Determination of Ketoprofen in Biological Fluids by Reversed-Phase Chromatography

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Abstract □ A sensitive and specific high-performance liquid chromatographic method was developed for the determination of ketoprofen [2-(3-benzoylphenyl)propionic acid] in plasma and urine. The method includes an extraction of the drug and the internal standard [2-(4-benzoylphenyl)butyric acid] into ether from acidified plasma. The organic phase is evaporated, and the residue is dissolved in the mobile phase (acetonitrile–0.02 *M* phosphate buffer, pH 3) (45:55). A 20-μl aliquot is analyzed on a reversed-phase column. The accuracy is within 1.5% for therapeutic concentrations, and the coefficients of variation are 5.5 and 3.4% for 2 and 10 μg/ml, respectively. For the urine assay, the accuracy is within 3%, and the coefficients of variation are 1.9 and 1.7% for 3 and 50 μg/ml, respectively. This method was applied successfully to the determination of ketoprofen in humans for pharmacokinetic studies.

Keyphrases □ Ketoprofen—high-performance liquid chromatographic analysis, human plasma and urine □ Anti-inflammatory agents—ketoprofen, high-performance liquid chromatographic analysis, human plasma and urine □ High-performance liquid chromatography—analysis, ketoprofen, human plasma and urine

Ketoprofen [2-(3-benzoylphenyl)propionic acid, I] is a benzophenone derivative with anti-inflammatory activity (1). Clinical proof of its therapeutic efficacy (2) has resulted in its wide use in rheumatology, leading to numerous clinical (3–5) and pharmacokinetic (6–9) studies.

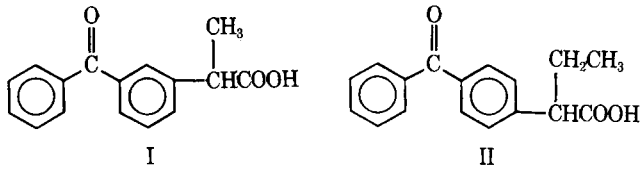
Several methods for the determination of ketoprofen in biological fluids have been reported. Colorimetry and polarography (10) are simple but cannot be applied easily to plasma or serum. TLC (11) is rapid but lacks sensitivity. Methyl (10) and trimethylsilyl esters of ketoprofen (12) can be separated using GLC, and this method can be considered as a reference due to its sensitivity. However, the technique is relatively complex and time consuming, and the resulting precision is only ~10%.

A new rapid and accurate analytical method for ketoprofen was described recently, which involves the determination of its methyl ester by high-performance liquid chromatography (HPLC) (13). This report describes the development of a precise and rapid HPLC assay for determining ketoprofen in biological fluids without derivatization.

EXPERIMENTAL

Reagents and Solvents—Acetonitrile[1], phosphoric acid[1], monobasic potassium phosphate[2], sodium hydroxide[2], acetone[2], sodium sulfate[2] (anhydrous), hydrochloric acid[2], sodium nitrate[1], and ether[2] were analytical grade and were used without further purification. Water was deionized. Ketoprofen and the internal standard [2-(4-benzoylphenyl)-butyric acid, II] were synthesized[3].

HPLC mobile phases of pH 3, 4, 5, 6, and 7 were made from phosphoric acid–monobasic potassium phosphate or monobasic potassium phosphate–dibasic sodium phosphate aqueous buffer solutions diluted with various volumes of acetonitrile (30–70%). The pH of the resulting solutions was adjusted with either 10% NaOH or 10% phosphoric acid. The final phosphate-ion concentration was 0.02 *M*.



I II

Apparatus—The liquid chromatograph[4], equipped with a 254-nm UV detector[5], was operated at ambient temperature. The output of the detector was displayed on a recorder[6] having 10-mv full-scale deflection. The output signal also was fed to an electronic integrator[7] for quantification.

