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ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PIROXICAM IN OINTMENTS AND PLASMA

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ABSTRACT

A new, simple and precise method using ion-pair high-performance liquid chromatography was developed for the determination of piroxicam in ointment and plasma. A reversed-phase system was used, consisting of a 5-μm NOVA-PAK C_{18} column with acetonitrile-water (42:58 v/v) containing 0.01M tetrabutylammonium phosphate and adjusted to pH 7.5 by phosphoric acid as the mobile phase. The flow rate was 0.8 ml/min and the effluent was monitored at 355 nm. The sensitivities of this method were 2 ng/ml levels of piroxicam in ointment and plasma samples using indomethacin as internal standard.

INTRODUCTION

Piroxicam (4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1, 2-benzo thiazine- 3-carboxamide-1, 1-dioxide) is a non-steroidal drug possessing anti-inflammatory, analgesic and antipyretic activities. It has been indicated for

the symptomatic treatment of rheumatoid arthritis and other inflammatory disorders (1).

Numerous approaches have been described for the analysis of this drug. Among these, spectrophotometric (2,3), fluorometric(2,3), high-performance thin-layer chromatographic(4) and high-performance liquid chromatographic (5,6,7,8) methods have been developed for the determination of piroxicam in biological fluids. High-performance liquid chromatography (HPLC) as a determinative technique appears to offer the best approach (5,6,7,8). As reported here, piroxicam and its metabolite were determinated by reversed-phase chromatography using ion suppression technique in acidic eluent to maintain the non-ionic free acid form of piroxicam. However, the HPLC method had some problem in theoretical plates and in peak separation of determination.

In recent years, the reversed-phase ion-pair chromatographic method has been increasingly used for the analysis of ionic compound since the capacity factor of the ionic compounds is increased by forming ion complexes with the pairing reagent. A literature survey also indicated that there was no a technique of ion-pairing reversed-phase HPLC for piroxicam. Therefore, in the present study the technique of ion-pairing reversed-phase HPLC for determination of piroxicam in ointment and plasma has been further investigated.

EXPERIMENTAL

Reagents and Materials

Piroxicam (4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1, 2-benzo thiazine-3-carboxamide-1,1-dioxide) was bought from Industrie Chimiche Farmaceutiche Itatiane S.P.A. and indomethacin used as internal standard, was bought from Simitomo Chemicals.

Acetonitrile, ethyl ether, tetrahydrofuran, acetic acid were of LC grade, tetrabutylammonium phosphate used as Pair-Ion Chromatographic reagent (PIC-A) produed from Waters, U.S.A. and the other reagent were of guaranted reagent grade.

Chromatography

A Waters Associate Model 450 analytical liquid chromatography (HPLC) equipped with a 15 cm x 3.9 mm I.D. 5- m NOVA-PAK C_{18} column (Waters Assoc., part No.086344) was fitted with a Waters Model 440 UV detector. A mobile phase of acetonitrile-0.01M PIC-A reagent (42:58 v/v) mixture was filtered, degassed, and used at a flow-rate of 0.8 ml/min. The aqueous solution of mobile phase was adjusted to pH= 7.5 with phosphoric acid. The effluent stream was monitored at 355 nm wavelength on the detectors.

A Waters 740 Data Module was used to calculate the result, its attenuation was fitted at 32 and the chart speed of the integrator was maintained at 0.5 cm/min.

Ointment Preparation and Extraction

The reagents and preparation of the piroxicam O/W type ointment formulated in Table 1, were essentially the same as that described previously (9).

