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METHOD DEVELOPMENT, VALIDATION AND OPTIMIZATION FOR SIMULTANEOUS ESTIMATION OF CELECOXIB AND PIPERINE BY RP-HPLC

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Abstract:

It is essential to study the stability of celecoxib in presence of piperine. The stability analysis of the drugs individually (celecoxib/piperine) and in combination (celecoxib with piperine) was carried out at different pH conditions (4.5 and 7.4 pH). Reverse Phase High Performance liquid chromatography method was developed for celecoxib and piperine in combined form. The method was optimized and developed using the design of experiments (DOE). For the optimization, the Design expert trial version 13 was used. The developed method was analyzed using Zodiac C-8 Luna, 5 µm, 100Å, and 150 mm column and methanol: acetonitrile (30:70) with 0.1% of trifluoroacetic acid as mobile phase (1 ml/minute, flow rate) and detected at a wavelength of 342 nm for piperine and 253 nm for celecoxib. Retention time of piperine and celecoxib was found to be 4.751 and 6.685 minutes respectively. The correlation coefficient of both drugs was found to be 0.999. The accuracy of piperine was found to be 99.07-100.61% whereas for celecoxib, it was 99.90-100.31. Overall % RSD was found to be less than 2%. Results showed that the celecoxib stability decreases slightly in the presence of piperine. The method was validated according to the International Conference on Harmonization guidelines and found to be reproducible with peaks showing good resolution with short retention time and can be used for simultaneous estimation of of celecoxib and piperine.

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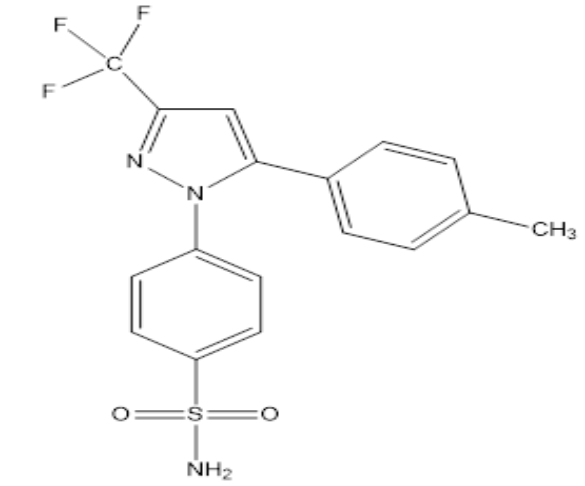
1. INTRODUCTION:

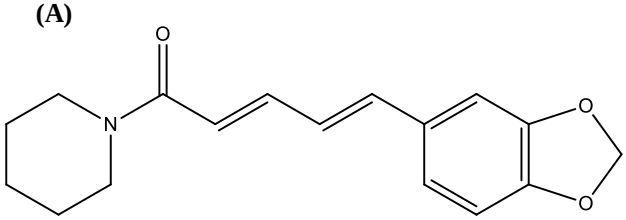
Celecoxib (CXB), a selective COX-2 inhibitor NSAID, has exhibited prominent anti inflammatory and anti-proliferative potential against numerous cancers. However, its low bioavailability and long term exposure related cardiovascular side effects, limit its clinical application. In order to overcome these limitations, natural bioactive compounds with lower toxicity profile are used in combination with therapeutic drugs. Therfore, in this study Piperine (PIP), a natural chemo-preventive agent possessing drug bioavailability enhancing properties, was considered to be used in combination with low doses of CXB.

The study results says that PIP as a bioenhancer increased the oral bioavailability of CXB (129%). The IC50 of CXB and PIP were evaluated to select doses for combination treatment of HT-29 cells. The drug combinations having combination index (CI) less than 1 were screened using CompuSyn software. These combinations were significantly cytotoxic to HT-29 cells but IEC-6 were least effected. Further, the mechanism behind CXB and PIP mediated cell death was explored. The co-treatment led to reactive oxygen species generation, mitochondrial dysfunction, caspase activation and enhanced apoptosis in HT-29 cells. Additionally, the combination treatment synergistically modulated Wnt/β-catenin pathway, downregulated the stemness markers and boosted therapeutic response in CT26 syngeneic Balb/c mice (Srivastava et al., (2021).
Testing the stability of the particular drug in the presence of other drugs plays a major role in the combination of drug treatment. The main aim of performing stability analysis is to analyze the quality of the drug and their degradation pattern in the presence of other drugs, with respect to time, temperature, and at different pH conditions. Analyzing the stability of the drugs in combined form gives us an insight into the treatment period, storage conditions, and shelf life. Many stability indicating methods have been reported in the literature for various combinations of drugs but none of the literature has reported the stability analysis of celecoxib and piperine in a combined form through Reverse Phase High Performance liquid chromatography (RP-HPLC).

In the present study, an attempt has been made to develop and validate a method for the simultaneous estimation of celecoxib and piperine, It can also be applied for routine analysis of either one or of any combinations of these drugs in dosage forms.



(A)



(B)

Fig 1: Structure of (A) Celecoxib (B) Piperine

2. MATERIAL AND METHODS:

Instruments

UV visible spectrophotometer (UV-Shimadzu 1800) was used and HPLC of Shimadzu SCL-10A$_{VP}$ inbuilt with binary pump (LC-10AT$_{VP}$) and UV detector (SPD-10A$_{VP}$), Rheodyne 20µl loop capacity manual injector (P/N 77251) was used throughout the analysis. The LC-Solution software was used to interpret the HPLC reports. Zodiac C8 (5µm; 150 x 4.6 mm ID.) column was purchased from Zodiac life sciences. (Wardha, India) and was used throughout the analysis. Digital weighing balance (ME-204) purchased from Mettler-Toledo (USA), Ultra-sonicator Labman® purchased from UltraChrom Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50 µ micro-syringe was purchased from Hamilton USA. 0.20µ and 0.45µ nylon membrane filters were purchased from Phenomenex® Mumbai, India.

Materials and reagents

Celecoxib was purchased from Yarrow Chem Pvt Ltd, while the piperine was purchased from Biomed Ingredients Pvt Ltd, Goa. Polyvinyl alcohol (PVA, Mw = 30,000–70,000) and trifluoroacetic acid was supplied by S.D Fine Chem, Mumbai. HPLC-grade

methanol, acetonitrile, ethanol, acetone, demineralized Milli-Q® water were obtained from Merck lifesciences Pvt Ltd. UV visible spectrophotometer (UV-Shimadzu 1800), FTIR (Shimadzu), Sonicator (9L250H, PCI) were used.

Preparation of standard stock solutions

Standard stock solutions of celecoxib and piperine were prepared separately by transferring 10 mg of drug in a 10 ml volumetric flask. Volume was made upto 10 ml mark by using diluent to obtain 1000µg/ml standard stock solution. Pipette out 0.5 ml from standard stock solution and dilute it upto 10 ml mark to obtain 50µg/ml of ceecoxib and 50µg/ml of piperine solution. Further concentrations were made by serial dilution.

3. OPTIMIZATION AND METHOD DEVELOPMENT

Software and statistical data analysis

The chromatographs were processed using Shimadzu's LC Solutions software. Design expert 13 trial version was used to design the experiments. Percentage relative standard deviation (%RSD) and the linear regression analysis were calculated using the method of least squares.

