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Chromatographic separation of the optical isomers of naproxen

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ABSTRACT

Naproxen, (*S*)-6-methoxy-α-methyl-2-naphthyleneacetic acid, is a non-steroidal anti-inflammatory drug, which is administered as a single enantiomer. Two different approaches applying high-performance liquid chromatography for the determination of the optical purity of naproxen are described. One method involves the derivatization of naproxen with 3,5-dinitroaniline and separation of the amide derivatives on a (*R*)-N-(2-naphthyl)alanine chiral stationary phase. The second method is a direct separation of naproxen optical isomers using α-1-acid glycoprotein columns. The latter method has been studied in detail to address the effects of temperature, ionic strength, pH and concentration of mobile phase buffer on enantiomeric resolution.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the determination of the optical purity of chiral substances is now a well developed analytical technique. The past 15 years have given rise to a profusion of new analytical developments such as chiral stationary phases (CSPs), derivatizing reagents, and chiral mobile phase additives, all of which have been proven effective in achieving chiral separation.

Four major approaches involving the separation of optical isomers by HPLC are: (1) direct separation on a CSP, (2) derivatization with an achiral reagent and separation on a CSP, (3) direct separation on an achiral stationary phase with the use of a chiral mobile phase additive, and (4) derivatization with a chiral reagent and separation of the resulting diastereomers using an achiral support. Similar techniques for the determination of optical purity by gas chromatography (GC) have also been reported.

Many of the separation types described above have been successfully applied to separate the optical isomers of the α-methylarylacetic acid series of non-steroidal anti-inflammatory drugs (NSAIDs), such as naproxen and ibuprofen. The majority of NSAIDs have one chiral center α to the carboxylic acid moiety, and most NSAIDs in this category are administered as the racemic mixture, with the exception of naproxen, which is administered as the resolved (*S*) enantiomer.

The majority of enantiomeric resolutions of NSAIDs reported in the literature are achieved by formation of diastereomeric derivatives with an optically active reagent and subsequent separation using either gas chromatography (GC) or HPLC. Separation via the diastereomeric amide derivatives is by far the most applied method. Of all the applicable chiral amines used as derivatizing reagents, α-methylbenzylamine has been the most widely published in conjunction with GC [1–6] or HPLC [7–10] analysis. The use of a number of other chiral amines e.g. L-1-(4-dimethyl-amino-1-naphthyl)ethylamine (DANE) and L-leucinamide in such applications has also been extensively reported [11–32] and, in many cases, these diastereomeric separations are preferred to the direct resolution on chiral columns. Other reported diastereomeric separations of the optical isomers of NSAIDs have included ester derivatives [9,33].

Derivatization of NSAIDs with various achiral amines combined with separation on CSPs has been applied to effect chiral separations of NSAIDs on numerous occasions [34–41]. The majority of these separations have been accomplished on "Pirkle-type" CSPs [34,35,37–41]. Other CSPs used have included cellulose based CSPs [39] and a CSP which contained either (R)- or (S)-1-(α-naphthyl)ethylamine with (S)-valine chemically bonded to γ-aminopropyl silanized silica [36]. Chiral separations of ester derivatives of NSAIDs have also been reported [34,42] using the Pirkle-type CSPs.

Protein based CSPs offered direct chiral separations of NSAIDs [43,44] while ion-pair chromatography employing chiral amine counter-ions on an achiral support [45,46] failed to facilitate enantiomeric resolution of naproxen. Nonetheless, a CSP that contains acetylquinine bonded to silica has been reported to separate the optical isomers of naproxen with the addition of quinine in the mobile phase [47,48].

Mixed advantages and disadvantages may be identified with the methods reported to date, therefore, method development was continued in order to develop a rugged chiral separation method for naproxen with good sensitivity and high efficiency. An important criterion is that this method can be easily adopted in a quality control environment. Two suitable methods considered to be most rugged are reported in this manuscript.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Spectra-Physics Model 8100 liquid chromatograph equipped with a Valco fixed-loop injector and a Kratos 757 UV detector. The detector output was monitored using a Spectra-Physics SP 4200 recording integrator.

Materials

Naproxen, its enantiomer and the racemic mixture were synthesized by the Institute of Organic Chemistry, Syntex Research (Palo Alto, CA, U.S.A.). 3,5-Dinitroaniline (98% +) and N,N-dimethyloctylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). Thionyl chloride was obtained from J. T. Baker (Phillipsburgh, NJ. U.S.A.). HPLC-grade hexane, isopropanol, acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). NaH_2PO_4, Na_2HPO_4,

and NaCl were obtained from Mallinckrodt (Paris, KY, U.S.A.). Doubly distilled deionized water was used throughout the experiments.

