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Determination by high-performance liquid chromatography of ketoprofen in vitro in rat skin permeation samples

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Abstract

A direct, simple and rapid high-performance liquid chromatographic method has been developed for the determination of ketoprofen with ibuprofen as internal standard. Samples were chromatographed on a 5 μm Kromasil 100 C_{18} column. The mobile phase was a mixture of acetonitrile–0.01 *M* KH_2PO_4 adjusted to pH 1.5 with orthophosphoric acid 85% (60:40, v/v). Detection was at 260 nm and the run time was 10 min. The detector response was found to be linear in the concentration range 0.02 to 40 μg/ml. This HPLC assay has been applied to measure the ''in vitro'' percutaneous penetration of ketoprofen through rat skin.

Keywords: Pharmaceutical analysis; Ketoprofen; Ibuprofen; Nonsteroidal anti-inflammatory drugs

1. Introduction

Ketoprofen, an aryl propionic acid derivate, is a potent nonsteroidal anti-inflammatory agent which also has analgesic and antipyretic activity [1]. This drug has been widely used for the treatment of rheumatoid arthritis, osteoarthritis [2], ankylosing spondylitis and gout [3].

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

Numerous chromatographic methods have been published for quantify ketoprofen in different medias; serum [5], urine [1], plasma [6–9], and also in pharmaceuticals [10]. The amount of drug in these samples was determined using double beam UV spectrophotometry [3], thin-layer chromatography (TLC) [11], capillary electrophoresis [12], gas chromatography (GC) [5], electrospray ionization mass spectrometry (ESI-MS) [13] and liquid chromatography with UV detection [1,2,4]. In general, high-performance liquid chromatography (HPLC) has been the most employed method to measure ketoprofen, although most of these methods present insufficient sensitivity, inadequate reproducibility or chromatographic interferences and are not sufficiently sensitive for the determination of ketoprofen in early stages of the percutaneous absorption process. The developed HPLC assay method offers detection

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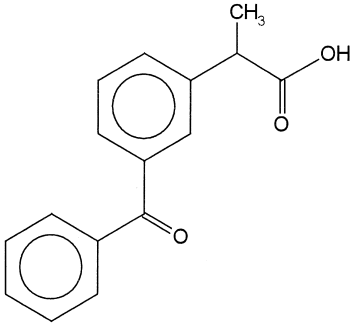
and quantification limits lower than other published methods which are used for measuring the percutaneous penetration without interferences.

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

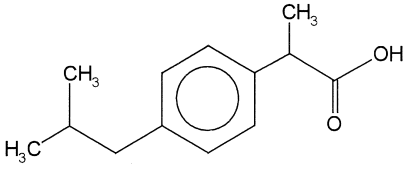
2. Experimental

2.1. Chemicals, reagents and solutions

Ketoprofen was a gift from Menarini (Barcelona, Spain) and ibuprofen (internal standard, I.S.) was kindly supplied by Bayer (Barcelona, Spain) (Fig. 1). Carbopol ETD 2020 was obtained from BFGoodrich (Cleveland, OH, USA). Triethanolamine 99% and propylene glycol were purchased from Roig-Pharma (Barcelona, Spain). Acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). Orthophosphoric acid 85%, potassium dihydrogenphosphate and disodium hydrogenphosphate dihydrate were of analytical grade from Merck.



(A)



(B)

Fig. 1. Chemical structures of (A) ketoprofen and (B) ibuprofen.

2.2. Standard solutions and samples

A stock solution of ketoprofen with a concentration of 40 μg/ml was prepared by dissolving 10 mg of ketoprofen in a phosphate buffer, pH 7.2. Nine standard solutions (0.02, 0.04, 0.2, 0.4, 2, 4, 10, 20 and 30 μg/ml) were made by further dilution of the stock solution with appropriate volumes of the phosphate buffer.

The internal standard stock solution of ibuprofen (2 mg/ml) was prepared in methanol.

