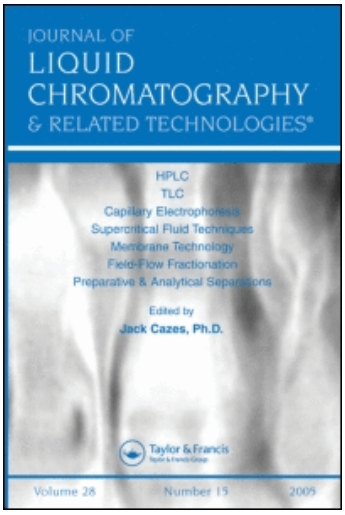

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Wanwimolruk, S. , Wanwimolruk, S. Z. and Zoest, A. R.(1991) 'Sensitive HPLC Assay for Ketoprofen in Human Plasma and its Application to Pharmacokinetic Study', Journal of Liquid Chromatography & Related Technologies, 14: 20, 3685 — 3694
To link to this Article: DOI: 10.1080/01483919108049486
URL: http://dx.doi.org/10.1080/01483919108049486

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(20), 3685-3694 (1991)

SENSITIVE HPLC ASSAY FOR KETOPROFEN IN HUMAN PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ABSTRACT

A selective and sensitive HPLC method was developed for the analysis of ketoprofen in human plasma. The assay involves an extraction of the drug and the internal standard (piroxicam) into diethyl ether from acidified plasma and then back-extracted into a small volume of alkaline aqueous solution before injection onto the HPLC column. A microbore column (2 mm I.D. x 10 cm) packed with a C18 reversed-phase material (5 μm ODS Hypersil) was used. The chromatographic separation was accomplished with a mobile phase comprising a mixture of acetonitrile-methanol-water (15 : 20 : 65, v/v) containing 10 mM Na_2HPO_4 buffer, pH 4. The mobile phase was pumped at a flow rate of 0.5 ml/min. The eluant was monitored at 258 nm. With this procedure coefficients of variation were less than 10%. The detection limit was 0.05 μg/ml (i.e., 50 ng/ml) of plasma. The highly sensitive nature of this method was applied successfully to the determination of ketoprofen in human plasma for pharmacokinetic studies.

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INTRODUCTION

Ketoprofen, 2-(3-benzoylphenyl)propionic acid, is a potent non-steroidal anti-inflammatory drug (NSAID). It is a widely used NSAID for the treatment of rheumatoid arthritis and osteoarthritis as well as in the treatment of various painful conditions (1,2). Clinical proof of therapeutic efficacy of ketoprofen has lead to numerous clinical and pharmacokinetic studies.

Several methods for determination of ketoprofen in plasma have been reported (3). The more tedious gas-liquid chromatographic methods have been largely superseded by assays based on high-performance liquid chromatography (HPLC) (3-9). However, these were unsuitable in our hands for the analysis of ketoprofen due to lack of sensitivity and chromatographic interference from endogenous substances in plasma. Thus the following assay for ketoprofen was developed. The applicability of this procedure is demonstrated by the analysis of plasma samples from subjects receiving oral ketoprofen in a bioavailability study.

MATERIALS AND METHODS

Materials and Reagents

Ketoprofen was supplied by Pacific Pharmaceuticals Ltd, Auckland, New Zealand. The internal standard, piroxicam was a gift from Pfizer Co. Ltd. (Auckland, New Zealand). HPLC-grade acetonitrile, methanol and diethyl ether were purchased from BDH Chemicals Ltd (Poole, UK). All chemicals used were of analytical grade. Water was double glass distilled and MilliQ® filtered. Glassware was cleaned and silinized with 0.05% Aquasil® (Pierce Chemical Co., Rockford, IL, USA).

Instrumentation

The HPLC system consisted of a Perkin Elmer pump, model 250 (The Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a Waters WISP 712B auto-injector (Waters Associate, Milford, MA, USA) installing with a refrigerated sample compartment. A C18 reversed-phase microbore column (2 mm I.D. x 10 cm) packed with 5 μm ODS Hypersil (Shandon, London, UK) was used. The column efficiency was over 4,000 plates per 10 cm. The eluate was monitored by

a variable wavelength UV detector (Spectroflow 757, Kratos Analytical Instrument, NJ, USA), operated at 258 nm using a setting of 0.1 a.u.f.s. The detector flow cell volume was 12μl with 8 mm light pathlength. Chromatograms were recorded on a Hitachi D-2500 (Kyoto, Japan) integrator at an attenuation of 7 (i.e., 128 mV).

