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CHROMATOGRAPHIC DETERMINATION OF CURCUMIN IN THE PRESENCE OF ITS DEGRADATION PRODUCTS BY HPLC

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Keywords:

Reversed phase-HPLC, Curcumin, stability, analysis, solubility, chromatography, Forced degradation, ICH guidelines

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ABSTRACT: A simple, selective, precise, and stability-indicating RP-HPLC method has been developed for the determination of Curcumin in bulk forms and solid dosage formulations. The HPLC separation was achieved on Waters Spherisorb ODS2 column (4.6mm × 250mm, 5µm particle size) using a mobile phase of methanol-water (77:33 v/v, pH 3) at a flow rate of 1ml/min and spectrophotometric detection at 419 nm. The method was validated for specificity, linearity, accuracy, precision, solution stability, robustness, system suitability, and ruggedness. The detector response for the Curcumin was linear over the selected concentration range from 1 to 10 µg/ml, with a correlation coefficient of 0.9993. The accuracy was between 99.27& 101.97%. The precision (R.S.D.) among the six sample preparations was 1.27%. The LOD and LOQ are 0.092 and 0.278 µg/ml, respectively. The recovery of curcumin was about 100.12%. The drug was subjected to acid and alkali hydrolysis, oxidation, wet heat treatment, and photodegradation. The drug undergoes degradation under former conditions, which indicates that the drug is susceptible to acid and base hydrolysis, wet heat, oxidation, and photo-oxidation. Thus, this method could be utilized to effectively separate the drug from its degradation products.

INTRODUCTION: The powdered dry rhizome of the plant *Curcuma longa*, commonly called turmeric, is widely used as a coloring agent and spice in many food items [1]. It has also been used for centuries as a traditional remedy for the treatment of inflammation, pancreatic cancer, multiple myeloma, colon cancer, psoriasis, myelo-dysplastic syndromes, Alzheimer's disease, and other diseases [2]. The rhizome contains a yellow pigment, which is composed of curcumin (CUR I), mono-demethoxycurcumin (CUR II), and bis-demethoxycurcumin (CUR III).

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They are commonly known as 'curciminoids' and have been reported to possess anti-oxidative, anti-inflammatory, and anti-carcinogenic properties [3].

Commercially available curcumin consists of a mixture of naturally occurring curcuminoids with curcumin as the main constituent [4]. Since the curcuminoid pigments vary in chemical structures, it is possible that the chemical and color characteristics, as well as the functional properties, will vary among the pigments [5]. Pure Curcumin, mono-demethoxycurcumin & bis-demethoxy-curcumin are scarce, expensive, and not easily available [6]. Therefore, it is important to obtain pure pigments and to characterize them individually to provide subsidies for the determination of each curcuminoid pigment [7]. Several studies were undertaken to separate curcuminoid pigments by thin-layer chromatography (TLC) and column

chromatography (CC), in which the stationary phase mostly used, was silica gel (60G) with different solvent systems including benzene, ethyl acetate, ethanol, chloroform, acetic acid, hexane and methanol for TLC and benzene, water, toluene and ethyl acetate for column chromatography [8].

However, poor resolution and curcumin bands with only up to 80% of purity were obtained. Furthermore, no separation was obtained for demethoxycurcumin and bisdemethoxycurcumin [9].

Research on dosage form with herbal drugs as raw material nowadays focused on isolation, identification, and pharmacological study of the active substance, while quantitative analysis of the active substance in the herbal drug that might be unstable after the distribution is rarely found. Most of the chromatographic methods reported for the quantification of curcuminoid pigments were by high-performance thin-layer chromatography (HPTLC). They have accuracy and simplicity but lacks precision, maybe due to interference by other pigments present in the plant. Very little information is available on the determination of curcuminoids in turmeric using HPLC [10]. It should be the first choice in medicinal and aromatic plant research programs as most of the phytochemicals are complex biological materials. It has advantages in terms of flexibility, parallel analysis of a large number of samples, and different modes of development [11].

