 Method Validation

3.4.1 Linearity

The linear regression data for the calibration curves (n-6) as shown in Table 1. The method was found to be linear in the concentration ranges 200-700 ng per spot with respect to peak height and peak area, Correlation of Coefficient (r) = 0.997, *Y= 1219.416+ 1.017*. No significant difference was observed in the slopes of standard curves. (Figure 2) shows the linearity curve of standard piperine.

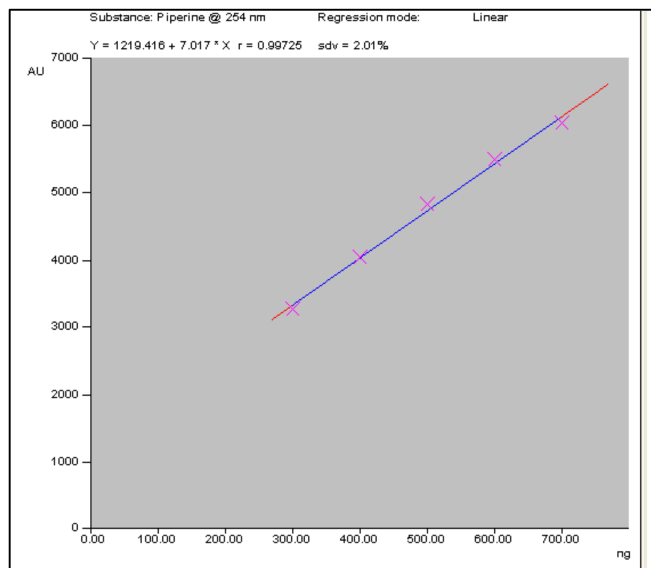


Fig 2: Linearity curve of Standard Piperine (n-6)

Table 1: Linearity regression data for calibration curve.

Parameters	Value
Linearity Range	200-700 ng
Correlation of Coefficient (r)	0.99725
Slope	1219.416

3.4.2 Precision

The repeatability study results were expressed in terms of %RSD and depicted in Table 2. This revealed intra and inter day variation of piperine at different concentration levels of 200-700 ng/spot (n-6). Table 3 shows the results of the Inter and intra-day precision of HPTLC method at different concentration levels of 200-700 ng/spot.

Table 2: Results of repeatability (n-3).

S. no	Amount (ng)	SD	%RSD
1	200	85.93631	1.76
2	300	83.37662	1.71
3	400	81.50253	1.33

SD= standard deviation, RSD= relative standard deviation

Table 3: Inter and intraday precision of HPTLC method (n-6).

S. no	Amount ng/spot	Inter day precision SD	%RSD	Intraday precision SD	% RSD
1	200	74.47413	1.49	72.45935	1.82
2	300	78.05666	1.60	81.231161	1.69
3	400	65.04249	1.34	56.2838	1.21
4	500	46.17877	0.79	103.3624	1.57
5	600	84.04884	1.27	74.21572	1.53
6	700	213.8899	1.93	101.8746	1.72

SD= standard deviation, RSD= relative standard deviation

Table 4: Method performance parameters for validation of piperine and *piper longum* extract

Parameters	Method
Selectivity	Selective
Specificity	Specific
Linear range (ng/spot)	200-700
Correlation coefficient (r)	0.99725
Linear regression equation $Y= mX + c$	Y= 1219.416 + 7.017x
LOD (ng/spot)	5.7
LOQ (ng/spot)	17.27
Repeatability (%RSD, n-6)	1.33-1.76
Inter day (n-6)	0.79-1.93
Intraday (n-6)	1.21-1.82

3.4.3 Limit of Detection and limit of quantification

Detection limit and quantification limit was calculated by the method

LOD = 3.3 x σ/S, LOQ = 10 x σ/S

Where, σ = standard deviation of the response, S = slope of the calibration curve. LOD and LOQ were calculated by these methods and found LOD 5.7 ng and LOQ 17.27 ng respectively.