Chiral HPLC columns

The chiral HPLC columns used were: (1) Pirkle covalent (R)-N-(3,5-dinitroben-zoyl)phenylglycine (Regis, Morton Grove, IL, U.S.A.); (2) Pirkle covalent (R)-N-(2-naphthyl)alanine (Regis); (3) Cyclobond β-cyclodextrin (Astec, Whippany, NJ, U.S.A.); (4) Resolvosil bovine serum albumin (Macherey-Nagel, Düren, Germany); (5) Enantiopak α_1-acid glycoprotein (AGP) (LKB, Bromma, Sweden); (6) Chiral AGP (ChromTech, Stockholm, Sweden); (7) Chiralpak OT(+) (J. T. Baker); (8) Chiralcel OB (J. T. Baker); (9) Chiralcel OD (J. T. Baker); (10) Chiralcel OJ (J. T. Baker); (11) Chiralcel CA-1 (J. T. Baker).

Derivatization procedure

The 3,5-dinitroanilide derivative of naproxen was prepared by converting the acid functionality of naproxen into the acid chloride with thionyl chloride followed by the addition of excess 3,5-dinitroaniline.

Naproxen acid chloride: naproxen (0.05 g, 0.22 mmol) was gently refluxed in 5.0 ml of freshly distilled thionyl chloride for 5 min. The excess thionyl chloride was then removed, *in vacuo*, to give a viscous yellow oil.

Naproxen 3,5-dinitroanilide: the acid chloride of naproxen was diluted with 5.0 ml of dry dichloromethane and (0.037 g, 0.24 mmol) of 3,5-dinitroaniline was added.
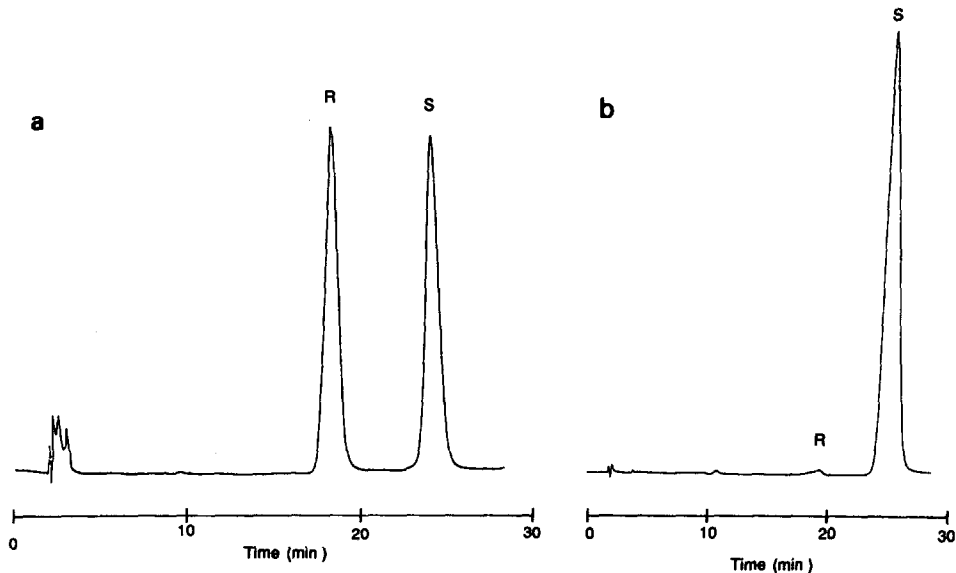

Fig. 1 (a). Chromatographic separation of the 3,5-dinitroanilide derivatives of R,S-naproxen on the (R)-N-(2-naphthyl)alanine CSP. Mobile phase: hexane–isopropanol (90:10). Flow-rate: 2.0 ml/min. UV detection at 254 nm. Temperature: ambient. (b) Chromatographic separation of the 3,5-dinitroanilide derivative of S-naproxen containing *ca.* 1.0% R-naproxen on the (R)-N-2-(naphthyl)alanine CSP. Chromatographic conditions same as in Fig. 1a.

The reaction mixture was stirred at room temperature for 10 min. A 1.0 ml aliquot of the reaction mixture was diluted to 50 ml with mobile phase and injected onto the HPLC system.