Standard and stock solutions of ketoprofen and ibuprofen 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 260 nm. 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, 250×4 mm base stable column packed with 5 μm C_{18} silica reversed-phase particles (Kromasil 100 C_{18}). This column was obtained from Tecnokroma (Barcelona, Spain). The mobile phase was a mixture of acetonitrile–0.01 *M* potassium phosphate adjusted to pH 1.5 with orthophosphoric acid (60:40, v/v). The mobile phase was filtered though 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 0.02, 0.04, 0.2, 0.4, 2, 4, 10, 20, 30 and 40 μg/ml for ketoprofen. The standard solutions for calibration curve were prepared by adding 1 ml of each ketoprofen standard solution to drug-free matrix into a 15-ml glass tube. Previously, 250 μl of

internal standard solution (2 mg/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 square linear regression analysis (weighting $1/x^2$). Peak area ratios of ketoprofen and ibuprofen versus the corresponding drug concentration were plotted.

The linearity of the method was confirmed by comparing the slopes, 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 that of ketoprofen and I.S.

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 RSD of replicate measurements. To be acceptable, the measures should be lower than 10% at all concentrations.

This study was developed with four concentrations of ketoprofen in phosphate buffer (0.02, 0.2, 10 and 30 μg/ml). Several aliquots of each sample 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 analyte in the sample. A Student *t*-test was performed to compare the obtained values with zero.

2.8. Determination of the limit of quantitation (LOQ) and detection (LOD)

LOD was defined as the sample concentration resulting in a peak area of three-times the noise level. LOQ was defined as the lowest drug concentration, which can be determined with an accuracy and precision <20%. In this work the LOD of the assay method was determined by analysis of the peak baseline noise in 10 blank samples.

2.9. Ruggedness

For ruggedness studies, different reversed-phase columns, such as LiChrospher C_{18} Select B (5 μm particle size; 25×0.40 cm; Merck) and Spherisorb C_{18} (15×0.46 cm; Tecnokroma) were used. Similarly, the influence of mobile phase (percentage of acetonitrile ranged from 1 to 5%) and column temperature (35°C or 45°C) on the analytical procedure was also evaluated.

2.10. Preparation of ketoprofen gels

Gels were prepared by dispersing 1% (w/w) Carbopol ETD 2020 in a mixture of water–propylene glycol (60:40, w/w) with 1% (w/w) ketoprofen, under magnetic stirring. The dispersion was then neutralized (pH 7.2) by the addition of triethanolamine and stored at room temperature for 24 h prior to use.

2.11. Application of the method to "in vitro" permeation studies

This assay was utilized to determine the in vitro percutaneous penetration of ketoprofen through abdominal rat skin (Wistar males 200–250 g) using the automatic Franz-type diffusion cells (Microette, Hanson Research, USA) in order to obtain the concentration–time profiles of this drug.

Under ether anesthesia, the abdominal hair of the rats was shaved using an electric razor. The abdominal skin 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. 0.6 g of the gel was placed in the donor compartment.

Sample collection times were 2, 4, 6, 8, 10, 12 and 14 h, 0.4 ml samples were taken from the receptor compartment and replaced by 0.5 ml volume of the 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 absorption of ketoprofen ''in vitro'' in transdermal delivery systems. These systems avoid the undesirable side effects observed following non-steroidal anti-inflammatory agent (NAISD) administration by oral route. Methods employed for the quantification of NAISDs from the samples obtained in the absorption ''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 for the receptor medium of Franz diffusion cells in the skin permeation studies.

For this reason, we have employed a Kromasil 100 C_{18} column of 250×4 mm I.D. in order to increase the number of theoretical plates. 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, we have adjusted mobile phase pH to 1.5, a pH value in which ketoprofen is fully unionised ($pK_a=5.02$). Consequently, the retention of ketoprofen in the stationary phase increases (the k' values were, respectively, 1.79 and 4.35 for ketoprofen and I.S.), and acceptable asymmetry coefficients were obtained (the asymmetry coefficients were 0.85 for ketoprofen and 0.84 for I.S.).

