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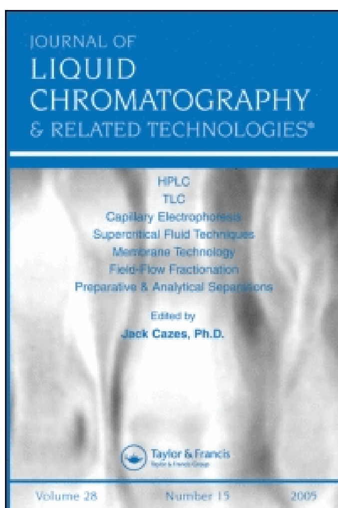

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LC-UV METHOD DEVELOPMENT AND VALIDATION FOR THE NON STEROIDAL ANTI-INFLAMMATORY AGENT TENOXICAM

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LC-UV METHOD DEVELOPMENT AND VALIDATION FOR THE NON STEROIDAL ANTI-INFLAMMATORY AGENT TENOXICAM

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□ *A rapid and sensitive reversed phase high performance liquid chromatographic (HPLC) method was developed and validated for the analysis of tenoxicam in raw material and its pharmaceutical formulation.*

The analysis was carried out on a reversed phase C_{18} column, using mixtures of buffer/acetonitrile (40:60, v/v) with flow rate was of 1 mL min^{-1}. The method was validated statistically for its linearity (correlation coefficient = 0.9983), accuracy, robustness, and intermediate precision. An experimental design was used during validation to evaluate method robustness and for the determination of intermediate precision. To test robustness, four factors were considered, mainly, percentage of organic modifier in the mobile phase, pH, flow rate, and different wavelengths. An increase of the flow rate results in a decrease of the drug concentration found, while the percentage of organic modifier, pH, and wavelength have no significant effect on the response. For intermediate precision the factors examined were multiple analysts, multiple instruments, and multiple days. The RSD value (0.49%, n = 24) indicated a good precision of the analytical method. Due to its simplicity, accuracy, sensitivity, and precision the method may be used for routine quality control analysis.

Keywords high performance liquid chromatography (HPLC), pharmaceutical analysis, tenoxicam

INTRODUCTION

Tenoxicam (4-hydroxy-2-methyl-N-2-pyridinyl-2H-thieno[2,3-e]-1,2-thiazine-3-carboxamide 1,1-dioxide) is a non-steroidal anti-inflammatory drug (NSAID) with analgesic, antipyretic properties and also inhibits platelet aggregation. It is a new thienothiazine derivative (Fig. 1) belonging to the

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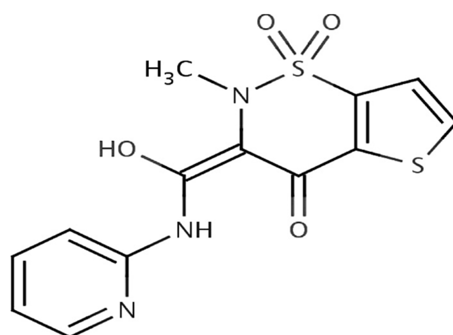

FIGURE 1 Chemical structure of tenoxicam.

chemical class of oxicams, which possess a potent inhibition effect on prostaglandin biosynthesis. On extra vascular administration, tenoxicam is absorbed in an unchanged form, after oral administration it is absorbed completely, whereas absorption after rectal administration is approximately 80%.[1]

Young et al.[2] reported on the quantitative determination of tenoxicam in human plasma by liquid chromatography with tandem mass spectrometry. Tenoxicam plasma levels were also determined by the RP-HPLC method with UV detection that employed piroxicam as an internal standard.[3] The human bioavailabilty of tenoxicam after intramascular administration was reported by Stebler and Guentert.[4]

Several other analytical methods have been described for *in vitro* and *in vivo* determination of tenoxicam.[5–9]

The aim of this investigation is to develop a rapid and sensitive HPLC method with UV detection, useful for routine control of tenoxicam in pharmaceutical formulation and in pharmacokinetic studies. The method was validated for linearity, accuracy, precision, and robustness. An experimental design was used during validation to evaluate both the robustness and the determination of intermediate precision.

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EXPERIMENTAL

Apparatus

Different HPLC systems were used at two laboratories involved in this study. The specifics are provided below.