The analytical procedure of ointment in this report was that 200 mg ointment dissolved in ether-cyclohexane (1:1 v/v) and then 5 ml of Na_2CO_3-$NaHCO_3$ buffer (pH=10.2) was added, vigorously shaken for 1 min, and the sample was centrifuged at 3000 rpm for 30 min. The upper layer was eliminated by suction and 50µl portion of down clear layer was accurately transferred into another tube, acidified with 1 ml of Na_2HPO_4-citric acid buffer (pH=3) and extracted with 5 ml of ether-cyclohexane (1:1 v/v) by mechanical centrifugation shaking for 30 min. After

TABLE 1

Piroxicam o/w Type Ointment Formulation

Piroxicam	2.86%
Cetyl alcohol	5.55%
Stearyl alcohol	5.55%
White vaselin	12.40%
Liquid paraffin	18.56%
Sod. lauryl sulfate	1.30%
Propylene glycol	10.41%
Water	43.37%

for 10 min at 3000 rpm, 1 ml of the ether-cyclohexane phase was transferred to another tube and 20µl of internal standard (2mg/ml) was added. The mixture was evaporated to dryness on a water bath at 40°C. The residue was redissolved in 1 ml mobile phase and shaken for 30 sec by a vortex mixer, then 10 µl of this solution was injected into the HPLC.

Plasma Sample Preparation and Extraction

A 30 µl of internal standard solution (2 mg indomethacin/ml acetonitrile) and 1 ml of 0.01M hydrochloric acid were added to 0.2 ml of plasma. The mixture was extracted with 5 ml of ether-cyclohexane (1:1 v/v) in a 12 ml glass tube, which was shaken gently for 20 min. After centrifugation for 10 min at 3000 rpm, the 4 ml organic phase was transferred to another tube for evaporation by N_2 gas at 40°C water bath. The residue was redissolved in 1 ml of mobile phase by vortexing. An aliquot of 5 µl was injected into the HPLC.

RESULT AND DISCUSSION

Since piroxicam is a weak acidic compound, the HPLC determination of piroxicam is unsuitable by typical con-

ditions of reversed-phase chromatography using neutral eluent. Therefore, a compositive modification of the eluent is definitely required to improve the resolution of piroxicam for better chromatographic properties. In previous study (6,7,8), the ion-suppression method that the mobile phase consisted of acetic acid and acetonitrile had been used to suppress the ionization of piroxicam and reduce peak tailing. In order to obtain higher resolution for piroxicam, ion-pairing complex of piroxicam with counter-ion in mobile phase was approached, and the mixing ratio of acetonitrile and water, the concentration of counter-ion and the pH of the mobile phase were investigated to find the optimum conditions for determination of piroxicam.

Indomethacin was used as internal standard (10), and tetrabutylammonium phosphate was examined as the counter-ion (11). The relationship between the capacity factor (K') and the counter-ion concentration of tetrabutylammonium was shown in Fig.1. The effect of counter-ion concentration in the acetonitrile-water (42:58 v/v) mobile phase on the chromatography of piroxicam was evaluatead from 0.002 to 0.02 M, while maintaining all other variables constant. The capacity factor (K') of piroxicam and indomethacin increased as the concentration of tetrabutylammonium increased. The concentration of tetrabutylammonium was selected at 0.01 M because adequate retention and resolution for piroxicam was obtained at that level.

The effect of ratio of acetonitrile and 0.01 M pairing ion reagent on the capacity factor of piroxicam was shown in Fig.2. By lowering the percentage of acetonitrile in the mobile phase would prolong the retention time of piroxicam and indomethacin, and 42% of acetonitrile in the mobile phase was selected for this analysis. In this condition the peaks of piroxicam and indomethacin were separated completely.