Under the chromatographic conditions used, ketoprofen and ibuprofen (I.S.) have retention times of 3.7±0.004 and 7.2±0.012 min, respectively. Representative chromatograms are shown in Fig. 2 for samples obtained in an absorption ''in vitro'' study. There was clear resolution of the compounds of interest ($R_s=6.92$) without no endogenous sources of interference. Ibuprofen was acceptable as I.S. because it exhibits similar chromatographic properties to ketoprofen. Moreover, neither interferences from excipients or endogenous compounds of skin were observed in any experiment.

The HPLC method described here enables the measurement of ketoprofen in Franz diffusion tests. Other methods proposed by previous authors are also able to detect ketoprofen in these kind of samples [14], although many of these methods fail to determine ketoprofen in early stages of the percutaneous absorption process. Only a gel permeation chromatographic method published by Fujii et al. [15] in 1997, is adequate for this purpose. Nevertheless, gel permeation chromatography is a technique more expensive and inadequate for examining a large number of dirty samples.

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

The assays exhibited linearity between the response (y) and the corresponding concentration of ketoprofen (x), over the 0.02–40 μg/ml range in the samples (typical equation: $y=-0.098x+1.34\cdot10^{-5}$). The results by least-square linear regression analysis show that correlation coefficients of all standard curves were ≥0.998. 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 are 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 and intercept equal to 0 (Student t-test).

Accuracy values were within acceptable limits (Table 1). The results for within-day and between-