Lab. A: The HPLC 1 apparatus was a Merck Hitachi chromatographic system pump (L- 6200A) equipped with a suptumless injector (Rheodyne 7725). A UV detector (L-4000A) was used. Peak area integration were performed using a chromatographic data system (PE NELSON 1022 HPLC system manager program). A Vertex Hypersil reversed phase C_{18} column (15 cm × 4.6 mm i.d., particle size 5 μm) was utilized.

Lab. B: The HPLC 2 apparatus was a Waters chromatographic system pump (Waters 510 HPLC) equipped with a septumless injector (Rheodyne 7725). A UV detector (Waters 486 Tunable Absorbance Detector) was used. Peak area integration was performed using a chromatographic data system (Waters 746 Data Model). A Vertex Hypersil reversed phase C_{18} column (15 cm × 4.6 mm i.d., particle size 5 μm) was used. The experimental design was produced, and statistical analysis of the data was performed, by Nemrod software[10] (LPRAI, Universite de Marseille III, France).

Reagents

Acetonitrile was purchased from Scharlau (Barcelona, Spain). Water used in the mobile phase was deionized, distilled, and filtered through a 0.45 μm Millipore filter (Sartorius, Germany) under vacuum before use. Chloroacetic acid (Panreac, Barcelona, Spain) was analytical grade. Mobiflex® 20 mg tablets and tenoxicam reference standard were supplied by the quality control Laboratories, Ministry of Health, (Amman – Jordan).

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Preparation of Standard/Sample Solutions

An accurate weighed sample of tenoxicam reference standard equivalent to 100 mg was dissolved into a 100 mL volumetric flask using mobile phase to make a solution (1 mg mL^{-1}) as a stock solution. From this solution, a dilution of 0.166, 0.210, 0.238, 0.320, 0.360 mg mL^{-1}) were prepared as working standard solutions in 100 mL volumetric flasks using mobile phase as solvent.

Calibration Curve

The calibration curve was plotted with five different concentrations of tenoxicam standard solution. The final concentrations of tenoxicam were 0.166, 0.210, 0.238, 0.320, and 0.360 mg mL^{-1}, respectively. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. Before injecting solutions, the column was equilibrated for at least 45 min with the mobile phase flowing through the system. Three determinations were carried out for each solution. Peak areas were recorded for all the solutions. The correlation graph was constructed by plotting peak areas obtained at the optimum wavelength of detection versus the injected amounts.

Chromatographic Conditions

The mobile phase was a mixture of 4.0 gm chloroacetic acid in 400 mL of purified water, pH = 3.0 was adjusted by ammonium hydroxide and

acetonitrile (400:600, v/v). The flow rate was 1.0 mL/min. The UV detector wavelength was set at 254 nm and an attenuation of 1.0 a.u.f.s was used.

RESULTS AND DISCUSSION

A chromatogram of tenoxicam is shown in Fig. 2. The substance is well resolved with retention time of 2.43 min. by using the selected chromatographic conditions. The method was validated statistically for its linearity, accuracy, robustness, and precision.

Linearity

The linearity of peak area response versus concentrations was studied from 0.166, 0.210, 0.238, 0.320, 0.360 mg ml^{-1}, respectively, for tenoxicam. A linear response was observed over the examined concentration range. Table 1 summarizes the correlation coefficient, slope, and intercept.

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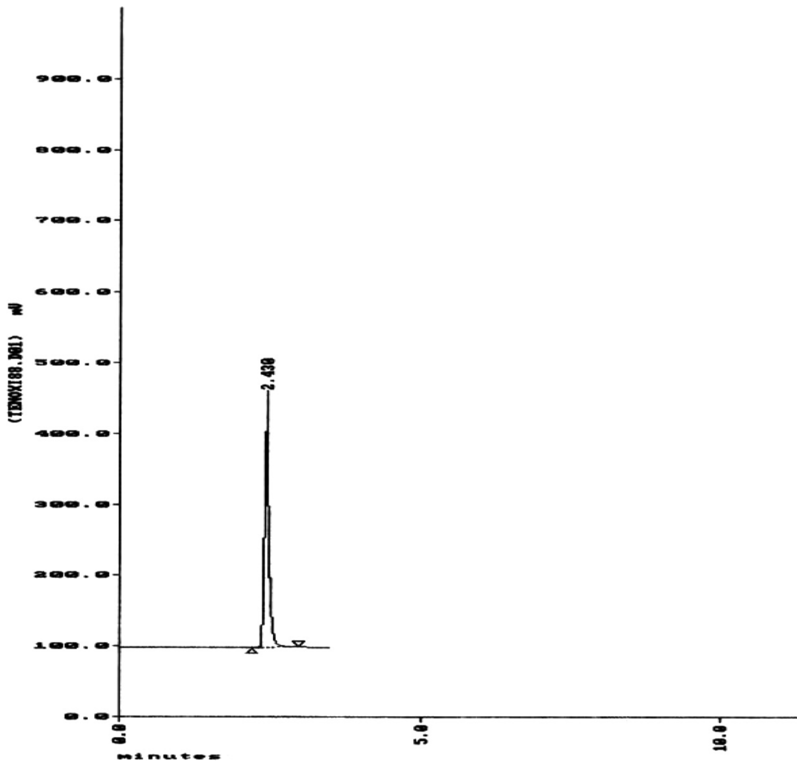