HPLC is now utilized frequently in the rapid plant screening [12]. It has become the workhorse of the pharmaceutical industries where it is used to identify, characterize, and purify molecules at all stages of a process from R&D to Quality Assurance and Validation [13]. Newer instrumentations and techniques have evolved that provide opportunities for improved methods, including improved analyte identification or detection limits, greater accuracy or precision or better return on investment [14]. The standard approach is to vary one instrument factor at a time and assess the effect of the changes on key performance requirements such as critical pair resolution and total assay time [15]. If no satisfactory result is obtained from changes to the selected factor, then a second factor is selected for study. This process is repeated until a method is obtained that meets the current performance requirements [16].

The main considerations for clinical applications of curcumin are its solubility and stability [17]. It has low solubility in water, at acidic & physiological pH, and rapid hydrolysis under alkaline conditions. It is also very susceptible to photochemical degradation [18]. The aim of the present work is to elucidate the inherent stability characteristics of the drug substance by subjecting it to the variety of suggested stress conditions as per International Conference on Harmonization (ICH) recommendations. Thus stability-indicating chromatographic methods are required for the determination of curcumin in the presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its bulk dosage forms [19]. The proposed HPLC method was validated in compliance with ICH guidelines and its updated international convention [20].

MATERIALS AND METHODS:

Materials: Curcumin was obtained from Loba chieme Mumbai, India, as a gift sample. Methanol and HPLC Water used were of analytical grade and were purchased from Merk Chemicals, India. Syringe Filter [MILLEX-HV (0.45µm)] and Millipore Filters [MILLIPORE GVWP04700, 0.45µm] are used. Three formulations collected from the market (A, B & C) with a drug equivalent to 500 mg curcumin. All the other chemicals and reagents used were of analytical grade.

Method Development:

HPLC Instrumentation and Chromatographic Conditions: Shimadzu HPLC system with CTO-10As up Column oven, SPD-M20A Prominence Diode array detector (D2 & W), DGU-20A5 Prominence Degasser, LC-20AD Prominence Liquid Chromatography and 10 µl Hamilton Syringe (Bonaduz, Schweiz). Data acquisition and analysis were carried out using LC Solution software.

Waters Spherisorb ODS2 column (4.6mm × 250mm, 5µm particle size) was used at 40 ºC temperature. The optimized mobile phase consisted of methanol-water (77:33, v/v) adjusted to a pH of 3 at a flow rate of 1ml/min with run time 10 min and spectrophotometric detection at 419 nm.

Standard Stock Solution: Stock solutions of curcumin containing 100 µg/ml were prepared in

methanol and stored in the refrigerator. Solutions were stable up to 3 weeks when stored in freezer or kept at room temperature in light-resistant amber color tightly stoppered volumetric flasks.

Method Optimization:

Effect of Stationary Phase: The chromatogram was recorded using the following column.

Hypersil ODS-2 columns (250 × 4.6 mm) 5µm. Waters Spherisorb ODS2 column (250 × 4.6 mm) 5µm.

Effect of Solvent: The three widely used organic modifiers for RPC are methanol, chloroform, acetonitrile, and tetrahydrofuran were used.

Effect of pH: The effect of pH on the chromatographic behavior of the drug was studied by varying pH at 2.4, 2.6, 3, 3.5, and 4 of respective Methanol: water solvent system.

Effect of Mobile Phase Ratio: The chromatogram was recorded by using a mobile phase containing 65%, 70%, and 77% of methanol in water (pH 3.0).

Effect of Flow Rate: The flow rate 1.0, 1.2, 1.4 ml/min were used and chromatogram was recorded.

