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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 310–314
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Chiral separation of ketoprofen on an achiral C8 column by HPLC using norvancomycin as chiral mobile phase additives

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Received 14 July 2005; received in revised form 25 October 2005; accepted 28 October 2005
Available online 2 December 2005

Abstract

A high-performance liquid chromatographic method for chiral separation of ketoprofen racemate was developed. (*R*)- and (*S*)-ketoprofen enantiomers were separated on a Hypersil BDS C8 column (150 mm × 4.6 mm i.d., 5 μm) at 25 °C, using acetonitrile–triethylamine acetate (TEAA) buffer (pH 5.2, 20 mM) (35:65, v/v) containing 2.0 mM norvancomycin as the mobile phase. Effects of norvancomycin concentration, content of acetonitrile and TEAA buffer pH on the enantioseparation were investigated. The method was validated for linearity, repeatability, limits of detection (LOD) and limits of quantification (LOQ). Calibration curves ($r^2 = 0.999$) were constructed in the range of 2.01–200.8 μg ml^{-1} for (*S*)-ketoprofen and 2.04–152.4 μg ml^{-1} for (*R*)-ketoprofen, respectively. Repeatability ($n = 5$) showed less than 2% relative standard deviation (R.S.D.). LOD and LOQ for the two enantiomers were found to be 0.20 and 0.78 ng for (*S*)-ketoprofen, 0.20 and 0.86 ng for (*R*)-ketoprofen, respectively. Norvancomycin and vancomycin as chiral mobile phase additives (CMPAs) in the chiral separation showed similar abilities of enantioseparation. However, to obtain the optimum enantioseparation, a lower concentration of norvancomycin than that of vancomycin is required.

Keywords: Chiral separation; Ketoprofen; Norvancomycin; Chiral mobile phase additives

1. Introduction

Chiral discrimination has been an issue in the development and use of pharmaceutical drugs because drug enantiomers may have different pharmacokinetic properties and produce different physiological responses. The administration of highly pure chiral drugs is a major goal of pharmaceutical industry to protect the client against strains caused by high drug concentration or toxic side effects.

Ketoprofen, (±)-(*R*,*S*)-2-(3-benzoylphenyl)-propionic acid, is a non-steroid anti-inflammatory drug (NSAID) that has analgesic, anti-inflammatory and antipyretic properties [1]. However, (*S*)-ketoprofen and (*R*)-ketoprofen display significantly different pharmacological activities. The (*S*)-enantiomer is used to reduce inflammation and relieve pains, while the (*R*)-enantiomer can be used as a toothpaste additive to prevent periodontal disease [2]. The (*R*)-enantiomer of the NSAID ketoprofen has been reported to convert into its antipode in human or animals bodies [3–5]. The enantiospecific metabolic invertion of (*R*)-ketoprofen to (*S*)-form in vivo, exhibits marked substrate and species variability [5]. Therefore, the determination of the two enantiomers in biological samples is very important. Additionally, the production of single enantiomer of this drug is expected to grow due to the pharmacological benefits of both enantiomers.

The literatures are rich with analytical methods for the determination of ketoprofen enantiomers in biological matrices and pharmaceutical formulations. Some of the direct approaches for the analysis of ketoprofen enantiomers by HPLC and CE were either using chiral stationary phases or adding chiral selector additives into mobile phase [6–12].

Vancomycin has been successfully used as chiral selector in LC and CE enantioseparations [13–19]. Kang et al. [14] reported

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doi:10.1016/j.jpba.2005.10.045

