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Direct Injection Analysis of Ketoprofen Enantiomers in Plasma Using Column-Switching High-Performance Liquid Chromatography System

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A high-performance liquid chromatography system was developed for the stereoselective determination of ketoprofen enantiomers in human plasma following direct sample injection. The system comprised of a pretreatment column and a chiral separation column connected in a series *via* a switching valve. When a 200 μl portion of human plasma containing a therapeutic level of ketoprofen was directly applied to the system, ketoprofen was adsorbed in the pretreatment column, while plasma proteins were excluded. After the elution of proteins from the pretreatment column, the valve was switched and ketoprofen was desorbed and transferred to the chiral separation column where the enantiomers were separated and determined by ultraviolet-absorption. The mobile phase conditions for the pretreatment and chiral separation were optimized, which enabled rapid and complete recovery followed by satisfactory separation of the enantiomers. The calibration line for each enantiomer showed good linearity in the range of 0.25—5 μg/ml with a detection limit of 0.02 μg/ml (signal to noise ratio (S/N) > 3), which was sufficient for practical demands. The precision test indicated that the coefficient of variation for five repeated determinations of (−) ketoprofen was 5.4% at 0.1 μg/ml and 1.4% at 1 μg/ml.

Keywords chiral separation; direct injection; column-switching; enantiomer; chiral stationary phase; HPLC ketoprofen; human plasma

It is roughly estimated that 50% of the drugs available for clinical use involve asymmetric carbon atom(s) in their molecules, and half of them come from natural sources. Therefore, synthetic chiral drugs occupy one forth of the total drugs, and 80% of the synthetic chiral drugs are used as racemates. As a result, racemic drugs account for about 20% of the total number of drugs. A number of investigations indicated a significant difference between enantiomers of a drug in their pharmacological activities, side-effects, and pharmacokinetic behaviors as well. Therefore, the stereoselective determination of drug enantiomers in plasma is of potential clinical importance.[1–3)]

The "in-line deproteinization" method by high-performance liquid chromatography (HPLC) has been recently developed, allowing for the direct injection of the plasma sample.[4–6)] It enables the separation of drugs from blood proteins, detection of drugs, and regeneration of the pre-column automatically by means of the column-switching technique.[7)] Enantiomeric separation has also been studied according to several different methods. One is the diastereomer derivatization method,[8–10)] in which enantiomers are derived to diastereomer by reaction with an optically active reagent, followed by separation on a conventional HPLC column (*e.g.* octadecyl silica). The direct resolution is attained by using a chirality-recognizable reagent as a stationary ligand[11–13)] or an additive to the mobile phase.[14,15)] The chiral separation of drugs and their metabolites in serum was also achieved by using achiral/chiral coupled-column HPLC.[16)] However, these methods can not immediately be applied to the plasma (or serum) samples which need tedious pretreatment for deproteinization, extraction and sometimes derivatization. These procedures often cause erroneous results.

Ketoprofen (2-(3-benzoylphenyl)propionic acid), a non-steroidal anti-inflammatory drug, having one asymmetric carbon atom is clinically used as a racemate, though *S*-isomer is known to be more potent than *R*-isomer. Since some 2-arylpropionic acid derivatives (profens) are known to undergo irreversible metabolic inversion from *R* to *S* isomer[17)] besides other options of metabolism, the investigation of their stereoselective pharmacokinetics is significant. The diastereomer derivatization method as mentioned above was reported to be applicable to the separation and determination of ketoprofen enantiomers in plasma.[9,10)]

The present paper deals with the development of an HPLC system for the simple and rapid stereoselective determination of racemic ketoprofen in human plasma following direct sample injection. The system involves the pretreatment column for the selective adsorption of the drug in plasma and the chiral separation column for the separation and determination of enantiomers. These columns are connected in a series *via* a column-switching valve.

Experimental

Reagents and Materials Racemic ketoprofen was supplied by Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan). Ammonium acetate, acetic acid, hydrochloric acid, sodium hydroxide and sodium chloride of guaranteed reagent grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan). Methanol of reagent grade was purchased from Wako and was distilled before use. Deionized and distilled water was further purified with a Milli-Q four houses water purification system.

