

THE DEVELOPMENT AND VALIDATION OF A RAPID HPLC METHOD FOR DETERMINATION OF PIROXICAM

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Abstract

The present paper presents the development and validation of a novel fast, sensitive and accurate HPLC-UV method for the quantitative determination of Piroxicam in bulk materials and pharmaceutical formulations. Development of the chromatographic method was based on a design of experiments (DOE) approach, using the Box-Behnken design in order to identify the significant parameters for the optimization, by simultaneously registering retention time, peak symmetry, and resolution of the chromatographic separation, number of theoretical plates and capacity factor of the first eluting peak as responses. Derringer's desirability function (d) was used in order to identify the areas in the design region where the process is likely to give the optimal results. The mobile phase consisting in a trifluoroacetic acid: acetonitrile mixture at a 60:40 (v/v) ratio, delivered isocratically at a flow rate of 1.1 mL/min, and a column temperature of 40°C were found to be the optimum experimental conditions for the separation.

The method was fully validated in accordance with the current ICH guidelines in terms of specificity, linearity, precision, accuracy, limits of detection/quantitation, and system suitability. The Piroxicam stability studies revealed that the active substance is stable in methanol solutions, as well as in acidic environment (in both mobile phase and in 0.1M HCl solution). The tested temperature conditions (from 4 to 37°C) had no significant impact on Piroxicam stability. However, direct exposure to sunlight induced an approximately 25% degradation within 4 hours. The method was found suitable for stability studies and for routine quality control analysis for products of similar type and composition.

Rezumat

Lucrarea de față prezintă dezvoltarea și validarea unei noi metode HPLC - UV, rapidă, sensibilă și precisă, pentru determinarea cantitativă a Piroxicamului în materiale vrac și formulări farmaceutice. Dezvoltarea metodei cromatografice de dozare s-a bazat pe un plan de experimente (DOE), folosind *design*-ul Box - Behnken pentru a identifica parametrii semnificativi pentru optimizarea, prin evaluarea în același timp a timpului de retenție, simetriei picului cromatografic, rezoluției separării cromatografice, numărului de talere teoretice și factorului de capacitate de la primul pic obținut prin eluarea analiților. Funcția dezirabilității lui Derringer (d) a fost utilizată pentru a identifica domeniile în planul de *design* în cazul în care procesul oferă rezultate optime. Faza mobilă a constat dintr-un amestec format din acid trifluoroacetic : acetonitril (v/v) 60:40, cu o eluție isocratică și un debit de 1,1 mL/min, o temperatură a coloanei de 40°C, acestea fiind condițiile experimentale de separare optime.

Metoda a fost validată în conformitate cu reglementările actuale ICH, în ceea ce privește specificitatea, linearitatea, precizia, acuratețea, limitele de detecție/cuantificare, în condițiile unui sistem adecvat ales. Studiile de stabilitate pentru piroxicam au arătat că substanța activă este stabilă în soluții metanolice, dar și în mediu acid (atât faza mobilă și în soluție 0,1M HCl). Condițiile de temperatură testate (4-37°C) au avut un impact semnificativ asupra stabilității piroxicamului. Cu toate acestea, expunerea directă la soare a condus la o degradare de aproximativ 25% în decurs de 4 ore. Metoda s-a demonstrat a fi potrivită pentru studiile de stabilitate și în analiza de rutină de control al calității pentru produsele de același tip și cu o compoziție similară.

Keywords: Piroxicam, HPLC, Design of Experiments, development

Introduction

Piroxicam (4-hydroxy-2-methyl-N-(pyridine-2-yl)-2H-1,2-benzothiazine-3-carboxamide-1,2-dioxide),

is a non-steroidal anti-inflammatory and analgesic drug of the oxicam class, often indicated in the treatment of rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, acute pain in

musculoskeletal disorders and acute gout [14]. The chemical structure of Piroxicam is presented in Figure 1.

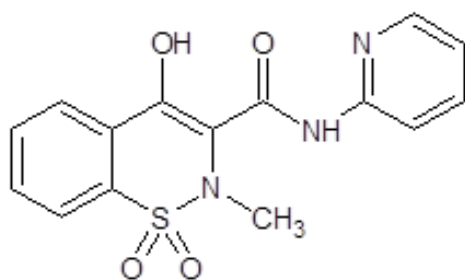


Figure 1.