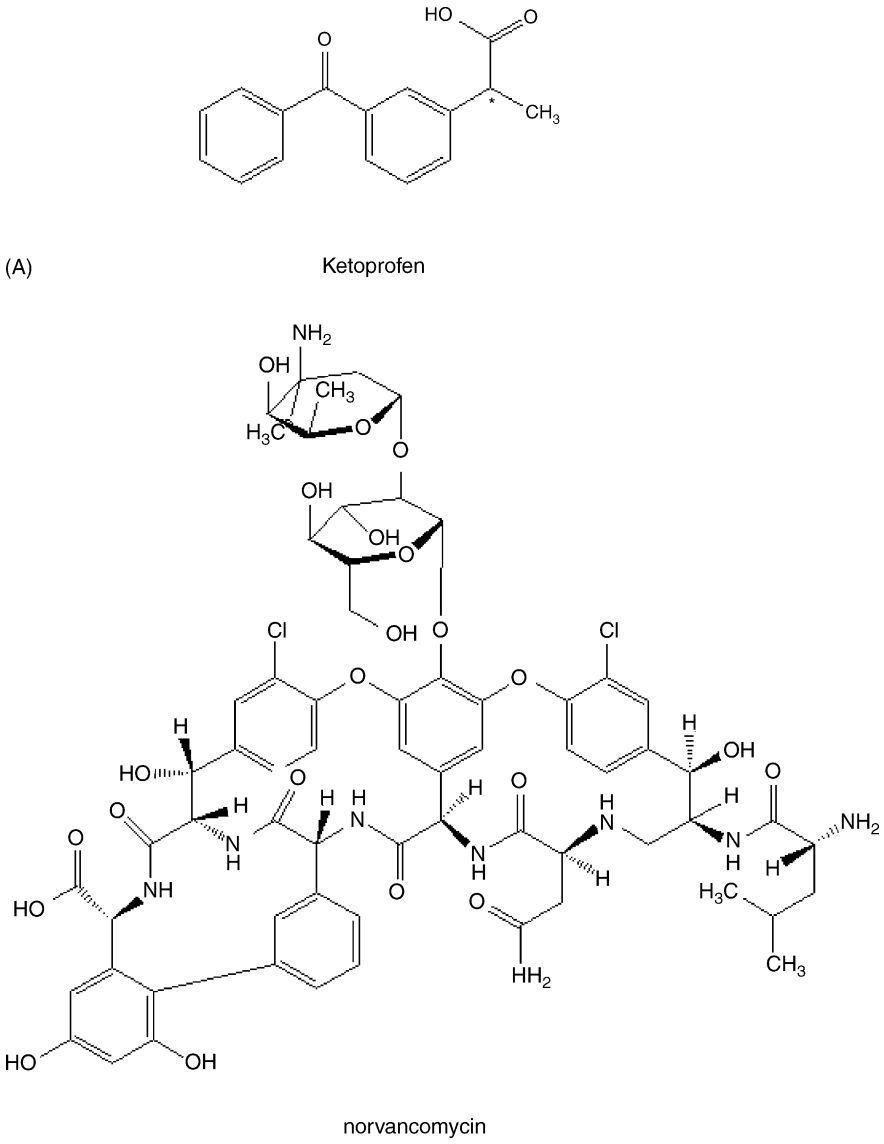

Fig. 1. Chemical structures of ketoprofen (A) and norvancomycin (B).

chiral separation of ketoprofen by CE using vancomycin as chiral additives. More recently, Fanali and co-workers [18] investigated the enantioseparation of ketoprofen and other basic and acidic compounds of pharmaceutical interest by nano-liquid chromatography using a vancomycin modified silica stationary phase.

Norvancomycin, as an analogue antibiotic of vancomycin, lacks a methyl group in its chemical structure and, consists of three fused macrocyclic rings, two side chains, a carbohydrate dimer and leucine (*N*-methyl-leucine in vancomycin) (Fig. 1), resulting in a notably higher chiral selectivity in chiral compounds containing a free carboxylic acid functional group. Norvancomycin bond-silica chiral stationary phase has been prepared and investigated by Wang and co-workers [19,20], and the advantages of norvancomycin used as chiral selector was illustrated.

In this paper, we developed a simple and precise method for chiral separation and determination of the (*S*)- and (*R*)-ketoprofen by HPLC on a C8 column using norvancomycin as chiral mobile phase additive (CMPA). Furthermore, the chiral separations of the enantiomers based on norvancomycin and vancomycin as CMPAs were evaluated.

2. Experimental

2.1. Materials

(*S*)-, (*R*)-, (*S*,*R*)-ketoprofen and vancomycin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Norvancomycin hydrochloride and pharmaceutical raw material of ketoprofen were donated by North of China Pharmaceutical Co. (Shijiazhuang, China). HPLC grade acetonitrile and methanol was purchased from Fisher (New Jersey, USA). Triethylamine acetate and other chemicals of analytical grade were purchased from Beijing Chemical Reagent Co. (Beijing, China).