Apparatus The developed system is composed of two sub-systems, a sample pretreatment system and a chiral separation system. The flow diagram of the system is shown in Fig. 1. The pretreatment system consisted of a pump (Model SSP DM 3M-2047, Sanuki Kogyo Co., Tokyo, Japan), a sample injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.) equipped with a 500-μl sample loop, a pretreatment column (L-column, 4.6 mm i.d. × 30 mm, Chemical Inspection & Testing Institute, Tokyo, Japan) and a ultraviolet (UV) detector (SPD-2A, Shimadzu, Kyoto, Japan). The chiral separation system was composed of a pump unit (LC-6A, Shimadzu, Kyoto, Japan), a sample injector (Model 7125, Rheodyne) equipped with a 500-μl sample loop, a chiral separation column (SUMICHIRAL OA-2500, 4.6 mm i.d. × 250 mm, Sumika Chemical Analysis Service, Osaka, Japan) and a UV detector (Model UVIDEC-100III, Jasco, Tokyo, Japan). The two sub-systems were connected *via* a variable loop injector (Model VL-611, Jasco, Tokyo, Japan) which was used as a six-way switching valve. Chromatopac C-R3A (Shimdazu, Kyoto, Japan) was used for the data analyses.

The UV detector in the pretreatment system was used in order to inspect the performance of the pretreatment column. The sample injector in the chiral separation system was used only to examine the condition of the chiral separation column.

Preparation of Mobile Phase The mobile phase for the pretreatment system was 0.02 M ammonium acetate (pH 6.8). The mobile phase for the chiral separation system was a mixture of methanol and 0.6 M acetate buffer (95 : 5). The 0.6 M acetate buffer was prepared by diluting 1 M acetate buffer (pH 4.0) which was a mixture of 1 M acetic acid and 1 M ammonium acetate (2 : 8).

Preparation of Standard Solutions of Ketoprofen Enantiomers A 20 μl of 1% methanol solution of (±)ketoprofen was applied to the chiral separation column under the mobile phase condition shown in Table I (pretreatment column was not used). The enantiomers were eluted in the retention order of (−)ketoprofen < (+)ketoprofen from OA-2500 column, and in the reversed order from OA-2500(S) (*S*-isomer of OA-2500) column. The eluent containing an enantiomer eluted first from respective columns was fractionated and rechromatographed under the same condition. The result indicated that the fraction from OA-2500 column and that from OA-2500(S) column contained optically pure (−)ketoprofen at a concentration of 126 μg/ml and (+)ketoprofen at 95.4 μg/ml, respectively. The assays were performed using the same HPLC condition according to the fact that (+)ketoprofen was a 50 : 50 racemic mixture and the enantiomers had the same molar UV-response. The fractions thus obtained were used as standard stock solutions of (−) and (+)ketoprofen. The standard aqueous solutions and standard plasma solutions containing known amounts of (+) or (−)ketoprofen were prepared from these stock solutions.

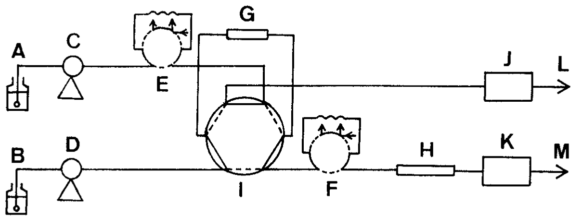

Fig. 1. Flow Diagrams of the On-line HPLC System for the Chiral Separation of an Enantiomeric Drug Following Direct Injection of Plasma Sample

(A) mobile phase for pretreatment; (B) mobile phase for chiral separation; (C, D) pump; (E, F) sample injector; (G) pretreatment column; (H) chiral separation column; (I) six-way switching valve; (J, K) detector; (L, M) waste. Dotted lines in the switching valve show the flow of the "pretreatment" mode, and solid lines show the flow of the "chiral separation" mode.

TABLE I. HPLC Condition of Column Switching System

Pretreatment	Column	L-column (4.6 mm i.d. × 30 mm)
	Column temperature	Room temperature
	Detection	UV (262 nm)
	Mobile phase	0.02 M Ammonium acetate buffer, pH 6.8
	Flow rate	1.0 ml/min
Chiral separation	Column	SUMICHIRAL OA-2500 (4.6 mm i.d. × 250 mm)
	Column temperature	Room temperature
	Detection	UV (262 nm)
	Mobile phase	Methanol–water (95 : 5, v/v) containing 0.03 M acetate buffer, apparent pH 6.2
	Flow rate	1.0 ml/min

Determination of Ketoprofen Enantiomers The plasma solution containing ketoprofen enantiomers was injected into a sample injector ((E) in Fig. 1). Ketoprofen bound to plasma proteins was released and retained on the pretreatment column (G) together with initially free ketoprofen, while proteins were excluded. Ketoprofen was eluted from the pretreatment column and transferred into the chiral separation column (H) by switching the valve (I). Ketoprofen enantiomers were separated and determined by a UV detector (K).