Chemical structure of Piroxicam

A literature survey revealed various assays for determining piroxicam in pharmaceutical dosage forms, including spectrophotometry [4, 9, 10, 16, 18], thin-layer chromatography with densitometry [1, 21] or mass spectrometric detection [6], and voltammetry [3, 19, 22]. However, among all quantitative methods, HPLC is the most popular technique, different detection methods being employed, such as: UV [2, 5, 7, 13, 17, 20, 23, 24], fluorimetry [11], mass spectroscopy (MS) [8] or nuclear magnetic resonance (NMR) [23].

The present study presents the development and validation of a new simple HPLC-UV method for the simultaneous assay of piroxicam from bulk material and pharmaceutical formulations, based on a modern design of experiments (DOE) approach. A Box-Behnken experimental design was used to build the mathematical models and to choose the significant parameters for the HPLC method optimization by simultaneously registering the retention time, resolution and capacity factor and peak symmetry as responses. Selection of the optimum experimental conditions in terms of mobile phase composition, column temperature and flow rate was made by using Derringer's desirability function.

Materials and Methods

Chemicals

Piroxicam and tenoxicam (internal standard), European Pharmacopoeia Reference Standards, were used for preparation of stock and standards solutions. HPLC grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany), whereas HPLC grade trifluoroacetic acid was provided by Sigma-Aldrich (St. Louis, MO, SUA). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was obtained within the laboratory using a TKA GenPure system. All other reagents were of analytical grade, purchased from different

commercial suppliers and used without further purification.

Stock and working standard solutions

The Piroxicam stock solution was prepared at a concentration of 100 µg/mL in methanol. Working dilutions from the stock solution were freshly prepared when needed. Seven spiked calibration standards were prepared in mobile phase, with the analyte at the concentrations of: 0.25, 0.5, 1, 2.5, 5, 10 and 25 µg/mL. Three quality control samples used in the accuracy and precision evaluation (QCs) were used, with the levels of Piroxicam being 0.8, 4 and 20 µg/mL.

The stock solution of the internal standard (Tenoxicam) was prepared in methanol, and added in each analyzed sample at a final concentration of 20 µg/mL.

Quantitative HPLC analysis

The quantitative analysis was performed on a Waters liquid chromatographic system (Waters, Milford, MA, USA) equipped with a 600 E Multisolvant Delivery System, 486 UV detector, Waters AF in line degasser and Waters 717 plus autosampler. The output signal was monitored at $\lambda=330$ nm and processed using Empower software (Waters, Milford, MA, USA).

The chromatographic separation was carried out on a Hypersil Gold, 5-µm 150 x 4 mm column (Thermo Fisher Scientific, Waltham, MA, USA). Volumes of 10 µL from all standard and tested solutions were injected onto the column. The mobile phase consisting in a trifluoroacetic acid: acetonitrile mixture at a 60:40 (v/v) ratio was delivered isocratically at a flow rate of 1.1 mL/min. The chromatographic procedures were conducted at 40°C.

The optimum composition of mobile phase, flow rate and column temperature were determined by means of experimental design.

Optimization of the HPLC method

In the preliminary studies a one-factor-at-a-time (OFAT) approach was used in order to select the stationary phase, as well as the nature of both aqueous component and organic modifier of the mobile phase.

Different aqueous solutions such as 0.1% trifluoroacetic acid, 0.1% phosphoric acid, 0.025M potassium phosphate buffer with pH ranging between 2.0 and 6.5 were tested. The results suggested that a pH above 3.5 was associated with broadening of the chromatographic peaks, and therefore decreasing the chromatographic method performance. The best results in terms of both symmetry and resolution were obtained using 0.1% trifluoroacetic acid solution. The use of methanol of mixtures of acetonitrile and methanol also led to wider chromatographic peaks compared to acetonitrile alone, therefore the selected

composition of the mobile phase was 0.1% trifluoroacetic acid: acetonitrile.

Further optimization of the chromatographic method involved the investigation of quantitative effects of different experimental variables on the performance of chromatographic separation using a DOE approach. The Box-Behnken design approach of response surface methodology (RSM) was used in order to study the individual and combined effects of three independent factors, *i.e.* the percent of organic modifier in the mobile phase (X_1), flow rate (X_2) and column temperature (X_3) on Piroxicam peak retention time (Y_1), asymmetry (Y_2), resolution of the chromatographic separation (Y_3), number of theoretical plates - N (Y_4) as well as the capacity factor of the first eluting peak (Tenoxicam) - k' (Y_5).

