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Chiral Separation of Ketoprofen Racemate by using Chirex® 3005 and Kromasil® CHI-II Chiral Column

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Abstract–Ketoprofen is a non-steroid anti-inflammatory drug (NSAID) that has analgesic, anti-inflammatory and antipyretic properties, of which pharmacological activity has been contributed mainly by the S-ketoprofen enantiomer. To gain highly purified S-ketoprofen enantiomer from ketoprofen racemate, suitable stationary phase was investigated by comparing R-type naphthylglycine and 3,5-dinitrobenzoic acid of Chirex® 3005 and O,O'-bis(4-tert-butyl-benzoyl)-N and N'-diallyl-L-tartardiamide of Kromasil® CHI-II. S-ketoprofen enantiomer was successfully isolated by using the Kromasil column as well as the mobile phase of hexane/tert-butyl methyl ether/acetic acid (v/v)= 60/40/0.1. Equilibrium constants of R- and S-ketoprofen were obtained by pulsed injection method (PIM) at various concentrations of ketoprofen racemate and loading sample amounts, which serve the basic information on overloading sample volume and amounts. A volume of 100 μl containing 3.0 mg of ketoprofen racemate to Kromasil column (250 mm×4.6 mm) resulted in 85% recovery of injected sample under an high concentration (30 mg/ml) feed condition.

Key words: Ketoprofen Racemate, HPLC, Chiral Separation, Enantiomer, Fractionation

INTRODUCTION

Most biochemicals exist in mirror image as optical isomers, and carry out various functions *in vivo* [Morrison et al., 1992]. Ketoprofen, a phenylpropionic acid derivative, is a non-steroid anti-inflammatory drug (NSAID) that has analgesic, anti-inflammatory and antipyretic properties [Blanco et al., 1988; Jagota et al., 1992]. Pharmacological activity has been contributed mainly by the S-form, while R-form is either inactive or reduced active form compared to S-form [Pietta et al., 1987; Meyring et al., 2000]. It is difficult to separate a chiral compound such as ketoprofen to each enantiomer by ordinary separation method, because stereo-structure is related to its molecular structure [Kakodkar et al., 1979]. The importance of chiral compounds is obvious; however, their importance is increased due to the demands of the pharmaceutical industry. Several chiral separation methods have been reported in the literature in which diasteromeric derivatives were formed prior to HPLC separation and analysis [Sallustio et al., 1986; Boisvert et al., 1997]. And membranes which had novel carrier system were utilized to separate enantiomer from racemic mixture [Oshima et al., 2003]. But, these methods are generally time-consuming and are prone to inter-conversion of enantiomers. Therefore, direct separation of each enantiomer by using a chiral stationary phase (CSP) was preferred [Wainer, 1993; Wright et al., 1993].

The direct separation of ketoprofen enantiomers has been successfully accomplished by using various types of CSP, but some columns are not available commercially, because others may require prior derivation to enhance resolution [Menzel-Soglowek et al., 1990; Van Overbeke et al., 1994; Grubb et al., 1996]. With the present demand, many chiral stationary phases are distributed by manufacturers like Merck, Daicel and so on [Francotte et al., 1997; Schuring et al., 1997; Gindy et al., 2001].

This paper describes the separation of S-ketoprofen enantiomer from ketoprofen racemate by using Chirex® and Kromasil® chiral columns. These two columns have chiral selective functional groups for effective combination of carboxyl group of ketoprofen with chiral center. Chiral stationary phase (CSP), mobile phase composition and sample concentrations were changed in this study to get a suitable separation condition under overloading experiments. Therefore, the overloading technique utilized in this paper is capable of separating ketoprofen enantiomers by using a commercially available chiral column in the laboratory. In addition, we can determine the relationship between recovery yield and sample concentration in fractionation experiments.