Chromatography

Naproxen 3,5-dinitroanilide: the separation of the 3,5-dinitroanilide of naproxen was accomplished using a Pirkle covalent (*R*)-N-(2-naphthyl)alanine (250 × 4.6 mm I.D.) HPLC column (Fig. 1). The mobile phase was hexane–isopropanol (90:10, v/v) and the flow-rate was 2.0 ml/min. The column eluent was monitored at 263 nm and the chromatography was accomplished under ambient temperature conditions. Sample concentrations were approximately 0.2 mg/ml and the injection volume was 20 μl.

Naproxen: the direct separation of naproxen isomers was accomplished using the Enantiopak AGP (100 × 4.6 mm I.D.) column or a second generation, Chiral AGP (100 × 4 mm I.D.) column. Mobile phases consisted of phosphate buffers at different pHs and ionic strengths with isopropanol as the organic modifier. Flow-rates were maintained between 0.3 and 0.8 ml/min. The eluent was monitored at 263 nm and column temperature was maintained in the range of 5 to 40°C using water-jacket thermostated control. Sample concentrations were approximately 0.05 mg/ml and the injection volume was 20 μl.

RESULTS AND DISCUSSION

Initial reports in the literature on the use of CSPs for the separation of the optical isomers of naproxen and other NSAIDs involved formation of amide derivatives followed by separation on a CSP. Wainer and Doyle [34] resolved the optical isomers of naproxen (separation factor, α = 1.23) and other NSAIDs as the 1-naphthylenemethylamide derivatives using the Pirkle covalent (*R*)-N-3,5-(dinitrobenzoyl)phenylglycine (DNBPG) CSP. Nicoll-Griffith [38] reported the resolution of twelve amide derivatives of ibuprofen using the DNBPG CSP, and McDaniel and Snider [39] compared the Pirkle 3,5-DNBPG column and the chiralcel OC (cellulose triphenylcarbamate coated on macroporous silica) column for the separation of amide derivatives of ibuprofen and two other α-methylarylacetic acids.

Pirkle [37] recently introduced a new CSP, covalent (*R*)-N-(2-naphthyl)alanine and reported numerous separations of 3,5-dinitrophenyl derivatives of alcohols, amines and acids (ibuprofen, α = 1.33). Encouraged by the results obtained by Pirkle, the 3,5-dinitroanilide of *R,S*-naproxen was prepared and chromatographed using the (*R*)-N-(2-naphthyl)alanine CSP. Baseline resolution, α = 1.34 was achieved using the conditions described in Fig. 1a. The resolution between the naproxen isomer peaks could be improved by decreasing the amount of the organic modifier, isopropanol, but, at the same time, the isomer peaks broadened, making quantitation of the minor *R*-isomer more difficult. It was determined by derivatizing and chromatographing each individual isomer of naproxen that the elution order was the *R*-isomer first (retention time, t_R = 18 min) and the *S*-isomer second (t_R = 24 min).

The precision of this method was addressed by performing six replicate injections of the same reaction product of a sample of *S*-naproxen containing 1% of *R*-naproxen (Fig. 1b). The ratios of the two isomer peaks gave a standard deviation of

0.1%, demonstrating the chromatographic method to be precise and repeatable. The detection limit was determined to be ca. 0.1%.

Direct resolution of the optical isomers of naproxen and other NSAIDs was reported using the Enantiopak AGP CSP [45,46], wherein the enantioselectivity of strong acids such as naproxen was regulated by the addition of a tertiary amine, N,N-dimethyloctylamine (DMOA) to the mobile phase. In this laboratory, the baseline separation ($\alpha = 1.25$) of naproxen isomers with the Enantiopak column could only be achieved upon addition of DMOA to the mobile phase. An example of the best separation achieved with this column is illustrated in Fig. 2. However, the long retention times of up to 65 min, broad peaks and generally poor peak shape encountered with the Enantiopak CSP prohibited the quantitation of small ($<1.0\%$) amounts of the R-naproxen present in S-naproxen. Column to column variability was observed with the Enantiopak CSP and, in many cases severe peak fronting was observed for the Enantiopak CSP.

The difficulties experienced with the Enantiopak CSP were not observed when using a "second generation" AGP CSP, the Chiral AGP column. This CSP has been reported to give superior results [49], for a series of chiral dihydropyridine compounds as compared to the Enantiopak column and, appears to be more durable as well.