Method development considerations

To develop an RP-HPLC method, the physicochemical characteristics of the analytes (solubility, polarity, wavelength, etc.) were studied. Two stationary phases (C8 & C18) were kept under for consideration, stationary phase was chosen as C8 as it gave better separation between two peaks of celecoxib and piperine.

Chromatographic conditions

The mobile phase used for this study was methanol and acetonitrile (organic phase 30:70) and MilliQ water with 0.1% of trifluoroacetic acid (aqueous phase) . The solvents used for the analysis were filtered using a 0.45 µm membrane filter and degassed through an ultra-bath sonicator. Phenomenex Luna Zodiac C8, 4.6 × 150 mm, 5 µm, 100A◦ was used as the stationary phase. The analysis was carried out in gradient conditions with 1 ml min^{-1} flow rate with an injection volume of 20 µl. The wavelengths of the detectors used were 253 nm and 342 nm for celecoxib and piperine, respectively.

Design of experiment

The optimization of the RP-HPLC method was implemented using Box-Behnken design. Four parameters (Independent variables) concentration (%) of organic phase (A), flow rate (ml/min) (B), (%) of Buffer strength (C) and Column temp (D) at three different levels low (−1), medium (0), and high (+1) were implemented to find the optimum combination and is represented in Table 3. The design comprised of fifteen experimental runs, a standard concentration (celecoxib and piperine) 50 µg/ml was used for all fifteen experimental runs, which were analyzed for the BBD method. The chromatographic responses like retention time of piperine [Rt (PIP)], the retention time of celecoxib [Rt (CEL)], the peak area of piperine (PIP), the peak area of celecoxib (CEL), no. of theoreotical plates of piperine and celecoxib, tailing factor of piperine and celecoxib were studied and shown in Table 4.

Table 1. Box-Behnken design table incorporated with independent experimental variables and levels (coded)

Variables	Levels		
Independent	**-1**	**0**	**+1**
A- % organic phase (%)	45	50	55
B-Buffer strength (%)	0.05	0.1	0.15
C-Flow rate (ml/min)	0.8	1	1.2
D-Column temp. (^{0}C)	25	26	27

Dependent

Rt (PIP) (min) = Retention time piperine.
Rt (CEL) (min) = Retention time celecoxib.
Peak area (PIP) = Peak height of piperine.
Peak area (CEL) = Peak height of celecoxib.
T. plate (PIP) = Theoretical plate of piperine.
T. plate (CEL) = Theoretical plate of celecoxib.
Tailing factor (PIP) = Tailing factor of piperine.
Tailing factor (CEL) = Tailing factor of celecoxib.

Table 2: Various responses analyzed to obtain desired optimized condition.

Run	Resp 1: Rt (PIP) (min)	Resp 2: Rt (CEL) (min)	Resp 3: Peak area (PIP)	Resp 4: Peak area (CEL)	Resp 5: T. Plate (PIP)	Resp 6: T. Plate (CEL)	Resp 7: Tailing factor (PIP)	Resp 8: Tailing factor (CEL)
1	9.07	14.21	9156493	3112686	4438	9278	1.26	1.17
2	10.76	13.85	8967294	2745989	4362	9312	1.25	1.16
3	4.78	6.69	10358732	4002584	4834	9745	1.33	1.20
4	6.16	9.24	11267541	3657834	4852	9786	1.32	1.21
5	4.78	6.83	10257672	4173671	4666	9578	1.31	1.20
6	9.78	12.82	8941834	2767342	4889	9794	1.34	1.21
7	4.68	6.74	14563519	5854354	4904	9746	1.33	1.22
8	4.81	6.78	10467244	4267192	4682	9548	1.32	1.20
9	4.66	6.89	14249371	6046528	4429	9327	1.26	1.15
10	4.75	6.68	10358724	4002577	4678	9564	1.32	1.19
11	6.17	9.31	11134854	3678523	4384	9574	1.25	1.18
12	9.11	14.17	9171563	3153174	4472	9292	1.27	1.16
13	9.81	12.78	9013285	2889246	4918	9746	1.35	1.22
14	4.69	6.92	14178458	5935724	4384	9278	1.25	1.16
15	6.47	11.17	8983436	2989361	4856	9804	1.33	1.23

After 15 runs we found that the Zodiac C8 (5µ, 150 X 4.6mm. ID.) column type gave desirable results. To getting this appropriate optimized condition, the Design expert trial version-13 was used. We found that the run no.10 showed good resolution and minimum retention time and other parameters within limit. Therefore it was selected as optimized condition. Further we proceeded for the validation of the optimized condition.

4. OPTIMIZATION AND MODEL VALIDATION

Design expert 13 trial version was used for optimization and model validation. The quadratic polynomial equations were framed from the statistical significance of coefficients of both the main effects and interaction effects. The aptness of the designed model was validated by analyzing parameters like the coefficient of correlation (R^2), adjusted (R^2), and adequate precision. The possible interaction effect of the chosen factors was studied from the response surface plot, contour plot, and perturbation plot. The optimum chromatographic conditions were selected based on short analysis time, peak elution time being in range, and percentage area of the peak. Derringer's desirability function and Design space plot was carried out to show the optimum chromatographic conditions.

Validation of the method

The RP-HPLC method developed for this study was validated according to the ICH guidelines (ICH, 2005).

Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. Nine injections of test drug solutions at different level (80%, 100%, 120%) were injected and peak area, retention time was recorded. Mean peak area and % RSD was calculated. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at three concentration levels of 80%, 100% and 120%. The solutions were analyzed in triplicate at each level as per the proposed method.

Linearity

ICH states linearity as the ability to acquire test results of the dependent variable data being directly proportional to the sample concentration. It is mandatory to analyze within an appropriate range for which the response of the instrument should be proportional to the concentration of the drug. Normally, the value of co-relation coefficient (R^2) > 0.998 is acceptable. The standard solutions of the combination of drugs piperine and celecoxib were prepared by diluting the stock solution (1 mg/ml) with methanol. The concentration of standard solutions ranged from 1.56 to 50 µg/ml were analyzed and plotted with peak area value with respect to the concentration of the drug. The linearity was estimated by linear regression analysis which was calculated through the method of least squares.

Precision

Precision is estimated normally with three parameters: repeatability, intermediate precision, and

reproducibility. ICH allows exclusion of intermediate precision, provided if the results of reproducibility are proven very well. 50 µg/ml solution was used to perform precision studies.

Specificity

ICH defines specificity as the efficiency to measure unequivocally the analyte even in the presence of other components. It includes comparison of retention time obtained for Celecoxib and Piperine peaks in standard solution and test solution.

Limit of detection and limit of quantitation

Limit of detection defines the ability to detect the lowest concentration of the analyte from the sample where quantitation is not needed under certain conditions. Limit of quantitation deals with the quantitation of the lowest concentration of the analyte in the sample with precision and accuracy. It is estimated through the slope and SD of the response. The method was validated by analyzing 6 replicates of a combination of celecoxib and piperine drug standard (50 µg/ml) and the Limit of Detection (LOD) and Limit of Quantitation (LOQ) was calculated.

LOD = 3.3 σ/s and LOQ = 10 σ/s

where σ = the SD of the response & S = the slope.