EXPERIMENTAL PART

1. Materials

Bulk ketoprofen racemate was purchased from Kookjeon Co. (Korea) in powdered form, and standard S-ketoprofen (99%) was purchased from Sigma (USA) to identify S-ketoprofen enantiomer separated. Chiral columns used in experiments were Chirex® column No. 3005 (250 mm×4.6 mm, Phenomenex, USA) and Kromasil® CHI-II HPLC column (250 mm×4.6 mm, Eka-Nobel, Sweden). The Chirex column is packed with 5 mm of particle diameter which is covalent bonded with R-type naphthylglycine and 3,5-dinitrobenzoic acid. And Kromasil CHI-II column is packed with 10 μm of particle diameter and 100 Å of internal pore which is covalent bonded with O,O'-bis(4-tert-butyl-benzoyl)-N and N'-diallyl-L-tartardiamide. Ammonium acetate and acetic acid were purchased from Hayashi Pure Chemical Industries (Japan) for adjusting mobile phase property.

2. HPLC System

The HPLC system consisted of a solvent delivery pump (Model M-930, Young Lin, Korea), sample injection valve (Model 7725i,

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Table 1. Mobile phase compositions for two kinds of columns

Stationary phase	Base solvent	Modifier	Variables
Chirex column 3005	Methanol	Ammonium acetate	Modifier concentrations (mM) 0, 20, 30, 50, 80 mM
Kromasil CHI-II	n-hexane/t-BME	Acetic acid (0.1%(v/v))	Ratio of n-hexane/t-BME (v/v) 100/0, 80/20, 60/40, 40/60, 80/20, 0/100

v/v = volume %

Rheodyne, USA) and UV detector (M-720, Young Lin, Korea). Absorbance values were collected by a PC using a data acquisition system (Autochro-win, Young Lin, Korea). The column was equipped with a column heater (CH-30, Eppendorf, USA) to maintain constant temperature. Sample was loaded in various sample volume by using sample loops (10, 20, 50, 100 and 200 μl).

3. Selection of Mobile Phase

Mobile phase components such as methanol, hexane (HXN) and tert-butyl methyl ether (t-BME) purchased from Aldrich (USA) were filtered through a Millipore filter (Durapore membrane filters, 0.22 μm, GV, USA), and degassed by sonicator (Brasonic, Brason, USA) before use in the HPLC system. Mobile phases were prepared as shown in Table 1, and isocratic elution experiments of ketoprofen were performed by using chiral columns with the compositions in Table 1.

4. HPLC Experiments for Calculation of Adsorbed Amount and Overloading

In the case of the Chirex® 3005 column used, flow rate of mobile phase was settled on 0.5 ml/min, not to exceed 750 psi of back pressure of column. Kormasil® columns back pressure was maintained at 450 psi with 1.0 ml/min flow rate. Concentration and loading amounts of samples were varied to calculate adsorption capacity of each column. To calculate adsorbed amount of each ketoprofen enantiomer, pulse input method (PIM) was used. When a small amount of sample is loaded in the column, the adsorbed amount in the column is expressed as follows:

$$q_{(stationary\ phase)} = KC_{(mobile\ phase)} \quad (1)$$

where, q means adsorbed amount of ketoprofen enantiomer in stationary phase, K is the equilibrium constant and C is the concentration of ketoprofen enantiomer in mobile phase.

Retention time is related to K as in the following equation:

$$t_R(\text{retention time}) = t_0 \cdot \left(1 + \frac{1-\varepsilon}{\varepsilon} \cdot K\right) \quad (2)$$

where, t_0 means zero retention time which is the retention time of does not retains component with stationary phase, and can be gained from experiment. ε is void fraction of column, and can be calculated by Eq. (3).

$$\varepsilon = t_0 \cdot Q/V \quad (3)$$

where, Q means flow rate of mobile phase, and V does total volume of column.

From the retention time, the equilibrium constant can be calculated by Eq. (2) using retention time of each enantiomer. In general, as loading sample concentration is increased, the peak retention time decreases owing to non-linear behavior in a column. PIM is a method for the calculation of equilibrium constant in that $q_{(stationary\ phase)}$ can be obtained by Eq. (1) with the K value obtained experimentally from t_R in Eq. (2).