The optimized HPLC conditions for the column-switching system are shown in Table I. The time programs of column-switching and sample injection are shown in Fig. 2. The "pretreatment period" is the time period between the sample injection and valve-switching, during which bound ketoprofen was released from protein and the total amounts were adsorbed in the pretreatment column. The "chiral separation period" is the time period between valve-switching and the complete elution of ketoprofen enantiomers from the chiral separation column, during which the adsorbed enantiomers are back-flashed from the pretreatment column into the chiral separation column and determined separately by the UV-adsorption at 262 nm. These periods are controlled in accordance with the time sequence of valve-switching as given in Fig. 2, where I and II specify the time periods during which the mobile phases flow through, respectively, the dotted line and the solid line shown in switching valve (I) of Fig. 1. The time period of II was selected as 5 min, which is sufficiently long to recover total amounts of ketoprofen. As is found later, in Fig. 7, ketoprofen enantiomers which are adsorbed to the pretreatment column can be very rapidly eluted out after valve-switching, giving almost the same retention times and peak areas with those of standard ketoprofen in an aqueous solution. According to the time program in Fig. 2, the injection of plasma samples can be repeated four times in 90 min, though it takes 40 min for a single analysis.

Results and Discussion

Elution Profile of Human Plasma and Ketoprofen on Pretreatment Column The packing material of the pretreatment column used in this study is the porous silica gel with the internal and external surfaces of pores covalently bonded with octadecyl and glycerylpropyl groups, respectively. When the plasma sample is injected, proteins are readily size-excluded without adsorption to the external surface, while a small molecule drug can penetrate into the pore and be retained on the internal surface. Therefore, this material can be used as a preextraction column for the on-line HPLC analysis of the drug in plasma.[18,19]

Figure 3 shows the chromatogram of human plasma injected into the pretreatment column, where 0.02 M ammonium acetate buffer (pH 6.8) was used as the mobile phase because it barely denatures proteins and is miscible with the mobile phase used for the subsequent chiral separation. Plasma proteins were eluted out completely within 15 min, while ketoprofen did not emerge for at least

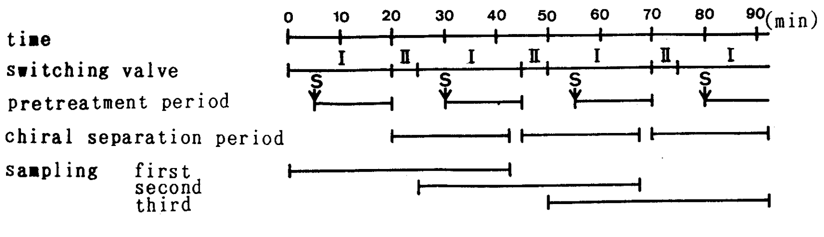

Fig. 2. Time Program of Column-Switching

I: time period during which pretreatment column is disconnected from chiral separation column. II: time period during which pretreatment column is connected to chiral separation column. S: sample injection.

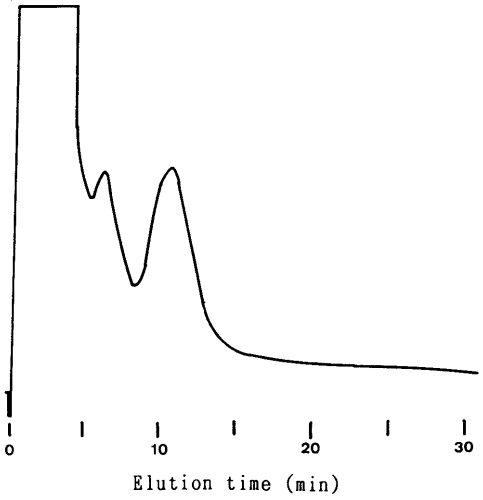

Fig. 3. Elution Profile of Human Plasma on the Pretreatment Column

Sample size: 200 μl. Conditions, see Table I.