FIGURE 2 Chromatogram of a solution containing tenoxicam.

TABLE 1 Calibration Curve of Tenoxicam

Concentration mg/mL	Area	Slope	Intercept	r^2
0.166	972706	5863301	15018	0.9983
0.210	1239452			
0.238	1394778			
0.320	1875145			
0.360	2109416			

Accuracy/Recovery

Accuracy was studied using simulated preparations at three different concentrations, corresponding to 0.164, 0.261, and 0.324 $mg\,mL^{-1}$. Recovery data obtained were within the range 99.91–100.38% and RSD was 0.11%, Table 2, satisfying the acceptance criteria for the study.

Repeatability

The system repeatability was assessed from twelve replicate injections of a sample solution of tenoxicam at the analytical concentration of $\sim$0.2766 $mg\,mL^{-1}$. The RSD for the active principle was found to be 0.45%, Table 3.

Robustness Testing

Robustness testing was performed in order to obtain information about those critical parameters affecting the response (peak area). The robustness of a method can be tested using an experimental design in order to study the simultaneous variation of the factors.[11]

As a result of data analysis, one is able to indicate which of the tested factors are not robust for the considered response. When factors that are not robust are detected one can decide to change the method or to control the factor in question more strictly.[11,12]

To carry out robustness testing with an experimental design, it is necessary to select the factors and the levels at which to test them, followed by the

TABLE 2 Accuracy/Recovery for Tenoxicam

Concentration (mg/mL)	n	Recovery (%)	RSD (%)
0.164	4	99.91	0.06
0.261	4	100.27	0.16
0.324	4	100.38	0.13
Mean		100.19	0.11

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TABLE 3 Repeatability of the Method

Trial	Actual Concentration	Recovery (%)	
1	0.2740	99.1	
2	0.2745	99.2	
3	0.2758	99.7	
4	0.2756	99.6	
5	0.2774	100.3	
6	0.2757	99.7	
7	0.2761	99.8	
8	0.2767	100.0	
9	0.2779	100.5	
10	0.2774	100.3	
11	0.2768	100.1	
12	0.2773	100.3	
		Mean	99.9%
		R.S.D	0.45%

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selection of the suitable design, which depends on the postulated relationship. In general, linear models are sufficient and advisable because of the small experimental domain and for the reduction in the number of experiments. For each controlled factor, it is necessary to know its optimized value in order to define the interval within it can be controlled.

In the assessment of the HPLC method for tenoxicam all the studied factors during the optimization process (organic modifier percentage, $\boldsymbol{b}_1$; pH, $\boldsymbol{b}_2$; wave length λ, $\boldsymbol{b}_3$; flow rate, $\boldsymbol{b}_4$) were considered.

The experimental domain of selected variables is reported in Table 4. The ranges examined were small deviations from the method settings and the considered response was the peak area.

A linear relationship (Eq. (1)) with four variables was postulated and Plackett-Burman design was chosen for the coefficients evaluation,[13]

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 \quad (1)$$

To test the model linearity, four experiments at the optimized conditions, corresponding to the center of experimental domain, were carried out. The experimental matrix is reported in Table 5.

