WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 5, 1262-1276. Research Article ISSN 2277– 7105

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CURCUMIN AND PIPERINE IN BULK MIXTURE.

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Article Received on
16 March 2016,
Revised on 05 April 2016,
Accepted on 25 April 2016
DOI: 10.20959/wjpr20165-6200

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ABSTRACT

A new RP-HPLC method has been developed and validated for simultaneous estimation of Curcumin and Piperine, present method is found to be simple, rapid, precise, accurate, specific and stability indicating. Column used was Synchronis C-18 (250 mm x 4.6 mm 5 µm), mobile phase consist of water (0.1%acetic acid): Acetonitrile in 40:60v/v ratio to achieve good resolution and retention of analytes and its impurities. Detector linearity was established in concentration range curcumin 25-125 µg/ml, piperine 0.25-1.25µg/ml. The regression coefficient was 0.9979 for curcumin and 0.999 for piperine. The analyte was exposed to stress conditions such as acid, base, oxidation, neutral and sunlight as per ICH guidelines recommendations. Method was validated in terms of linearity, specificity, precision, accuracy, limit of detection and limit of quantification, results were found to be satisfactory and offers good column life.

KEYWORDS: Curcumin, Piperine, Stability, RP-HPLC.

INTRODUCTION

Stability studies are aimed to provide confirmation that product remains within established specifications to ensure identity, strength, quality and purity. Stability study data also help us providing effect of various environmental factors including - pH changes like acidic, basic or neutral, temperature, humidity and light on drug as long term effects.

Stability study even provide data on degradation products formed under various conditions, help indentifying impurities and even one can know how two or more drugs in combination interact with each other, whether they are compatible with each other in altered environmental conditions. Also one can know interaction between drug and excipients. Stability defines or interpret time and condition under which drug will remain under predefined limits for all its possible characters.

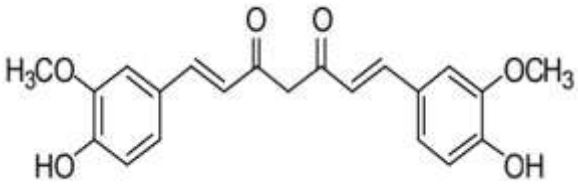
In order to carry out acceptable, systematic, reproducible and compliant stability program we need to strictly follow various regulatory guidelines. Besides all guidelines ICH (International Conference on Harmonization) guidelines are mostly followed.

ICH guideline Q1A "Stability Testing of New Drug Substances and Products"[1] encovers testing of drugs character which are likely to alter in various condition and can affect quality, safety and efficacy of drug. Entire stability testing should be done by a validated stability indicating method.[2]

Curcumin is a polyphenolic, fluorescent constituent more specifically a terpenoid derived from Curcuma longa categorized as a spice. Curcumin is used widely since long time as a dietary supplement, coloring agent and for curing wide range of disease as it shows wide range of therapeutic effect including bioavailability enhancer, anti-inflammatory[3], antifungal[4], antibacterial[5], anticancer[6], anti-fertility[7], anti-diabetic[8], antioxidant[9], antiamoebic[10], anti-HIV[11], anti spasmodic[12], nematocidal.[13]

Safe dose was found to be 8g/day.[14-16]

Curcumin IUPAC name is (1, 7-Bis-(4-Hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione, having molecular formula $C_{21}H_{20}O_6$ and molecular weight 368.37g/mol and melts at 182-184ºC[17], Curcumin structure is shown in figure-1.



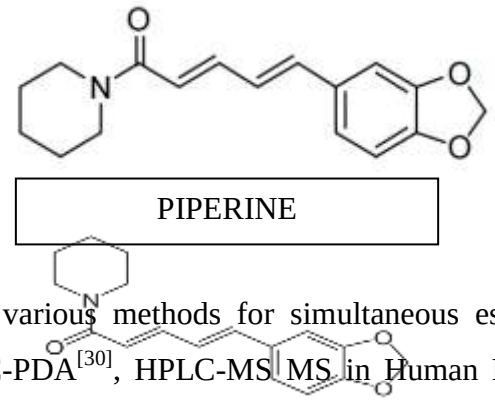
CURCUMIN

Figure-1[18]

