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Sensitive LC determination of piroxicam after in vitro transdermal permeation studies

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Abstract

A direct, very sensitive, simple and rapid high-performance liquid chromatographic (HPLC) method for the determination of piroxicam, with tenoxicam as internal standard, has been developed and validated. Samples were chromatographed on a 5 μm Scharlau C_{18} column. The mobile phase was a mixture of acetonitrile–acetic acid 4% (pH 2.8) (45:55, v/v). Detection was at 354 nm and the run time was 7 min. The limit of detection was 0.025 μg/ml. The detector response was found to be linear in the concentration range 0.05–9 μg/ml. This HPLC assay has been applied to measure the 'in vitro' percutaneous permeation of piroxicam through abdominal hairless rat skin, using Franz-type diffusion cells, in order to obtain the concentration–time profiles of piroxicam. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Piroxicam (4-hydroxy-2-methyl-*N*-(2-pyridyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-di-oxide) is a non-steroidal anti-inflammatory and analgesic agent. This drug has been widely used in the treatment of rheumatoid arthritis and other inflammatory disorders [1]. Its efficacy has been demonstrated, in humans, in the treatment of various inflammatory diseases and arthropathies.

Piroxicam is well absorbed following oral administration; however, its use has been associated with a number of undesirable side effects on the stomach: nausea, dyspepsia, diarrhoea, constipation and some renal ones. Besides, it can cause gastric mucosal damage, which may result in ulceration and/ or bleeding [2]. Consequently, its topical administration can be useful to reduce such systemic adverse actions [3]. Because piroxicam is not easily absorbed through the skin, many attempts have been made using different vehicles and penetration enhancers to increase its percutaneous permeation [4–7].

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In previous works, the amount of drug in the samples was determined using double-beam UV spectrophotometry [8,9], spectrofluorometry [10], thin-layer chromatography [11], capillary electrophoresis [12] and liquid chromatography with UV detection [13–15].

Chromatography could be performed on biological samples containing piroxicam without chemical modification of the drug. Furthermore, they were simpler analytically, more rapid and, as they incorporated internal standards, they were also more accurate. In general, high-performance liquid chromatography (HPLC) has been the most employed method to measure piroxicam. In this way, numerous chromatographic methods have been published for quantify piroxicam in different medias (phosphate buffer, plasma, urine, bile and different tissues) [14–18], although most of these HPLC methods presented insufficient sensitivity or chromatographic interferences between the drug and endogenous compounds of the skin for percutaneous permeation studies. Some authors [19,20] have already described suitable methods for the determination of piroxicam in transdermal permeation studies, although with these methods tailing factors >1 were observed under these chromatographic conditions unless pair ionic agents or organic modifiers (i.e. isopropilamine) were used. Moreover, most of these methods involved sample pretreatment with different extraction procedures, which must be avoided when a great number of samples have to be analyzed, as accounted in permeation studies. This report described a rapid and sensitive method for the determination of piroxicam in buffer solution and it can be employed to measure the percutaneous permeation using very diluted samples.

This method has been successfully applied to determine the concentration of piroxicam obtained in the 'in vitro' percutaneous permeation studies, through hairless rat skin, using the automatic Franz-type diffusion cells.

2. Materials and methods

2.1. Materials

Piroxicam was a gift from Industrial Kern Española, S.A. (Barcelona, Spain) and tenoxicam (internal standard (IS)) was kindly supplied by Roche (Barcelona, Spain) (Fig. 1). Carbopol ETD 2001®, trietanolamine 85%, and propylene glycol USP were supplied by Roig Pharma S.A. (Barcelona, Spain). Acetonitrile (HPLC grade), glacial acetic acid (HPLC grade) and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). Orthophosphoric acid (85%), potassium dihydrogenphosphate and disodium hydrogenphosphate dihydrate were analytical grade from Merck.