30 min. Therefore, it was decided to connect the pretreatment column to the chiral separation column by switching the line at 15 min after the injection of the plasma sample into the pretreatment column. The column-switching at this time kept plasma proteins from entering the chiral separation column. However, since a small amount of uncertain endogenous plasma components accumulated due to the repeated injections of plasma sample (total volume about 12 ml), it was recommended to clean the column filter in the ultrasonic cleaner and/or to occasionally renew the pretreatment column.

Optimization of Chiral Separation Condition The packing material of the chiral separation column has (*R*)-*N*-(3,5-dinitrobenzoyl)-1-naphthylglycine phase covalently bonded to aminopropyl silica. This column can separate various enantiomers without derivatization.[20)]

Effects of the salt concentration in the mobile phase on capacity factor(k'), theoretical plate number(n), separation factor(α) and resolutions(Rs) of ketoprofen enantiomers were investigated using a chiral separation column alone where the apparent pH of the mobile phase was kept at 6.3. As shown in Fig. 4, the k' values of both (−) and (+)ketoprofen decreased with an increasing salt concentration, while n and α values were almost unchanged. The Rs value increased from 1.1 to 1.5 with a decreasing salt concentration from 0.05 to 0.01 M.

Figure 5 shows the effect of methanol concentration in the mobile phase on the k', n, α and Rs values. The Rs value increased gradually with an increasing k' value as the methanol concentration decreased from 97 to 70%, while α and n values suffered little change.

Figure 6 shows the effect of the pH of the mobile phase on the k', n, α and Rs values, where other conditions were kept constant as given in the legend. The pH value was measured on a glass electrode which had been standardized using aqueous buffer solutions. The abscissa of Fig. 6, therefore, shows the apparent pH value. It is found that k', n, α and Rs values were almost unaffected by the apparent pH of the mobile phase, though k', n and Rs showed small maximum values at around pH 6 to 7 for some uncertain reason.

From these results, the mobile phase conditions for the

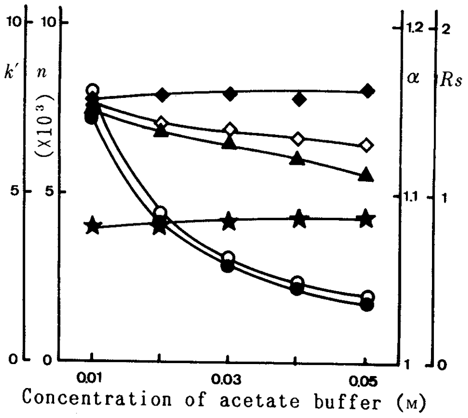

Fig. 4. Effect of Salt Concentration on Capacity Factors (k'), Theoretical Plate Number (n), Separation Factor (α) and Resolutions (Rs) of Ketoprofen Enantiomers

●, k' of (−)ketoprofen; ○, k' of (+)ketoprofen; ◆, n of (−)ketoprofen; ◇, n of (+)ketoprofen; ★, α of (±)ketoprofen; ▲, Rs of (±)ketoprofen.

Mobile phase: methanol–water (95:5, v/v) containing 0.01—0.05 M acetate buffer (mixture of acetic acid and ammonium acetate), apparent pH 6.3. Flow rate: 1.0 ml/min. Stationary phase: SUMICHIRAL OA-2500 packed in a 4.6 mm i.d. × 250 mm column. Detection: UV-258 nm. Sample: 10 μl of 10 μg/ml aqueous solution of (±)ketoprofen.

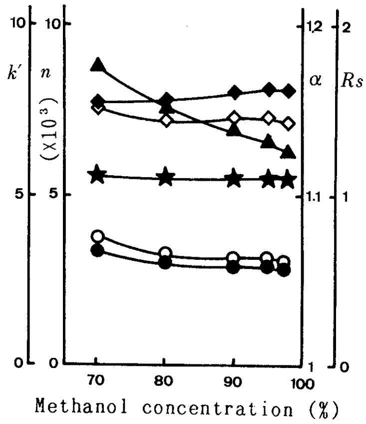

Fig. 5. Effect of Methanol Concentration on k', n, α and Rs of Ketoprofen Enantiomers

●, k' of (−)ketoprofen; ○, k' of (+)ketoprofen; ◆, n of (−)ketoprofen; ◇, n of (+)ketoprofen; ★, α of (±)ketoprofen; ▲, Rs of (±)ketoprofen.