Curcumin is yellow powder[19], fluorescent in nature and was soluble in oil, ethanol, dimethylsulfoxide and acetone, practically insoluble in water and ether.[19]

Piperine is potent, pungent[20] alkaloid recovered from Piper longum and Piper nigrum also called black pepper and is also categorized as spice.[21] Piperine obtained from herbal source shows 98% purity.[22,23] Piperine show therapeutic action including CNS depressant[21,24], antipyretic[21,24], anti-inflammatory[21-24], antioxidant[21,24], cytoprotective, antiulcer, anticonvulsant[25], showed good results as bioavailability enhancer[26,27] and as an insecticide.[28]

Piperine IUPAC name is 1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine[29], with molecular formula $C_{17}H_{19}NO_3$, having molecular weight 285.34g/mol and melts at 281-283ºC. Piperine is highly soluble in alcohol, ether and chloroform, slightly soluble in water.[20] Piperine structure is shown in figure-2.[18]



PIPERINE

Literature review showed various methods for simultaneous estimation of curcumin and piperine include RP-HPLC-PDA[30], HPLC-MS MS in Human Plasma[18], HPTLC[31], RP-HPLC in aqueous humor of rabbit.[32] Analytical method reported for stability studies of curcumin alone include HPLC-UV and with Silymarin in HPLC.[33] Similarly for stress degradation study for Piperine include LC/Q-TOF- dual ESI MS[34] alone and RP-HPLC method for Piperine with Rifampicin[35] or Aconitine.[36]

Most of these methods are for determination of either curcumin and piperine separately or in combination with other drug. No method is available so far showing simultaneous stability indicating study of curcumin and piperine, this provoked us to perform present study which aims at new validated stability indicating RP-HPLC method development for simultaneous estimation of curcumin according to ICH recommended guidelines.

MATERIALS AND METHODS

Materials

Working standards include Curcumin procured from TCI Chemicals and Piperine purchased from Sigma Aldrich.

Reagents include Acetonitrile HPLC grade (SD fine chem), Glacial acetic acid HPLC grade (spectrochem pvt. ltd., Mumbai), Hydrochloric acid (Allied Chemicals laboratories), Sodium Hydroxide flakes LOBA Chemie, Hydrogen peroxide solution 30% w/v (Fisher scientific), Double distilled water. All other chemicals and reagents used were HPLC grade unless otherwise indicated and 0.2µm Nylon filter (Pall life science, Mumbai, India).

Instrumentation and chromatographic condition

Instrumentation

Instruments used in the study were

For RP-HPLC SHIMADZU LC-20AT Prominence was used. Detection was performed with SHIMADZU SPD-20A Prominence UV/VIS Detector.

Sample was injected using Rheodyne 7725 injector valve with fixed loop at 20µl.

Data acquisition and integration was performed using Spinchrom software (Spincho biotech, Vadodara).

An ultrasonicator DTC 503 (Ultrasonics Selec, Vetra, Italy) was used for degassing the mobile phase.

Chromatographic condition

Chromatographic separation of Curcumin and Piperine and that of their degradants were obtained by using Synchronis C-18 (250 mm x 4.6 mm 5 µm) column. Mobile phase used was): Acetonitrile: Water (0.1% Acetic acid, pH 3.2 in 60:40 v/v), water filtered through 0.2µm Nylon filter and degassing was performed by using ultrasonicator by 1 cycle of 5 minutes. Mobile phase pumping through column was performed at 1ml/min, injection volume 20µl. Detection wavelength was selected as 343nm. Each analysis was completed within 10 minutes.

Standard Solutions

Preparation of standard solutions

Stock solution of curcumin 1µg/ml was prepared by dissolving 10mg curcumin in 10 ml methanol (stock 1a), similarly stock solution piperine 0.1µg/ml was prepared by dissolving 5 mg in 50 ml methanol (stock 1b).

Working standard solutions curcumin (25-125µg/ml) and piperine(0.25-1.25µg/ml) mixture was prepared by withdrawing suitable aliquots of corresponding stock 1a for curcumin and stock 1b for piperine in same volumetric flask and making up to the mark with mobile phase.