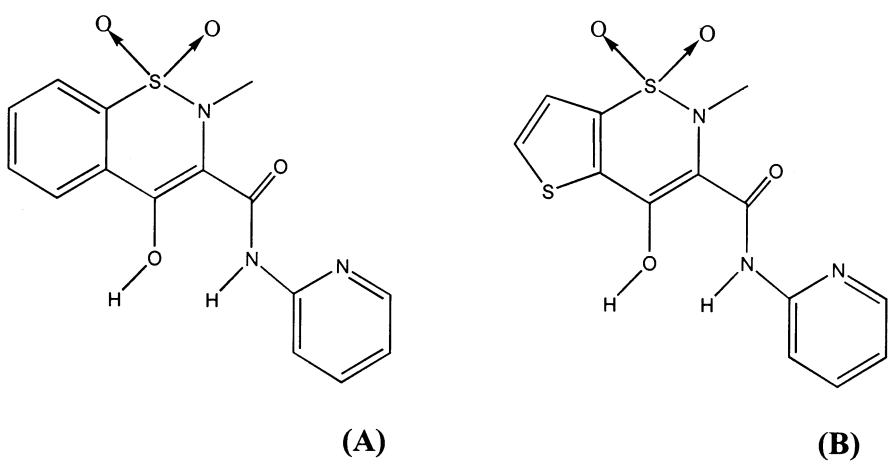

Fig. 1. Chemical structures of (A) piroxicam and (B) tenoxicam.

2.2. Standard solutions and samples

A stock solution of piroxicam with a concentration of 10 μg/ml was prepared by dissolving 10 mg piroxicam in a phosphate buffer (pH 7.4). Nine standard solutions (0.05, 0.1, 0.2, 0.5, 1, 3, 5, 7 and 9 μg/ml) were made by further dilution of the stock solution with appropriate volumes of the phosphate buffer.

The internal standard stock solution of tenoxicam (50 μg/ml) was prepared in methanol. Standard and stock solutions of piroxicam and tenoxicam were stored at 4 °C.

2.3. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Hewlett-Packard (Waldbronn, Germany) system equipped with a HP 1050 quaternary pump; a HP 1050 autosampler and a HP 1050 diode-array detector set at 354 nm (λ_{max}). Data acquisition and treatment were performed with a Hewlett-Packard computer using ChemStation G2170 AA. Separation was carried out at 40 °C on a reversed-phase, 12×0.46 cm base stable column packed with 5 μm C_{18} silica reverse particles (Scharlau C_{18}). This column was obtained from OPPAC (Pamplona, Spain). The mobile phase was a mixture of acetonitrile–acetic acid glacial 4% (pH 2.8) (45:55, v/v). The mobile phase was filtered through a 0.45 μm pore-size membrane filter. The flow rate was 1 ml/min. The injection volume was 100 μl.

2.4. Instrument calibration

Calibration curves were prepared using concentrations of 0.05, 0.1, 0.2, 0.5, 1, 3, 5, 7 and 9 μg/ml piroxicam. The standard solutions for calibration curve were prepared by adding 250 μl each piroxicam standard solution to drug-free matrix into a 700 μl glass tube. Previously, 50 μl internal standard (50 μg/ml) was spiked into this glass tube and evaporated to dryness under reduced pressure (Rotary evaporator, Model 4322000; Labconco, USA).

Calibration curves were determined by least-squares linear regression analysis (weighting, $1/x^2$). Peak area ratios of piroxicam and tenoxicam versus the corresponding drug concentration were plotted.

The linearity of the method was confirmed by statistical comparison among the slopes obtained, the intercepts of calibration curves with zero and the correlation coefficients with 1. Moreover, a Student t-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones.

2.5. Specificity

The specificity of the assay was verified against endogenous compounds of skin. Several blank skin samples from different rats were tested for the absence of interfering compounds. The retention times of endogenous compounds in the matrix were compared with those of piroxicam and IS.

2.6. Precision

In this work, precision of the method was tested as both within-day and between-day reproducibilities of the assay. Precision of a method was expressed as the relative standard deviation (RSD) of replicate measurements. To be acceptable, the measures should be lower than 10% at all concentrations [21,22].