The experimental design for the optimization along with statistical analysis of data was performed with Design-Expert 9.0 software, Trial Version (Stat Ease Stat-Ease, Inc., Minneapolis, MN, USA).

The minimum and maximum contents of acetonitrile (X_1) were fixed as 25% and 45%, respectively. Likewise, the column temperature (X_2) was varied between 30°C and 50°C, and the flow rate (X_3) between 0.8 mL/min and 1.2 mL/min.

The design matrix for the Box-Behnken study also included five replicates of the central point, resulting in a total of 17 analytical experiments. The experiments were performed in randomized order to minimize the effects of uncontrolled variables that may influence the results. The range and the levels of experimental investigated variables are presented in Table I.

Table I
Factors and levels of the Box-Behnken experimental plan

Factors	Code	Range and levels		
		-1	0	+1
% organic modifier (acetonitrile)	X_1	25	35	45
Column temperature (°C)	X_2	30	40	50
Flow (mL/min)	X_3	0.8	1	1.2

The resulting experimental data were analysed to fit the following second-order polynomial model:

$$Y = b_0 + \sum_{i=0}^n b_i X_i + \sum_{i=0}^n b_{ii} X_i^2 + \sum_{i < j}^n b_{ij} X_i X_j + \varepsilon$$

where Y is the measured response, b represents the coefficients calculated by multiple regression analysis, X_i represents the main effects of the independent variables, $X_i X_j$ the interaction terms between variables, X_i^2 quadratic expressions of the independent variables (included into the model in

order investigate nonlinearity) and ε represents the random error.

The quality of the fitted model was expressed by the coefficient of determination (R^2) and its statistical significance was checked by F-test and P-value test. The statistical analysis of the model was performed by using the analysis of variance (ANOVA).

Validation of the HPLC method

The HPLC method was validated in accordance with the International Conference on Harmonization (ICH) regulations Q2(R1) [12] in terms of specificity, linearity, precision (repeatability and intermediate precision) and accuracy. Additional studies were performed in order to evaluate Piroxicam photostability and stability under acidic conditions.

The specificity was evaluated *versus* interferences from the matrix components in the drug-free media. Piroxicam linearity assessment was performed by using seven concentration levels, in the range 0.25-25 µg/mL. The analyte to internal standard peak area ratio was plotted against the corresponding analyte concentration in order to obtain the calibration curve, and the determination coefficient (R^2) was calculated by least squares linear regression analysis. All analyses were performed in triplicate.

The sensitivity of the analytical method was evaluated by determining the detection limit (LOD) and quantitation limit (LOQ) using the signal-to-noise ratio method, *i.e.* the concentrations yielding to signal-to-noise ratios of 3:1 and 10:1 were considered as LOD and LOQ, respectively.

Precision was evaluated for repeatability and intermediate reproducibility on spiked samples, at three different concentration levels (QC samples). Precision was assessed by means of the RSD% of the analyte concentration resulting from interpolation on the calibration curve. Repeatability study was performed using 5 replicates from each QC sample analyzed within a single day experimental session, whereas intermediate reproducibility was tested by means of five different samples processed in different experimental sessions.

The accuracy was evaluated based on the bias (%) between the concentration values determined for the QC samples and their nominal values.

Results and Discussion

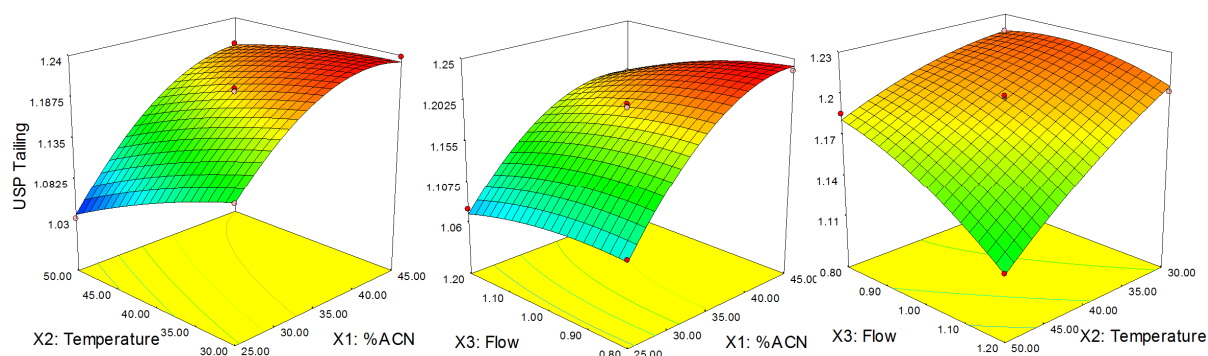
A total of seventeen tests (including five replicates of the center point) were carried out in random order, in accordance with the Box-Behnken design (BBD) (Table II).