Preparation of sample solution

Forced degradation of Curcumin and Piperine

Stock solution: Stock solution of binary mixture of Curcumin and Piperine was prepared in 1:1 ratio of Curcumin and Piperine by dissolving in methanol, individual methanolic stock of curcumin and piperine are also prepared.

1.) Acidic hydrolysis

1ml of above methanolic stock solution of binary mixture was taken in 10 ml volumetric flask and was made up to 10 ml with 2N HCl. The mixture was refluxed for 5 hours at 80ºC on oil bath. Reflux condition was maintained under dark in order to prevent photo-degradation. Suitable aliquots of degradation samples were taken periodically and neutralized with NaOH, assay was performed after suitable dilutions with mobile phase. 1ml from individual curcumin and piperine stock was taken separately and treated similar to binary mixture.

2.) Basic hydrolysis

1ml of above methanolic stock solution of binary mixture was taken in 10 ml volumetric flask and was made up to 10 ml with 0.2N NaOH. The mixture was refluxed for 2 hours at 80ºC on oil bath. Reflux condition was maintained under dark in order to prevent photolytic degradation. Suitable aliquots of degradation samples were taken periodically and neutralized with HCl, assay was performed after suitable dilutions with mobile phase. 1ml from individual curcumin and piperine stock were taken separately and treated similar to binary mixture.

3.) Oxidative hydrolysis

1ml of above methanolic stock solution of binary mixture was taken in 10 ml volumetric flask and was made up to 10 ml with 1.5%w/v H2O2 The mixture was refluxed for 4 hours at 80ºC on oil bath. Reflux condition was maintained under dark in order to prevent photolytic degradation. Suitable aliquots of degradation samples were taken periodically and assayed after suitable dilutions with mobile phase. 1ml from individual curcumin and piperine stock were taken separately and treated similar to binary mixture.

4.) Neutral hydrolysis

1ml of above methanolic stock solution of binary mixture was taken in 10 ml volumetric flask and was made up to 10 ml with water. The mixture was refluxed for 12 hours at 80ºC on oil bath. Reflux condition was maintained under dark in order to prevent photolytic degradation. Suitable aliquots of degradation samples were taken periodically assay was performed after suitable dilutions with mobile phase. 1ml from individual curcumin and piperine stock is taken separately and treated similar to binary mixture.

5.) Photochemical degradation

Photolytic degradation study was performed by exposing 1ml of methanolic stock of binary mixture to direct sunlight for 7 hours. Suitable aliquots of degradation samples were taken periodically and assayed after suitable dilutions with mobile phase. 1ml from individual curcumin and piperine stock were taken separately and treated similar to binary mixture.

6.) Thermal degradation

Thermal degradation was performed by placing binary mixture of compound in 1:1 ratio in solid form at 80ºC in hot air oven for 72 hours in dark condition. Curcumin and Piperine were also placed separately in oven. Fixed amount of API was weighed periodically and assayed after suitable neutralization with mobile phase.

Validation of RP-HPLC method for Curcumin and Piperine in bulk drug

Method Validation was performed in accordance with ICH guidelines.

Calibration curve (Linearity and Range)

Working standard solutions were prepared by accurately measuring aliquots from individual stock 1a and stock 1b in separate series of 10 ml volumetric flask and diluted up to mark with mobile phase such that resultant concentration comes out to be 25-125µg/ml in curcumin

containing flask and 0.25-0.125µg/ml in piperine containing flask. 20µl of triplicate injections were given at 1ml/min flow rate, detected at 343nm. Calibration curve was obtained for both drugs separately by plotting Area under peak versus concentration.

Table-1: ***Caliberation curve analysis for Curcumin and Piperine of developed method*.**

Parameter	Curcumin	Piperine
Linearity Range (µg/ml)	25-125	0.25-1.25
Detection Wavelength (nm)	343	343
Slope± RSD	0.7068	0.7070
Intercept±RSD	0.5634	0.6483
Correlation Coefficient	0.9979	0.997

RSD= Relative Standard Deviation (n=3).