Chromatographic Conditions

Analysis of the samples of ketoprofen was performed using a mobile phase consisting of a mixture of acetonitrile-methanol-water (15 : 20 : 65, v/v) containing 10 mM Na_2HPO_4, and adjusted to pH 4 with orthophosphoric acid. The flow rate of the mobile phase was 0.5 ml/min (pressure 84 kg/cm^2). Separations were done at room temperature.

Analytical Procedure and Sample Preparation

Stock solutions of ketoprofen were prepared in methanol. Known concentrations of ketoprofen in plasma were prepared by diluting a stock standard solution (0.1-1 ml) with drug-free plasma (10 to 100 ml). The final ketoprofen concentrations in the plasma standards were 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 μg/ml ketoprofen in plasma. The internal standard solutions of piroxicam of 25 μg/ml was prepared in water fresh each day of analysis.

To 1 ml of plasma in a silinized centrifuge tube, 100 μl of the internal standard solution (25 μg/ml piroxicam) and 100 μl of 4 N HCl were added. The contents were then shaken with 7 ml of diethyl ether for 10 minutes. The samples were centrifuged at 4°C for 10 minutes at 1500 g to separate the phases. The organic layer was transferred to a clean tapered glass centrifuge tube containing 200 μl of 0.1 M NaOH. The mixture was shaken for 15 minutes and centrifuged (1500 g, 4°C) for 10 minutes. The organic layer was aspirated, and 50 μl of 0.5 M H_3PO_4 was added to the remaining aqueous phase. Then the aqueous solution was transferred to the autosampler plastic vials and 70 μl was injected onto the HPLC column. The samples were stored at 4°C until injection.

Recovery

The assay recovery of ketoprofen from plasma was determined at 0.5 and 10 μg/ml. Absolute recovery was calculated by comparing the peak heights from 6 extracted plasma samples with those obtained by direct injection of the pure drug

standard of ketoprofen. The absolute recovery of the internal standard (piroxicam) was assessed using the same procedure with piroxicam at 5 μg/ml.

Calibration Curve

Standards corresponding to 0, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 μg/ml of ketoprofen were prepared in plasma. The sample analysis was performed as described above and standard curves were run daily. Quantitation is based on peak height ratios (ketoprofen/internal standard). An unweighted least-squared regression was fitted to each individual calibration curve.

Clinical Study: Bioavailability of a Sustained Release Ketoprofen Capsule

This study was conducted to determine the relative bioavailability of a new sustained release (SR) ketoprofen capsule (Pacific Pharmaceuticals Ltd, New Zealand), thereafter noted **Pac**. This was compared to the currently marketed formulation, Orudis SR (May & Baker Pharmaceuticals, Australia), thereafter noted **Oru**. The study was approved by the local Ethics Committee. Each subject gave his signed informed consent. A single oral dose of ketoprofen 200 mg **Pac** and **Oru** 200 mg capsule was given to 18 healthy male volunteers once every 24 hours for 5 days in a randomized two way crossover study. Blood samples were collected prior to the drug administration every day for 4 days at the time before dosing. On day 5, a series of blood samples were drawn through a venous catheter at intervals over 24 hours after the final (5th) dose of ketoprofen. Plasma was separated by centrifugation (400 g for 10 min) and kept frozen at -70°C until analysis.

Statistical Analysis

Student *t*-test was used throughout the study unless otherwise stated.

RESULTS AND DISCUSSION

The liquid chromatographic separation of ketoprofen and the internal standard (piroxicam) from the endogenous plasma peaks was accomplished using a reversed-phase C18 microbore column with an aqueous acetonitrile/methanol mobile phase. A mobile phase of acetonitrile-methanol-water (15 : 20 : 65, v/v)

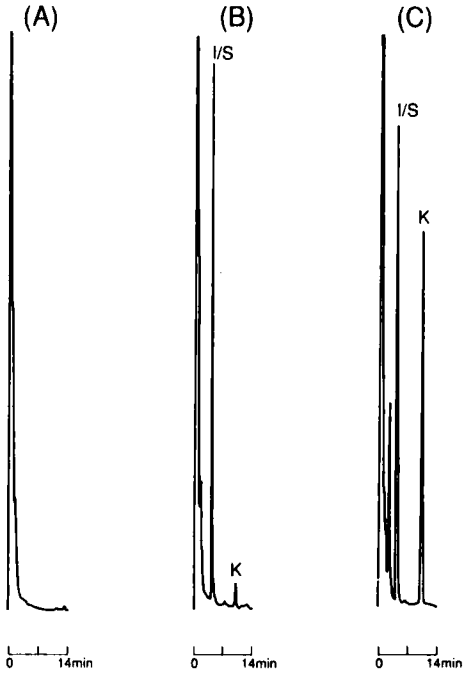

FIGURE 1

Typical chromatograms of human plasma: (A): blank plasma; (B): blank plasma spiked with 0.05 μg/ml ketoprofen and the internal standard (piroxicam); (C): plasma with 2.3 μg/ml ketoprofen taken from a healthy volunteer 7 hours after an oral dose of 200 mg sustained release ketoprofen capsule on day 5 of the clinical study.

