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Note

Highly sensitive determination of ketoprofen in human serum and urine and its application to pharmacokinetic study

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Pharmacokinetic study of the percutaneous absorption of ketoprofen, a non-steroidal anti-inflammatory agent, requires a highly reliable assay method to evaluate its bioavailability [1]. Our preliminary experiments showed that a normal dose of the ointment preparation afforded only a 3% recovery of the drug itself and its glucuronide conjugate in urine. Procedures reported so far [2—6] show that considerable amounts of contaminants in serum extracts make it difficult for an accurate calibration to be made on the basis of peak-height ratio. Better extraction conditions, such as rapid-flow fractionation (RFF) [7], afforded satisfactory results for only urine specimens and serum containing the drug in high concentrations. But, in the case of low concentrations, a multi-column developing technique utilizing both normal- and reversed-phase chromatography proved entirely sufficient for eliminating residual, overlapping, endogenous materials. We thus developed a more accurate chromatographic technique applicable to the assay of this drug in serum and urine in concentrations as low as 2 ng/ml.

EXPERIMENTAL

Chemicals

All reagents and organic solvents were of analytical or reagent grade (Wako, Osaka, Japan). Ketoprofen crystals and oral capsule preparations (containing 50 mg of agent per capsule) were supplied by Chugai (Tokyo, Japan), and naproxen crystals by Tanabe (Osaka, Japan).

Single oral application of ketoprofen capsules and sample collections

Two capsules, each containing 50 mg of ketoprofen, were orally administered 2 h after eating. Blood samples were collected prior to the administration and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 24 h after the administration. On each occasion, 5 ml of venous blood were withdrawn from an elbow vein, and serum samples were obtained by centrifugation for 10 min at 2000 g. Urine specimens were collected immediately after taking the blood samples. Serum samples and combined urine were stored at $-20°C$ until analysis.

Extraction of ketoprofen from serum samples

To a 0.5-ml serum aliquot were added 100 ng of naproxen dissolved in ethanol, and the mixture was acidified with 50 μl of 1 M hydrochloric acid. Extraction procedures were those of the RFF technique [7] using a diatomaceous-earth short column (3-ml glass tube, Kusano Scientific, Tokyo, Japan) and an elution solvent consisting of methanol—diethyl ether (1:99). Ketoprofen and naproxen were enriched in 7 ml of the solvent and then evaporated to dryness. The residue was redissolved in 20 μl of methanol and dichloromethane (1:10) for high-performance liquid chromatographic (HPLC) analysis. In the case of serum samples collected 24 h after the administration, a 5-ml aliquot was used for the analysis.

Extraction of ketoprofen from urine specimens

Free ketoprofen. A 0.5-ml aliquot was spiked with 100 ng of naproxen, followed by acidification with 50 μl of 1 M hydrochloric acid. The resulting solution was treated by the RFF technique, using 7 ml of the methanol—diethyl ether mixture (1:99) as the extraction solvent.

Total ketoprofen. A 0.5-ml aliquot was spiked with 5 μg of naproxen, made more basic with 50 μl of 5% sodium hydroxide solution and allowed to stand for 10 min so that the hydrolysis reaction of ketoprofen glucuronide could proceed to completion. The resulting mixture was acidified with 100 μl of 1 M hydrochloric acid and extracted as described above, using 7 ml of the extraction solvent.

Determination of ketoprofen by high-performance liquid chromatography

Apparatus. The HPLC apparatus was equipped with a reciprocal pump (LC-5A, Shimadzu, Tokyo, Japan), a UV detector (SPD-2A, Shimadzu) set at 254 nm, a loop injector (Model 7125, Rheodyne, CA, U.S.A.) and pen recorder (U-228, Nippon Denki, Tokyo, Japan). Two analytical columns were used for the determination. A silica-gel column (ERC-Silica 1161, particle size 5 μm,

10 cm × 6 mm I.D., Erma, Tokyo, Japan) was used for determination of urine ketoprofen and higher levels of ketoprofen (> 100 ng) in the 0.5-ml serum samples. The column was prewashed with 0.5 ml of 5% sulphuric acid to make the surface pH less than 7.0. A reversed-phase column (Finepack Sil C_{18}, particle size 10 μm, 25 cm × 4 mm I.D., Jasco, Tokyo, Japan) was used to determine lower ketoprofen levels (< 100 ng) in the 0.5-ml serum samples, for which the two-column developing technique was used to eliminate the background peaks overlapping those of ketoprofen and naproxen on the first silica-gel chromatogram.