2.2. Apparatus and HPLC conditions

An Agilent 1100 HPLC system (Agilent, USA) with a quaternary pump and a variable wavelength UV detector was employed. Deionized water from a Milli-Q system (Millipore, USA) was used for preparation the buffer and sample solutions.

HPLC conditions were: column, Hypersil BDS C8 column (150 mm × 4.6 mm, 5 μm); mobile phase, acetonitrile–20 mM TEAA buffer (35:65, v/v, pH 5.2) containing 2.0 mM norvancomycin; flow rate, 1.0 ml min^{-1}; detection, 290 nm; temperature, 25 °C; load amount, 50 μg ml^{-1} (10 μl).

2.3. Preparation of norvancomycin and working standard solution

Norvancomycin was obtained by adjusting pH of norvancomycin hydrochloride aqueous solution to 7.5 with 0.1 M NaOH solution. The precipitated off-white solid was recovered by filtration and dried in a vacuum oven at 60 °C overnight.

Working standard solutions were prepared using mobile phase (except norvancomycin) by adding known masses of (*R*)- or (*S*)-ketoprofen. The levels of the calibration standards were 2.0, 5.0, 10, 20, 50, 80, 100, 150, and 200 μg ml^{-1} for (*S*)-ketoprofen and 2.0, 5.0, 8.0, 10, 20, 50, 80, 100, and 150 μg ml^{-1} for (*R*)-ketoprofen, respectively. All solutions and samples were filtered through a 0.45 μm filter (Millipore, USA) prior to use. Racemate solution was prepared by dissolving the ketoprofen racemate 5.0 mg in 100 ml mobile phase.

2.4. HPLC analysis of (S)-ketoprofen capsules

Commercial pharmaceutical raw material (25 mg) of (*S*)-ketoprofen capsules were dissolved in 20 mM NaOH aqueous solution (10 ml) and then oscillated in a sonicator for 10 min. One milliliter of the aqueous solution was sequentially diluted to 100 ml with the mobile phase (except norvancomycin). The obtained solution was filtered through a 0.45 μm membrane filter, and then 10 μl of this solution was injected into Agilent 1100 HPLC system at 25 °C.

3. Results and discussion

3.1. Chiral separation

For HPLC analysis, 10 μl of the racemate of ketoprofen solution (50 μg ml^{-1}) was injected into Agilent 1100 HPLC system at 25 °C. Because norvancomycin has strong UV absorbance at shorter wavelength, a detection wavelength of 290 nm (where ketoprofen has strong absorbance) was selected, escaping the interferences of background UV absorbance and therefore improving the detection sensitivity. Retention factors were calculated from the equation $k = (t_R - t_0)/t_0$, where t_R is the retention time of retained analytes and t_0 is the retention time of the void marker (acetone). The enantioselectivity (α) is calculated from the equation $\alpha = k_R/k_S$, where k_R and k_S are retention factors of (*R*)- and (*S*)-ketoprofen, respectively.

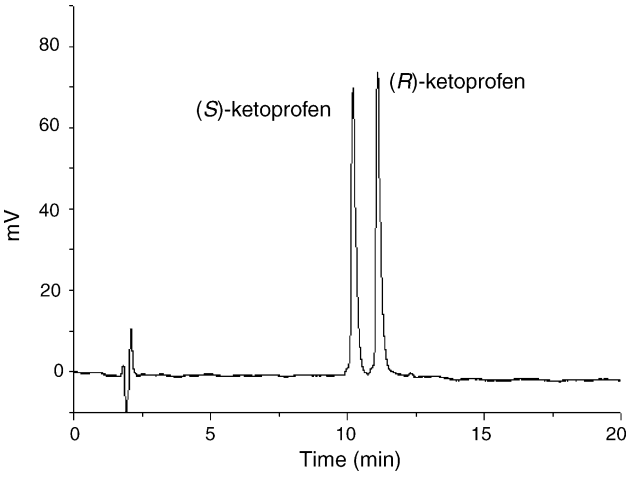

Fig. 2. Chromatogram of enantioseparation of (*R*,*S*)-ketoprofen. HPLC conditions: column, Hypersil BDS C8 column (150 mm × 4.6 mm i.d., 5 μm); mobile phase, acetonitrile–20 mM TEAA buffer (35:65, v/v, pH 5.2) containing 2.0 mM norvancomycin; flow rate, 1.0 ml min^{-1}; detection, 290 nm; temperature, 25 °C; load amount, 50 μg ml^{-1} (10 μl).