This study was developed with three concentrations of piroxicam in phosphate buffer (0.05, 1, and 9 μg/ml). Several aliquots of each samples were tested the same day to determine the within-day reproducibility. Aliquots of the same sample were tested once a day, during different days, to determine between-day reproducibility.

2.7. Accuracy

Accuracy of the assay method was defined as the percentage of recovery by the assay of the known added amount of analyte in the sample. To be acceptable, measures should be within $\pm$ 10% at all concentrations [21,22].

2.8. Determination of the limits of quantification and detection

The limit of quantification (LOQ) was defined as the lowest drug concentration, which can be determined with an accuracy and precision $<$ 20%. The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. In this work, the LOD of the assay method was determined by analysis of the peak baseline noise in ten blank samples [21,22].

2.9. Preparation of piroxicam gels

Gels were prepared by dispersing 1% (w/w) Carbopol ETD 2001® in a mixture of water–propylene glycol (60:40, w/w) with 1% (w/w) piroxicam, under magnetic stirring. The pH of the carbopol dispersions was adjusted to 7.4 with triethanolamine and stored at room temperature for 24 h prior to use.

2.10. Application of the method to 'in vitro' permeation studies

This assay was employed to determine the 'in vitro' percutaneous permeation of piroxicam through abdominal hairless rat skin (OFA-hr/hr males, 200–250 g) using Franz-type diffusion cells (Microette, Hanson Research, USA) to obtain the concentration–time profiles of this drug.

Under CO_2 asphyxiation, the abdominal hair of the rats was surgically removed from the animal and the adhering subcutaneous fat was carefully cleaned. To remove extraneous debris and leachable enzymes, the dermal side of the skin was in contact with a saline solution for 1 h before being mounted on the automatic Franz-type diffusion cell, with the stratum corneum facing the donor compartment. Then, 0.5 g gels were placed in the donor compartment.

Sample collection times were 4, 8, 12, 16, 19, 22 and 25 h. The 0.4 ml samples were taken from the receptor compartment and replaced by 0.5 ml volume of the fresh buffer. Samples were filtered and analyzed immediately.

The flux, J, was determined from the slope of the steady-state portion of the amount of the drug permeated versus time plot and the lag time from the x intercept. The permeability coefficient, P, was estimated from the flux and the donor drug concentration.

3. Results and discussion

In this paper, we have described a specific HPLC method suitable to evaluate the percutaneous permeation of piroxicam ' in vitro' in transdermal delivery systems. These systems avoid the undesirable side effects observed following non-steroidal anti-inflammatory agent (NSAID) administration by oral route.

Methods employed for the quantification of NSAIDs from the samples obtained in the permeation 'in vitro' studies have to be specific, since these kind of samples were usually contaminated with skin endogenous compounds. Moreover, these methods also have to show enough sensitivity, due to the little volume of sample obtained from the receptor medium of Franz diffusion cells in the skin permeation studies.

This HPLC method was based on that developed by Troconiz et al. [16], although some modifications were made to obtain better sensibility for the very diluted samples employed in the permeation experiments. For this reason, we have used a Scharlau C_{18} column of 12×0.26 cm^2 instead of Nucleosil C_{18}, to increase the number of theoretical plates and achieve better resolution of the peaks. This column is a pH-stable column packed with a high-performance stationary phase, a monofunctional phase bonded to extremely pure spherical silica particles that exhibits good peak shape. In addition, the pH of the mobile phase was 2.8, a pH value in which piroxicam is fully unionised ($pK_{a1} = 1.86$). The acetic acid, in this case, was able to avoid the pK_{a2} of piroxicam ($pK_{a1} = 1.86$ and $pK_{a2} = 5.46$). On the other hand, the addition to the mobile phase of a carboxylic acid, as acetic acid, reduced the secondary retention of acidic samples in the stationary phase. Consequently, the retention of piroxicam in the stationary phase increases (the k' values were,