3.4.4 Specificity

The specificity of the method was ascertained by analyzing standard piperine and extracts. The spot for piperine in the sample was confirmed by comparing the R_f and spectra of the spot with that of sample extract of *Piper longum*. The peak purity of piperine was assessed by comparing the spectra at three different levels, i.e., peak start, peak middle and peak end positions of the spot/ bands. (Figure 3) shows overlay spectrum of standard piperine over extract.

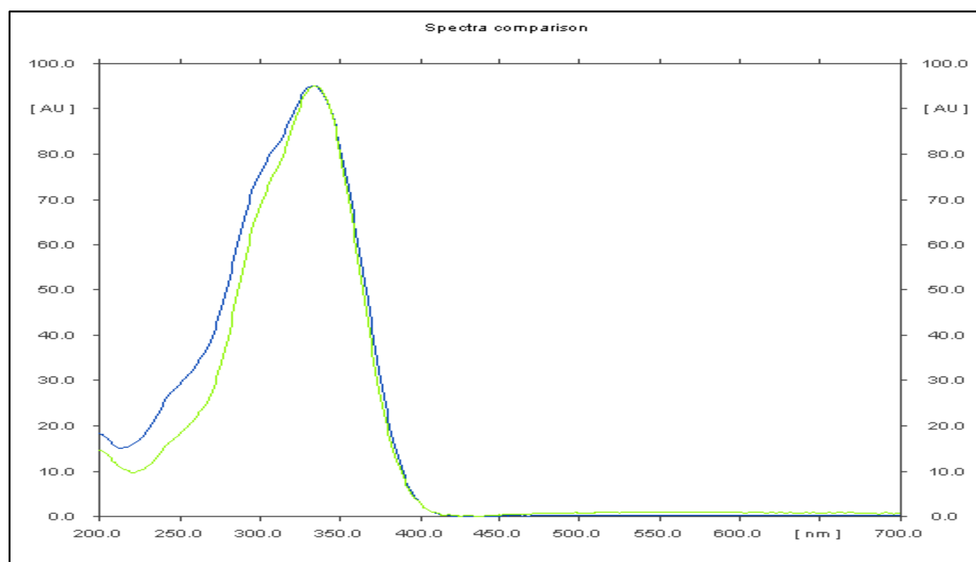


Fig 3: Overlay spectrum of standard piperine over extract.

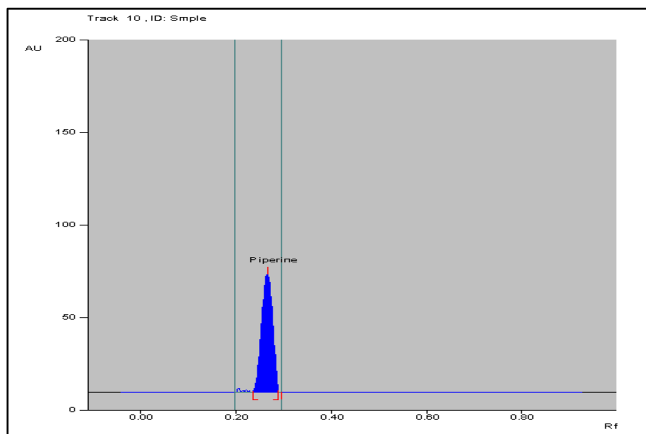
3.5 Forced Degradation Studies

Acid, base, hydrogen peroxide and light degraded samples showed well separated spots of pure piperine from some additional peaks of degradation. Table 5 summarized with the details of degradation products with their R_f values. Chromatogram of base hydrolysis, acid hydrolysis shows degradation of standard piperine with degradation product peak at R_f value 0.37.