RESULTS AND DISCUSSION

1. Effects of Mobile Phase Composition on the Resolutions of Ketoprofen

To separate S- and R-ketoprofen efficiently, Phenomenex Chirex® 3005 and Kromasil® CHI-II columns were tested at different mobile phase composition. When ketoprofen racemate was injected into the Chirex® 3005 column, the peak of S-ketoprofen eluted later than

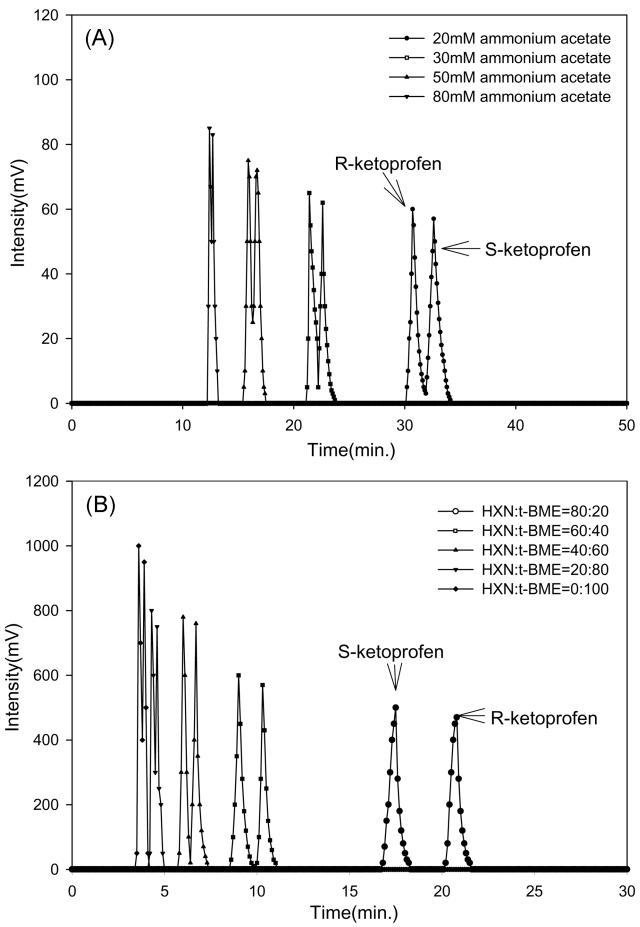

Fig. 1. Chromatograms of ketoprofen racemate from different columns and mobile phase compositions (sample loading amount=20 μl, concentration=0.5 mg/ml, temperature=40 °C; A : Chirex column, ammonium acetate concentration (1 : 20 mM, 2 : 30 mM, 3 : 50 mM, 4 : 80 mM), flow rate= 0.5 ml/min; B : Kromasil column hexane/t-BME (1 : 80/20, 2 : 60/40, 3 : 40/60, 4 : 20/80, 5 : 0/100), flow rate=1.0 ml/min).

Table 2. The effects of mobile phase compositions on selectivity (α) and resolution (R) in two chiral columns

Column	Chirex 3005					Kromasil CHI-II					
Comp.	Ammonium acetate in methanol (mM)					Hexane/t-butylmethyl ether acetic acid (v/v)					
Conc.	0	20	30	50	80	100/0/0.1	80/20/0.1	60/40/0.1	40/60/0.1	20/80/0.1	0/100/0.1
a	-	1.076	1.078	1.087	1.081	-	1.210	1.209	1.209	1.201	1.208
R	-	1.210	1.188	1.157	0.935	-	7.204	1.600	1.204	0.686	0.374

Injection volume=20 μl, temperature=40 °C, λ=254 nm and sample concentration=1.0 mg/ml

that of R-ketoprofen (Fig. 1A). As shown in Table 2, no peak was eluted in the case that pure methanol was used. However, ketoprofen peaks emerged as soon as ammonium acetate was added in methanol as shown in Fig. 1A. The effect of ammonium acetate increase is that selectivity and resolution of ketoprofen racemate decrease together and retention time of ketoprofen is diminished. Hexane with 0.1% (v/v) of acetic acid leads to no peak in the chromatograms of Fig. 1B, so t-BME was added to change the composition of solvent. A composition of 60/40/0.1 (%v/v) of hexane, t-BME and acetic acid was selected as an optimum composition for overloading experiments by observing the chromatograms in Fig. 1B to separate ketoprofen in the Kromasil column. The Kromasil CHI-II column showed shorter retention time, better selectivity and resolution than that of the Chirex 3005 column, and the back pressure of the Kromasil was lower than that of the Chirex 3005 column, even with high flow rate of the mobile phase. Although the composition of hexane/t-BME/acetic acid=80/20/0.1 (Fig. 1B) is better than that of 60/40/0.1 (No. 4 in Fig. 1B) in resolution, the former composition is not adopted due to the low solubility of ketoprofen in mobile phase and long retention time. S-ketoprofen was eluted earlier than R-ketoprofen in Kromasil column in contrast to the elution profile of Chirex column.