TABLE 4 Method Settings and Range Investigated During Robustness Testing

Variable	Optimized Value	Range Investigated
Mobile phase 0.02 M Na_2HPO_4:CH_3CN	60:40	55:45–65:35
pH of mobile phase	3	2.9–3.1
Flow rate (mL min^{-1})	1	0.9–1.1
λ(nm)	254	253–255

TABLE 5 Experimental Matrix for Robustness Testing

Exp. No.	Run Order	b1	b2	b3	b4
1	5	1	1	1	−1
2	6	−1	1	1	1
3	8	−1	−1	1	1
4	12	1	−1	−1	1
5	1	−1	1	−1	−1
6	3	1	−1	1	−1
7	11	1	1	−1	1
8	9	−1	−1	−1	−1
9	4	0	0	0	0
10	2	0	0	0	0
11	7	0	0	0	0
12	10	0	0	0	0

The regression model assumed was found not significant by means of analysis of variance, but the graphic analysis of effects (Fig. 3) pointed out that the factor flow rate caused statistically significant variation of the response. Graphic analysis of effects is a tool of experimental design in which the numerical values of the effects are displayed. This analysis requires the construction of a bar graph in which the length of each bar is proportional to the absolute affect value. The effects that exceed the reference lines, corresponding to the 95% confidence interval, are those significant for the response.[14–18] In this case, the variation in flow rate was significant and exerts critical effects on the response. In summary,

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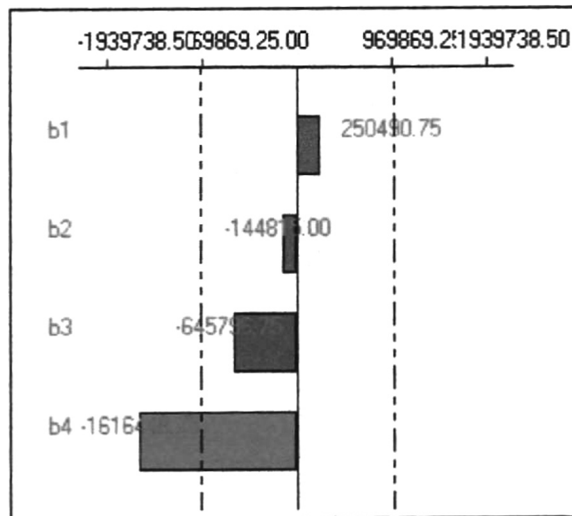

FIGURE 3 Graphic analysis of effect for the response peak area during robustness test.

the method can be considered robust, but a precautionary statement about flow rates have to be included in the procedure.

Intermediate Precision

The intermediate precision is a measure of precision between repeatability and reproducibility. It is obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory.[11] Because these parameters influence the response together, it is advisable to study these effects simultaneously. In this case, the factors considered were the analyst (analyst 1 and analyst 2), the instrument (HPLC 1 and HPLC 2), and the day (day 1 and day 2). A linear model ($y = b_0 + b_1x_1 + b_2x_2 + b_3x_3$) was assumed and a full factorial design 2^3 was employed to estimate the model coefficients.[19] The considered response was the tenoxicam amount found. Each experiment was repeated three times in order to evaluate the experimental error.

The analyses were carried out in a randomized order according to the experimental plan reported in Table 6. The level of tenoxicam was

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TABLE 6 Experimental Plan for Intermediate Precision and Obtained Responses

Analyst	Day	Instrument	Response (%)
1	1	HPLC1	101.21
1	1	HPLC1	101.11
1	1	HPLC1	100.79
1	2	HPLC1	100.58
1	2	HPLC1	100.86
1	2	HPLC1	100.50
2	1	HPLC1	100.99
2	1	HPLC1	100.73
2	1	HPLC1	101.14
2	2	HPLC1	100.84
2	2	HPLC1	100.99
2	2	HPLC1	100.50
1	1	HPLC2	99.98
1	1	HPLC2	99.60
1	1	HPLC2	100.53
1	2	HPLC2	100.32
1	2	HPLC2	99.94
1	2	HPLC2	99.83
2	1	HPLC2	100.78
2	1	HPLC2	100.97
2	1	HPLC2	101.20
2	2	HPLC2	99.51
2	2	HPLC2	100.44
2	2	HPLC2	100.35

$\sim 0.2766\,mg\,mL^{-1}$. The regression model was found not significant, thus indicating that no factor did influence the response. Furthermore, the RSD found in this condition (0.49%, n = 24) was acceptable with respect to RSD (0.45%, n = 12) found in the repeatability study.

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