Table II

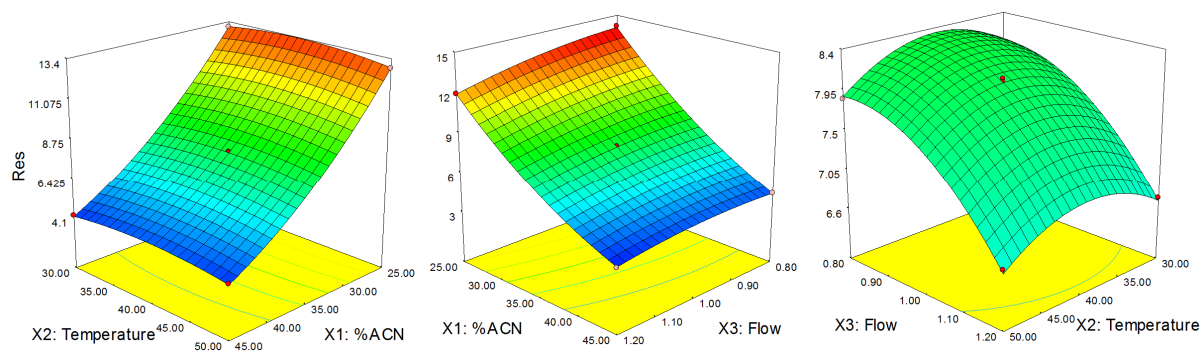
Run no.	X_1	X_2	X_3	$Rt(Y_1)$	Tailing factor (Y_2)	Resolution (Y_3)	Plate count (Y_4)	k' (Y_5)
1	35	30	1.2	3.24	1.20	6.72	4990	1.31
2	25	50	1	7.37	1.03	12.86	7178	4.26
3	35	40	1	4.80	1.20	8.08	6803	2.43
4	45	40	1.2	2.23	1.19	3.78	3970	0.59
5	35	50	0.8	4.65	1.19	7.86	6782	2.32
6	35	50	1.2	3.10	1.12	6.66	5200	1.21
7	35	30	0.8	4.88	1.22	8.03	6782	2.49
8	45	30	1	2.74	1.24	4.21	4663	0.95
9	25	40	1.2	6.33	1.08	11.98	6249	3.52
10	35	40	1	4.80	1.20	8.04	6850	2.43
11	35	40	1	4.80	1.20	8.04	6875	2.43
12	25	40	0.8	10.04	1.09	14.11	8018	6.17
13	35	40	1	4.81	1.20	7.99	6791	2.44
14	45	50	1	2.63	1.20	4.15	4772	0.88
15	45	40	0.8	3.30	1.24	4.44	5587	1.35
16	35	40	1	4.81	1.20	8.04	6800	2.43
17	25	30	1	8.09	1.13	13.07	7058	4.78

The relationship between the dependent and independent variables is further illustrated using the response surface methodology (RSM). The

resulting graphs for three of the selected responses are presented in Figures 2-4. In all representations, one factor was kept constant at its center value.

**Figure 2.**

Response surface plots representing peak asymmetry (Y_2) as a function of % organic modifier (X_1), column temperature (X_2) and flow rate (X_3)

**Figure 3.**

Response surface plots representing resolution of the chromatographic separation (Y_3) as a function of % organic modifier (X_1), column temperature (X_2) and flow rate (X_3)

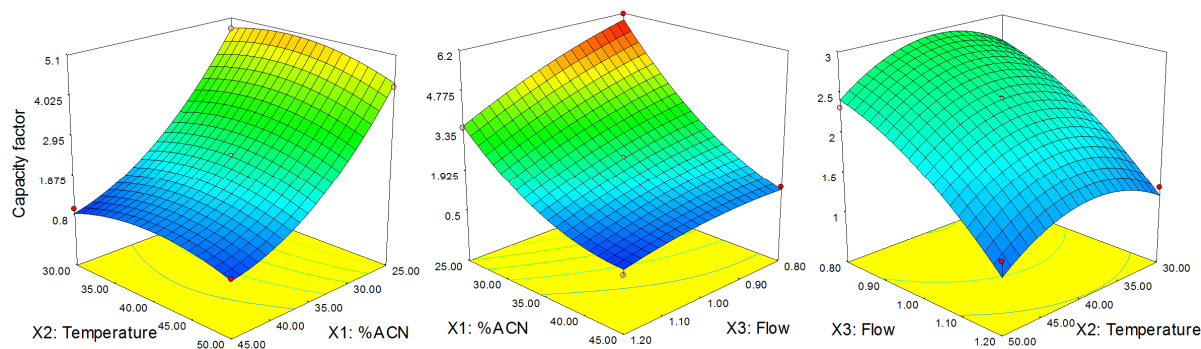


Figure 4.