2. Calculation of Adsorbed Amount

To calculate adsorption capacity of each column, sample concentrations were varied from 0.5 to 50 mg/ml. The chromatograms of ketoprofen racemate in the Chirex column are shown in Fig. 2A. When the concentration of ketoprofen racemate exceeded 5 mg/ml, peaks were eluted in an asymmetric shape. Such phenomenon is caused by non-linear behavior of the adsorption isotherm, because the adsorption-desorption speed of the solute is not equal between stationary phase and mobile phase. Fig. 2B shows chromatograms when the Kromasil column is used as the stationary phase. As the concentration of ketoprofen goes over 10 mg/ml (No. 4), the chromatogram shows asymmetric peaks. From the chromatograms in Fig. 2A and Fig. 2B, we can calculate adsorbed amount of R- and S-ketoprofen enantiomer by PIM using the equilibrium constant calculated by Eq. (2) and (1). In Table 3, the amount of adsorbed ketoprofen enantiomer in stationary phase is listed, and Fig. 3A and Fig. 3B show adsorption isotherms of ketoprofen enantiomer in Chirex and Kromasil columns. In the case of the Chirex column, as the concentration of ketoprofen racemate is beyond 10 mg/ml, the slope of equilibrium isotherm declines. Although, the Chirex column has a higher capacity of ketoprofen than that of the Kromasil, but selectivity and resolution of ketoprofen racemate is lower than that of the Kromasil (Table 2). Retention time of ketoprofen in the Chirex column is longer than that of the Kromasil column, and the low pressure drop of the column is higher than that of the