Procedures. A sample solution was injected into the silica-gel column in a mobile phase of acetic acid—2-propanol—dichloromethane—n-hexane (0.2:0.8:4:95) at a flow-rate of 1 ml/min. After obtaining the peak-height ratio of ketoprofen and naproxen, the concentration of ketoprofen was calibrated by the calibration curve: $y = 20.37 x + 0.04$ ($r = 0.9980$), where y is the concentration of ketoprofen per millilitre of serum or urine, and x is the peak-height ratio. Linearity of the calibration curve was assumed for injected amounts from 100 ng to 2 μg. However, an unknown acidic material in the serum sample appeared at the same chromatographic position as that of ketoprofen. When the serum concentration of ketoprofen obtained by the silica-gel column was lower than 200 ng/ml, the eluent containing ketoprofen and naproxen were recollected in accordance with their capacity ratios (k') of 4.00 and 2.15, respectively. The combined eluents were evaporated to dryness, dissolved in 20 μl of 10% methanol in dichloromethane, and then chromatographed on the reversed-phase column. The mobile phase for reversed-phase chromatography was 60% methanol containing 0.2% acetic acid, and the flow-rate was 1 ml/min. A calibration curve was made on the basis of peak-height ratio and the correlation equation was $y = 22.22 x - 0.06$ ($r = 0.9990$). Keto-profen determination by the two-column developing technique improved the detection limit to as low as 2 ng/ml of serum. The capacity ratios on the reversed-phase chromatogram were 2.48 for ketoprofen and 3.43 for naproxen, and the signal-to-noise ratio at the detection limit was 4.

RESULTS

Recovery of ketoprofen from the serum sample was 94 ± 4%. This deviation was easily corrected by the addition of naproxen as an internal standard for the determination; a calibration curve made from the peak-height ratios showed a correlation coefficient of 0.9980. It should be noted, however, that ketoprofen tended to adhere to the inner wall of the glass syringe and, consequently, an internal standard having characteristics similar to ketoprofen had to be used. Among the compounds tested, naproxen was found to be the most suitable for quantification in our chromatographic operations. A typical chromatogram is illustrated in Fig. 1.

Fig. 2 shows the results obtained for the serum sample containing less than 200 ng/ml ketoprofen. The chromatogram obtained by the silica-gel column (Fig. 2A) clearly shows a significant number of overlapping peaks at the same position as that of either ketoprofen or naproxen. An accurate determination was subsequently carried out by reversed-phase chromatography and the results
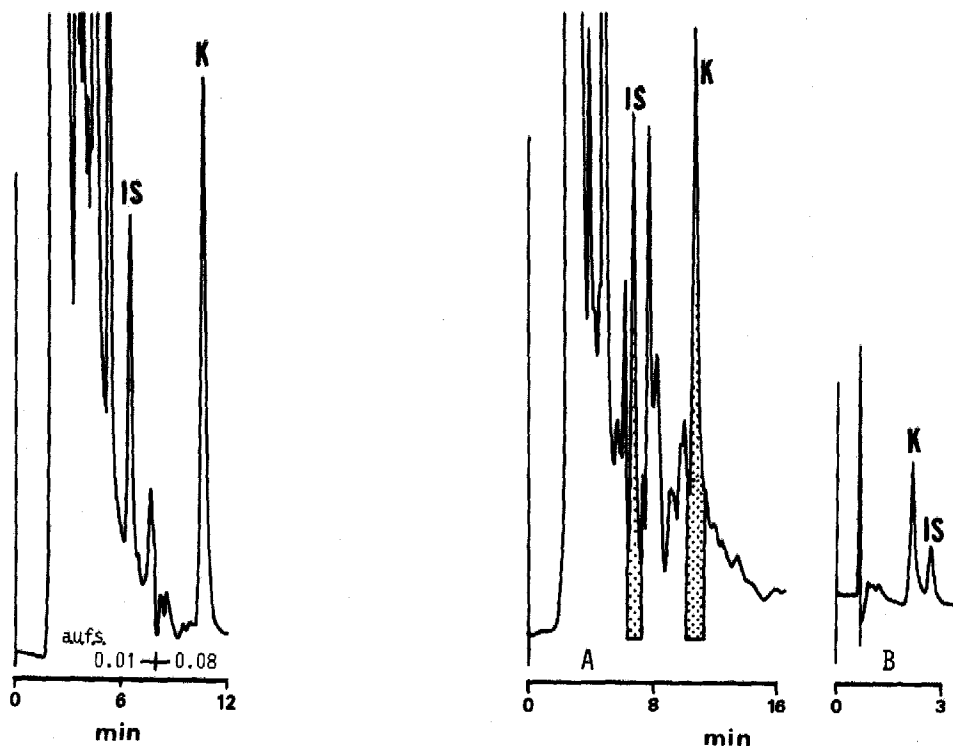
Fig. 1. Chromatogram of serum ketoprofen after oral administration of two tablets containing 100 mg of the agent. A whole extract from a 0.5-ml serum sample was injected into the silica-gel column. The mobile phase consisted of acetic acid—2-propanol—dichloromethane—n-hexane (0.2:0.8:4:95) and the flow-rate was 1 ml/min. The a.u.f.s. was changed at the assigned position from 0.01 to 0.08. The amount of internal standard was 100 ng and the ketoprofen determination could be carried out directly using the peak-height ratio obtained at a wavelength of 245 nm. Calculated amount of ketoprofen is 268 ng/ml serum. Peaks: K = ketoprofen; IS = naproxen, the internal standard.