Response surface plots representing the capacity factor k' (Y_5) as a function of % organic modifier (X_1), column temperature (X_2) and flow rate (X_3)

The coefficients of the second-order polynomial model were estimated by the least square regression analysis, and the function of responses related to the three selected factors was obtained.

By applying ANOVA for the regression equations, all the resulting models were found to be significant, thus useful in predicting the effects of the factors on the selected responses. The magnitudes of the coefficients in the regression equations were used as the basis for judging statistical significance and illustrating the relative effects of linear, quadratic effects, as well as of the interactions between the parameters. The p value

was used as indicator of the significance of each coefficient, a p value smaller than 0.05 being considered significant.

The values of R^2 shown in Table III indicate a good fitting, suggesting that the model adequately fits the real relationship between the parameters chosen in this study and is adequate for prediction within the range of experimental variables ($R^2 > 0.9$ in all cases). The coefficient of determination values listed in the table represent the “adjusted R^2 ” values, obtained after the nonsignificant terms ($p > 0.05$) were removed from the models through the “backward elimination” process.

Table III

Results of the regression analysis for the selected responses

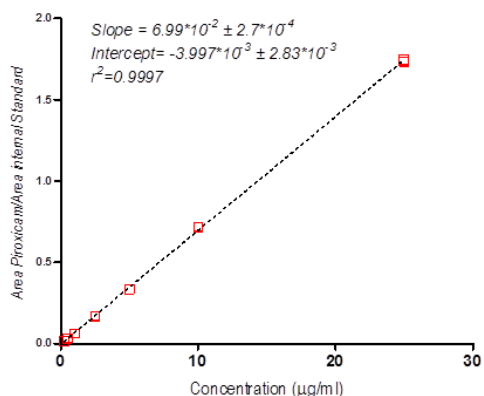
Response	b_0	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{11}	b_{22}	b_{33}	R^2
Y_1	4.80	-2.62	-0.15	-1.00	0.15	0.66	0.02	0.96	-0.55	-0.29	0.9869
$p =$		< 0.01	0.12	< 0.01	0.24	< 0.01	0.87	< 0.01	< 0.01	0.045	
Y_2	1.20	0.07	-0.03	-0.02	0.02	-0.01	-0.01	-0.04	-0.01	-0.01	0.9923
$p =$		< 0.01	< 0.01	< 0.01	< 0.01	0.017	< 0.01	< 0.01	0.035	0.009	
Y_3	8.04	-4.43	-0.06	-0.66	0.04	0.37	0.03	0.90	-0.36	-0.36	0.9996
$p =$		< 0.01	0.022	< 0.01	0.250	< 0.01	0.385	< 0.01	< 0.01	< 0.01	
Y_4	6824	-1189	55	-845	-3	38	52	-444	-462	-423	0.9993
$p =$		< 0.01	< 0.01	< 0.01	0.871	0.041	0.011	< 0.01	< 0.01	< 0.01	
Y_5	2.43	-1.87	-0.11	-0.71	0.11	0.47	0.02	0.68	-0.39	-0.20	0.9869
$p =$		< 0.01	0.121	< 0.01	0.239	< 0.01	0.866	< 0.01	< 0.01	0.045	

Legend: $p < 0.01$, $0.01 \leq p < 0.05$, $0.05 \leq p < 0.10$, $p \geq 0.10$

The retention related parameters (Y_1 , Y_3 and Y_5) are negatively influenced by all the selected variables; however, the influence of temperature is less significant than that of acetonitrile % and flow rate. Although all the selected variables significantly influence peak symmetry (Y_2), the values obtained for the USP peak tailing ranged from 1.03 to 1.24, being therefore within the acceptable limits for all determinations. On the other hand, the interactive factors had significant influence on the selected variables.