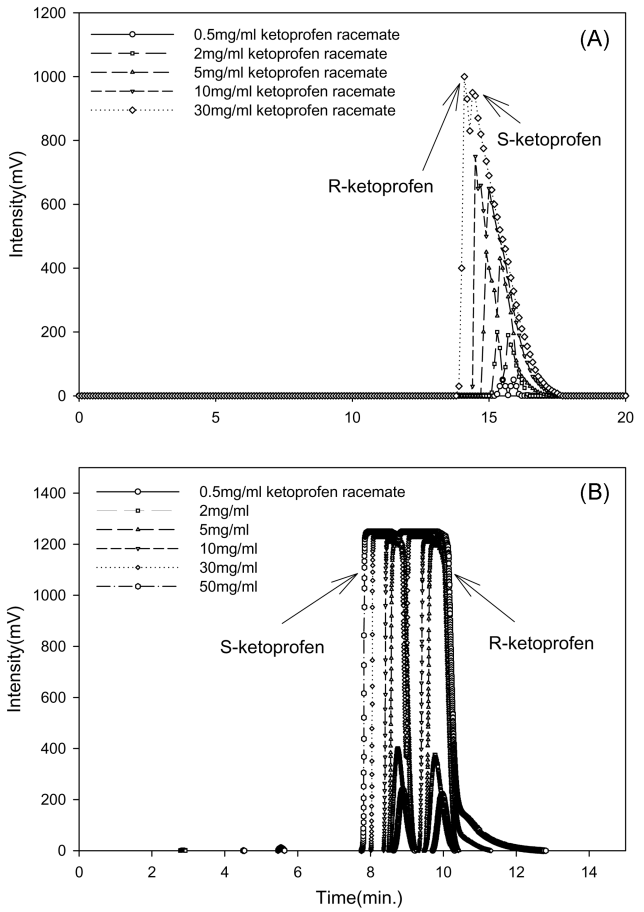

Fig. 2. Chromatograms of ketoprofen racemate at different sample concentrations (sample loading volume=20 μl, temperature=40 °C; A : Chirex column with 50 mM ammonium acetate methanol, flow rate=0.5 ml/min; 1 : 0.5 mg/ml, 2 : 2 mg/ml, 3 : 5mg/ml, 4 : 10 mg/ml, 5 : 30 mg/ml; B : Kromasil column with hexane/t-BME=60/40; flow rate=1.0 ml/min; 1 : 0.5 mg/ml, 2 : 2 mg/ml, 3 : 5 mg/ml, 4 : 10 mg/ml, 5 : 30 mg/ml, 6 : 50 mg/ml).

Kromasil column even with low flow rate of the mobile phase. Therefore, the Kromasil® CHI-II column was further utilized for overloading experiments.

3. Recovery of S-Ketoprofen Enantiomer in Overloading Experiments

In order to determine suitable loading volume of ketoprofen racemate, we have performed HPLC experiments with different sample volumes. Fig. 4 shows elution profiles with different sample volumes. Sample volumes and concentrations were varied for main-

Table 3. Adsorbed amount of ketoprofen enantiomer (R, S) at stationary phase

C_R=C_S (mg/ml)	Chirex 3005 column		Kromasil CHI-II column	
	$\overline{C}_R$ (mg/ml)	$\overline{C}_S$ (mg/ml)	$\overline{C}_R$ (mg/ml)	$\overline{C}_S$ (mg/ml)
0.25	2.1	2.2	0.7	0.7
0.5	4.0	4.2	3.1	2.7
2.5	17.1	17.9	7.7	6.7
5.0	29.8	31.2	15.1	12.9
15	54.5	55.5	44.3	37.7
25	69.9	73.1	71.0	59.3

Injection volume=20 μl, temperature=40 °C, λ=254 nm, C_i=concentration of each enantiomer in mobile phase, $\overline{C}_i$=concentration of each enantiomer in stationary phase.

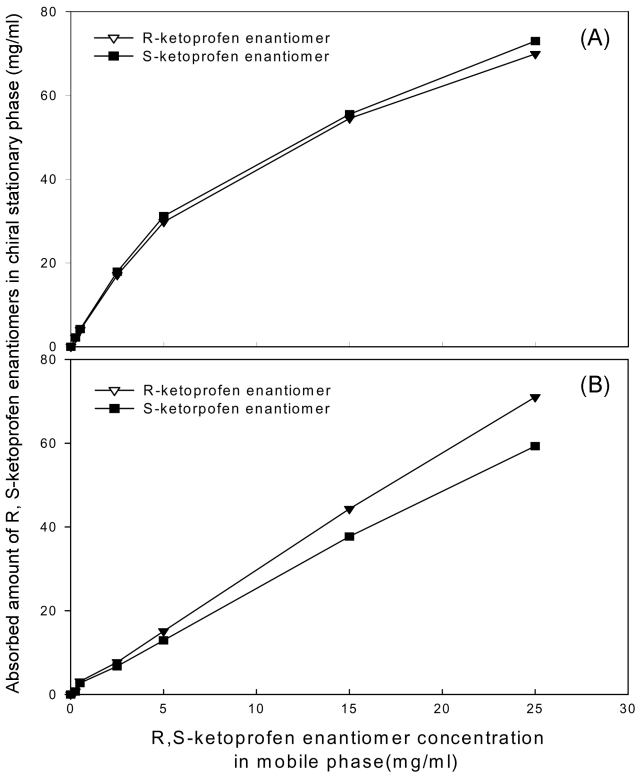

Fig. 3. Adsorption amount of each enantiomer of two chiral columns (A : Chirex column, B : Kromasil column).

