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A New Stability-Indicating HPLC Method for Simultaneous Determination of Curcumin and Celecoxib at Single Wavelength: an Application to Nanoparticulate Formulation

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Abstract

A simple, precise, isocratic, reverse phase high performance liquid chromatography (HPLC) method was developed for the simultaneous determination of curcuminoids: viz. curcumin(C), desmethoxycurcumin (DMC), bisdesmethoxycurcumin (BDMC) and celecoxib at single wavelength using a Agilent RP C18, 4.6 mm × 150 mm, 5 µm XDB column. The run time was 18 min. The influence of mobile phase composition, injection volume, mobile phase pH, flow rate, temperature, and detector wavelength on resolution was investigated. The method was validated with respect to precision, accuracy, and linearity. The LOD and LOQ were found to be 0.3 and 1 µg/mL, respectively for curcumin and 0.03 and 0.1 µg/mL for respectively for celecoxib. Linearity range was from 1- 20 µg/mL for curcumin and from 0.1-2 µg/mL for celecoxib. Further, the proposed method was found to be reproducible and convenient for stability-indicating analysis of curcumin–celecoxib pH sensitive nanoparticles.

Keywords: High performance liquid chromatography; Method validation; Stability indicating; Curcumin; Celecoxib; Nanoparticles

Introduction

It is a well known fact curcumin; a coloring principle in turmeric has emerged as the most exploited phyto-constituents for its wide spectrum of biological activities. Mostly, it is available in the market as a mixture of three different constituents, commonly known as curcuminoids. These curcuminoids constitute majorly 75% curcumin (curcumin I), followed by 20% desmethoxycurcumin (curcumin II) and to smaller extent bisdesmethoxycurcumin (curcumin III, 5%) (Figure 1) [1]. Research over the last two decades has shown curcumin to be a potent antioxidant, anti-inflammatory, anti-proliferative, anti-metastatic, anti-angiogenic, anti-diabetic, hepatoprotective, antiatherosclerotic, anti-thrombotic, and anti-arthritic agent [2]. Structurally, curcumin is 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, desmethoxycurcumin is 1,6-Heptadiene-3,5-dione,1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl) and bisdesmethoxycurcumin is 1,7-bis (4-hydroxyphenyl) hepta-1,6-diene-3,5-dione (Figure 1) [1,3]. These three molecules show very small chemical modifications with respect to their number of methoxy groups (none for bisdesmethoxycurcumin, one for desmethoxycurcumin and two for curcumin) and the presence of methoxy groups in the ortho position on the aromatic ring, [4]. However, these modifications influence the hydrophobic nature of three curcuminoids which is in the order of curcumin > desmethoxycurcumin > bisdesmethoxycurcumin, with curcumin being the relatively most hydrophobic among the three curcuminoids.

Celecoxib, 4-[5-(4-methylphenyl)-3-trifluoromethyl-1H-pyrazol-1yl] benzene sulphonamide, is a 1, 5-diaryl-substituted pyrazole with a pK$_a$ of 11.1 (Figure 2). Celecoxib was the first specific inhibitor of cyclooxygenase-2 (COX-2) with little or no effect on the gastrointestinal (GI) tract and kidney. It has been recommended for the treatment of rheumatoid arthritis, and osteoarthritis for its anti-inflammatory activity. Besides this, it also exerts analgesic and antipyretic, and does not inhibit platelet aggregation. It has also been used for treatment of colon cancer, ultraviolet (UV) light-induced skin cancer and breast cancer [5,6].

Mechanistically, both curcumin and celecoxib utilize a different pathway to inhibit COX-2. It is believed that curcumin down-regulates COX-2 mRNA and protein levels, while celecoxib inhibits COX-2 activity directly by binding to its active site. Hence, the synergistic use of these drugs could augment the anti-inflammatory activity. This has

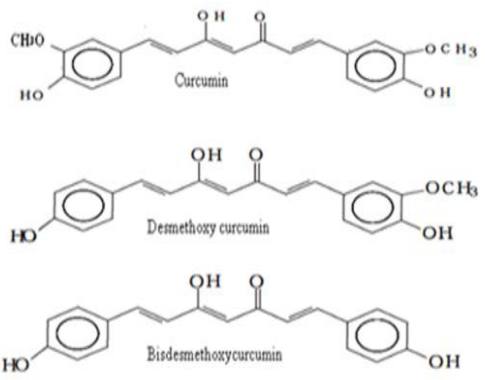

Figure 1: Chemical structure of curcuminoids: Curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