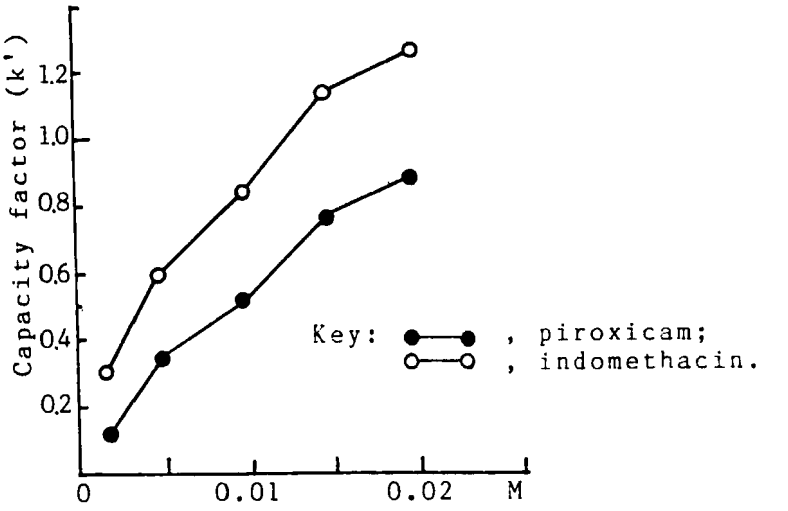

FIGURE 1
Influence of tetrabutylammonium (paired-ion) concentration on the capacity factor (k') of piroxicam and indomethacin.

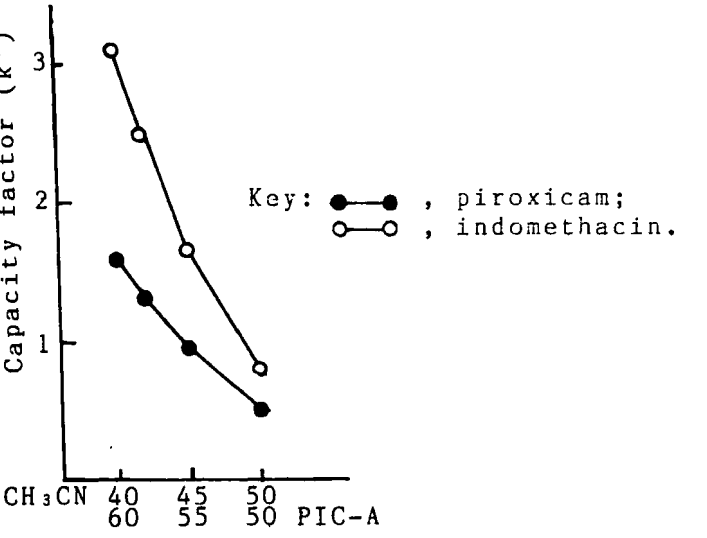

FIGURE 2
Influence of the mixing ratio of CH_3CN and tetrabutylammonium (PIC-A) reagent on the capacity factor (k') of piroxicam and indomethacin.

The pairing effect occurs when a molecule dissociates into ions in the mobile phase. Piroxicam dissociates near pKa 5.5 (9) and indomethacin dissociates near pKa 4.2(10). Therefore, the effect of variation in the mobile phase pH on the chromatogram was evaluated for the pH range. The relationships between pH and peak height are shown in Fig.3. The peak height of piroxicam and indomethacin increased as the pH value of mobile phase increased from 6.5 to 7.5. Good peak shapes of piroxicam and indomethacin were obtained at pH = 7.5.

Based on the above optimization of conditions for the mobile phase, a typical chromatogram of piroxicam is shown in Fig.4. The peak shape of piroxicam and indomethacin on the chromatogram could be improved by comparisonwith the previous study (7,8). The retention time of piroxicam and indomethacin were 3.29 and 4.94 min, respectively.

Under the chromatographic conditions described above, Fig.4 gives typical chromatograms for indomethacin and

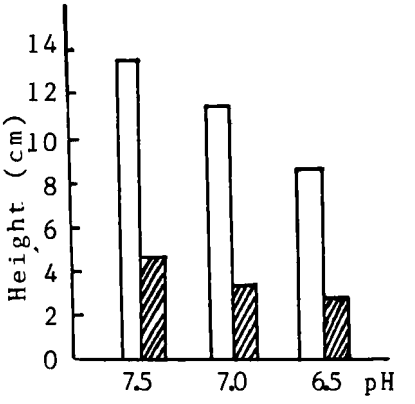

FIGURE 3
Influence of the pH value of mobile phase on the peak high of piroxicam and indomethacin.
Key: ▭ , piroxicam;
▨ , indomethacin.