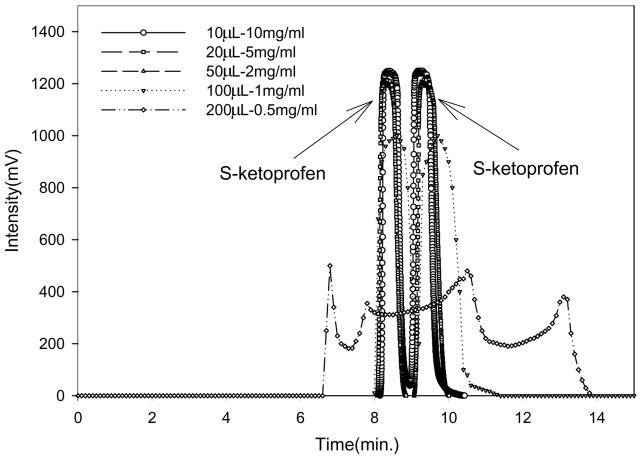

Fig. 4. Chromatograms under conditions of equal loading amount and different loading volumes at Kromasil column (1 : 10 μl of 10 mg/ml, 2 : 20 μl of 5 mg/ml, 3 : 50 μl of 2 mg/ml, 4 : 100 μl of 1 mg/ml, 5 : 200 μl of 0.5 mg/ml, flow rate=1.0 ml/min, temperature=40 °C, mobile phase=HXN/t-BME/Acetic acid=60/40/0.1, wavelength=254 nm).

taining the same quantity of ketoprofen in injection samples. While sample volume increased, peak width was gradually broadened. When sample volume was over 100 μl, the chromatogram spread out too much at 200 μl and so we chose 100 μl as the maximum sample loading volume.

The concentration of ketoprofen racemate was so high that absorbance value of the UV detector was over its limit at 254 nm of wavelength. Therefore, we measured the absorbance of ketoprofen at a different wavelength. The maximum peak of absorbance of ketoprofen was measured at 254 nm; at the other side that of ketoprofen showed 10% of the absorbance at 350 nm (Fig. 5). So the wavelength of the UV detector was varied to 350 nm.

Chromatograms from overloading experiments are illustrated in Fig. 6. Due to high concentration of ketoprofen, the peak of each enantiomer is fused at the middle of two peaks. In this case, we collected 100 μl of samples containing separated ketoprofen enantiomer every 6 seconds from the elution stream, and reanalyzed the fractions with analytical condition. When 100 μl of 10 mg/ml sample was loaded, the peak of S-ketoprofen was eluted at 7.2 min and then 11 fractions, which were collected at the discharged stream from the detector, were pooled to determine average concentration

Table 4. Results of fractionation experiments under various sample concentrations

Sample conc. of ketoprofen racemate	Loading amount (S-form)	Fraction No.	Pooled fraction	Concentration of recovered S-form	Recovered amount (Yields)
10 mg/ml	0.5 mg per a batch	30 (7 min 12sec after injection)	No. 1-11 (1.1 ml)	0.4 mg/ml	0.44 mg (86%)
30 mg/ml	1.5 mg per a batch	30 (6 min 30sec after injection)	No. 1-8 (0.8 ml)	1.6 mg/ml	1.28 mg (85%)
50 mg/ml	2.5 mg per a batch	30 (6 min 18sec after injection)	No. 1-3 (0.3 ml)	5.3 mg/ml	1.59 mg (64%)

Injection volume=100 μl, flow rate=1.0 ml/min, fraction volume=100 μl (6 sec.), temperature=40 °C, mobile phase (HXN/t-BME/acetic acid)=60/40/0.1, λ=350 nm.

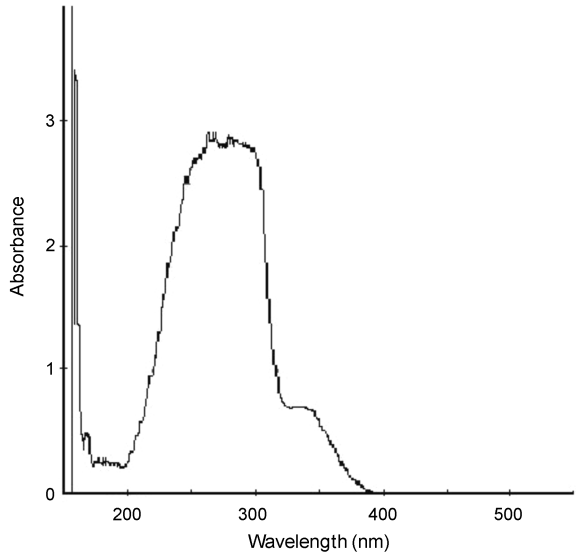

Fig. 5. Absorbance of ketoprofen at different wavelength of UV; sample concentration=30 mg/ml.