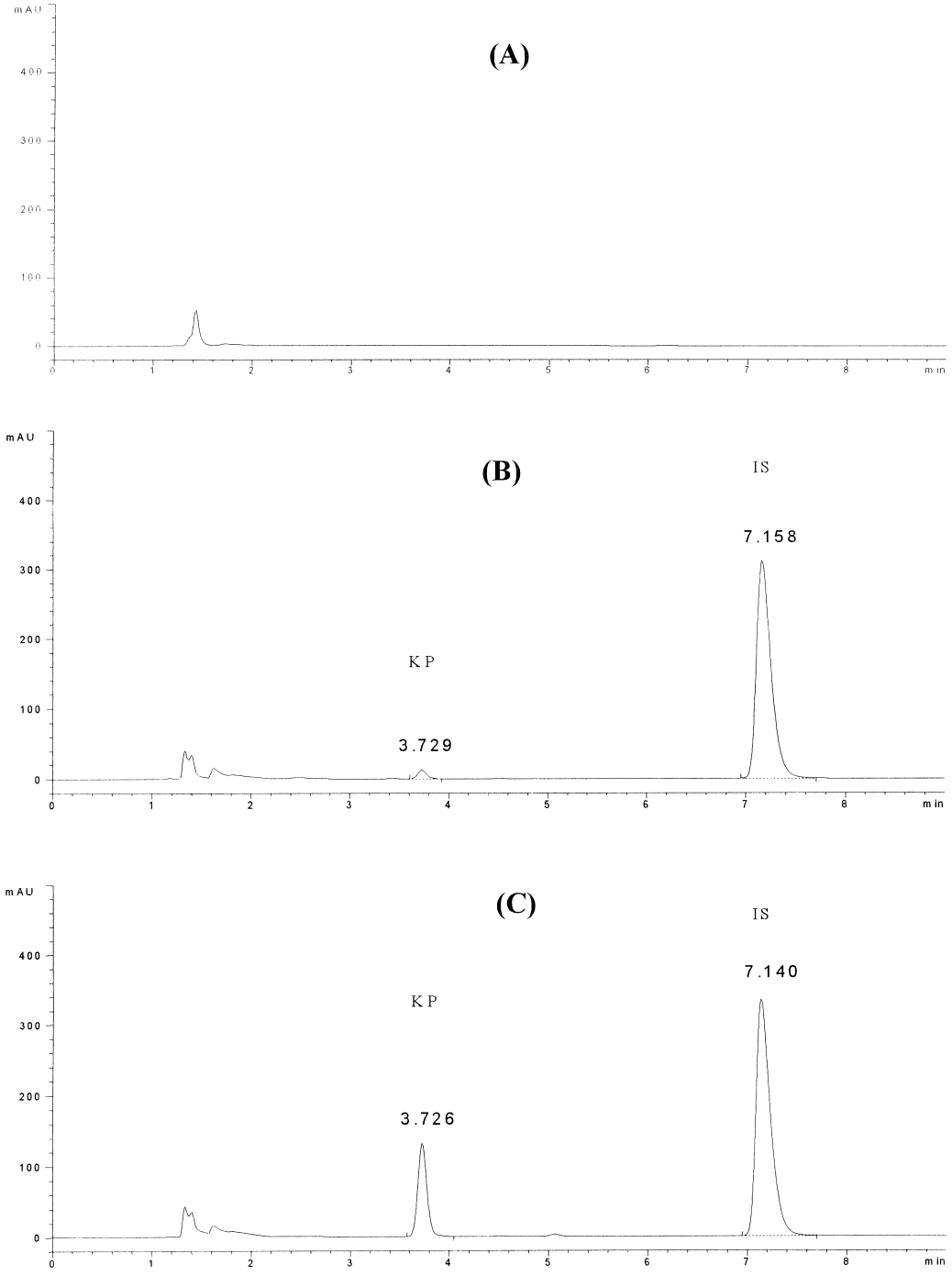


Fig. 2. Chromatograms resulting from the analysis of blank sample (A) and samples taken from an experiment 2 h (B) and 14 h (C) after a topical formulation of ketoprofen had been applied to the skin. I.S.=ibuprofen, KP=ketoprofen.

Table 1
Accuracy of the HPLC method for determining ketoprofen concentrations in phosphate buffer, pH 7.2[a]

Concentration added (μg/ml)	Concentration found, $n=10$ (mean±SD) (μg/ml)	t_{exp}
0.02	0.02±0.0003	1.89
0.2	0.20±0.0044	0.34
10	9.82±0.1060	0.10
30	30.62±0.7466	1.97

[a] The t value ($P=0.05$) is 2.26.

day precision are presented in Table 2 for our sample and the values were below 2.6 and 3.5%, respectively. The precision was characterized by RSD.

The LOD of ketoprofen was 0.003 μg/ml, a value smaller than that reported by Lovlin et al. [16] in plasma (0.015 μg/ml). The estimated LOQ was 0.02 μg/ml which is lower than described by others authors [8,11,16] (0.32 μg/ml [8], 1 μg/ml [11], 50 μg/ml [16]) and was confirmed for our samples. The mean assay result was 0.02 μg/ml ($n=10$), with RSD<5%.

For the ruggedness study, different analytical columns (LiChrospher Select B and Spherisorb C_{18}) were successfully used with no significant variations in the chromatography results. If the temperature of the column was decreased to 35°C or increased to 45°C, the elution times were not modified. Slight modifications in the percentage of acetonitrile (5%) in the mobile phase not significantly alter the ketoprofen and I.S. retention times. Variance in the flow-rate resulted in changes in the retention times, prolonging the chromatogram time. Nevertheless, no single parameter, extended to the specified limits, resulted in a dramatic adverse effect on the system suitability.

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 penetration of ketoprofen through abdominal rat skin. Ketoprofen flux value at steady state from this formulation was found to be $(40.44\pm3.97)\cdot10^{-2}$ μg/cm^2 h and the permeability coefficient value calculated was $(0.40\pm0.04)\cdot10^{-4}$ cm/h. For this gel, a lag time period of 3.23±0.25 h was observed.

In summary, a simple chromatographic method has been developed for the rapid and precise determination of ketoprofen in samples obtained in the ''in vitro'' skin permeation studies without any extraction procedure.

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Table 2
Between- and within-day variabilities of the HPLC method for determining ketoprofen concentrations in phosphate buffer, pH 7.2

Concentration added (μg/ml)	Between-day variability ($n=10$)		Within-day variability ($n=4$)	
	Concentration found (mean±SD) (μg/ml)	RSD (%)	Concentration found (mean±SD) (μg/ml)	RSD (%)
0.02	0.02±0.001	2.86	0.02±0.001	2.58
0.2	0.20±0.007	3.52	0.20±0.003	1.46
10	9.73±0.194	1.99	9.71±0.047	0.48
30	30.36±0.998	3.29	29.79±0.370	1.24

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