The 10-cm × 4.6-mm i.d. stainless steel columns were packed with Lichrosorb RP 18[2] (5 μm) or RP 8[2] (5 μm) by a balanced-density slurry technique (14). A 20-μl loop injection valve[8] was used to introduce samples into the chromatographic system. The efficiency of newly made columns was tested with a standard mixture using sodium nitrate as an unretained marker. The solvent system was methanol–water (80:20). Under these conditions, the column efficiency was 24,000 theoretical plates for benzene and 21,000 for anthracene.

Stock Solutions—The ketoprofen stock solution (10 mg/100 ml) was prepared by dissolving I in acetone. The internal standard stock solution (10 mg/100 ml) was prepared by dissolving II in acetone. A 10-fold dilution of these two solutions was made before use.

Calculations—The plasma and urine concentrations were calculated using the internal standard method.

Chromatographic Conditions—Columns were preconditioned with the mobile phase until a stable recorder baseline was obtained. The flow rate then was adjusted to 1 ml/min, generating a pressure of nearly 1000

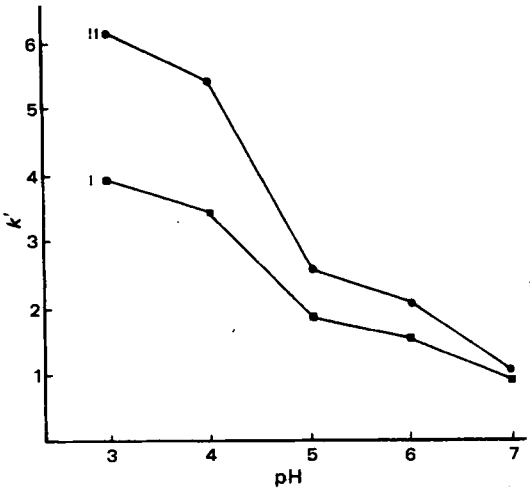


Figure 1—*Effect of variation of the solvent pH on the capacity factors, k', of ketoprofen (I) and the internal standard (II) on a Lichrosorb RP 18 column; the mobile phase was acetonitrile–0.020 M phosphate buffer (40:60).*

[1] Prolabo, Paris, France.
[2] E. Merck, Darmstadt, West Germany.
[3] Specia, Paris, France.
[4] Chromatem 38, Touzart and Matignon, Paris, France.
[5] Model 153, Altex, Berkeley, Calif.
[6] Model PE 1286, Sefram, Paris, France.
[7] Model 3385 A, Hewlett-Packard, Orsay, France.
[8] Model 70-10, Rheodyne, Berkeley, Calif.

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Table I—Chromatographic Parameters of I and II on Lichrosorb RP 18 and Lichrosorb RP 8 [a]

Compound	Lichrosorb RP 18		Lichrosorb RP 8	
	I	II	I	II
Retention time (T_R)	5.2	7	4.2	5.65
Capacity factor[b] (k')	3.3	4.83	3.2	4.65
Theoretical plates[b] (N)	1260	1380	2940	3200
Resolution[b] (R)	2.42		3.72	
Selectivity[b] (α)	1.46		1.45	

[a] Chromatographic conditions included a mobile phase of 45% acetonitrile–55% phosphate buffer (0.02 M, pH 3), a flow rate of 1 ml/min, and a pressure of 1100 psi. [b] For calculations, see C. F. Simpson, "Practical High Performance Liquid Chromatography," Heyden & Son, Philadelphia, Pa., 1976, pp. 7–12.

Table II—Reproducibility and Accuracy [a] for the Plasma Assay [b]

I Added, μg/ml	I Measured, μg/ml	Mean Error	Relative Error, %	CV, %
2	1.98 ± 0.11	0.02	1.01	5.5
5	5.13 ± 0.21	0.13	2.60	4.2
10	10.15 ± 0.34	0.15	1.50	3.4

[a] For chromatographic conditions, see Table I. [b] n = 10.

psi. The mobile phase solutions were prepared daily and degassed by sonification.