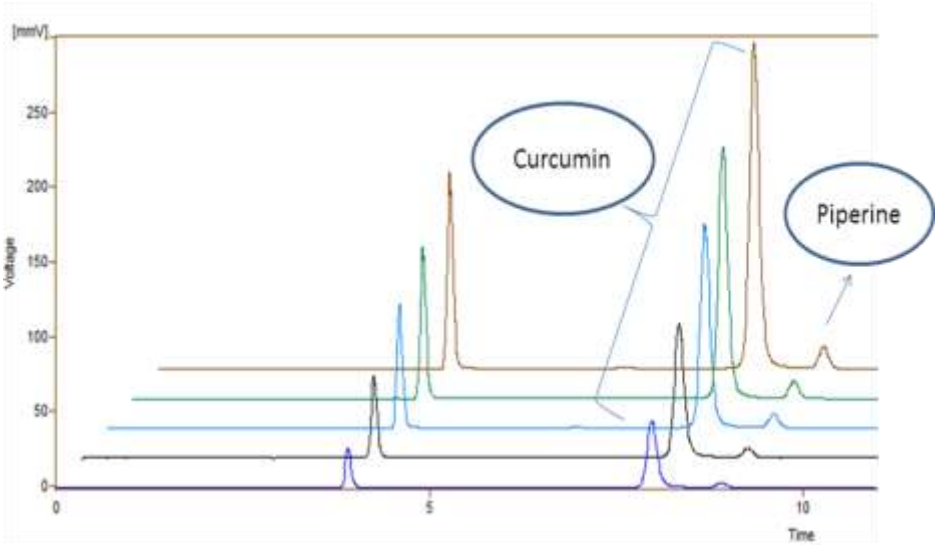


Figure-3: 3D chromatogram showing peaks of Curcumin (25-125µg/ml) and Piperine (0.25-1.125µg/ml) at different concentrations.

Precision

a.) ***Method Precision (Repeatability)***

To check instrumental precision a binary mixture containing Curcumin (75µg/ml) and Piperine (0.75µg/ml) was repeatedly injected (n=6). Results were expressed as %RSD.

b.) ***Intermediate Precision (Reproducibility)***

Intraday precision was determined by analyzing standard binary mixture solution 3 times a day in a triplicate.

Interday precision was determined by analyzing standard binary mixture for 5 consecutive days. Results were expressed as %RSD.

Accuracy

Accuracy was determined by recovery study which was performed by spiking bulk drug mixture with standard API Curcumin and Piperine at 3 levels- 80%, 100%, 120%.

Each level analysis was performed by three replicates. Spiked standards were obtained by taking into account bulk drug mixture without standard spiking and applying there values to regression equations of calibration curves.

Limit of detection (LOD)

LOD of the drug were calculated using the following equation given below as per International Conference on Harmonization (ICH) guidelines

$LOD = 3.3 \times \sigma/S.$

Where σ = the standard deviation of the response and S = the slope of the regression equation.

Limit of quantitation (LOQ)

LOQ of the drug were calculated using the following equation given below as per International Conference on Harmonization (ICH) guidelines

$LOQ = 10 \times \sigma/S.$

Where σ = the standard deviation of the response and S = the slope of the regression equation.

Table-2: ***Results of Validation Parameters for Proposed RP-HPLC method.***

Parameter	Curcumin	Piperine
Limit of Detection (LOD)	8.4167	0.18628
Limit of Quantitaion (LOQ)	25.505	0.56450
Accuracy%	99.36-100.586%	98.77-100.157%
Repeatability(RSD*%, n=5)	0.342	0.298
Precision Interday (n=3) Intraday (n=3)	 1.462 0.268	 1.0227 0.1326

Robustness

Robustness is measured by making small changes in method parameters. Method is considered robust when it remains unaffected by small changes in parameters. Three factors are varied at 3 levels which include (a) Flow rate (0.9, 1, 1.1), (b) Ratio of Acetonitrile in

mobile phase (58%, 60%, 62%), (c) Detection wavelength (341nm, 343nm, 345nm). One factor was changed at a time. 3 replicate injection of bulk drug mixture at 3 consecutive levels were injected. Results were reported in terms of Relative Standard Deviation (RSD).