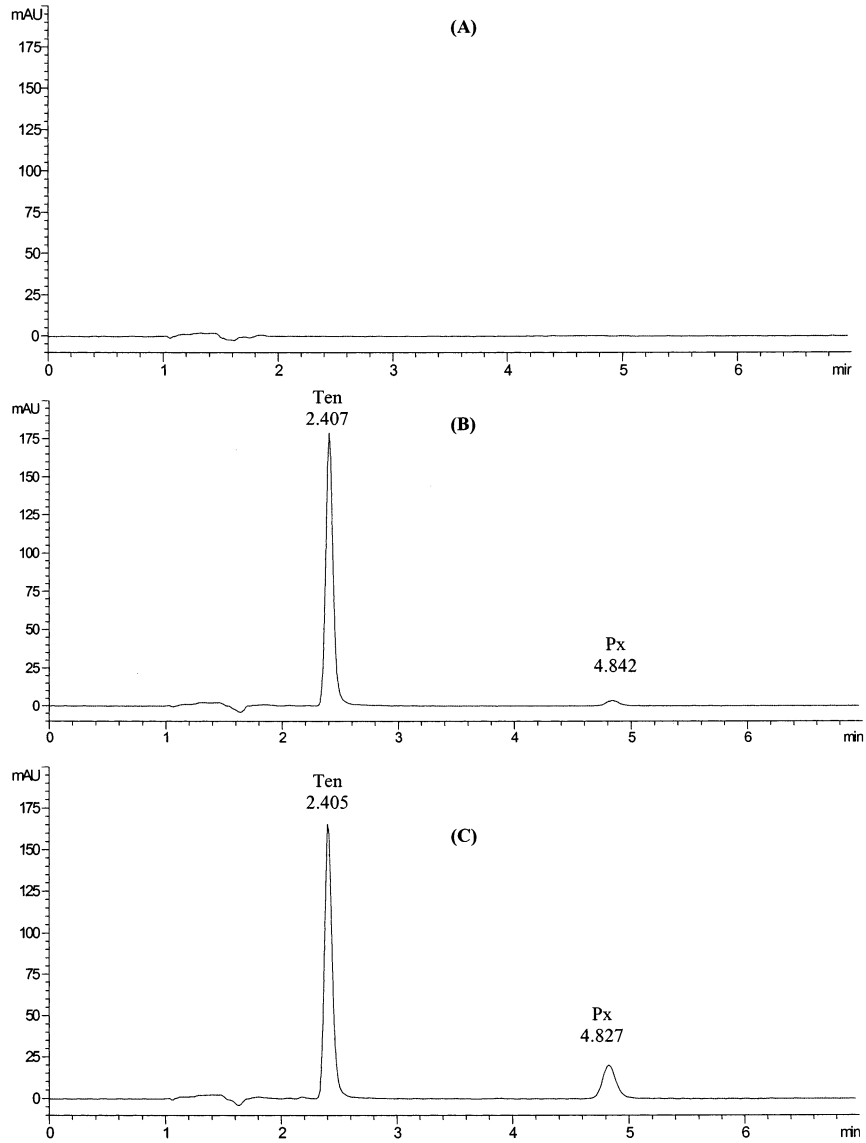

Fig. 2. Chromatograms obtained from the analysis of a blank sample (A), and samples after 8 h (B) and 25 h (C) in a piroxicam transdermal permeation study. Ten, Tenoxicam; Px, piroxicam.

respectively, 1.07 and 3.53 for IS and piroxicam), and acceptable asymmetry coefficients was obtained (the asymmetry coefficients were 0.83 ± 0.041 ($n = 5$) for piroxicam and 0.80 ± 0.057 ($n = 5$) for IS).