Robustness

Robustness of the method was carried out by deliberately made small changes in the flow rate, wavelength and effect of concentration using 50µg/ml standard solutions.

i. Flow rate (+ 0.1ml /min) i.e, 1.1 ml/min
ii. Flow rate (- 0.1ml /min) i.e, 0.9 ml/min
iii. Effect of solvent concentration (+ 2 %) i.e, 52%
iv. Effect of solvent concentration (- 2 %) i.e, 48%
v. Change in wavelength (+2 nm) i.e, 255 nm & 344 nm
vi. Change in wavelength (-2 nm) i.e, 251 nm & 340 nm

Combination of celecoxib and piperine stability analysis

About 1 mg of both the drugs were weighed accurately and dissolved in 1 ml of Phosphate Buffer saline (PBS) (4.5 & 7.4 pH) and kept in a rotating shaker for 24 hours at 37°C. The samples were centrifuged at 10,000 rpm and the supernatant was taken as samples and they were analyzed through RP-HPLC using the validated method for a time period of 48 hours.

5. RESULT AND DISCUSSION:

Identification of Standard Drug by FTIR spectra

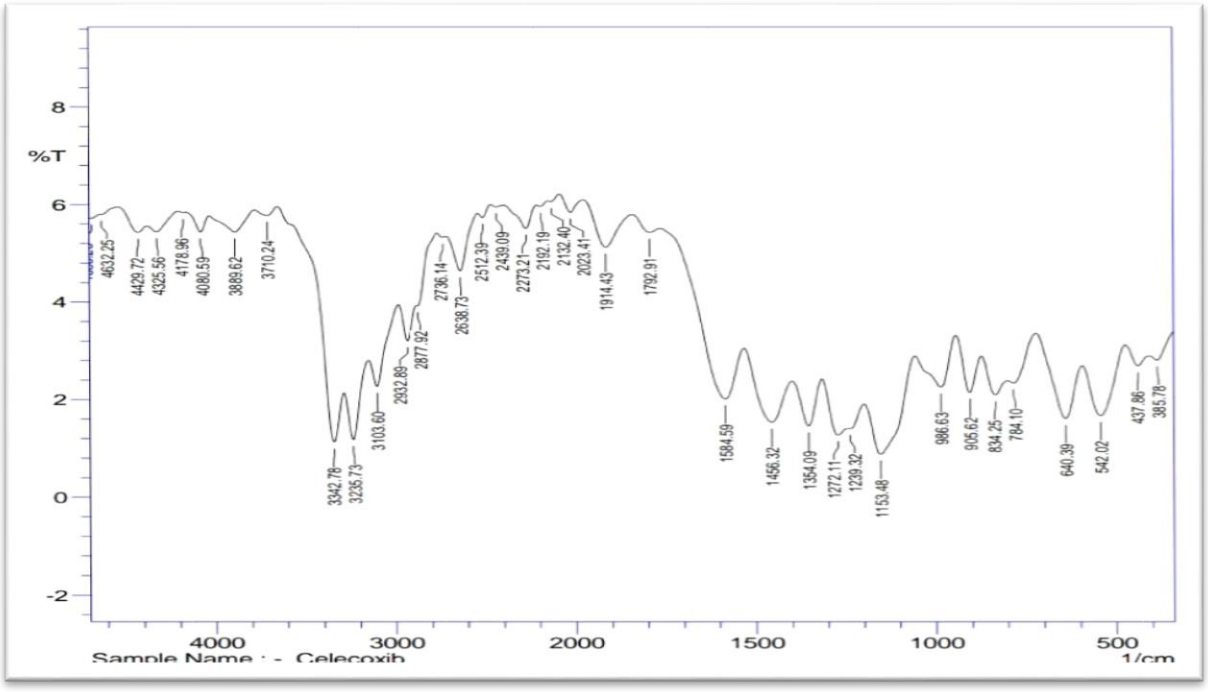


Fig 2: FTIR spectra of standard celecoxib

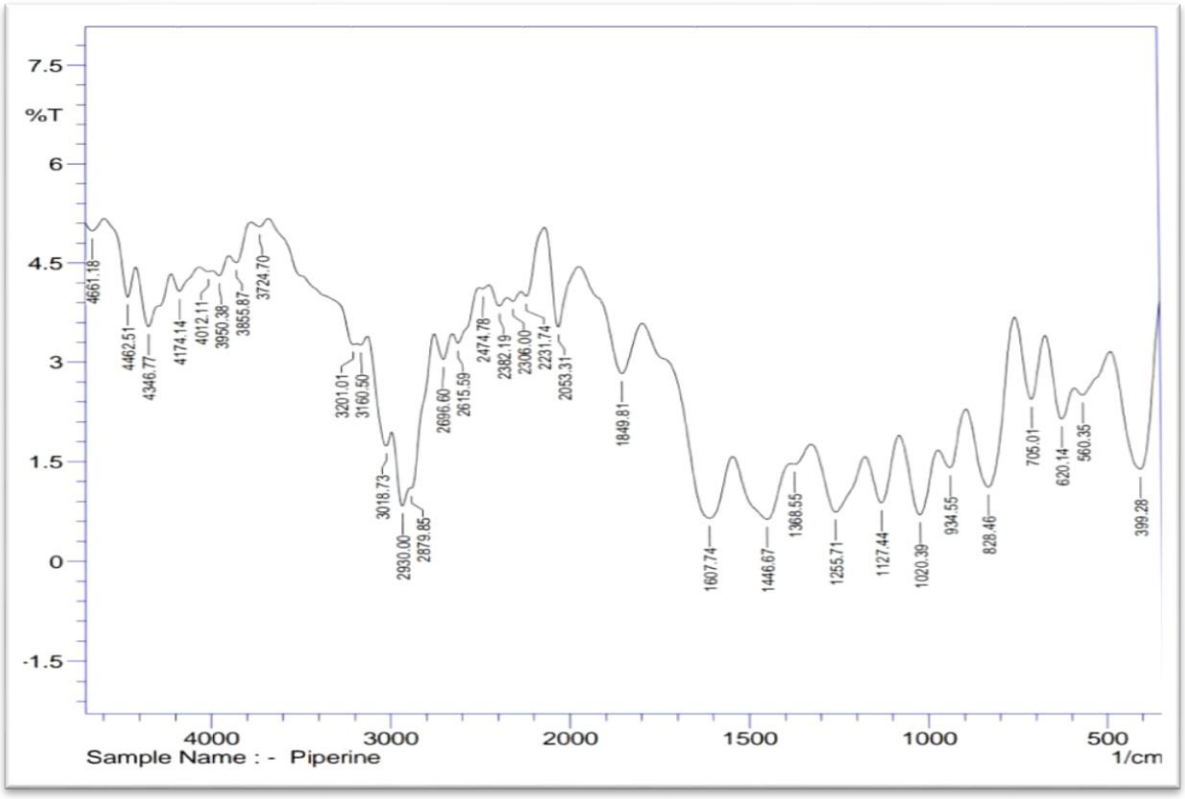


Fig 3: FTIR spectra of standard piperine.

We observed the various peaks of different wave number on FTIR spectra of celecoxib. We found the functional group like C=C, C-N, C-F, N-H, C-H showing stetching vibrations and C-H group with bending vibrations. We found that the required functional groups were present in the standard drug sample of celecoxib.