Method Validation: Validation of the developed HPLC method was carried out as per the International Conference on Harmonization (ICH) guidelines Q2 (R1) [21,] and accordingly, the parameters evaluated were:

Sensitivity: The sensitivity of the method was determined with respect to the limit of detection (LOD) and limit of quantification (LOQ). Series of concentrations of drug solutions (0.05–10 µg/ml) were applied on the column and analyzed to determine LOD and LOQ. The limit of detection (LOD) and limit of quantification (LOQ) were respectively determined at a signal-to-noise ratio (S/N) of 3 and 10 [22].

LOD and LOQ were experimentally verified by diluting the known concentration of curcumin until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Linearity and Calibration Curve: Linearity of the method was evaluated by constructing calibration curves at ten concentration levels. The standard solutions were prepared by dilution of the stock solution with methanol to reach a concentration range 1-10 µg/ml for HPLC determination. Twenty microliters of the solution were injected under operating chromatographic conditions described above.

The calibration graph was constructed by plotting peak area versus concentration of curcumin, and the regression equation was calculated [23].

Accuracy: Accuracy of the method was evaluated by carrying the recovery study at three levels. The analyzed samples were spiked with an extra 50, 100, and 150% of the standard curcumin, and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels in the formulations [24].

Precision: Precision was evaluated in terms of Intraday and Interday precisions. System repeatability was determined by six replicate applications and six-fold measurement of 5µg/ml concentration. The intra- and inter-day variation for the determination of curcumin was carried out at three different concentration levels of 2.5, 5, and 7.5 µg/ml, respectively.

Robustness: To evaluate the HPLC method robustness a few parameters were deliberately varied. Thus the pH of the mobile phase, column temperature, and flow rate was varied. By introducing small changes in the established parameters, the effects on the results were examined [25].

Specificity: A stock solution containing 50 mg curcumin in 50 ml methanol was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating the property and specificity of the proposed method. The specificity was done by degrading the drug through the following procedure:

Preparation of Acid and Base Induced Degradation Product: To 10 ml of methanolic stock solution, 10 ml each of 0.1 N HCl and 0.1N NaOH were added separately. These mixtures were refluxed for 8 h at 70 °C in the dark in order to exclude the possible degradative effect of light.

Preparation of Hydrogen Peroxide Induced Degradation Product: To 10 ml of methanolic stock solution, 10 ml of 3.0% hydrogen peroxide was added. The solution was heated in a boiling water bath for 10 min to completely remove the excess of hydrogen peroxide and then refluxed for 8 h at 70 ℃ in the dark in order to exclude the possible degradative effect of light.

Preparation of Wet Heat-Induced Degradation Product: The stock solution was refluxed for 8 h on a boiling water bath for wet heat-induced degradation. Refluxing is done in the dark in order to exclude the possible degradative effect of light.

Preparation of Photochemical Degradation Product: The photochemical stability of the drug was studied by exposing the stock solution to direct sunlight for 24 h in the open air.

System Suitability Tests: System suitability parameters like accuracy, precision, tailing factor, number of theoretical of plates, etc. were calculated and compared with the standard values.

Analysis of the Curcumin in Prepared Formulation: The content of curcumin in tablets (labeled claim: 500 mg per tablet) were determined by powdering twenty tablets, and powder equivalent to 10 mg of curcumin was weighed. The drug from the powder was extracted with methanol [26]. For the complete extraction of the drug, it was sonicated for 30 min, and volume was made up to 100 ml. The resulting solution was centrifuged at 2500 rpm for 10 min, and the supernatant was analyzed for drug content. The 2.5 ml of the filtered solution was further diluted in 10 ml of methanol. The resulting solution was then injected in the HPLC system. The analysis was repeated in triplicate. The possibility of interference of the excipients in the analysis of curcumin was studied.

System Suitability: System suitability is defined as, the checking of a system, before or during an analysis of unknowns, to ensure system performance. A data from five injections of system precision were utilized for calculating system suitability parameters like %RSD, Tailing factor, and theoretical plates.