Fig. 2 represents the chromatogram of enantioseparation of (*R*,*S*)-ketoprofen on the achiral Hypersil BDS C8 column using norvancomycin as the chiral mobile phase. The enantiomers of (*R*,*S*)-ketoprofen were baseline chiral separated within 15 min and the resolution was 1.9. The retention factors for (*S*)- and (*R*)-ketoprofen were 5.22 and 6.32, respectively.

3.2. Effects on enantioseparation

The effects of the mobile phase composition were investigated. Firstly, it was found that a variation of the acetonitrile content had a substantial effect on the enantioselectivity. Fig. 3A shows the effect of the concentration of acetonitrile in the mobile phase on the enantioseparation, when 20 mM TEAA buffer and 2.0 mM norvancomycin were fixed in the mobile phase. Both retention factors for (*R*)-enantiomer (k_R) and (*S*)-enantiomer (k_S) significantly decreased with the increase in acetonitrile concentration in the mobile phase. However, the maximum enantioselectivity ($\alpha = 1.24$) and maximum resolution (R_S) were found at acetonitrile concentration of 40% (v/v) and 35% (v/v), respectively. So the solvent system of acetonitrile–20 mM TEAA aqueous buffer (35:65, v/v) was selected.

Subsequently, the effects of pH of TEAA buffers on chiral separation were investigated. Fig. 3B indicates both k_R and k_S are sharply decreased with the increase of pH at ranges from 3.5 to 7.0. The ideal acidity of the TEAA buffer was found to be pH 5.2, where both satisfactory enantioselectivity and good resolution could be achieved.

The concentration of norvancomycin in the mobile phase could influence both the resolution and the enantioselectivity. In Fig. 5, both R_S and α are shown to increase with an increase in norvancomycin concentration from 0.5 to 2.0 mM, followed by a slight decrease at norvancomycin concentration between 2.5 and 5.0 mM. Overall, good enantioselectivity and satisfactory resolution were simultaneously found at the concentration level of 2.0 mM.

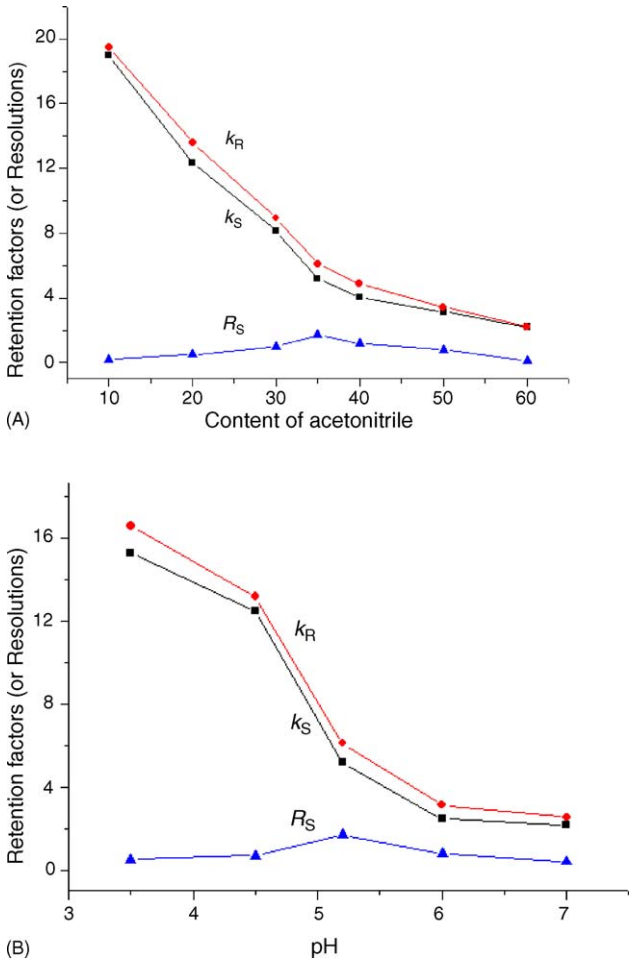

Fig. 3. Effects of content of acetonitrile (A) and pH of buffer (B) in the mobile phase on chiral separation.