We also observed the various peaks of different wave number on FTIR spectra of piperine. We found the functional group like C=C, C-N, C=O, C-O, C-H, =C-H showing stetching vibrations. We observed that the required functional groups were present in the standard drug sample of piperine.

Determination of λ_{max} by UV

After serial dilution of the standard stock solution (1000µg/ml), the 10µg/ml solution was analyzed on UV spectrometer, the λ_{max} of celecoxib was found at 253 nm while the λ_{max} of piperine was found at 342 nm.

Solubility of standard drug

To confirm the ingredients of the nanoformulation, the solubility of drugs in different types of solid lipids, liquid lipids and surfactants was checked. The solubility of piperine and celecoxib was found in methanol, acetonitrile and ethanol,etc.

Simultaneous estimation of celecoxib and piperine RP HPLC method optimization and validation

The total fifteen experimental runs designed using BBD were analyzed for their chromatographic response. The interaction effect of the independent variables on chromatographic response is studied using three-dimensional response surface plots. The probability p lies below < 0.05 for most of the chromatographic responses and the correlation coefficient and adjusted R2 values were found to be high which infers the model chosen is significant. Figure 4 shows the effect of the organic phase and flow rate, Figure 5 shows the effect of the flow rate and buffer strength.

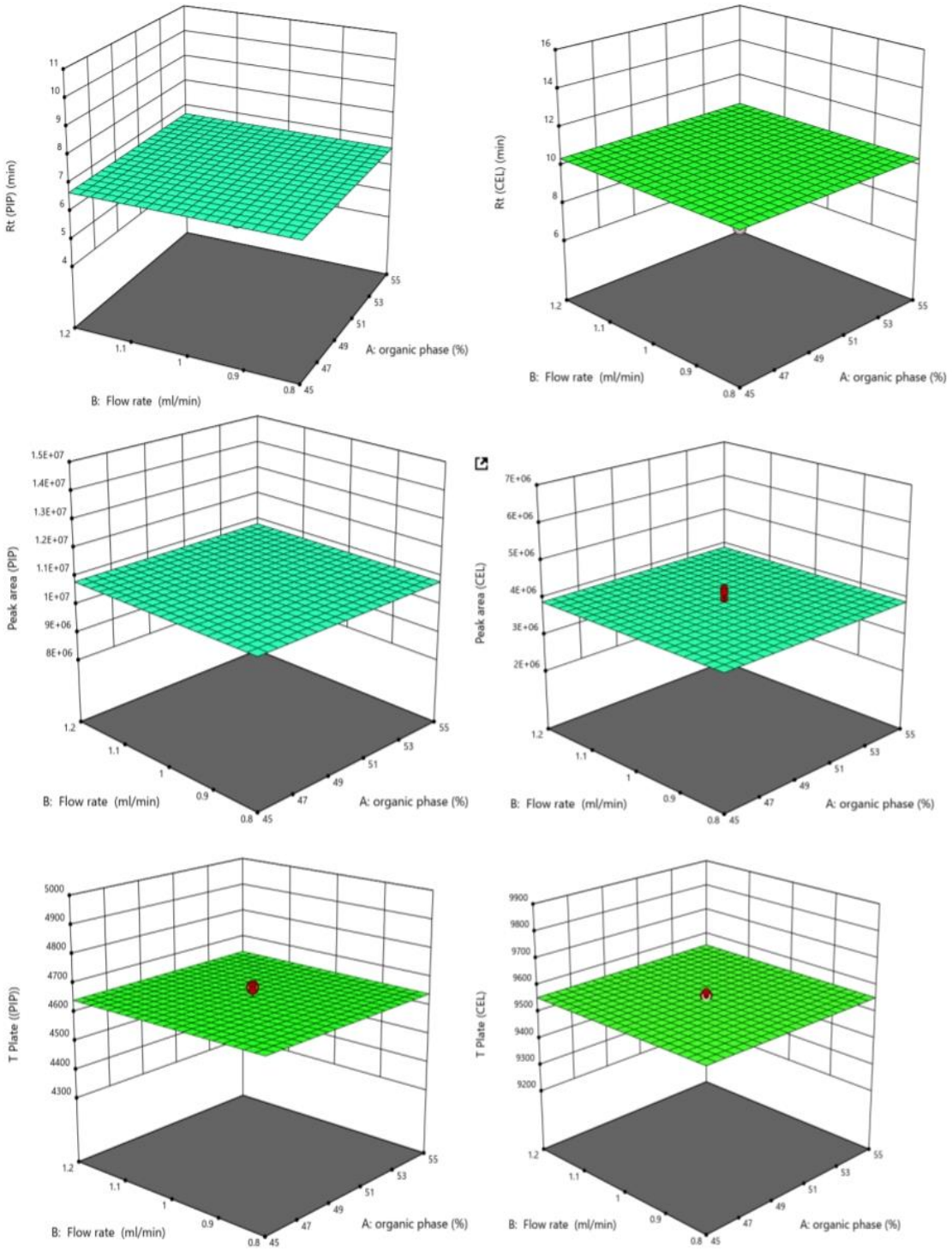


Fig 4: Response surface plots showing the impact of (A) organic phase(%) and (B) Flow rate(ml/min) on Rt(PIP), Rt(CEL), Peak area(PIP), Peak area(CEL), T.Plate(PIP), T.Plate(CEL).