Mobile phase: methanol–water (70:30 to 97:3, v/v) containing 0.03 M acetate buffer (apparent pH 6.1±0.2). Other conditions are the same as in Fig. 4.

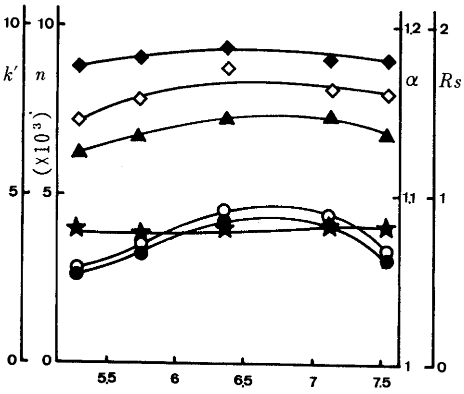

Fig. 6. Effect of pH on k', n, α and Rs of Ketoprofen Enantiomers

●, k' of (−)ketoprofen; ○, k' of (+)ketoprofen; ◆, n of (−)ketoprofen; ◇, n of (+)ketoprofen; ★, α of (±)ketoprofen; ▲, Rs of (±)ketoprofen.

Mobile phase: methanol–water (95:5, v/v) containing 0.03 M acetate buffer (apparent pH 5.3—7.5, ionic strength 0.028). Other conditions are the same as in Fig. 4.

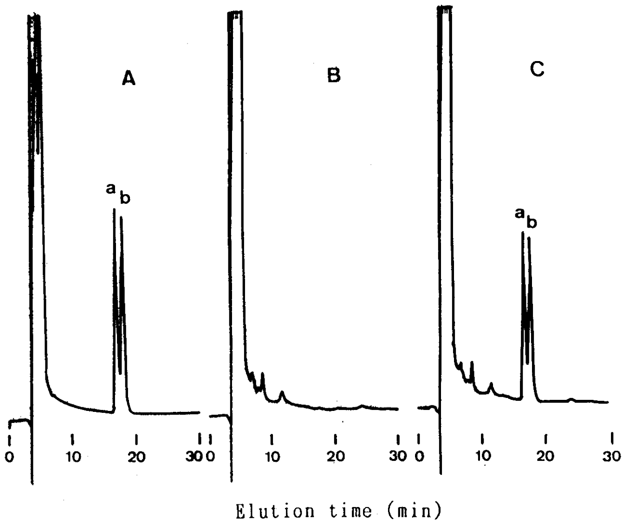

Fig. 7. Chiral Separation of Ketoprofen Enantiomers Following Direct Injection of Plasma into Column-Switching HPLC System

Peak a, (−)ketoprofen; peak b, (+)ketoprofen. Sample: A, 1 µg/ml aqueous solution of (±)ketoprofen; B, blank human plasma; C, 1 µg/ml plasma solution of (±)ketoprofen. Sample size: 200 µl. HPLC conditions, see Table I.

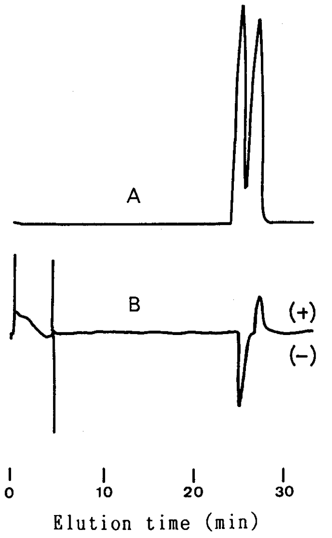

Fig. 8. Chiral Separation Chromatogram of (±)Ketoprofen Detected by (A) UV Absorption (262 nm) and (B) Optical Rotation

Sample: 20 µl of 10 mg/ml methanol solution of (±)ketoprofen. Mobile phase: methanol–water (95:5, v/v) containing 0.01 M ammonium acetate buffer. Other conditions are the same as in Table I (pretreatment column was not used).