RESULTS AND DISCUSSION: To develop the HPLC method of analysis for curcumin for routine analysis, Reverse phase chromatography (RPC) was chosen because of its recommended use for ionic and moderate to non-polar compounds. Among the reverse phase columns, **the** C18 column was selected because of its wide applicability and more stability than short-chain bonded phases. Reversed-phase chromatography is not only simple, convenient, but performance is better in terms of efficiency, stability, and reproducibility. In addition to these, detection is easier, especially for UV detectors, due to the use of solvents. Also, RPC is nowadays the best starting point for the separation of a mixture of compounds.

Waters Spherisorb ODS2 column (4.6mm × 250mm, 5µm particle size) was used at 40ºC temperature as it gave desirable retention and symmetrical peak. The selection of the mobile phase was carried out such that it should be sufficiently transparent at the wavelength of detection, *i.e.*, minimum absorbance. The mobile phase that gives a sharp and well-defined peak with appropriate and significant retention value for curcumin was selected. Various mobile phases such as acetonitrile-methanol, methanol-chloroform, methanol-water, methanol- tetrahydrofuran, acetonitrile-water, and methanol-acetonitrile-glacial acetic acid were evaluated in different proportions. Among these, the solvent system comprising of methanol: water (77:33 v/v) at pH 3.0, gave good separation of curcumin from its matrix. It also gave a good resolution of analyte from excipients used in various formulations.

Analysis of the Drug: Melting point of curcumin was found to be 183 ℃, and it was freely soluble in methanol, chloroform, ethanol, and acetone but practically insoluble in water. Spectral scan – λ_{max} of curcumin was found to be at 419 nm. A representative scan and purity peak index of curcumin are shown in **Fig. 1** and **2**, respectively.

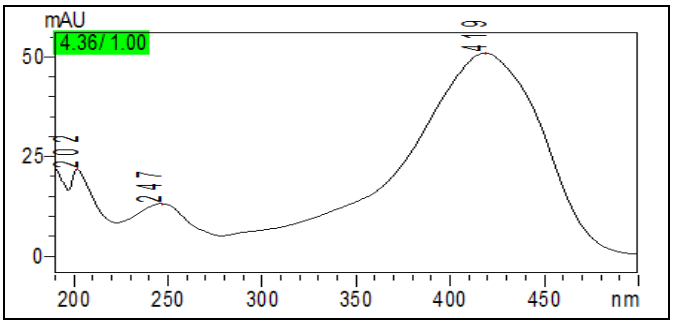

FIG. 1: SPECTRAL SCAN OF CURCUMIN ON HPLC

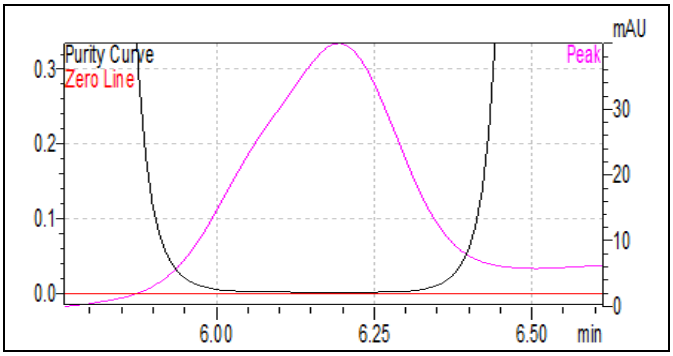

FIG. 2: PURITY PEAK INDEX OF CURCUMIN ON HPLC

A well defined chromatographic peak of Curcumin was exhibited with a retention time of 4.41 min **Fig. 3**. The flow rate of 1ml/min, gives an advantage for the less consumption of the mobile phase with good peaks.