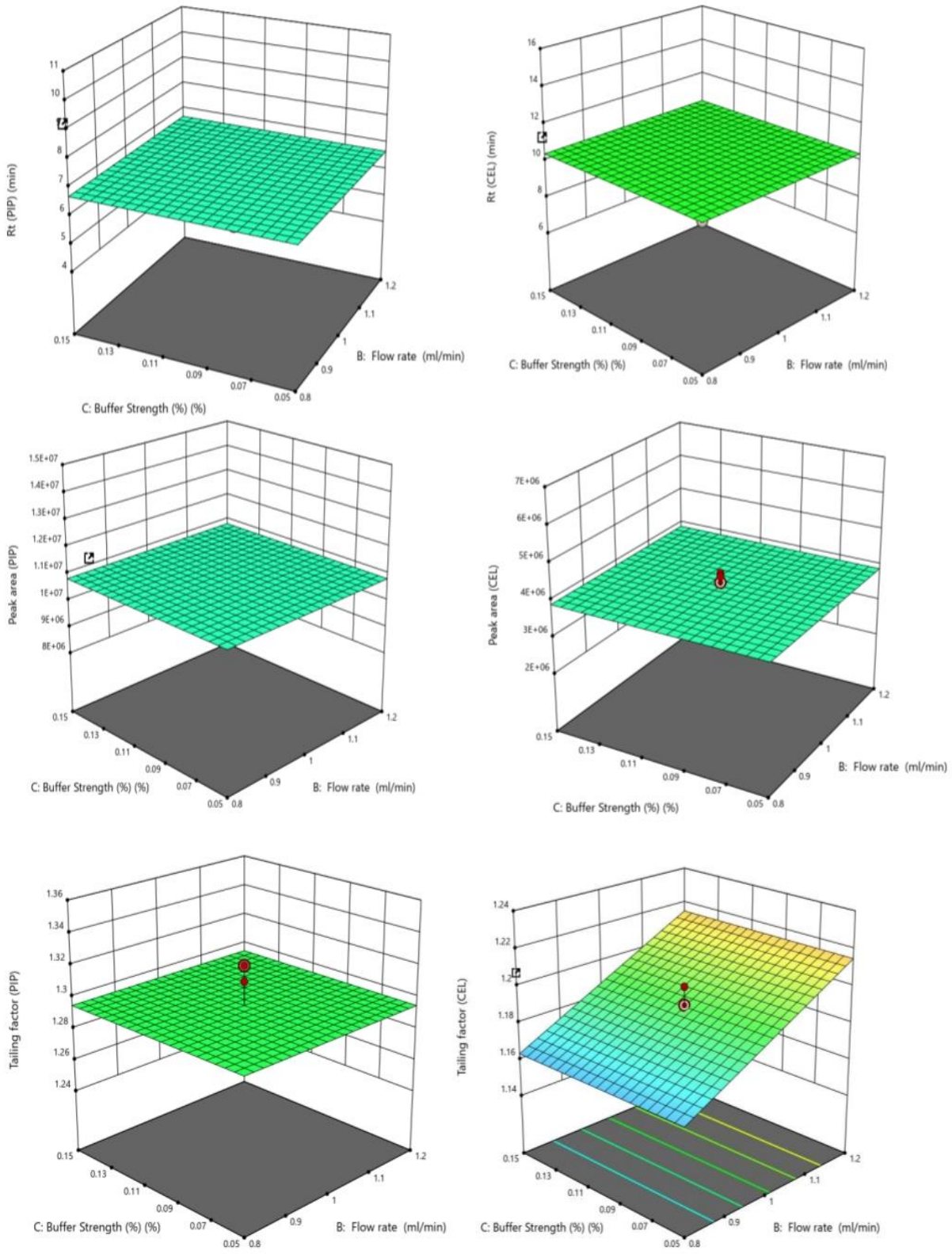


Fig 5: Response surface plots showing the impact of (B) Flow rate(ml/min) and (C) Buffer strength on Rt(PIP), Rt(CEL), Peak area(PIP), Peak area(CEL), Tailing factor(PIP), Tailing factor (CEL).

Optimization

Peak separation within range of all parameters and with short run time is the main criteria for optimization. According to the literature, Derringer's desirability function, D value nearly 1 indicates that the obtained chromatographic response values are close to the target value. By following the conditions based on the selected criteria, the optimization procedure was conducted. Figure 6 shows the desirability of chromatographic response at the optimized conditions showing the D value 1 confirms the optimized method is in the desirability range.

Both the main and the interaction effect are studied using the full quadratic equations. where A: (%) organic phase, B: buffer strength (%) and C: flow rate (ml/min). The positive and negative values in the quadratic equation symbolize the positive and converse effects of the independent parameters and experimental response. From the equations, buffer strength (B) has positive effects on Rt (PIP), Rt (CEL), Peak Area (PIP) & Peak Area(CEL), Organic phase (A) shows favorable response on T.Plate (CEL) and T.Plate (PIP) while flow rate (C) shows a positive effect on Peak Area(PIP) & Peak area(CEL). Supplementary Information explains how independent variable levels have an impact on chromatographic responses through perturbation plots.

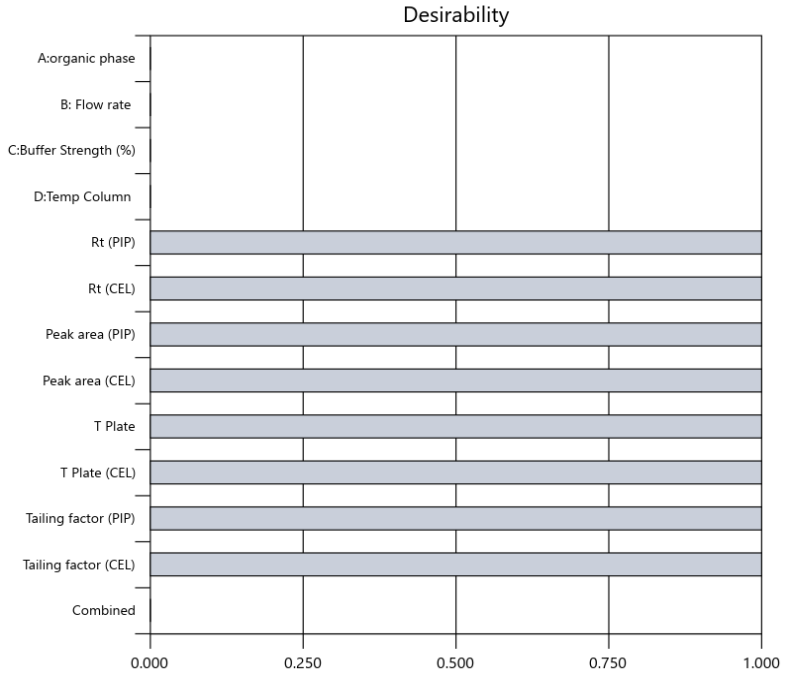


Fig 6: Desirability bar graph of responses for optimized chromatographic condition.

The optimized chromatographic condition for the chromatographic separations of the drugs celecoxib and piperine was achieved using reverse-phase phenomenex® Luna C8, 4.6 × 150 mm, 5 µm. The drugs were eluted with a mobile phase consisting of methanol; acetonitrile (30:70) and MilliQ water with 0.1% of trifluoroacetic acid at a flow rate of 1 ml/min. The drug piperine eluted with a Rt of 4.75 minutes which was detected at 342 nm, while celecoxib eluted with a Rt of 6.68 minutes which was detected at 253 nm. The chromatogram Figure 7 shows two distinct clear peaks with no additional peaks. The RP-HPLC method can segregate clearly at low concentrations as well as in mixture of compounds (Hakkimane et al., 2017).