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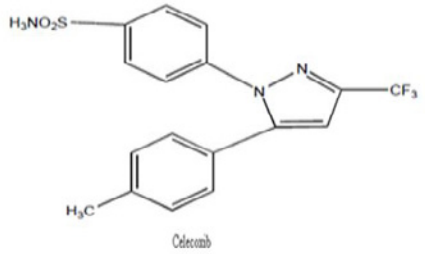

Figure 2: Chemical structure of celecoxib.

been demonstrated in osteoarthritis synovial adherent cells wherein curcumin synergistically enhances the growth inhibitory and pro-apoptotic effects of celecoxib [7].

Considering these benefits, it is worthwhile to explore the combination of both these drugs in treatment of inflammatory conditions. However, there is a dearth of suitable techniques for testing this combination in pharmaceutical dosage form. Although, there are many reported methods for the determination of curcumin [8–15] or celecoxib [16–22] in pharmaceutical dosage forms and biological fluids individually. The absence of suitable stability indicating method for the simultaneous determination of curcumin and celecoxib in presence of their degradants and their associated main impurities forms the underlying basis for this investigation.

To our knowledge, this is the first report of a stability indicating method for the simultaneous determination of both curcumin and celecoxib in nanoparticulate formulations. The present manuscript describes a simple, rapid, precise and accurate, reverse-phase stability-indicating HPLC method for the simultaneous determination of curcumin and celecoxib in the nanoparticulate formulation.

Experimental

Materials and reagents

Curcumin (95% purity) and Celecoxib (99% purity) were obtained as gift samples from Konark Herbals Ltd, India and Cadila Pharmaceuticals Ltd., Ahmedabad, India respectively. HPLC grade acetonitrile, acetic acid, and triethanolamine were purchased from s.d. fine Chemicals (Mumbai, India). 0.45 µm membranes were purchased from Pall Life Sciences. All other chemicals used were of analytical grade unless otherwise indicated.

Chromatographic system and conditions

The HPLC system consisted of Plus Intelligent LC pump PU-2080 from Jasco (Tokyo, Japan) equipped with a Jasco UV-2075 Intelligent UV/Vis detector and a Rheodyne 7725 injector (Rheodyne, Cotati, CA, USA). The output signal was monitored and processed using a Jasco Chroma Pass Chromatography Data System Software (Version 1.8.6.1). Chromatographic separation was achieved on a 5 µm Agilent RP C_{18} XDB column (4.6 mm × 150 mm). The mobile phase employed comprised of Solvent A: Water (1% acetic acid); adjusted to pH of 3.0 using 50% triethanolamine and Solvent B: Acetonitrile. Prior to use, water was filtered through a 0.45 µm filter membrane. Mobile phase was pumped through the column at the flow rate of 1.50 mL/min. The injection volume was 20 µL. The analytes were analyzed at single wavelength; 254 nm for curcumin, celecoxib and their associated degradation products.

Preparation of standard and sample solutions

Stock solutions of curcumin (200 µg/mL) and celecoxib (100 µg/mL) were separately prepared by dissolving accurately weighed 10 mg of curcumin and 5 mg of celecoxib in methanol using 50 mL volumetric flask. Standard solutions were prepared by dilution of the diluted stock solution with methanol to obtain solutions in the concentration range of 1-20 µg/mL and 0.1-2 µg/mL for curcumin and celecoxib respectively in 10 mL volumetric flask.

Forced degradation of curcumin and celecoxib

The standard solutions for the following studies were prepared by subjecting 1 mL of curcumin and 0.2 mL of celecoxib stock solutions (previously described) to various forced degradation conditions to provide an indication of the stability indicating property and specificity of the proposed method. Prior to analysis, the resultant mixture was diluted with methanol to give solutions containing curcumin and celecoxib in the concentrations of 20 and 2 µg/mL respectively.

Preparation of acid- and base-induced degradation product

To 1 mL of methanolic stock solution of curcumin and 0.2 mL of methanolic stock solution of celecoxib, 1 mL of 0.1 N HCl and 1 mL of 0.1 N NaOH were added separately. The acidic mixture was heated for 30 min at 80°C, and basic mixture was heated for 10 min at 80°C. Forced degradation in acidic and basic media was performed in 10 mL amber volumetric flasks in order to exclude the possible degradative effect due to light. The neutralized solutions were injected in triplicate and chromatograms were run as described.