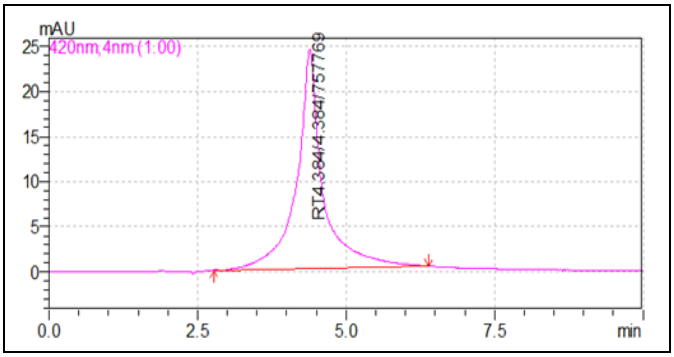

FIG. 3: CHROMATOGRAM OF CURCUMIN AT 5 µg/ml

Analytical Method Validation Parameters: The method was validated as per ICH guidelines (Q2 (R1).

Linearity and Range: Linearity of an analytical method is its ability, within a given range, to obtain test results that are directly, or through a mathematical transformation, proportional to the concentration of the analyte. Good linear correlations were obtained between peak and concentration in the selected range of 1 – 10 µg/ml. Characteristic parameters are Slope ± S.D. 0.1265 ± 0.15, Intercept ± S.D. 0.0174 ± 0.27, the regression coefficient of 0.9991 and correlation coefficient of 0.9995 between the standard drug concentration and corresponding mean absorbance show a good linearity of the standard curve.

Precision: The precision of an analytical method expresses the degree of scattering between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Intraday precision refers to the use of analytical procedures within a laboratory over a short period of time using the same operator with the same equipment, whereas Interday precision involves estimation of variations in analysis when a method is used within a laboratory on different days, by different analysts. Repeatability (intraday) was assessed by analyzing these three different Concentrations (2.5, 5.0, 7.5 µg/ml), three times a day. Intermediate precision (Interday) was established by analyzing these three different concentrations (2.5, 5.0, 7.5 µg/ml) three times a day for at least three different days **Table 1**.

As per ICH guidelines (Q2 R1), the relative standard deviation for each mixture was calculated. The method passed the test as the RSD was less than 2%.

TABLE 1: INTRA- AND INTERPRECISION STUDIES (*n* = 3)

Amount of drug injected (µg /ml)	Amount of drug detected (µg, mean ± SD)	%RSD
Intraday (*n* = 5)		
2.5	2.404 (0.029)	1.218
5	5.251 (0.071)	1.359
7.5	7.624 (0.059)	0.774
Interday (*n* = 5)		
2.5	2.410(0.037)	1.533
5	5.166 (0.021)	0.410
7.5	7.510 (0.147)	1.957

Accuracy: Accuracy of an analytical method is the closeness of test results to the true value. It was determined by the application of the analytical procedure to recovery studies, where a known amount of standard is spiked in pre-analyzed samples solutions. The % recovery for the standard addition analysis method for all the three concentration levels (2.5, 5.0, 7.5 µg/ml), ranged from 99.27 to 101.97 with a confidence interval ranging from ± 0.218 to ± 0.653 showing that any small change in the drug concentration can be accurately determined with high accuracy. The results obtained from the standard addition and reference analysis method ware also found supporting the accuracy of the proposed method.

Specificity: The sample was degraded by subjecting the drug to acid hydrolysis **Fig. 4**, alkali hydrolysis **Fig. 5**, oxidation **Fig. 6**, wet heat treatment **Fig. 7** and photodegradation **Fig. 8**. The drug undergoes degradation under former conditions. The results of forced degradation are summarized in **Table 2**.