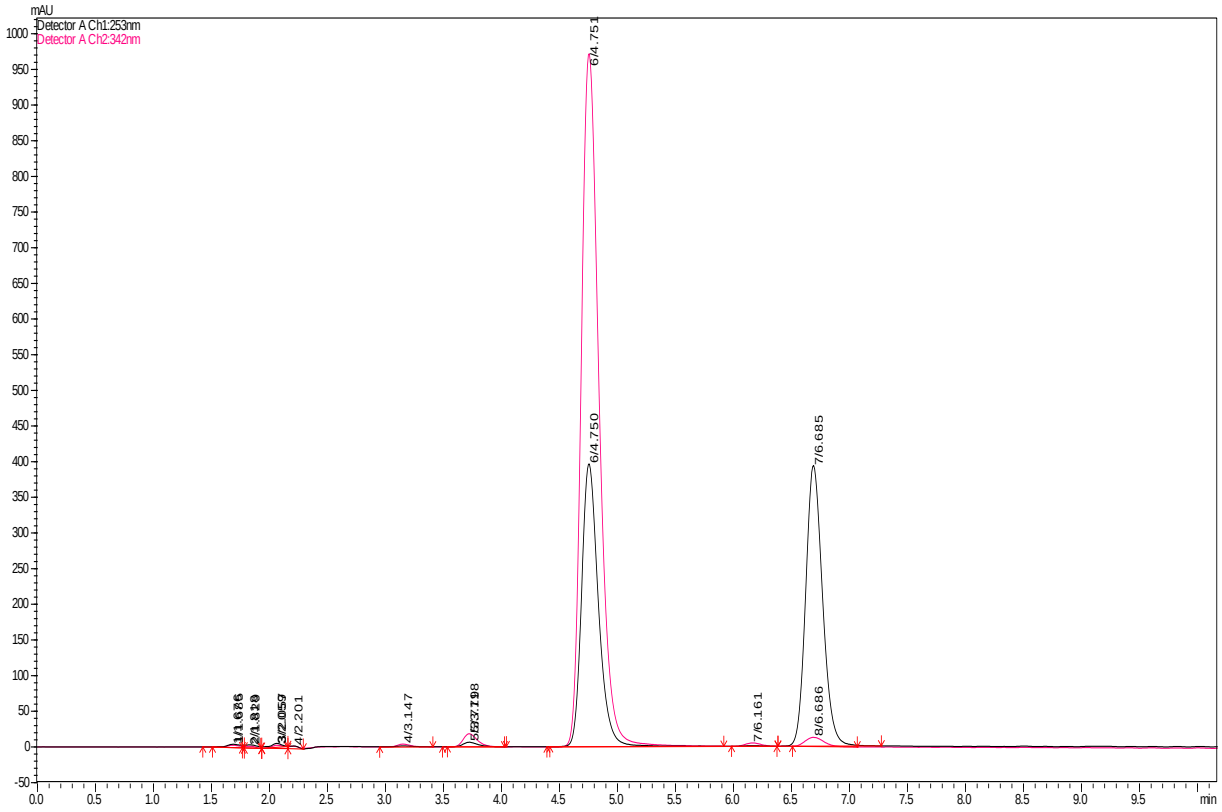


Fig 7: Method development for simultaneous estimation of piperine and celecoxib

Table 3: Result of parameters at 342 nm

Analyte	Ret. Time	Area	Height	Area%	T.Plate	Resolution	k'	Tailing F.	Separation
PIP	4.751	10358724	971766	95.8552	4608.853	4.01	1.835	1.323	1.506

Table 4: Result of parameters at 253 nm

Analyte	Ret. Time	Area	Height	Area%	T.Plate	Resolution	k'	Tailing F.	Separation
CEL	6.685	4002577	393561	48.7972	9510.257	7.158	2.967	1.199	1.631

Method validation

Linearity

The linearity of the optimized RP-HPLC method for the combination of drugs celecoxib and piperine was established by plotting the area value of the chromatogram with respect to different concentrations ranging from 1.56 -50 µg/ml. Table 7 shows the R^2 (correlation coefficient) of drugs celecoxib and piperine to be linear with a value of 0.999 exhibiting a good relationship between concentration range and peak area.

Table 5: Linearity data of piperine and celecoxib

Drug name: Piperine		
Sr no.	**Concentration (µg/ml)**	**Area**
1	50	5591387
2	25	2906436
3	12.5	1513107
4	6.25	807313
5	3.12	425489
6	1.65	237511
Regression Equation		y = 110348x + 103139
Correlation coefficient (R^2)		0.9997
Std. error of intercept		21913.37836
Std. Dev. Of intercept		53676.59552

Name of Drug Celecoxib		
Sr no.	**Concentration (µg.mL^{-1})**	**Area**
1	50	3719977
2	25	1816390
3	12.5	984164
4	6.25	495502
5	3.12	258005
6	1.65	143121
Regression Equation		y = 73448x + 30786
Correlation coefficient (R^2)		0.9996
Std. error of intercept		18270.0401
Std. Dev. Of intercept		44752.27583

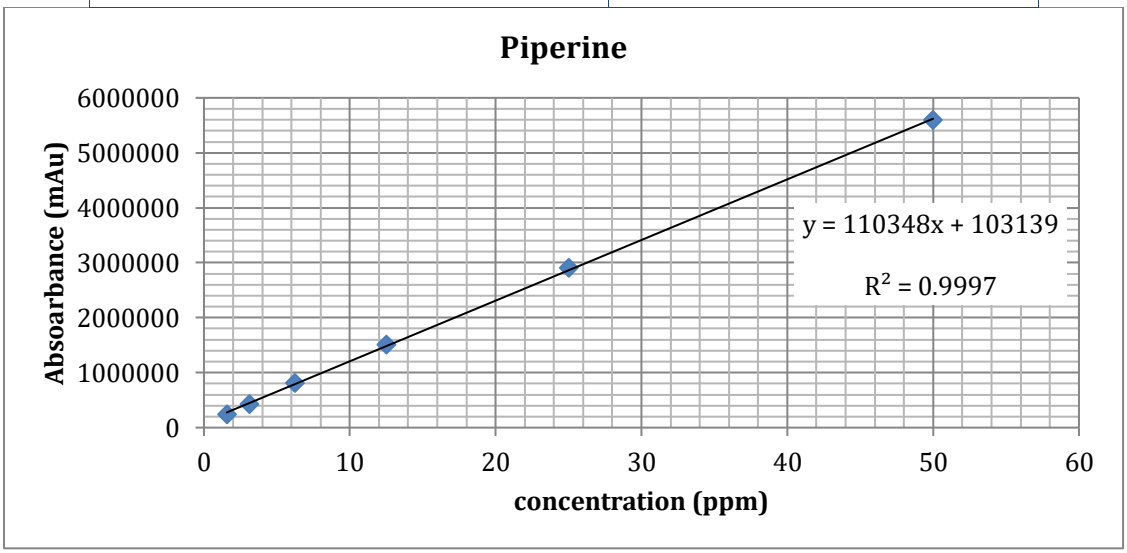


Fig 8: Calibration curve of Piperine.

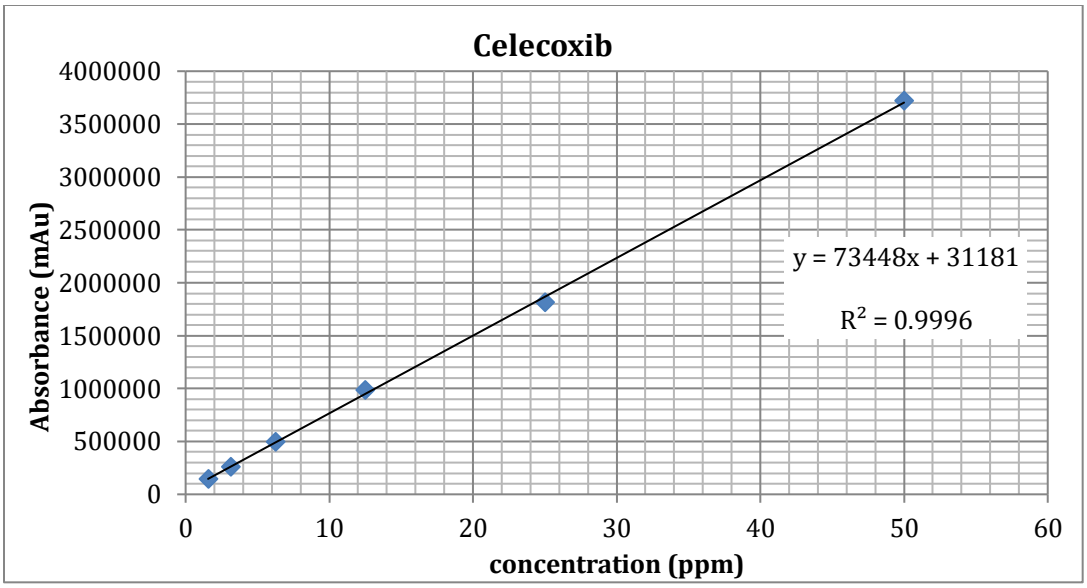


Fig 9: Calibration curve of Celecoxib.