Fig. 2. Two-column chromatograms of serum ketoprofen 24 h after oral administration. A whole extract was injected into the silica-gel column (A) and the dotted area was recovered from the eluent so as to make a final determination of ketoprofen with reversed-phase chromatography. Calculated amount of ketoprofen is 52 ng/ml serum. The mobile phase for the reversed-phase column (B) was 60% methanol containing 0.2% acetic acid. The flow-rate was 2 ml/min and the a.u.f.s. was 0.01.

are shown in Fig. 2B. It is clear that nearly all contaminating peaks have been eliminated. The detection limit was as low as 2 ng/ml of serum for a 1-ng injection, with a signal-to-noise ratio of 4 and an a.u.f.s. of 0.005. A concentration time curve of serum ketoprofen is shown in Fig. 3.

In the urine analysis, these rather time-consuming two-column methods were not required for the detection of ketoprofen in concentrations as low as 2 ng/ml; only the silica-gel column was used. A typical chromatogram is shown in Fig. 4. Analytical recovery of ketoprofen was almost quantitative. Biological recovery of the drug in urine (free and conjugated forms) was as much as 69.5%, based on the dose of the agent.
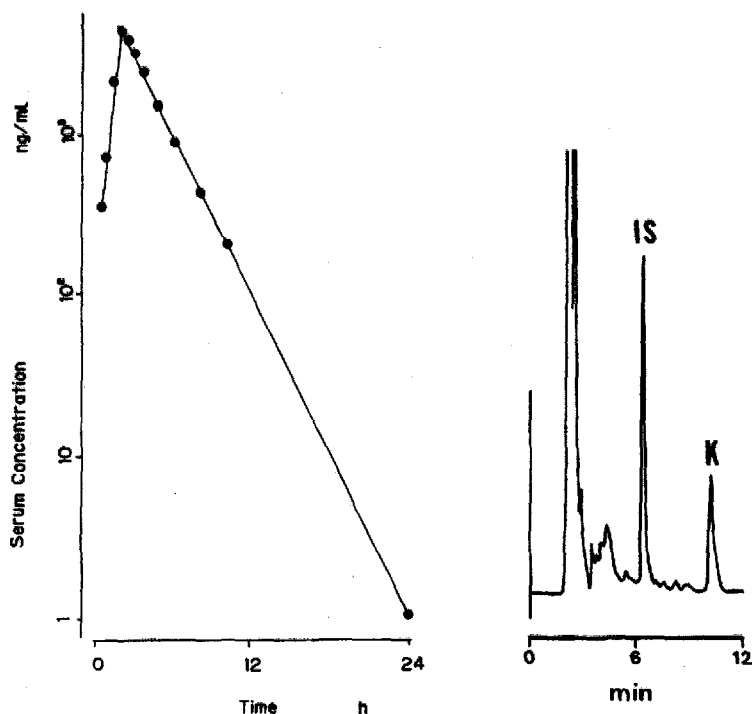
Fig. 3. Changes in the serum concentration of ketoprofen after oral administration of a 100-mg dose. Good linearity was observed for the elimination phase of the agent up to 24 h from the onset of the test. The average elimination half-life of four healthy volunteers was 1.96 ± 0.15 h.

Fig. 4. Chromatogram of urine ketoprofen after oral administration. A whole extract from the 0.5-ml specimen containing 100 ng of the internal standard was injected into the silica-gel column. The a.u.f.s. was 0.01; for other conditions, see Fig. 1. Background peaks were almost negligible in the direct determination of ketoprofen using the peak-height ratio. Calculated amount of ketoprofen is 8 ng/ml of urine. Peaks: K = ketoprofen; IS = naproxen, the internal standard.

DISCUSSION

During the past ten years, chromatographic determination has become a very reliable technique for the pharmacokinetic study of various drugs. In most such cases, however, the plasma levels were rather high, usually of microgram order for therapeutic uses. But recently, the application of certain internal medicines in transdermal dosage forms [8] has resulted in lower concentrations of drugs in the blood and urine, thus necessitating a more accurate analytical technique.

We have already reported the RFF technique for the restricted extraction of target molecules, using minimal but sufficient solvent polarities [7]. The solvent system of 1% methanol in diethyl ether was thus optimized so as to extract ketoprofen and naproxen separately from other contaminants. In the present research, we were not able to improve conventional reversed-phase chromatography for detection of ketoprofen in low concentrations. Consequently, we developed a method of chromatography using the silica-gel

column. By this technique, our method was found adequate for determining as little as 200 ng/ml in the blood and urine, as shown in Figs. 1 and 4. To determine serum ketoprofen at concentrations lower than 200 ng/ml, this method, combined with reversed-phase chromatography, afforded satisfactory results (Fig. 2).

Our procedures were applied to the pharmacokinetic study of oral ketoprofen administration to clarify drug elimination profiles for very low concentrations of ketoprofen prior to percutaneous experiments. The concentration-time curve in Fig. 3 shows that the serum ketoprofen concentration decreases with logarithmic linearity within a wide range of four logarithmic scales. This was also confirmed by the results obtained from three volunteers. The average half-life of the agent in the blood was calculated to be 1.96 ± 0.15 h. Other pharmacokinetic parameters agreed well with literature values [9]. On the basis of these results, our method can be applied to a comparative study of the oral and percutaneous pharmacokinetics of ketoprofen.

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