TABLE 2: FORCE DEGRADATION STUDY OF CURCUMIN AND CURCUMIN COMPLEX IN DIFFERENT CONDITIONS

S. no.	Sample exposure condition	No. of degradation products (Rs [a] value)	Curcumin remained (µg/5µg) (±SD, n=4)	Recovery (%)
1	Curcumin-day light	3(0, 3.94, 2.25)	3.61(0.56)	72.2
2	Curcumin complex-day light	3(0, 3.01, 2.08)	3.52(0.94)	70.4
3	Curcumin-acid	2(0, 6.39)	4.57(0.67)	91.4
4	Curcumin complex-acid	2(0, 6.61)	4.42(0.59)	88.4
5	Curcumin-base	3(0, 4.15, 2.3)	3.64(0.78)	72.8
6	Curcumin complex-base	3(0, 4.07, 2.44)	3.51(0.45)	70.2
7	Curcumin-H_2O	2(0, 6.94)	3.54(0.54)	70.8
8	Curcumin complex- H_2O	2(0, 7.12)	3.52(0.39)	70.4
9	Curcumin- H_2O_2	2(0, 6.51)	4.36(0.74)	87.2
10	Curcumin complex- H_2O_2	2(0, 6.14)	4.17(0.85)	83.4

[a] *Rs* indicates Resolution (Rs = 2.0 used as a minimum to account for day to day variability, non-ideal peak shapes and differences in peak sizes.)

The presence of degraded products in the formulation does not interfere with the drug peak. Hence, the method is specific.