Accuracy

Percentage drug accuracy of three different concentrations; 80%, 100% and 120% (injected thrice) to estimate the piperine and celecoxib from nanoformulation and results obtained have been reported in Table. The accuracy of the method was determined by calculating the recovery of piperine and celecoxib by the spiked method.

For the both the drug, the values of standard deviation were satisfactory and the %recovery were found close to 100%. The %RSD value was found less than 2% which indicates the accuracy of the method.

In case of drug piperine, the Std concentration was taken as 10 ppm, while for drug celecoxib, it was taken as 100 ppm.

Standard:

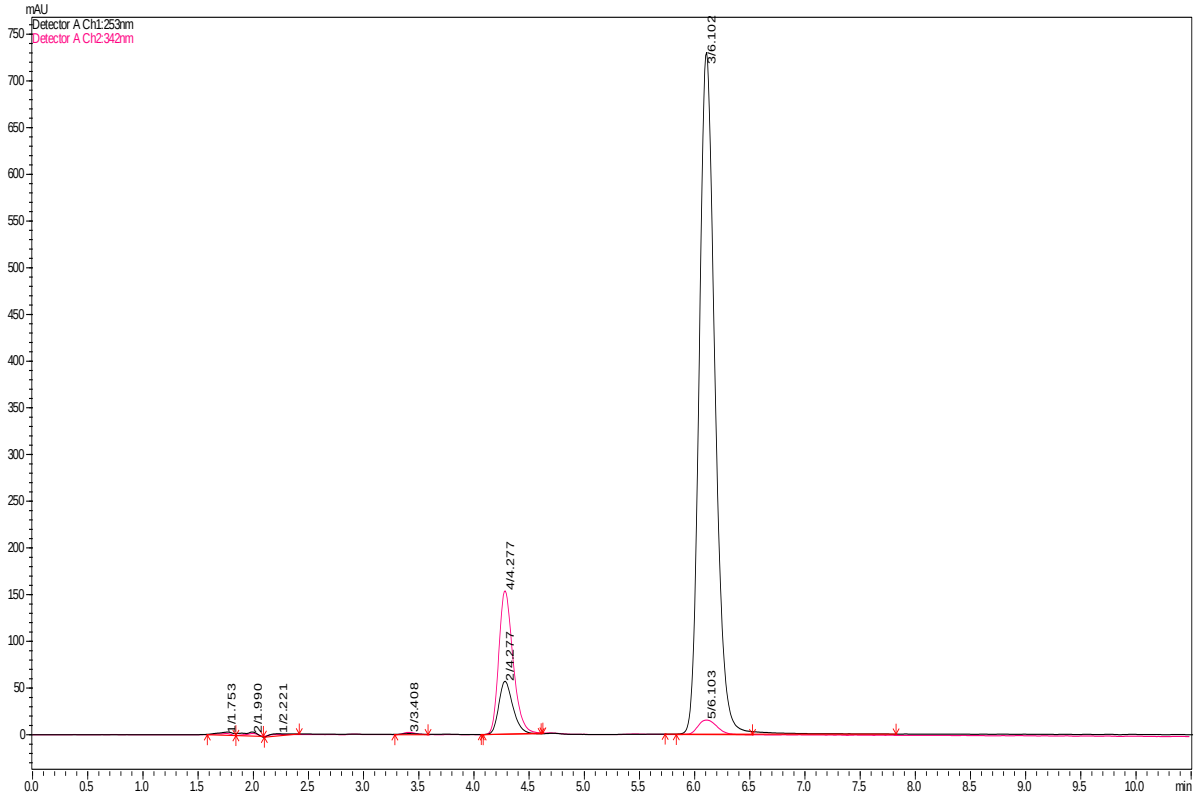


Fig 10: Piperine (10 ppm) and Celecoxib (100 ppm) standard HPLC analysis

Table 6: Peak area of Std piperine

Drug name: Piperine		
Std conc. (%)	**Std (ppm)**	**Peak area**
100%	**10 ppm**	1381263

Table 7: Drug recovery data of Piperine.

Drug name: Celecoxib								
Conc. (%)	**Std (ppm)**	**Amount added (ppm)**	**Peak area**	**Recovery (ppm)**	**Recovery (%)**	**Mean (%)**	**± SD**	**% RSD**
80%	10	8	2492901	18.04	100.22	100.61	0.39	0.39
	10	8	2512367	18.18	101.00			
	10	8	2501782	18.11	100.61			
100%	10	10	2710823	19.62	98.10	99.07	1.63	1.65
	10	10	2789276	20.19	100.95			
	10	10	2711256	19.63	98.15			
120%	10	12	3087621	22.35	101.59	100.36	1.09	1.09
	10	12	3023781	21.89	99.50			
	10	12	3042874	22.02	100.00			

The %RSD was found less than 2% and in range of 0.39%. to 1.65%.

Table 8: Peak area of Std celecoxib

Drug name: Celecoxib		
Std conc. (%)	**Std (ppm)**	**Peak area**
100%	**100 ppm**	7284333

Table 9: Drug recovery data of Celecoxib.

Drug name: Celecoxib								
Conc. (%)	**Std (ppm)**	**Amount added (ppm)**	**Peak area**	**Recovery (ppm)**	**Recovery (%)**	**Mean (%)**	**± SD**	**% RSD**
80%	100	80	13097356	179.80	99.88	99.90	0.26	0.25
	100	80	13134816	180.31	100.17			
	100	80	13107248	179.94	99.66			
100%	100	100	14586726	200.25	100.12	100.31	0.64	0.64
	100	100	14719679	202.07	101.03			
	100	100	14538256	199.58	99.79			
120%	100	120	15978126	219.35	99.70	100.31	0.69	0.70
	100	120	16049837	220.33	100.15			
	100	120	16197092	222.35	101.07			

The %RSD was found less than 2% and in range of 0.25%. to 0.70%

Precision

In intraday precision, nine injections of each drug of concentration 50 ppm were injected on the same day at different time. In interday precision, nine injections of each drug of concentration 50 ppm were injected on the three consecutive days at same time. Precision is estimated normally with three parameters: repeatability, intermediate precision, and reproducibility.

a) Intraday precision

Intraday precision of Piperine

Table10: Intraday precision data of piperine at 50 ppm.

Drug Name: Piperine					
Sr no.	**Conc. (ppm)**	**Peak area**	**Mean**	**± SD**	**%RSD**
1.	50 ppm	10778076	10553676.33	194350.54	1.84
	50 ppm	10439084			
	50 ppm	10443869			
2.	50 ppm	10440808	10475366.33	59480.46	0.57
	50 ppm	10544048			
	50 ppm	10441243			
3.	50 ppm	10544530	10507203.00	49524.66	0.47
	50 ppm	10451020			
	50 ppm	10526059			

The %RSD was found less than 2% and in the range of 0.47% to 1.84%.