Plasma Extraction—One milliliter of plasma, 1 ml of diluted internal standard, and 1 ml of 1 N HCl were transferred to a 35-ml screw-capped centrifuge tube and extracted by shaking with 25 ml of ether for 5 min.

The organic and aqueous phases were separated by centrifugation for 3 min at 2000 rpm. The ether layer was then pipetted carefully into a 35-ml conical centrifuge tube, dried over anhydrous sodium sulfate, and evaporated to dryness under a nitrogen stream at 40°. The sample residue was reconstituted with various volumes of the mobile phase (200–500 μl), depending on the drug concentration. Aliquots of 20 μl then were injected onto the column.

Urine Extraction—To determine the total ketoprofen excreted in urine, any conjugated drug first was hydrolyzed in an alkaline medium (12). Then, 0.1–0.5 ml of urine, 1 ml of 1 N NaOH, 1 ml of the internal standard stock solution (10 mg/100 ml), and 25 ml of ether were transferred to a 35-ml screw-capped centrifuge tube. The sample was agitated for 5 min, and the organic phase was discarded. The aqueous phase, after acidification with 2 ml of 1 N HCl, was treated similarly to the plasma. The sample residue then was reconstituted with 1–2 ml of the mobile phase, and 20-μl aliquots were injected into the chromatograph.

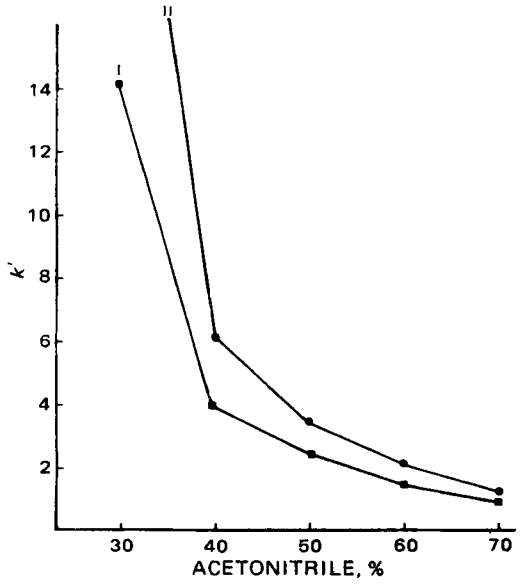

Figure 2—*Effect of the acetonitrile content at pH 3 upon the capacity factors, k', of ketoprofen (I) and the internal standard (II) on a Lichrosorb RP 18 column.*

Table III—Urine Assay Precision Data [a]

I Added, μg/ml	n	I Measured, μg/ml	Mean Error	Relative Error, %	CV, %
3	5	3.09 ± 0.06	0.09	3	1.94
9	6	8.87 ± 0.31	0.13	2.6	3.49
30	5	29.03 ± 0.72	0.97	3.23	2.48
50	5	48.99 ± 0.85	1.00	2.00	1.79

[a] For chromatographic conditions, see Table I.

Table IV—Retention Times and Column Efficiency of I, II, and Selected Drugs [a]

Compound	Retention Time, min	N^b
Aspirin	2.5	1860
I	4.2	2940
II	5.6	3200
Fenoprofen	7.6	2770
Metiazinic acid	8.6	2340
Indomethacin	9.1	3180
Niflumic acid	9.4 (tailing)	1640
Ibuprofen	10.7	2800
Protizinic acid	11.2	2570
Mefenamic acid[c]	15.8 (tailing)	1790
Flufenamic acid[c]	18.30	2010

[a] For chromatographic conditions, see Table I. [b] Theoretical plates. [c] The detection wavelength was 280 nm.

RESULTS AND DISCUSSION

Reversed-phase chromatography on modified silica (C_{18} or C_8) has replaced ion-exchange chromatography for the separation of many polar ionogenic compounds. The technique, called solvophobic chromatography, was developed by Horváth *et al.* (15, 16). Ketoprofen, a drug with a polar carboxylic functional group, can be determined by such a method.