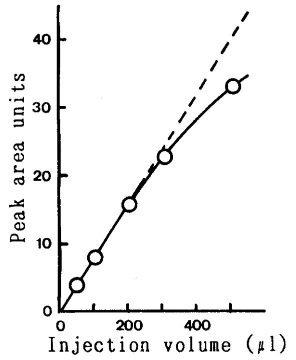

Fig. 9. Relationship between Peak Area of (−)Ketoprofen in Plasma and Injection Volume

Sample: 1 µg/ml plasma solution of (−)ketoprofen. Other conditions are same as in Table I.

chiral separation were optimized as shown in Table I. It was also found as mentioned later that ketoprofen adsorbed in the pretreatment column was rapidly and completely eluted out by this mobile phase.

Determination of Ketoprofen Enantiomers in Plasma Figure 7 shows the chromatograms obtained by injecting a 200 µl portion of (A) aqueous solution (1 µg/ml) of (±)ketoprofen, (B) blank human plasma and (C) plasma solution (1 µg/ml) of (±)ketoprofen. The peaks due to the degradation products[21] were not found in Fig. 7A, where the bulky peaks appearing at void volume were due to the change in the solvent system by column-switching, because these were also obtained even in the absence of a sample. The rat plasma obtained 0.5 h after the i.v. administration of 0.1 mg/kg *R*(−)ketoprofen indicated a separation profile similar to that of Fig. 7C.

The optical assignment was performed using an optical rotation detector (Chira Monitor Model 750/25, Applied Chromatography System Ltd., England). Figure 8 shows the typical chromatograms of (±)ketoprofen separated on the chiral separation column (OA-2500) and detected by both the optical rotation detector and the UV detector. (−)Ketoprofen was eluted faster than (+)ketoprofen. The injection of (+) and (−)ketoprofen in plasma and in purified water confirmed the purity of the respective enantiomers. The retention times of (+) and (−)ketoprofen in Fig. 7A agreed well with those in Fig. 7C and also with those obtained by injecting standard samples directly into the chiral separation column. The enantiomers gave the same peak area recorded on the integrated data analyzer in Fig. 7A and 7C. These results indicated that total ketoprofen was recovered from the plasma and rapidly submitted to chiral separation, and that chiral inversion and difference in the recovery between enantiomers did not occur during these processes. The metabolites of ketoprofen, except for the chiral inversion product, did not interfere with the separation of unchanged enantiomers. Those metabolites, such as glucuronide and oxidation products,[17] are more hydrophilic than ketoprofen and hence were expected to pass through the pretreatment column together with plasma proteins.

Ketoprofen is known to bind strongly with plasma proteins (bound fraction about 99%).[22] The bound drug is released from proteins by dilution with the mobile phase in the pretreatment column. Therefore, the injection of an excess volume of the plasma sample may cause the loss of drug during pretreatment.[23] Figure 9 shows the relationship between the injection volume of the plasma sample containing 1 µg/ml (−)ketoprofen and the peak area. The linear relation was obtained in the range of an injection volume lower than 200 µl. This means that when the injection volume was relatively small, bound ketoprofen was rapidly released and separated from plasma proteins. Thus, total ketoprofen was completely adsorbed to the pretreatment column. However, deviation from linearity found in Fig. 9 indicates that when a large volume of plasma (more than 300 µl) was injected, a part of the bound ketoprofen escaped together with plasma proteins. The consequent losses of ketoprofen were 9 and 29% of the total, when 300 and 500 µl portions of the plasma sample were injected, respectively. These results suggest that the injection volume of the plasma sample should not exceed 200 μl under the conditions shown in Table I.

The calibration line obtained by injecting 200 μl of 0.25 to 5 μg/ml plasma solution of (−)ketoprofen indicated good linearity with correlation coefficients higher than 0.999. The recovery of ketoprofen was estimated as the ratio of the slopes of the calibration lines of plasma solution and aqueous solution. The result that the ratio was almost equal to unity confirmed 100% recovery of the ketoprofen from the plasma.

The detection limit calculated for the signal to a noise ratio $(S/N)>3$ was 0.02 μg/ml for both ketoprofen enantiomers in the plasma. This is sufficient for the determination of ketoprofen enantiomer concentrations encountered in the clinical samples. The coefficient of variation (within run) for the five repeated determinations of 0.1 and 1 μg/ml (−)ketoprofen in plasma were 5.4 and 1.4%, respectively.

The present system allows for the accurate and fast determination of ketoprofen enantiomers with easy operation and no manual pretreatment of the plasma. This system can be applied to a wide range of enantiomeric drugs by modification of the conditions.

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