Peaks: I/S = Internal standard (piroxicam); K = Ketoprofen.

containing 10 mM Na_2HPO_4 (pH 4) gave a well resolved, sharp peak for ketoprofen and the internal standard. Figure 1 shows chromatograms of blank plasma, plasma spiked with 0.05 μg/ml of ketoprofen and a typical subject's plasma chromatogram 7 hours after the 5th dose of ketoprofen once a day. Under these chromatographic conditions, no endogenous sources of interference were observed. The peaks corresponding to piroxicam (internal standard) and

TABLE 1

Within-run Reproducibility and Precision of the Assay for Ketoprofen in plasma

Spiked Concentration (μg/ml)	n	Observed Concentration[1] (μg/ml)	C.V. (%)	Accuracy[2] (%)
0.05	6	0.055 ± 0.005	9.1	110
2.5	5	2.80 ± 0.22	7.9	112
10	5	9.40 ± 0.45	4.8	94

[1] Results given are mean ± S.D.

[2] Accuracy (%) = $\frac{\text{Observed concentration}}{\text{Spiked concentration}} \times 100$

ketoprofen were well resolved with retention times of 3.9 and 8.9 minutes, respectively (Figure 1B and 1C).

The mean recovery of ketoprofen from plasma was 81.3 ± 4.5% (S.D.) at 0.5 μg/ml and 87.2 ± 3% at 10 μg/ml (n=6). The recovery of the internal standard was also satisfactory at the concentration used with a recovery of 98.1 ± 4% (n=6).

The standard curve of ketoprofen was linear over the concentration range of 0.05 to 10 μg/ml with the square of the correlation coefficient (r^2) greater than 0.99. The typical linear relationship for the calibration curve can be expressed by the equation : $y = 0.3205x$; where y is the peak height ratio and x is the plasma ketoprofen concentration (μg/ml). The intercept (a) in all calibration curves were found to be statistically insignificant ($p > 0.1$) and were thus not included for the calculations. The day-to-day coefficient of variation (C.V.) of the slope of the calibration curves of ketoprofen was 3.9% (n = 10).

Precision and accuracy of the assay were assessed in conjunction with the linearity study. The within-day (within-run) reproducibility and accuracy of the method are presented in Table 1. At all concentrations studied the C.V. was less than 10%. These results indicate good precision of the assay. The measured value for six replicates of 0.05 μg/ml plasma ketoprofen standards gave values of 0.061, 0.059, 0.054, 0.057, 0.049 and 0.051 μg/ml. This data gives a mean and S.D. of 0.055 ± 0.005 μg/ml. The C.V.of the assay at this concentration was

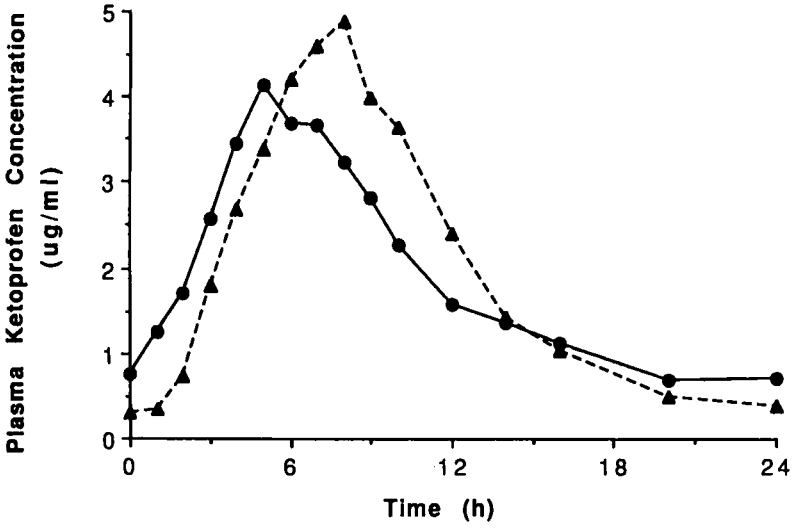

FIGURE 2

Mean plasma ketoprofen concentration-time profiles in 18 healthy male subjects on day 5 (i.e., during steady state) following once daily oral administration for 5 days of either 200 mg sustained release (SR) ketoprofen capsules (Pacific Pharmaceuticals, New Zealand, ●—●), or 200 mg SR Orudis capsules (May & Baker, Australia, ▲----▲).