Additionally, column temperature was also investigated and results indicated that the chiral separation was not significantly influenced by temperature in the range from 20 to 40 °C.

3.3. Method development and validation

Calibration curves were obtained by injecting each of 10 μl working standard solutions of the individual enantiomer at the concentrations mentioned in Section 2.3. Linearity was evaluated based on the correlations between the peak areas and the concentrations of the analytes. Results showed the linearity was acceptable in the range of 2.01–200.8 μg ml^{-1} for (*S*)-ketoprofen ($R^2 = 0.9992$) and 2.04–152.4 μg ml^{-1} for (*R*)-ketoprofen ($R^2 = 0.9997$), respectively.

Solutions of known concentration (typically 20 μg ml^{-1}) of (*R*)- and (*S*)-ketoprofen were injected five times, indicating the repeatability of the proposed method. The mean values for precision were 0.96% (R.S.D.) for (*R*)- and 1.27% (R.S.D.) for (*S*)-ketoprofen, respectively.

The limits of detection (LOD, $S/N = 3$) and quantification (LOQ, $S/N = 10$) for the two enantiomers were calculated experimentally from serial dilution. Results indicated that the values of LOD and LOQ for the two enantiomers were found to be 0.20 and 0.78 ng for (*S*)-ketoprofen, 0.20 and 0.86 ng for (*R*)-ketoprofen, respectively.

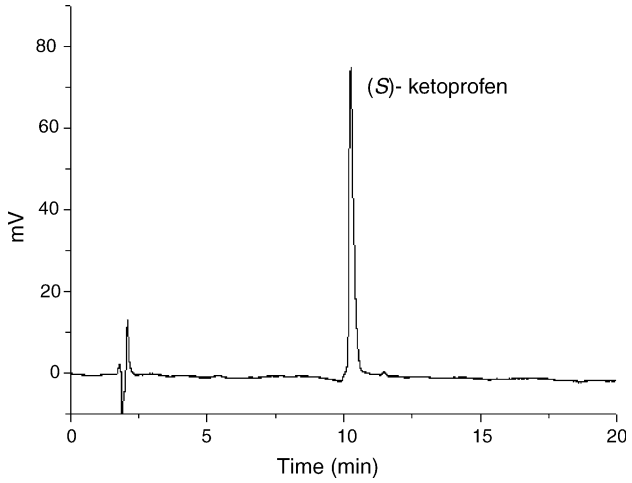

Fig. 4. Chromatogram of pharmaceutical (*S*)-ketoprofen formulation HPLC conditions: the same as Fig. 2, except for the load amount 25 μg ml^{-1} (10 μl).

The accuracy of the method was determined by investigating the recoveries of each enantiomer at five levels, i.e. 60, 80, 100, 120 and 140% of the known concentration, by spiking the sample solution (50 μg ml^{-1}) with the standard solutions. The recoveries of (*R*)- and (*S*)-ketoprofen were all found to be between 96.7 and 100.2%, and the average R.S.D. was below 2%.

3.4. Analysis of (S)-ketoprofen in pharmaceutical formulation

The proposed method was employed to determine the content of (*S*)-ketoprofen in its pharmaceutical capsules. Fig. 4 shows the chromatogram of the pharmaceutical (*S*)-ketoprofen capsules. As can be seen from the chromatogram, there are no obvious peaks corresponding to (*R*)-ketoprofen at the retention time 11.3 min, and most of the solvable subsidiary components have no (or weak) response at UV wavelength 290 nm. The average content of (*S*)-ketoprofen in the pharmaceutical raw materials was 99.24% with R.S.D. = 1.68% (n =6).

3.5. Evaluation of chiral separations based on norvancomycin and vancomycin as CMPAs

Since the two antibiotics are similar in their chemical structures, they are expected to have similar chromatographic properties and enantiorecognition abilities to those of vancomycin. The aim of this investigation was to obtain a better knowledge of the chiral recognition of norvancomycin.