Selection of the optimum experimental conditions of the HPLC analysis

When individual responses are being analysed, the model analysis indicates areas in the design region where the process is likely to give the optimal results. However, generally, when an analysis of multiple responses is performed, a compromise should be achieved between the targeted values for all selected responses. One approach in that direction is the use of Derringer's desirability function (d) [15]. The function has values between 0 and 1, significant for a response ranging from completely undesirable (0) to fully desired (1).

**Figure 7.**

Calibration curves for Piroxicam (n=3 replicates, range = 0.25-25 µg/mL)

The limit of quantification (LOQ), calculated based on a 10:1 signal-to-noise ratio was 0.087 µg/mL. No endogenous peaks or other degradation products interfered with the chromatographic separation.

Repeatability and intermediate reproducibility experiments resulted in RSD values ranging from 0.42 to 1.76 %. The bias (%) between the experimental and nominal concentrations of the quality control samples did not exceed 3 %. All these results indicated that precision and accuracy of the analysis is satisfactory (Table V). Although for quality control methods the use of an internal standard is not compulsory, the presence of Tenoxicam in the analysed samples led to a decrease in variability.

Table V

Accuracy and precision of the Piroxicam quantitative HPLC method

Conc (µg/mL)	Intra-day precision & accuracy			Inter-day precision & accuracy		
	Mean±SD	RSD (%)	Bias (%)	Mean±SD	RSD (%)	Bias (%)
0.8	0.795 ± 0.005	0.59	-0.60	0.802 ± 0.009	1.13	+0.23
4.0	3.880 ± 0.017	0.43	-2.99	3.934 ± 0.069	1.76	-1.66
20.0	19.956 ± 0.085	0.42	-0.22	19.955 ± 0.102	0.51	-0.23

Stability

Stability of Piroxicam in different experimental conditions was studied for both methanol stock solution and QC samples prepared by dilution of the stock solution with mobile phase, and 0.1 N HCl. The samples were maintained under different temperature and luminosity conditions, and analyzed at predefined time intervals. For

photolytic degradation studies, the stock solution was placed in direct sunlight for 4 hours. All analyses were performed in triplicate. Since no significant differences occurred between the different QC samples concentrations, a “global” mean recovery was calculated. The results of the stability studies are presented in Table VI.

Table VI

Piroxicam stability under different experimental conditions

Conditions	Time	Recovery (%)
Stock solution in methanol, room temperature, light protected	72 h	98.23 ± 0.64
Stock solution in methanol, room temperature, direct sunlight	4 h	75.24 ± 1.36
Stock solution in methanol, +4°C	72 h	99.54 ± 0.45
QC samples, diluted in mobile phase, ambient temperature, light protected	48 h	99.43 ± 0.62
QC samples, diluted in mobile phase, +4°C	48 h	98.81 ± 0.51
QC samples, diluted in 0.1N HCl solution, ambient temperature, light protected	48 h	99.76 ± 0.74
QC samples, diluted in 0.1N HCl solution, 37°C, light protected (simulated gastric conditions)	48 h	99.14 ± 0.86

The results emphasized that direct light exposure leads to a significant degradation of the drug substance, and a possible major source of error in the determinations. Therefore, during preparation, storage and analysis all samples were protected from light. No degradation product interfered with the Piroxicam or Internal standard peaks, thus making the method applicable for stability studies.

The proposed method was applied for the quantitative analysis of Piroxicam from commercially available pharmaceutical tablets containing 20 mg active ingredient. The results are provided in Table VII. The high percentage recoveries and low RSD (0.54 %) also confirm the applicability of the developed method in routine quality control analysis.

Table VII

Analysis of Piroxicam from a commercially available product

Labeled value (mg per tablet)	Found (mg)	Recovery ± SD (%)*
20	19.83	99.15 ± 0.058

* mean of five determinations

Conclusions

The paper describes the development and validation of a novel fast, sensitive and accurate HPLC method for the quantitative determination of Piroxicam in bulk and pharmaceutical formulations. The development of the HPLC method was performed using the Box-Behnken design approach and response surface methodology. The optimized method was fully validated in accordance with the current ICH guidelines in terms of specificity, linearity, precision, and accuracy, limits of detection/quantitation and system suitability. Extensive stability studies also revealed that Piroxicam is stable in methanol solutions, as well as in acidic environment (in both mobile phase and in 0.1H HCl acid solution), and the tested temperature conditions (from 4 to 37°C) had no significant impact on its stability. However, direct exposure to sunlight induced an approximately 25 % degradation within 4 hours. The method was found suitable for stability studies and for routine quality control analysis for products of similar type and composition.

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