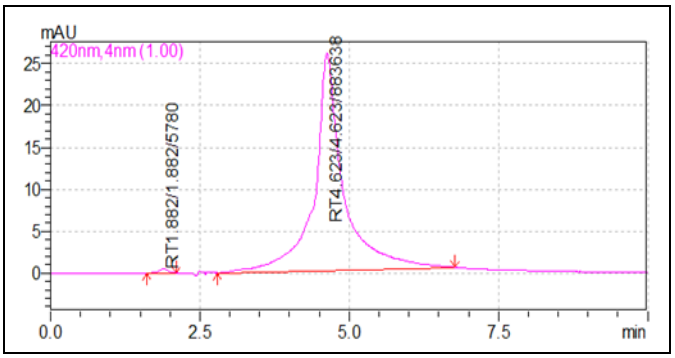

FIG. 4: CHROMATOGRAM OF ACID DEGRADATION PRODUCTS OF CURCUMIN

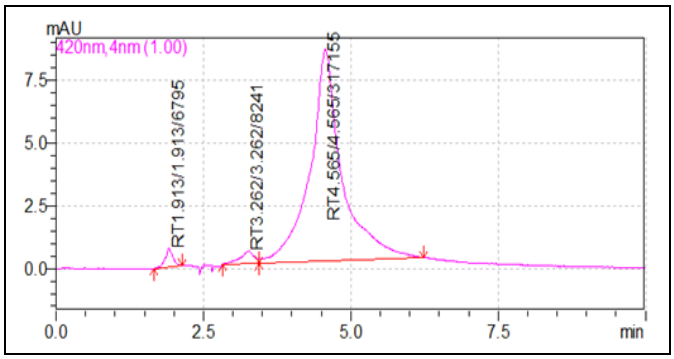

FIG. 5: CHROMATOGRAM OF BASE DEGRADATION PRODUCTS OF CURCUMIN

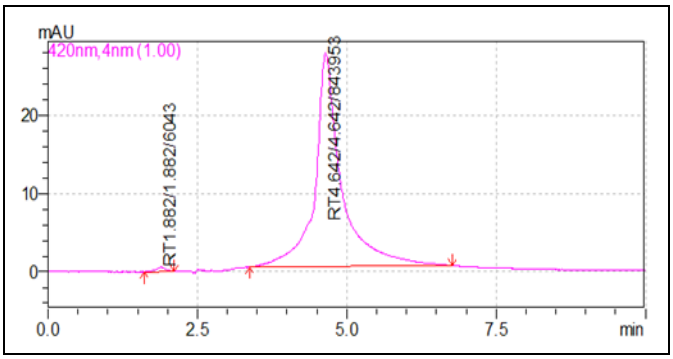

FIG. 6: CHROMATOGRAM OF H_2O_2 DEGRADATION PRODUCTS OF CURCUMIN

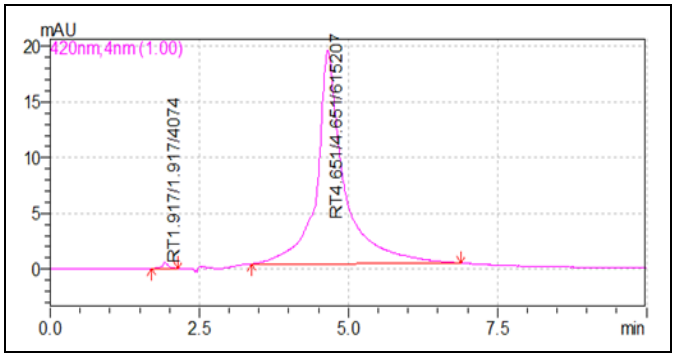

FIG. 7: CHROMATOGRAM OF H_2O DEGRADATION PRODUCTS OF CURCUMIN

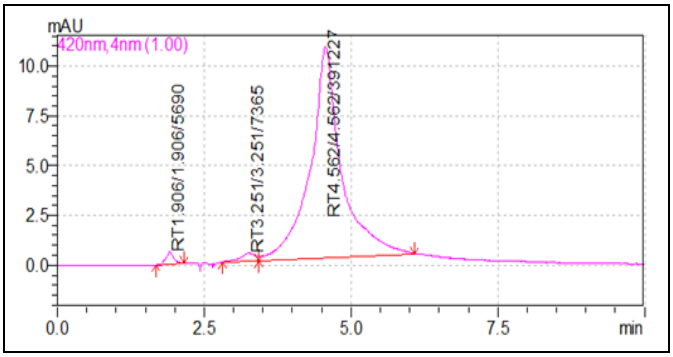

FIG. 8: CHROMATOGRAM OF LIGHT DEGRADATION PRODUCTS OF CURCUMIN

LOD/LOQ: Under the experimental conditions employed, LOD and LOQ were calculated according to the formulae:

$$LOD = 3.3\ \sigma / S = 0.092\ \mu g/ml$$

$$LOQ = 10\ \sigma / S = 0.278 \mu g/ml$$

Robustness: Slight changes in the flow rate (± 0.1ml/min), PH of the mobile phase (± 0.5) and column temperature (± 1nm) did not affect the chromatographic method so developed.

Application of the Validated HPLC Method on the Marketed Formulations: The marketed tablet formulations were analyzed by the proposed method. Results, presented in **Table 3**, showed that their % assay value ranging in between 99.39 to 99.75 with a maximum % confidence interval of ± 1.53.

TABLE 3: FORMULATION STUDY DATA FOR THREE DIFFERENT FORMULATIONS

S. no.	Brand Name	Amount labeled	Amount found	SD [a]	%RSD [b]	%Recovery
1	A	500	498.76	1.92	0.384	99.752
2	B	500	496.99	1.81	0.369	99.398
3	C	500	499.67	1.78	0.356	99.934

[a] SD indicates standard deviation & [b] % RSD indicates percentage relative standard deviation

System Suitability: All the values of parameter, i.e., precision (%RSD) of 1.32, the accuracy of 99.93%, theoretical plates/meter was 6963; tailing factor 1.13, and retention time 4.41 min of six replicate injections of system suitability was found to be within in the acceptable limits. It concluded that the method and systems are adequate for the analysis to be performed.

CONCLUSION: The analytical method developed for the first time on HPLC was simple, reliable, accurate, and reproducible. The method eliminates extraction steps, thus reduce analytical time, cost and minimize the extraction errors. Low cost, faster speed, satisfactory precision, and good specificity, to assess the analyte unequivocally in the presence of components, which may be expected to be present, are the main features of this method. The method was successfully validated as per ICH guidelines and can be conveniently employed for routine quality control analysis of curcumin in bulk drug, marketed tablets, and other formulations without any interference from excipients. The method was comparable to the existing methods in all respects, which analyze the drugs but in plasma.

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CONFLICTS OF INTEREST: The authors report no conflicts of interest.

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