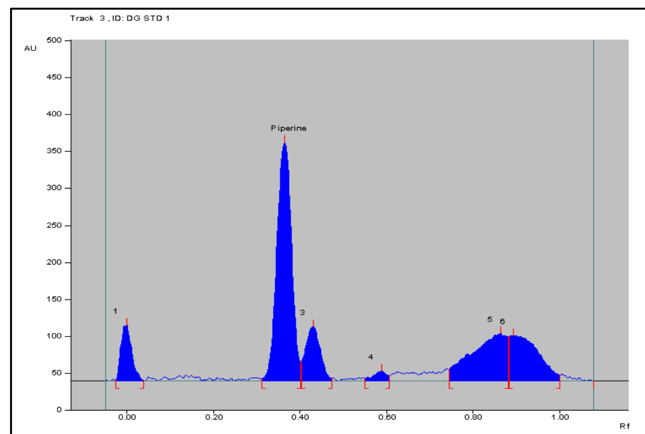
Degradation studies indicate that standard piperine was susceptible to alkali hydrolysis, acid hydrolysis and oxidative stress degradation, photolytic degradation and dry heat degradation. Standard piperine shows single sharp peak before degradation but it shows different contaminated peaks then degraded at different parameters. Fig. 4 shows comparisons of chromatogram of standard piperine before degradation and chromatogram of standard piperine after degradation parameters.

Table 5: Degradation studies of standard piperine at different parameters.

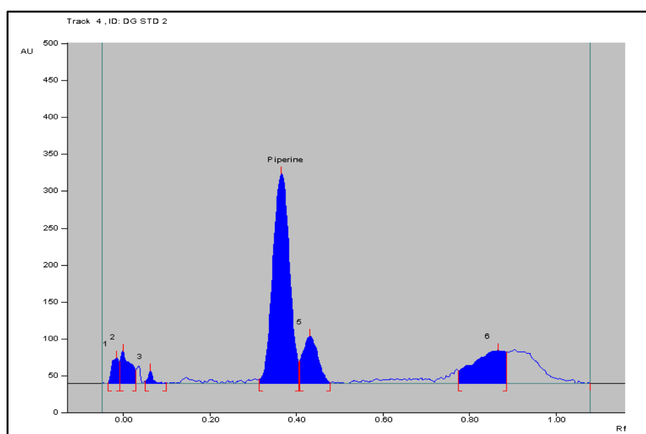
Sr. no	Sample	Parameters	Condition	Time	Rf value of standard piperine before degradation	Rf value of std. piperine after degradation
1	Piperine standard	Alkali hydrolysis	1 N NAOH at 80 ^{0}C in water bath	2 hrs	0.26	0.37
2	Piperine standard	Acid hydrolysis	1 N HCL at 80 ^{0}C in water bath	2 hrs	0.26	0.37
3	Piperine standard	Oxidative stress degradation	3% H2O2 at 80 ^{0}C in water bath	2 hrs	0.26	0.36
4	Piperine standard	Photolytic degradation	Photolytic, expose to UV	6 hrs	0.26	0.36
5	Piperine standard	Dry heat degradation	Dry heat, hot air oven at 80 ^{0}C	2 hrs	0.26	0.36



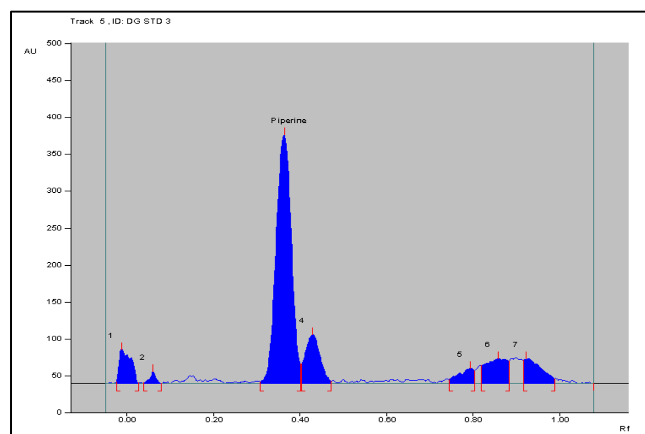
Peak of standard piperine before degradation,