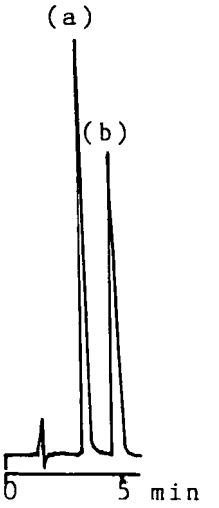

FIGURE 4
The trace of high-performance liquid chromatogram of piroxicam and indomethacin.
Peak (a): piroxicam;
(b): indomethacin.

piroxicam obtained from a spiked ointment sample. The linearity of the calibration curves, $Y= 0.0457X + 0.012$ ($r=0.9990$), for piroxicam in a plain ointment at concentrations ranging from 6 to 40 mg/g ointment was obtained from the observed value in Table 2. The precision and reproducibility are also summarized in Table 2. The coefficient of variation (C.V.) for these results was less than 5% for all the investigated.

During the development of this assay, a number of variations were tried. An analytical procedure for piroxicam ointment similar as that for indomethacin ointment was approached (12), but the residue of ointment base might precipitate in the mobile phase during through the column and the efficiency of the column was reduce. Therefore, the present analytical procedure for piroxicam

TABLE 2

Assay Precision and Reproducibility for Piroxicam Extracted from Ointment Base

Concentration (μg/ml)	Mean area ratio sample/I.S.(n=3)	S.D.	CV(%)
6	0.2599	0.0063	2.42
10	0.4988	0.0215	4.31
20	0.9195	0.0108	1.17
30	1.3948	0.0166	1.19
40	1.8302	0.0344	1.88

ointment was developed as stated under EXPERIMENTAL section in this study. The ether-cyclohexane (1:1 v/v) solvent used can dissolve all components of the ointment base and piroxicam. The Na_2CO_3-$NaHCO_3$ buffer (pH=10.2) was chosen as the first extracting solvent because of its ability to separate ionized piroxicam from the ointment base. During the Na_2HPO_4-citric acid buffer (pH=3) solution added to the first extracting solvent, the ionized piroxicam was changed to unionized piroxicam which was extracted by the second solvent of ether-cyclo hexane (1:1 v/v). This method gave a good recovery of this compound with essentially no interference from the ointment.

The present reversed-phase ion-pair chromatographic method was also applied to the plasma of rat to which piroxicam was added. Table 3 shows the linearity of the calibration curve (Y=0.0115X + 0.0188, r=0.9992) for piroxicam in rat plasma at concentration ranging from 5 to 75 μg/ml. The recovery and reproducibility are also summarized in Table 3. The coefficient of variation (C.V.) for these results was less than 5% for all all concentrations investigated.

TABLE 3

Assay Precision and Reproducibility for Piroxicam Extracted from Plasma of Rats

Concentration (μg/ml)	Mean area ratio sample/I.S.(n=3)	S.D.	CV(%)
5	0.0820	0.0040	4.88
15	0.1889	0.0039	2.06
25	0.3087	0.0151	4.90
50	0.5859	0.0127	2.17
75	0.8908	0.0176	1.97

The extraction of piroxicam from plasma samples was pH dependent. As shown in Table 4, it was found that the recovery of piroxicam could be improved when the 0.01M HCl solution was added to the plasma. Different organic solvents have been studied as extractive solvent and its results were shown in Table 5. It has been found that ether-cyclohexane (7:3 v/v) had the best extractive recovery of piroxicam. The next best were ether, benzene, ether-cyclohexane (6:4 v/v), ether-cyclohexane (5:5 v/v) and so on. Increasing the proportion of ether in the ether-cyclohexane mixture was found to increase the

TABLE 4

Extraction Recovery with Different Acidified Solutions

Acidified solution	Piroxicam added(μg)	n	Recovery (μg) mean (S.D.)	ratio
1M HCl	30	3	15.66 (0.89)	0.522
0.1M HCl	30	3	28.28 (0.64)	0.943
0.01M HCl	30	3	28.03 (1.82)	0.934
pH=2.2*	30	3	27.55 (1.20)	0.918
pH=3.0*	30	3	25.98 (2.44)	0.866

* Disodium hydrogen phosphate-citric acid buffer solution.