Preparation of hydrogen peroxide-induced degradation product

To 1 mL of methanolic stock solution of curcumin and 0.2 mL of methanolic stock solution of celecoxib, 1mL of hydrogen peroxide (3% v/v, H_2O_2) was added. This solution mixture was heated for 1 hour at 80°C. Forced degradation was performed in 10 mL amber volumetric flask in order to exclude the possibility of light induced degradation. The final solution was injected in triplicate and chromatogram was run as described previously.

Photochemical degradation product

1 mL of methanolic stock solution and 0.2 mL of methanolic stock solution were diluted to 10 mL with methanol to give concentrations of 20 and 2 µg/mL of curcumin and celecoxib respectively, and photochemical stability of drugs was studied by exposing the stock solution to direct sunlight for 30 minutes.

Heat-induced degradation product

1 mL of methanolic stock solution of curcumin and 0.2 mL of methanolic stock solution of celecoxib were taken in 10 mL amber volumetric flask and heated for 1 hour in water bath maintained at 80°C to study the heat degradation. The resultant cooled mixture was diluted to 10 mL with methanol, injected in triplicate and the chromatogram was run as described previously.

Method Validation

Accuracy and precision

Accuracy and precision determinations were carried out at 0.2, 10, 20 µg/mL and 0.2, 1, 2 µg/mL of curcumin and celecoxib concentrations respectively. At each level of the amount, six determinations were

performed, both intraday and interday variation were expressed in terms of % RSD.

Robustness of the method

By introducing small changes in the mobile phase composition, injection volume, mobile phase pH, flow rate, temperature and detector wavelength, the effects on the results were examined. In mobile phase; composition of solvent B was changed by 1%, injection volume was changed from 20 µL to 50 µL, pH of buffer was varied from 3.0 to 3.3, flow rate was changed by 0.3 units, effect of column temperature was studied at 30°C and 40°C, and detector wavelength was changed by 5 nm. For most of the above conditions, the components of the mobile phase were held constant. Robustness of the method was studied in triplicate at a concentration level of 20 and 2 µg/mL for curcumin and celecoxib respectively [23].

Limit of detection and limit of quantification

LOD and LOQ, blank methanol was injected six times following the same method as explained previously. The LOD and LOQ for curcumin and celecoxib were estimated at S/N of 3:1 and 10:1, respectively.

Linearity

Solutions for curcumin and celecoxib were prepared by diluting the stock solution to the required concentrations. Solutions were prepared from LOQ to 1, 2, 5, 10, 15, 20 µg/mL and 0.1, 0.2, 0.5, 1, 1.5, 2 µg/mL for curcumin and celecoxib respectively. Calibration curves were drawn by plotting the peak areas of curcumin and celecoxib against the corresponding concentration. The slope and *Y*-intercept of the calibration curves were calculated.

Solution stability

The solution stability of curcumin and celecoxib was carried out by leaving a combined spiked sample (20 µg/mL is of curcumin and 2 µg/mL of celecoxib) solution in a tightly capped volumetric flask at room temperature for 8 hours. Content of curcumin and celecoxib were determined by following the procedure as described previously.

Analysis of curcumin and celecoxib from pH sensitive nanoparticles

To determine the content of curcumin and celecoxib in freeze dried pH sensitive nanoparticles (label claim: 100 mg of curcumin and 10 mg of celecoxib), nanoparticles equivalent to 5 mg of curcumin and 0.5 mg of celecoxib were accurately weighed and transferred to a volumetric flask containing 50 mL methanol. To ensure complete extraction of drug, it was sonicated for 10 min. The resulting solution was filtered through 0.45 µm membrane and analyzed for drug content. Twenty microlitres (corresponding to 20 and 2 µg/mL for curcumin and celecoxib respectively) of filtered solution was injected and chromatogram was run as described previously. The analysis was repeated in six individual steps and the possibility of excipient interference in the analysis was also studied.