Intraday precision of Celecoxib

Table 11: Intraday precision data of celecoxib at 50 ppm

Drug Name: Celecoxib					
Sr no.	**Conc. (ppm)**	**Peak area**	**Mean**	**± SD**	**%RSD**
1.	50 ppm	3980285	3910604.00	60508.89	1.55
	50 ppm	3880207			
	50 ppm	3871320			
2.	50 ppm	3874947	3863243.67	16666.89	0.43
	50 ppm	3844161			
	50 ppm	3870623			
3.	50 ppm	3617772	3611838.33	7414.89	0.20
	50 ppm	3603526			
	50 ppm	3614217			

The %RSD was found less than 2% and in the range of 0.20% to 1.85%.

b) Interday (Intermediate) Precision

Interday precision of Piperine

Table 12: Interday precision data of piperine at 50 ppm.

Drug Name: Piperine					
Sr no.	**Conc. (ppm)**	**Peak area**	**Mean**	**± SD**	**%RSD**
1.	50 ppm	10778076	10553676.33	194350.5386	1.84
	50 ppm	10439084			
	50 ppm	10443869			
2.	50 ppm	10541108	10521466.33	37484.30	0.35
	50 ppm	10545048			
	50 ppm	10478243			
3.	50 ppm	10582530	10568603.00	75390.54	0.71
	50 ppm	10487220			
	50 ppm	10636059			

The %RSD was found less than 2% and in range of 0.35% to 1.84%.

Interday precision of Celecoxib

Table 13: Interday precision data of celecoxib at 50 ppm

Drug Name: Celecoxib					
Sr no.	**Conc. (ppm)**	**Peak area**	**Mean**	**± SD**	**%RSD**
1.	50 ppm	3980285	3910604.00	60508.89	1.55
	50 ppm	3880207			
	50 ppm	3871320			
2.	50 ppm	3664940	3669715.67	4621.59	0.12
	50 ppm	3674166			
	50 ppm	3670041			
3.	50 ppm	3690072	3657815.67	42201.52	1.15
	50 ppm	3673320			
	50 ppm	3610055			

The %RSD was found less than 2% and in range of 0.12%. to 1.55%.

Specificity

Table 14: Specificity data of piperine and celecoxib

Sr no.	Solution	Retention time
1.	Blank	0
2.	Piperine Standard	4.751
3.	Piperine Sample	4.682
4.	Celecoxib Standard	6.685
5.	Celecoxib Sample	6.722

Robustness

Robustness of HPLC method represents its ability to remain unaffected by small but deliberate variations in separation parameters to ascertain its reliability during routine analysis. In this method, robustness was established by making deliberate changes in the flow rate, concentration and wavelength.

Table 15: Robustness data of Piperine

Variables	Drug name: Piperine				
	t_R (min)	k'	T_f	Rs	N
Flow rate (+0.1 ml/min)	4.289	1.79	1.318	1.767	4436
Flow rate (-0.1 ml/min)	4.072	1.148	1.398	5.456	4222
ACN-MeOH (50 +2%)	4.428	1.164	-	3.603	4058
ACN-MeOH (50 -2%)	4.063	0.976	1.314	2.84	4494
wavelength (+2 nm)	4.742	1.79	1.317	3.992	4646
wavelength (+2 nm)	4.7	1.277	1.319	3.966	4673

Table 16: Robustness data of Celecoxib

Variables	Drug name: Celecoxib				
	tR (min)	k'	Tf	Rs	N
Flow rate (+0.1 ml/min)	6.099	2.956	1.202	7.068	8323
Flow rate (-0.1 ml/min)	7.151	2.767	1.206	7.053	10341
ACN-MeOH (50 +2%)	6.159	2.646	1.235	4.561	8068
ACN-MeOH (50 -2%)	7.652	2.726	1.198	6.762	13893
wavelength (+2 nm)	6.663	2.907	1.197	7.158	9606
wavelength (-2 nm)	6.589	2.892	1.202	7.085	9416

LOD and LOQ

From the calculation, the lowest detectable concentration was found to be 2.01 & 1.60 µg/ml for celecoxib and piperine respectively. The limit of quantitation was found to be 6.09 & 4.96 µg/ml for celecoxib and piperine respectively.

Observation table:

Table 17: Overall results of system suitability and validation parameters.

System suitability parameters	Piperine	Celecoxib	Acceptable Values
Theoretical plates (*N*)	4608	9510	≥ 2000
Capacity Factor (*K'*)	1.83	2.967	> 1
Resolution (*R*)	4.01	7.15	> 2
Separation factor (α)	1.506	1.631	> 1

Tailing factor (*T*)	1.323	1.199	< 2
Retention time (*tR*)	4.75 min.	6.68 min.	> k'
Wavelength of Detection (nm)	342 nm	253 nm	> 200 nm
Repeatability (% RSD)	1.29	1.12	< 2%
Intra-Day Precision (% RSD)	0.47-1.84	0.16-1.55	< 2%
Inter-Day Precision (% RSD)	0.35-1.84	0.13-1.78	< 2%
Accuracy (%)	99.07-100.61	99.90-100.31	98%-102%
Linearity range	1.56–50 µg/ml	1.56–50 µg/ml	NA
Regression equation	y =110348x + 103139	y = 73448x + 30786	NA
SE of intercept (Se)	21913.37836	18270.0401	NA
SD of intercept (Sa)	135512.951	43421.08825	NA
Correlation Coefficient (r2)	0.9997	0.9996	NA
LOQ (µg/ml)	4.86 µg/ml	6.09 µg/ml	NA
LOD (µg/ml)	1.60 µg/ml	2.01 µg/ml	NA

The above table shows the result of system suitability and validation parameters. All result were found within the acceptance criteria.

Stability analysis by RP-HPLC

The following results were obtained for piperine and celecoxib at P^H 4.5 and P^H 7.4 after 48 hour.

At P^H 4.5

Table 18: Peak area and % stability at P^H 4.5 after 48th hour.

Drug name	Peak area (0th hr)	Peak area (48th hr)	% stable
Piperine	10358724	447368	6.24%
Celecoxib	4002577	1543481	26.1%

At P^H 7.4

Table 19: Peak area and % stability at P^H 7.4 after 48th hour.

Drug name	Peak area (0th hr)	Peak area (48th hr)	% stable
Piperine	10358724	727948	11.32%
Celecoxib	4002577	1978343	33.98%

6. CONCLUSION:

A simple RP-HPLC method for simultaneous estimation of celecoxib and piperine was developed and optimized. Optimization of the method for the combination of drugs by the conventional trial and error method would have been a tedious job. Design of Experiments (DOE) made it simpler with a minimum number of experiments. The applied BBD design gave more information about the interactional effect of the independent variables to accomplish the desired chromatographic response. The optimized RP-HPLC method developed for the simultaneous analysis of celecoxib and piperine was found to be simple, precise, and reproducible. The validation of the RP-HPLC method was carried out according to ICH guidelines. The absence of significant interfering peaks and lower %RSD values (<1%) shows that the developed method was sensitive. Considerable low LOD and LOQ values determine the method is suitable for quantifying and the detection of low concentrations of drugs. The method developed was effectively applied for the stability analysis of celecoxib and piperine in combined form. Results showed that there was slight degradation of celecoxib in presence of piperine. More research work on this area is needed to figure out the reason behind the degradation of celecoxib in the presence of piperine. Hence the developed method can be implemented for the simultaneous analysis of celecoxib and piperine in pharmaceutical dosage forms in quality control.

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