TABLE 5

Extraction Recovery with Different Extraction Solvents

Extraction solvent	Piroxicam added(μg)	n	Recovery (μg) mean (S.D.)	ratio
Ether	30	3	29.03 (1.38)	0.968
Cyclohexane	30	3	14.48 (0.67)	0.583
Benzene	30	3	28.92 (0.53)	0.964
Ethylacetate	30	3	25.09 (1.04)	0.837
E:C* (3:7)	30	6	25.36 (1.36)	0.846
E:C (4:6)	30	6	26.92 (1.12)	0.897
E:C (5:5)	30	6	27.89 (2.18)	0.930
E:C (6:4)	30	6	27.68 (1.95)	0.933
E:C (7:3)	30	6	29.79 (1.76)	0.973

* Ether:Cyclohexane

extractive recovery of piroxicam. However, the mixing ratio (5:5 v/v) of ether-cyclohexane was selected for this analysis due to considering the toxicity of benzene, the convenience of performance and the volatility of ether.

Piroxicam (10 mg/kg) was given intravenously to rats and time courses for the concentration of piroxicam in the plasma of rats are shown in Fig.5. The plasma levels of piroxicam appear to be consistent with a two-compartment model. Table 6 summarizes the pharmacokinetic parameters generated from analysis of the data.

TABLE 6

The Pharmacokinetic Parameters of Piroxicam in Rat Following Intravenous Administration in Dose 10 mg/kg

$$Cp = A'e^{-\alpha t} + B'e^{-\beta t}$$

A'	30.16	K_{12}	0.333
B'	28.22	K_{21}	0.487
α	0.903	Ke	0.180
β	0.097	AUC	324.3
Cp	58.38	t1/2	7.140

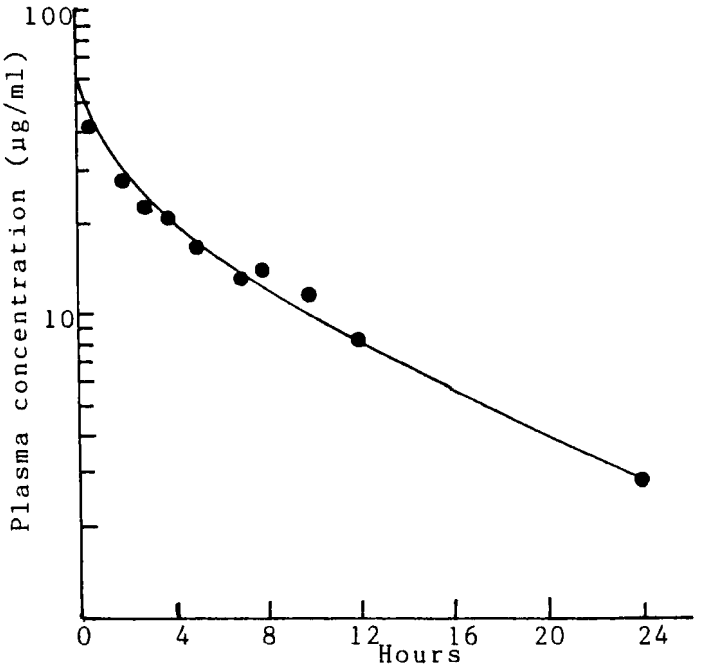

FIGURE 5

Plasma concentration-time curve for piroxicam (●—●) in plasma of rat after I.V. administration of 10 mg/kg of piroxicam. Curve for piroxicam calculated from the equation $Cp = 30.16\ e^{-0.903t} + 28.22\ e^{-0.097t}$, where Cp is piroxicam concentration in plasma.

In conclusion, it can be said the reversed-phase ion-pairing HPLC procedure described here provides a precise and accurate method for the determination of piroxicam in the dosage form of ointment and the biological fluid of plasma.

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