The internal standard for this determination was proposed by Populaire *et al.* (10). Its structure is very close to that of I, and its physicochemical properties also are similar. Preliminary studies showed that separation of I and II in biological fluids was possible with a mixture of methanol–acetonitrile–0.025 M phosphate buffer (30:15:45) at pH 3 on a 5-μm Lichrosorb RP 18 column. However, under such conditions, retention times were long (8 and 12.8 min, respectively), and the peaks were unacceptably broad.

To determine the optimum chromatographic conditions, variation of the capacity factor, k', with pH was measured on 5-μm Lichrosorb RP

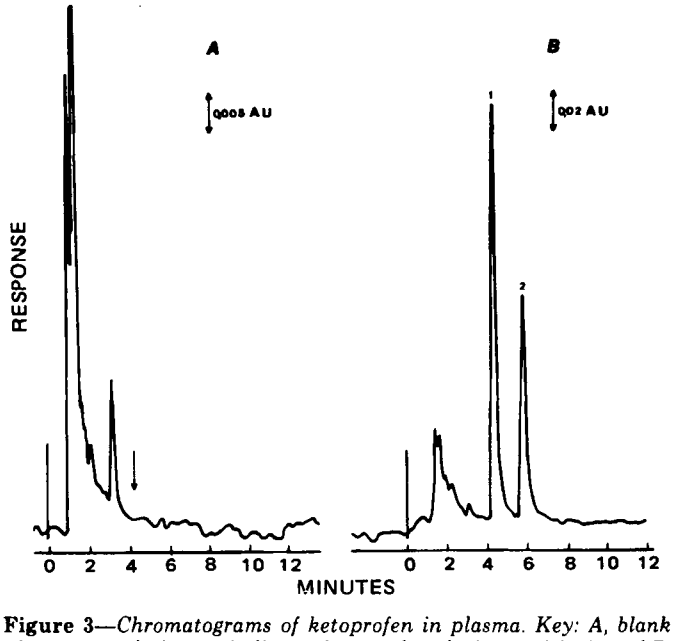

Figure 3—*Chromatograms of ketoprofen in plasma. Key: A, blank plasma sample (arrow indicates ketoprofen elution position); and B, plasma sample with 8.53 μg of ketoprofen/ml (peak 1) and 5 μg of the internal standard/ml (peak 2).*

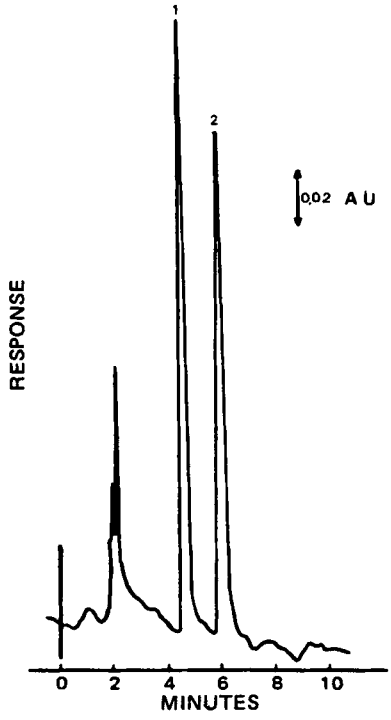

Figure 4—*Chromatogram of ketoprofen in urine for the 0–6-hr collection. The ketoprofen (peak 1) concentration was 189 μg/ml, and the internal standard (peak 2) concentration was 250 μg/ml.*

18 columns with an acetonitrile–phosphate buffer mobile phase. Figure 1 shows that the best separation was obtained at pH 3 ($k_I' = 3.89$, $k_{II}' = 6.10$, $R_S = 3.03$, and $\alpha = 1.57$). At pH 3, I and II were not ionized and thus showed a maximum affinity for the stationary phase. Moreover, Twitchett and Moffat (17) demonstrated that k' is influenced strongly by liposolubility. Figure 2 shows that an increase in the proportion of acetonitrile in the mobile phase decreases k'.