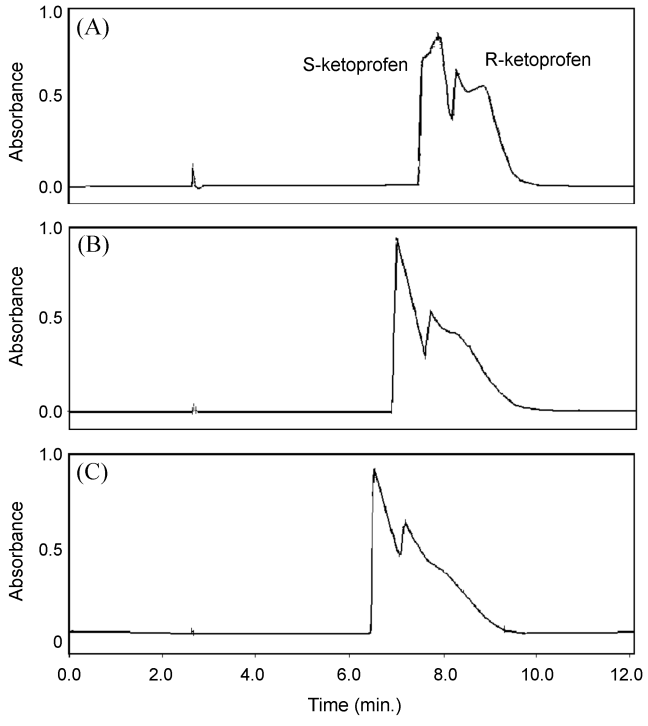

Fig. 6. Chromatograms under overloaded conditions; sample concentration : (A) =10 mg/ml, (B) =30 mg/ml, (C) =50 mg/ml, injection volume=100 μl, flow rate=1.0 ml/min, fraction volume=100 μl (6 sec), temperature=40 °C, mobile phase= HXN/t-BME/Acetic acid=60/40/0.1, wave length=350 nm.

by using analytical HPLC (Table 4). In this case, the pooled volume was 1.1 ml and the average concentration of that was 0.4 mg/ml. Recovery yield of S-ketoprofen enantiomer was 86% at the preparation. In this manner, sample concentration was increased to 30 mg/ml, and recovery yield in the second case was 85%. However, as the concentration of sample is increased to 50 mg/ml, the recovery yield decreases to 64% as in Table 4. This phenomenon could be explained by the non-linear behavior chromatography which was related with band broadening of chromatogram and shortening of elution time of the second peak, under overloaded conditions, where the fused region of two enantiomer peaks increases too much as shown in Fig. 6C.

CONCLUSIONS

In order to isolate S-ketoprofen enantiomer from racemate mixture, we selected the Kromasil CHI-II column (covalent bonded with O,O'-bis(4-tert-butylbenzoyl)-N and N'-diallyl-L-tartardiamide) as a stationary phase due to its resolution and low back pressure by comparing of two CSP columns between thte Chirex column No. 3005 (R-type naphthylglycine and 3,5-dinitrobenzoic acid) and the Kromasil CHI-II column. A suitable composition of mobile phase for isolation of ketoprofen in the chosen stationary phase was settled as hexane/t-BME/acetic acid=60/40/0.1 (%v/v). When the Kromasil® CHI-II column was used as stationary phase, non-linear behavior of column appeared over 10 mg/ml of sample concentration. Since the chromatogram of ketoprofen spread out when the loading sample volume exceeded 100 μl, sample loading volume was determined as 100 μl for overloading. In overloading experiments, S-ketoprofen could be isolated by fractionation of eluted peaks. The loaded amount of ketoprofen racemate was 3.0 mg per batch; we gained 85% yield in the Kromasil column (4.2 ml column volume), and the average concentration of pooled fractions was 1.6 mg/ml at the loading amount 3.0 mg.

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