When acetonitrile–TEAA buffer (pH 5.2, 20 mM) (35:65, v/v) was employed as the mobile phase and 2.0 mM vancomycin was added as CMPA, the retention times of (*R*)- and (*S*)-ketoprofen were prolonged about 20% than those produced by norvancomycin. To obtain the maximum enantioselectivity, 2.0 mM norvancomycin and 3.0 mM vancomycin was required in the mobile phase (Fig. 5).

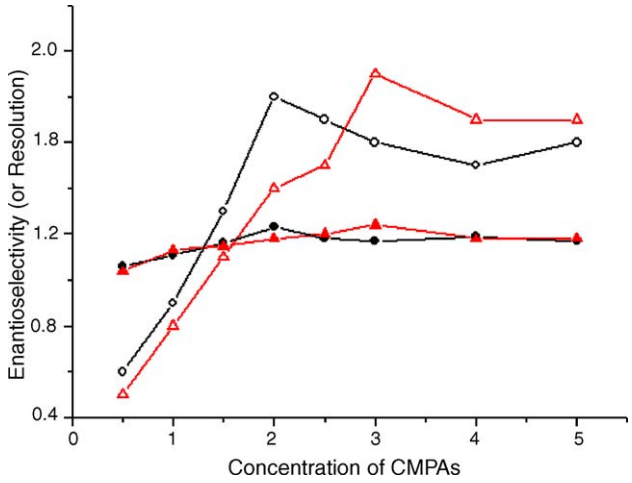

Fig. 5. Effects of concentration of norvancomycin and vancomycin in mobile phase on enantioselectivity (α) and resolution (R_S): (●) enantioselectivity (α) for norvancomycin, (◯) resolution (R_S) for norvancomycin, (▲) enantioselectivity (α) for vancomycin, and (△) resolution (R_S) for vancomycin.

As far as chemical structures are concerned, both vancomycin and norvancomycin consist of three fused macrocyclic rings formed by ether and peptide linkages, two side chains and a carbohydrate dimer. The enantioselectivity of vancomycin and norvancomycin should be related to their semi-rigid basket shaped aglycan structure and the chiral environment formed by 18 asymmetrical centers with various functional groups, which can provide the essential interactions for chiral recognition. Previous studies have shown that vancomycin and norvancomycin may form stable non-covalent dimmers or micelles in aqueous solutions [19–23]. The enantioseparation of ketoprofen was more likely obtained by forming different (*R*)-ketoprofen-norvancomycin and (*S*)-ketoprofen-norvancomycin complexes in the mobile phase through non-covalent interactions including hydrogen bonding, hydrophobic interaction, ionic bonding and π–π complexation, and so on. Furthermore, the reversed phase surface may be modified with norvancomycin, thus leading to a new dynamically coated stationary phase. This made the differences of chromatographic behaviors between (*R*)- and (*S*)-ketoprofen in the chiral recognition process. However, the differences of enantioseparation behaviors may originate from the leucine in norvancomycin, which is a *N*-methyl-leucine in vancomycin. The amidogen of leucine in norvancomycin has better hydrophilicity and more likely to undergo non-covalent interactions (e.g. hydrogen bonds and ion interactions) than *N*-methyl-leucine in vancomycin. Therefore, to obtain the proposed enantioselectivity, a lower concentration of norvancomycin should be required than vancomycin. More evidences and further experiments will be carried out to explain the observed phenomenon.

4. Conclusion

Enantiomers of (*R*,*S*)-ketoprofen were chiral separated on an achiral C8 column with norvancomycin added into the mobile phase as a chiral selector. This method was validated for linearity, repeatability, LOD and LOQ. Norvancomycin and vancomycin showed similar enantioseparation abilities as CMPAs in the chiral separation. However, optimum enantioseparation was achieved at lower norvancomycin concentration than that of vancomycin. In conclusion, this work has demonstrated that the optimized CMPA method was a useful and simple technique for chiral separation and determination of (*R*)- and (*S*)-ketoprofen. Additionally, this method has potential application in quality control of pharmaceutical formulations of ketoprofen.

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