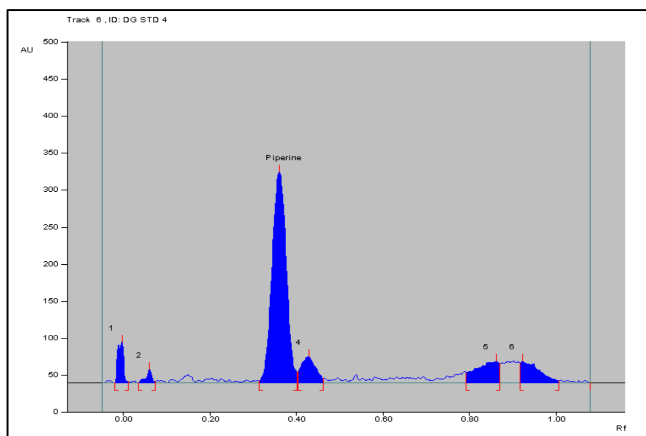
Standard piperine after alkali hydrolysis



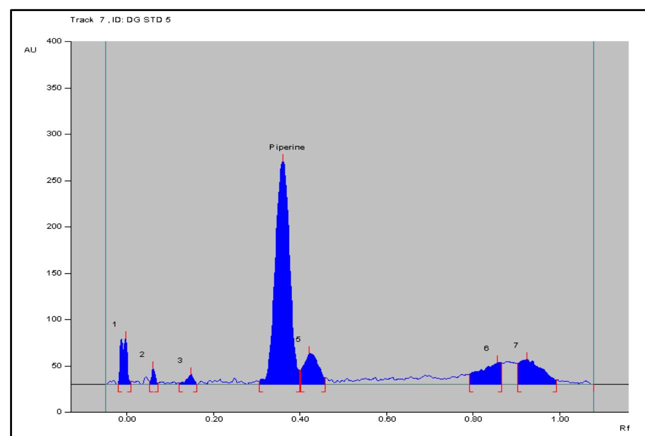
Standard piperine after acid hydrolysis



Standard piperine after oxidative hydrolysis



Standard piperine after photolytic degradation



Standard piperine after dry heat degradation

Fig 4: Chromatogram comparison of standard piperine before degradation and after degradation parameters.

4. Discussion

The present study is based on the development and validation of HPTLC protocol for the analysis of piperine and its degraded products. Piperine is the major phytoconstituent of *piper longum*. HPTLC nowadays used for analysis of herbal drugs and formulation because HPTLC consumes by far less amount of solvents and therefore can be regarded as more economic and environment friendly. In addition, it is a fast method of analysis allowing the simultaneous processing of large number of samples with no memory effect. Furthermore, it allows the detection of all compounds even those strongly retained on baseline. Indeed, HPTLC does not require elaborate treatment or the sophisticated experimental setup usually associated with HPLC methods of analysis. This work describes a simple, sensitive and robust HPTLC method for piperine to determine presence of piperine in extract of *Piper longum*. The proposed method met ICH validation acceptance criteria concerning; linearity, ranges, precision and accuracy, specificity. The selectivity of the proposed method was evaluated through the analysis of several laboratory prepared mixtures at different

ratios within the linearity ranges of the drugs. In addition, the applicability of the proposed method to real life situations was assessed through the analysis of commercially available piperine and *Piper longum* extract used in the formulation of Indian traditional medicines and herbal drugs. The present study confirms the development of validated, precise, specific, accurate and stability indicating HPTLC technique for standard piperine and extract of *Piper longum*. The present study was carried out to study the stability profile of important bioactive compound piperine and extract of *Piper nigrum.* Degraded samples indicated well separated piperine from its degraded product could be quantified easily. Piperine and *Piper longum* extract are also ingredients of many formulations used in the Indian traditional system of medicine, currently sold in market. Since stability of herbal extracts and bio active compounds is of prime concern to retain safety and efficacy of a drug, this degradation study indicates that standard piperine and *Piper longum* extract was susceptible to alkali hydrolysis, acid hydrolysis and oxidative stress degradation, photolytic degradation and dry heat degradation. The degraded products were easily reported in the developed HPTLC protocol. Study of degradation products i.e. impurity profile of piperine and *Piper longum* extract can be future scope.

5. Acknowledgements

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