Results and Discussion

Development of HPLC method

Initially methanol and water were tried in various ratios for each drug individually and in combination. Celecoxib showed splitting peak nature whereas curcumin was unable to show three separate peaks. Then methanol was replaced by acetonitrile and used in various ratios, with the result that splitting peaks were observed for both drugs. Then both the drugs were tried with combination of methanol and water (1% acetic acid; adjusted to pH of 3.0 using 50% triethanolamine) at various ratios, still curcumin was unable to separate into three peaks. Therefore, methanol was completely replaced with acetonitrile: water (1% acetic acid; adjusted to pH of 3.0 using 50% triethanolamine) in the ratio 45:55 (v/v) which exhibited good peak nature and peaks were found to be symmetrical at 254 nm. Tailing factor for both the drug peaks was less than 2% with good resolution (Figure 3).

The linear regression data for the calibration curves (n = 3) (Table 1) showed a good linear relationship over a concentration range 1- 20 µg/mL for curcumin and 0.1-2 µg/mL for celecoxib with respect to the peak area at 254 nm. No significant difference was observed in the slopes of standard curves (ANOVA, $P < 0.0001$).

Forced degradation of curcumin and celecoxib

The chromatograms of samples subjected to various forced degradation conditions showed well separated chromatograms of pure curcumin (C), desmethoxycurcumin (DMC), bisdesmethoxycurcumin (BDMC), as well as some degradation peaks at different retention time, and celecoxib was found to be stable when subjected to various forced degradation conditions, as shown in (Figure 4-8). The number of degradation products, drug content of curcumin, and percentage recovery were calculated and are listed in Table 2.

Acid- induced degradation product

In addition to the curcuminoids peaks, the chromatogram of acid degraded sample of curcumin showed three additional peaks at 0.96, 1.24 and 1.35 min. Post 30 min heating on water bath, the areas of the

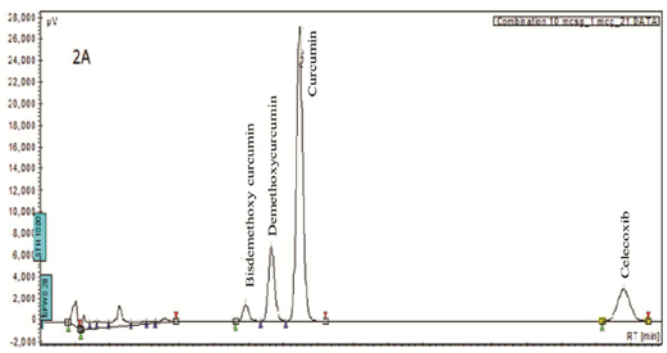

Figure 3: Chromatogram of curcumin 95% (20 µg/mL) and celecoxib (2 µg/mL) at λmax- 254 nm.

Parameter	Curcumin	Celecoxib
Linearity (µg/mL)	1-20	0.1 – 2
Slope	303.4 ± 4.898	702.6 ± 10.85
Y-intercept when X = 0.0	-43.52 ± 54.94	-1.790 ± 12.17
X-intercept when Y = 0.0	0.1434	0.002547
1/slope	0.003296	0.001423
95% Confidence Limit		
Slope	289.8 to 317.0	672.5 to 732.7
Y-intercept when X = 0.0	289.8 to 317.0	35.58 to 32.00
X-intercept when Y = 0.0	-0.3725 to 0.6244	0.04716 to 0.04901
R^2	0.999	0.999
P value	< 0.0001	< 0.0001

Table 1: Linear regression data for calibration curve (n=3).

acid degraded product peaks were found to be higher than the area of standard curcumin concentration (20 µg/mL) indicating that curcumin undergoes significant degradation under mild acidic conditions (0.1 N HCl) and celecoxib was found to be stable (Figure 4). Drug recovery of acid-degradation product is given in Table 2.

Base-induced degradation product

The chromatogram of base degraded sample showed additional peaks at 0.96, 1.26, and 1.36 min. The presence of additional degradation peaks at mild conditions (0.1N NaOH, 10 min exposure) reconfirmed the vulnerability of curcumin to degradation in basic conditions and celecoxib was found to be stable (Figure 5). Drug recovery of base degradation product is given in Table 2.

Hydrogen peroxide-induced degradation product

The chromatogram of curcumin treated with H_2O_2 (3% v/v) showed additional peaks at 1.06 and 1.35 min and celecoxib was stable under this condition (Figure 6). Drug recovery of oxidation degradation product is given in Table 2.

Photochemical degradation product

The chromatogram of curcumin exposed to photochemical degradation (sunlight for 30 min) showed additional peaks at 1.008 and 1.25 min. This indicates that the drug is unstable towards photochemical radiations for the exposure period under study and celecoxib was found to be stable towards light degradation (Figure 7). Drug recovery of photochemical degradation product is given in Table 2.