Table-3: ***Results of Robustness as Validation Parameter for Proposed RP-HPLC method.***

Factors	Rt (min) Curcumin	Rt (min) Piperine	Area (mV.s) Curcumin	Area (mV.s) Piperine
Flow Rate (ml/min) 0.9 1.0 1.1 Mean±SD	 8.910 8.077 7.297 8.0946±1.153	 9.923 8.973 8.140 9.012±1.288	 1567.902 1539.653 1525.227 1554.2606±1.6072	 112.879 108.612 103.439 106.6433±1.744
Ratio of ACN.(ml) 58 60 62 Mean±SD	 8.103 8.077 7.07 7.75±0.961	 9.090 8.973 8.047 8.7033±0.9282	 1621.148 1593.653 1606.37 1618.057±1.89678	 115.795 108.612 109.539 111.3153±1.03578
Wavelength (nm) 341 343 345 Mean±SD	 7.987 8.071 7.963 8.009±0.1004	 8.920 8.973 8.903 8.932	 1512.606 1539.653 1578.760 1547.0063±1.43901	 113.571 108.612 106.340 109.5076±1.74609

System Suitability Test (SST)

SST parameters like Retention time Rt, Theoretical Plates, Tailing factor, Capacity factor and Resolution were calculated from the chromatogram obtained by injecting suitable bulk drug mixture in replicate of 6 (n=6).

Table-4: ***System Suitability Test Parameters for Curcumin and Piperine for proposed RP-HPLC method.***

Parameter	Curcumin ± RSD	Piperine ± RSD
Retention Time Rt (min)	8.1485	9.0495
Theoretical plate	16722.833	19160.333
Tailing factor	1.2216	0.9906
Resolution	-	3.5096
Capacity factor	2.48	2.53

RESULTS AND DISCUSSION

Curcumin and Piperine chromatogram showed resolution less than two which was not acceptable, for this reason method was optimized, after large number of trials method

optimized was having mobile phase Acetonitrile: Water (0.1% Acetic acid) 60:40v/v, 20µl sample injection at 1ml/min flow rate, detection wavelength was 343nm. Acceptable separation and good peak symmetry for curcumin and piperine were obtained by proposed method and all validated parameters were found within ICH guidelines limitation.

Under acidic degradation studies curcumin was found to be almost stable and gave no characteristic degradant peak under acid strength 0.1N, 0.2N, 0.5N, 1N HCl, but lastly with 2N HCl curcumin gave one distinct and and two small degradant peaks with increasing intensity with time. Piperine do show decrease in peak intensity but no characteristic peak of piperine degradant was observed.

Acidic hydrolysis for 5 hours show more than 20% degradation of Piperine and Curcumin.