9.1% with accuracy of 110%. This C.V. is lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). A typical chromatogram of 0.05 μg/ml plasma ketoprofen standard is shown in Figure 1B. Thus, the MQC or the detection limit of sensitivity for this HPLC assay was assigned at 0.05 μg/ml (i.e., 50 ng/ml).

Plasma samples stored at -70°C for up to 2 months showed no signs of decomposition and there was no difference in the plasma ketoprofen concentrations between the fresh plasma samples and the stored plasma samples ($n=6$, $p > 0.4$). This indicates that the plasma samples containing ketoprofen can be stored under these conditions for at least 2 months without an appreciable decomposition.

TABLE 2

Summary of Ketoprofen Pharmacokinetics in the Bioavailability Study of Ketoprofen Capsule

Pharmacokinetic Parameter[1]	Ketoprofen 200 mg Sustained Release (SR) Capsule		ANOVA Test
	Ketoprofen (Pacific)	Orudis (May & Baker)	
AUC (μg hr/ml)	43.6 ± 13.3[2]	45.4 ± 11.3	NS[3]
C_{max} (μg/ml)	5.3 ± 1.9	7.0 ± 2.8	p = 0.01
C_{min} (μg/ml)	0.53 ± 0.18	0.21 ± 0.12	p = 0.001
DF[4]	2.62 ± 0.67	3.62 ± 1.11	p = 0.001
Relative bioavailability (Fr)[5]	0.96 ± 0.15	-	-

[1] AUC = area under the plasma concentration-time curve during the steady-state (i.e., 5th dose interval); C_{max} = peak plasma ketoprofen concentration at steady-state; C_{min} = minimum plasma ketoprofen concentration at steady-state.

[2] Results are given as mean ± S.D. (n = 18)

[3] NS = not significantly different (p > 0.05)

[4] DF = degree of fluctuation of plasma drug concentration at steady-state and defined as : DF = $(C_{max}-C_{min})/C_{av}$ where C_{av} = AUC/dosage interval (i.e., 24 hr).

[5] Relative bioavailability = AUC Pacific/AUC Orudis.

The present method was used to determine the plasma ketoprofen concentrations in plasma samples collected from subjects who participated in a bioavailability study of a new formulation of sustained release (SR) ketoprofen capsule. This study was designed to evaluate the relative bioavailability (Fr) of ketoprofen 200 mg **Pac** capsules relative to that of **Oru** 200 mg capsule, a currently marketed SR ketoprofen capsule formulation. Pharmacokinetics of ketoprofen during the steady state (i.e., day 5) after oral doses of each formulation once daily for 5 days have been studied in the 18 male healthy volunteers. The results of plasma ketoprofen concentration-time profiles during the course of study are shown in Figure 2. The comparison between the two capsule formulations in respect to area under the plasma concentration-time curve (AUC), the peak plasma ketoprofen concentration (C_{max}), the minimum plasma concentration at steady-state (C_{min}) and the degree of fluctuation (DF) after the 5th administration of the drug can be seen in Table 2.

No significant difference (p = 0.3) in the AUC was found between the two formulations tested. However, there were significant differences in C_{max}, C_{min} and DF between the two capsule formulations. The results obtained indicate that **Pac** capsules have less fluctuation of peak and trough plasma drug concentrations than that of **Oru** capsule at steady state. The relative bioavailability of the new ketoprofen 200 mg **Pac** capsule assessed by the comparison of AUC was 0.96 (Table 2). As there was no difference in the AUC between the two preparations, it can be concluded that **Pac** capsules are bioequivalent to **Oru** capsules.

In summary, the HPLC assay reported affords a sensitive and simple procedure for the determination of ketoprofen in human plasma suitable for use in clinical and pharmacokinetic studies. The use of a microbore column provides an additional feature to improve the sensitivity of the assay and is more economical as less mobile phase is consumed.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Arthritis Foundation of New Zealand. The author thanks Dr CT Hung who organised the clinical study.

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Received: March 18, 1991
Accepted: May 31, 1991