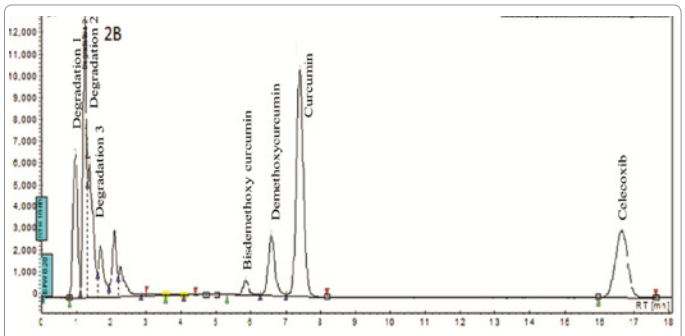

Figure 4: Chromatogram of acid (0.1 N HCl) treated curcumin (95%) and celecoxib.

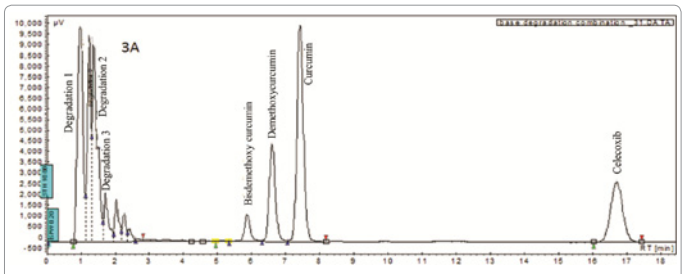

Figure 5: Chromatogram of base (0.1 N NaOH) treated curcumin (95%) and celecoxib.

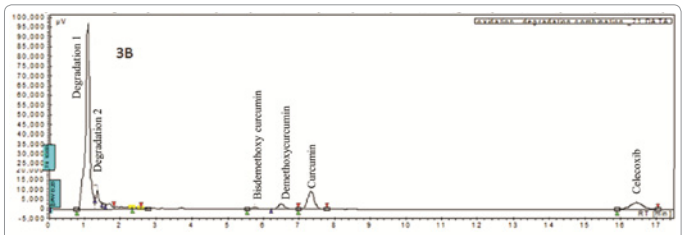

Figure 6: Chromatogram of oxidation (3% v/v H_2O_2), treated curcumin (95%) and celecoxib.

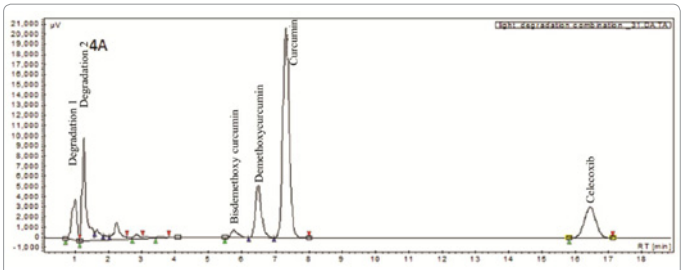

Figure 7: Chromatogram of photo degradation treated curcumin (95%) and celecoxib.

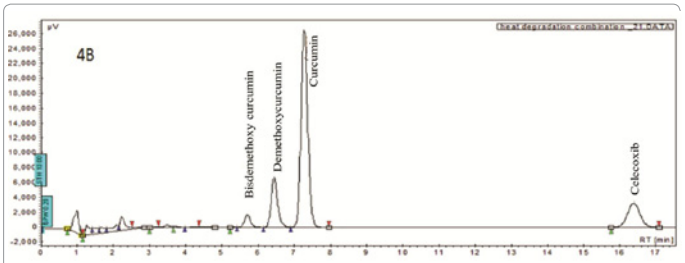

Figure 8: Chromatogram of heat degradation treated curcumin (95%) and celecoxib.

Heat degradation product

The samples subjected to heat (1 hour water bath at 80°C) conditions showed no additional peak for curcumin and celecoxib. This indicates that drugs were stable towards heat for the exposure period under study (Figure 8). Drug recovery of heat degradation product is given in Table 2.

Method Validation

Accuracy and precision

The accuracy and precision of the method were determined by spiking known amount of combined solution of curcumin and celecoxib in triplicate at low, medium and high levels of the specified limit. The measurement of the peak area showed low values of % RSD (< 2) for inter and intraday variation, which suggested an excellent accuracy and precision of the method (Table 3-6).