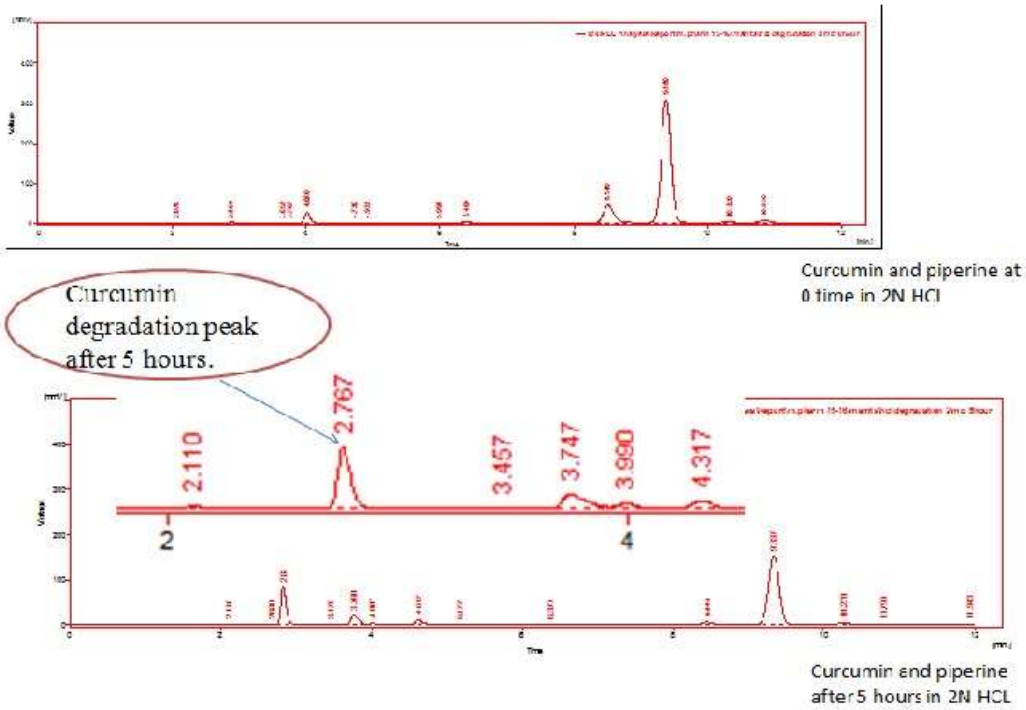


Figure-4: Chromatographic separation of Curcumin and Piperine from their acidic degradation peak.

Under basic hydrolysis Curcumin was found highly unstable (0.2N NaOH) and showed nearly 80% degradation within 30 minutes but piperine was found much stable to basic condition than curcumin hence basic condition was maintained for further two hours giving piperine degradant peak.

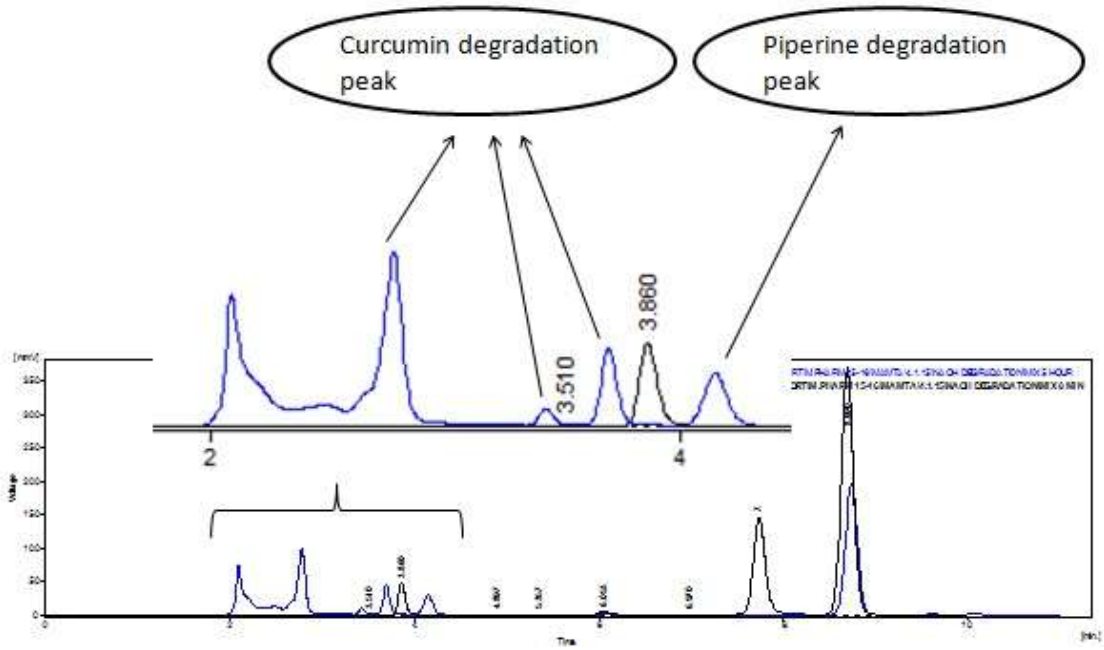


Figure-5: Chromatographic separation of Curcumin and Piperine from their basic degradation peak.

Oxidation was performed using hydrogen peroxide 3% w/v curcumin and piperine was found much unstable and hence milder stressed condition (1.5%H2O2) was tried giving good results. Curcumin oxidation gave a degradant peak with increasing concentration periodically while Piperine do show decrease in peak intensity with time but no degradant peak of Piperine was observed.