Results for the determination of I on Lichrosorb RP 18 under these conditions are summarized in Table I. Optimization of pH and liposolubility led to a shorter analysis time and better resolution. However, the efficiency of this column still was not sufficient and the peaks remained rather broad. A better separation subsequently was obtained using a 5-μm Lichrosorb RP 8 column. The right side of Table I shows that the chromatographic parameters for I and II were nearly the same for the two stationary phases, but resolution and efficiency were increased because of peak sharpness. Consequently, 5-μm Lichrosorb RP 8 was chosen as the stationary phase for the final determination.

Ether extracts of control plasma were free of endogenous UV (254-nm) absorbing peaks liable to interfere with the assay (Fig. 3). Figure 4, representing the chromatogram of a urine extract under these conditions, shows that ketoprofen and the internal standard were well resolved.

To verify the linearity of the chromatographic response, a plasma calibration curve was run in which the concentration of II was maintained at 5 μg/ml while that of I was varied from 1 to 10 μg/ml. A linear regression analysis of the data resulted in a good linear fit ($r^2 = 0.998$, intercept = 0.027, and slope = 0.200). For the calibration for urine, the concentration of I was varied from 10 to 150 μg/ml. Good linearity was observed ($r^2 = 0.999$, intercept = 0.04, and slope = 0.097).

Reproducibility and Accuracy—Reproducibility and accuracy of the HPLC method were studied by measuring the concentration of I in plasma samples spiked at 2, 5, and 10 μg/ml. The results (Table II) show that accuracy was within 4%. The assay precision for urine was determined for various ketoprofen concentrations (Table III). The lower limit of sensitivity was 0.04 μg/ml for plasma samples and 0.05 μg/ml for urine samples, both measured at a signal-to-noise ratio of 3:1.

Potential Interfering Substances—Retention times for several compounds that might potentially interfere with ketoprofen are shown in Table IV. The chromatographic system provided good specificity, and the column efficiency for these compounds was good. Moreover, no interference was found between ketoprofen and its hydroxylated metabolites since they were not extracted in the acidic medium.

Clinical Study—Figure 5 shows the plasma level in a human subject

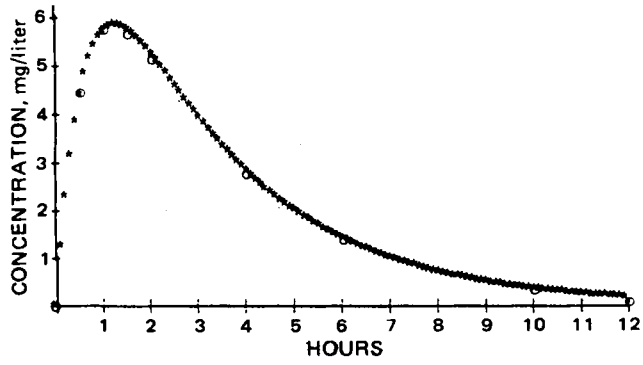

Figure 5—*Plasma decay for ketoprofen after a single oral dose of 100 mg ($K_e = 0.354\ hr^{-1}$ and $K_a = 1.54\ hr^{-1}$).*

following a single oral dose of 100 mg of I in tablet form. Blood samples were collected before medication and after 30 min and 1, 1.5, 2, 4, 6, 10, and 12 hr. The plasma levels of I were measured as described.

In this subject, a peak plasma level of 5.8 μg/ml was reached at 1 hr. The decrease in the plasma level followed a monoexponential decline with an elimination rate constant of 0.354 hr^{-1} and a half-life of 1.97 hr.

The urinary excretion levels of ketoprofen also were determined for the same subject. Urine was collected at intervals of 0–6, 6–12, and 12–24 hr during the first 24 hr following oral administration. A total of 61.2 ± 5.8% of the dose was excreted in 24 hr. These results agree closely with the data reported previously (5, 6, 18) and indicate that the method is well suited for the determination of plasma and urine levels of I in bioavailability and pharmacokinetic studies.

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