Robustness of the method

% RSD of peak areas was calculated for change in mobile phase composition, injection volume, mobile phase pH, flow rate, composition of Solvent B was changed by 1%, temperature, and detector wavelength at a combined concentration level of 20 and 2 µg/mL for curcumin and celecoxib respectively in triplicate. The symmetry (< 2), asymmetry (> 2) and % RSD (< 2), obtained after introducing small deliberate changes in the developed HPLC method indicated the robustness of the method.

Limit of detection and limit of quantification

LOD and LOQ were calculated by the method as described previously, and were found to be 0.3 and 1 µg/mL, and 0.03 and 0.1 µg/

	Curcumin					Celecoxib
Sr. No	Exposure condition	Time (h)	Drug remaining (µg/mL)	% of drug remaining	% RSD	Stability of celecoxib under subjected conditions
1	Photo degradation	0.5	15.33	76.65	0.82	Stable
2	Oxidation	1.0	6.60	33.00	1.25	Stable
3	Base degradation	0.6	7.60	38.01	0.69	Stable
4	Acid degradation	1.0	8.058	40.29	0.089	Stable
5	Heat degradation	1.0	20.04	100.203	1.69	Stable

Table 2: Forced degradation studies of curcumin (95 %) and celecoxib.

	Inter-day accuracy			Intra-day accuracy		
Amount of added drug (µg/mL)	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD
2	1.98	98.33	1.00	2.049	102.45	0.480
10	9.99	100.92	0.842	9.60	96.00	0.456
20	20.1	100.15	20.25	20.25	101.25	0.263

Table 3: Intra and inter-day accuracy and recovery studies of HPLC method (n=6): Curcumin.

				Inter - day precision Intra- day precision		
Amount of drug added (µg/mL)	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD
2	1.97	97.30	1.91	2.057	102.85	1.185
10	9.98	99.37	0.969	9.83	98.3	1.361
20	20.01	100.42	0.159	20.07	100.35	0.270

Table 4: Intra and inter-day precision and recovery studies of HPLC method (n=6): Curcumin.

Inter-day accuracy				Intra-day accuracy		
Amount of drug added (µg/mL)	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD
0.2	0.192	96.37	0.154	0.2	100.09	1.4
01	1.02	102.02	1.07	0.99	99.69	1.6
02	1.97	98.92	0.986	2.01	100.99	0.59

Table 5: Intra and inter-day accuracy and recovery studies of HPLC method (n=6): Celecoxib.

Intra-day precision				Inter-day precision		
Amount of drug added (µg/mL)	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD	Amount of drug remaining (µg/mL)	% of drug recovered	% RSD
0.2	0.192	96.22	0.25	0.198	99.00	0.67
01	1.004	100.42	0.63	1.06	106.00	0.254
02	1.970	98.97	0.241	1.98	99.00	0.18

Table 6: Intra-and inter-day precision and recovery studies of HPLC method (n=6): Celecoxib.

mL for curcumin and celecoxib respectively, which indicates adequate sensitivity of the method.

Solution stability

% RSD for combined solution of curcumin and celecoxib concentrations during solution stability experiments was within 1%. No significant changes were observed for the chromatograms of standard solution and the experimental solution. Further, absence of degradation peaks confirmed that the sample is stable in solvent used during the assay for 8 hours.

Analysis of curcumin and celecoxib from pH sensitive nanoparticles

In the chromatogram of curcumin-celecoxib samples extracted

from pH sensitive nanoparticles, separate peaks were observed corresponding to the three curcuminoids and celecoxib. There was no interference from the excipients commonly present in nanoparticles. The drug content was found to be 99.18 and 99.01% with a % RSD of 0.017 and 0.028 respectively for curcumin and celecoxib. It may therefore be inferred that degradation of curcumin and celecoxib did not occur in the formulations that were analyzed by this method. The low % RSD value indicated the suitability of this method for routine analysis of curcumin- celecoxib in pharmaceutical dosage forms.

Conclusions

The developed HPLC technique is precise, specific, accurate and stability- indicating for the determination of curcumin (95%) and celecoxib. Statistical analysis also proves that the method is reproducible and specific for the analysis of curcumin along with its co-purified derivatives (desmethoxycurcumin and bisdesmethoxycurcumin) and celecoxib. This method is versatile in separate analysis of curcumin and celecoxib in pH sensitive nanoparticles.

Acknowledgments

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