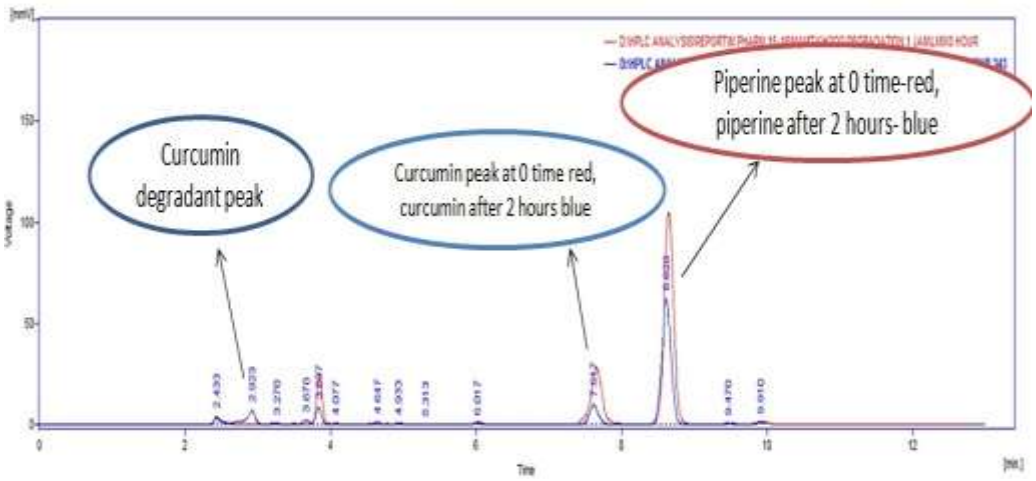


Figure-6: Chromatographic separation of Curcumin and Piperine from their oxidation degradation peak.

Under photo degradation piperine was found to be highly sensitive to light and gave three distinct degradation peak within 7 hours, Curcumin was much stable than piperine under photodegradation. Curcumin gave four to five less intense peaks after seven hours.

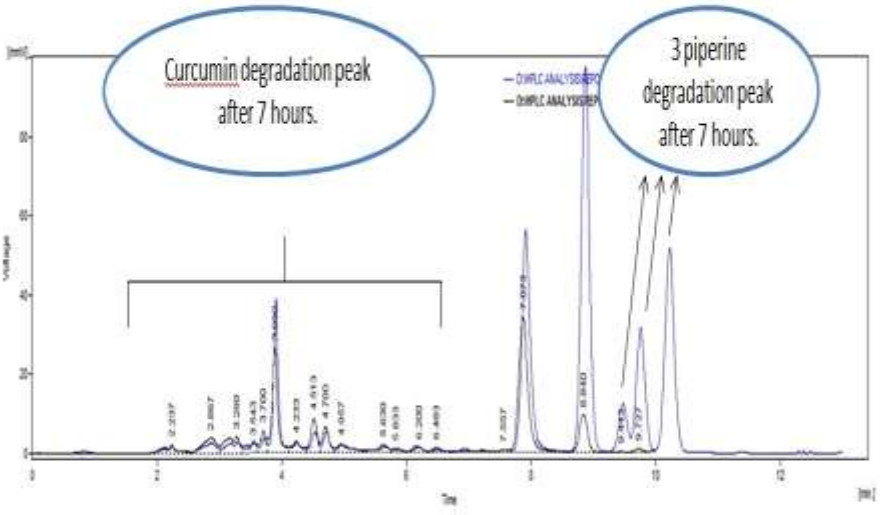


Figure-7: Chromatographic separation of Curcumin and Piperine from their photolytic degradation peak.

Both Curcumin and Piperine were found to thermally stable and gave no distinct peak after maintaining 72 hour thermally stressed condition. Also both drugs were found to be stable under neutral degradation giving no distinct peak after 12 hours study under stressed condition.

CONCLUSION

A rapid, convenient, accurate, precise method for simultaneous estimation and identification of Curcumin and Piperine in presence of their degradant product was developed. Both Curcumin and Piperine was found much stable in acidic condition, stability decreases as pH increases. Both drugs were found prone to oxidative hydrolysis but showed thermal stability. Piperine was found to be highly photosensitive as compared to Curcumin. Above developed method can be said to be more economical and applicable compared to other literature reported methods in terms of column life as no buffer is used as mobile phase component and as 0.1% Acetic acid is used this will confirm applicability of developed method for LC-MS for degradant identification and also can be used for routine quality control of Curcumin and Piperine in different laboratories.

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