Under the chromatographic conditions used, piroxicam and tenoxicam (IS) have retention times of 4.6 ± 0.004 and 2.3 ± 0.012 min, respectively. Representative chromatograms for samples obtained in a permeation study, after applying the topical formulation of piroxicam, are shown in Fig. 2. There was clear resolution of the compounds of interest ($R_s = 14.81 \pm 0.398$, $n = 5$) without no endogenous sources of interference. Tenoxicam was acceptable as IS because it exhibits similar chromatographic properties to

Table 1
Accuracy of the HPLC method for determinig piroxicam concentrations in phosphate buffer (pH 7.4) after transdermal permeation experiments

Concentration added (μg/ml)	Accuracy ($n = 5$)	
	Concentration found (mean ± S.D.) (μg/ml)	Accuracy (%)
0.05	0.048 ± 0.004	4
1	0.938 ± 0.023	6.2
9	9.546 ± 0.252	6

piroxicam. Moreover, neither interference from excipients or endogenous compounds of skin was observed in any experiment.

The assay performance of the present method was assessed by all following criteria: linearity, accuracy, precision, LOD, LOQ, stability and applicability in percutaneous permeation studies.

The assays exhibited linearity between the response (y) and the corresponding concentration of piroxicam (x), over the 0.05–10 μg/ml range in the samples (typical equation, $y = 0.351x + 0.00056$). The results by least-squares linear regression analysis showed that correlation coefficients of all standard curves were ≥ 0.997. For each point of calibration standards, the concentrations were back-calculated from the equation of the regression curves, and RSDs were computed. The obtained values were below 10% for all concentrations. For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from 0. Moreover, a linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope an intercept equal to 0 (Student t-test).

Accuracy values were within acceptable limits (Table 1). The results for within-day and between-day precision are presented in Table 2 for our samples and the values were below 10% in both cases.

The LOD of piroxicam was 0.025 μg/ml, a value smaller than that reported by Cerretani et al. [15] in plasma, muscle and skin (0.1 μg/ml), and by Avgerinos et al. [13] in human plasma and urine (0.05 μg/ml). The estimated LOQ in this study was found to be 0.05 μg/ml, a similar value to that previously reported by Cordero et al. [20] in skin permeation studies.

Stability studies carried out in phosphate buffer indicated that samples permeation were stable for at least several weeks when stored at 4 °C.

The applicability of this method has been demonstrated by the study of in vitro percutaneous permeation of piroxicam through abdominal hairless rat skin. Piroxicam flux value at steady state from the gel applied was 0.21 ± 0.03 μg cm^{-2} h^{-1}, and the permeability coefficient value calculated was 2.08 ± 0.27 cm h^{-1}. For this formulation, a lag time period of 4.83 ± 0.85 h was observed.

The HPLC method described here enables the measurements of piroxicam in percutaneous permeation tests through abdominal hairless rat skin. Other methods, proposed by previous authors, were also able to detect piroxicam in these kinds of samples, although in some cases, the drug was

Table 2
Between-day and within-day variabilities of the HPLC method for determining piroxicam concentrations in phosphate buffer (pH 7.4) after percutaneous permeation studies

Concentration added (μg/ml)	Between-day variability ($n = 10$)		Within-day variability ($n = 5$)	
	Concentration found (mean ± S.D.) (μg/ml)	RSD (%)	Concentration found (mean ± S.D.) (μg/ml)	RSD (%)
0.05	0.051 ± 0.005	9.8	0.048 ± 0.004	8
1	0.939 ± 0.034	3.6	0.938 ± 0.023	2.4
9	9.482 ± 0.627	6.6	9.546 ± 0.252	2.6

transported across a model synthetic membrane [23] and when the transport was across the skin, the limit of quantification was worse than ours [20].

4. Conclusions

A simple chromatographic method has been developed for the rapid and precise determination of piroxicam in phosphate buffer after its transdermal permeation.

The simplicity of the technique (without any extraction procedure), the shorter analysis time (<8 min) and the high sensitivity makes this technique particularly attractive for this purpose.

This method was sensitive, accurate and has a good level of precision. The results described in this paper show that this assay is suitable for the determination of piroxicam after skin permeation experiments through abdominal hairless rat skin.

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