The chromatographic conditions for the resolution of the optical isomers of naproxen with the Chiral AGP were optimized by changing mobile phase conditions such as pH, buffer strength, temperature and ionic strength. The optimal conditions as shown in Fig. 3a and b allowed for the baseline resolution ($\alpha = 1.71$) of the naproxen optical isomers in less than five minutes! The R-isomer was determined to elute first and the S-isomer second. As can be seen in Fig. 3a and b, the peak shapes are much improved from those obtained on the Enantiopak column (Fig. 2). In addition, quantitation of small amounts ($<1\%$) of R-naproxen was more feasible with the Chiral AGP column than with the Enantiopak column.

The effects of pH, temperature, ionic strength, buffer strength and organic modifier on retention times and resolution of the naproxen enantiomers were studied using the Chiral AGP column.
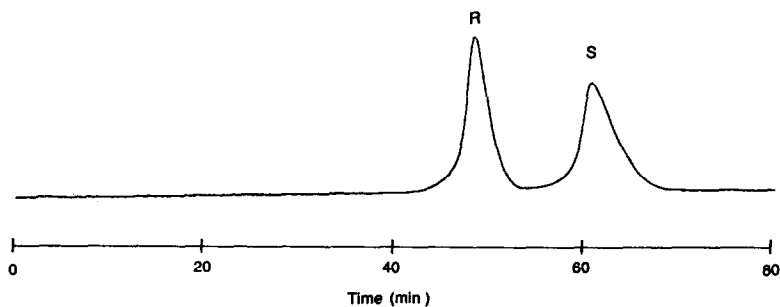

Fig. 2. Direct chromatographic separation of the optical isomers of naproxen on the Enantiopak AGP CSP. Mobile phase: 20 mM phosphate buffer with 8 mM DMOA at pH 7.0–isopropanol (99:1). Flow-rate: 0.3 ml/min. UV detection at 263 nm. Temperature: ambient. Sample loading: 1.0 μg.
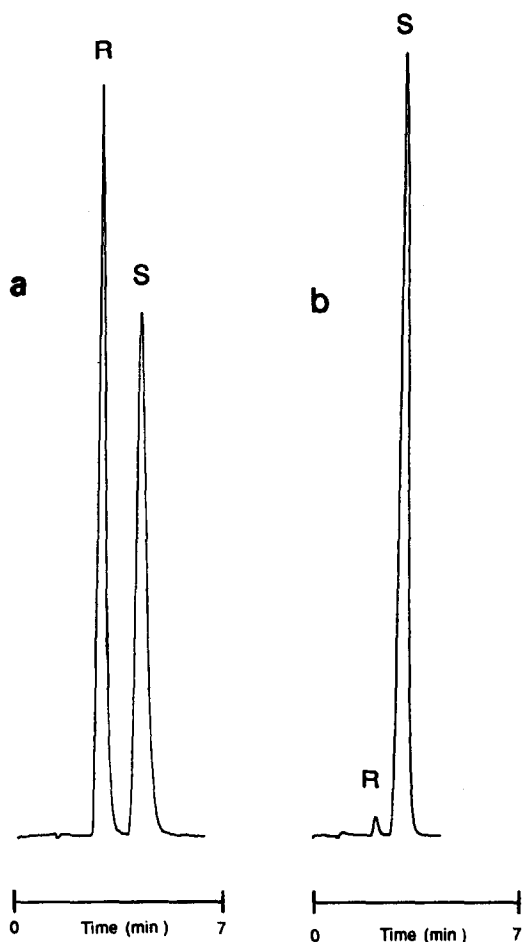
Fig. 3 (a). Direct chromatographic separation of the optical isomers of naproxen on the Chiral AGP CSP. Mobile phase: 4 mM phosphate buffer, pH 7.0–isopropanol (99.5:0.5). Flow-rate: 0.8 ml/min. UV detection at 263 nm. Temperature: ambient. Sample loading 1.0 μg. (b) Chromatographic separation of S-naproxen containing 1.5% R-naproxen on the Chiral AGP CSP. Chromatographic conditions as in (a).

Effect of buffer concentration

The effects of buffer strength were studied at pH 7.0 using six phosphate buffer concentrations ranging from 40 to 1 mM. All the mobile phases contained 0.5% isopropanol as the organic modifier. At higher buffer strengths, capacity factors (k') were increased, peak shape was broadened and α-values increased. The data are depicted in Fig. 4a and b. In general, the retention behavior of the S-isomer was slightly more sensitive than that of the R-isomer to changes in the buffer concentration.

Effect of pH and ionic strength

The effect of mobile phase pH was studied in the range between 5.0 and 7.5 while maintaining a 4.0 mM phosphate buffer concentration in a buffer–isopropanol
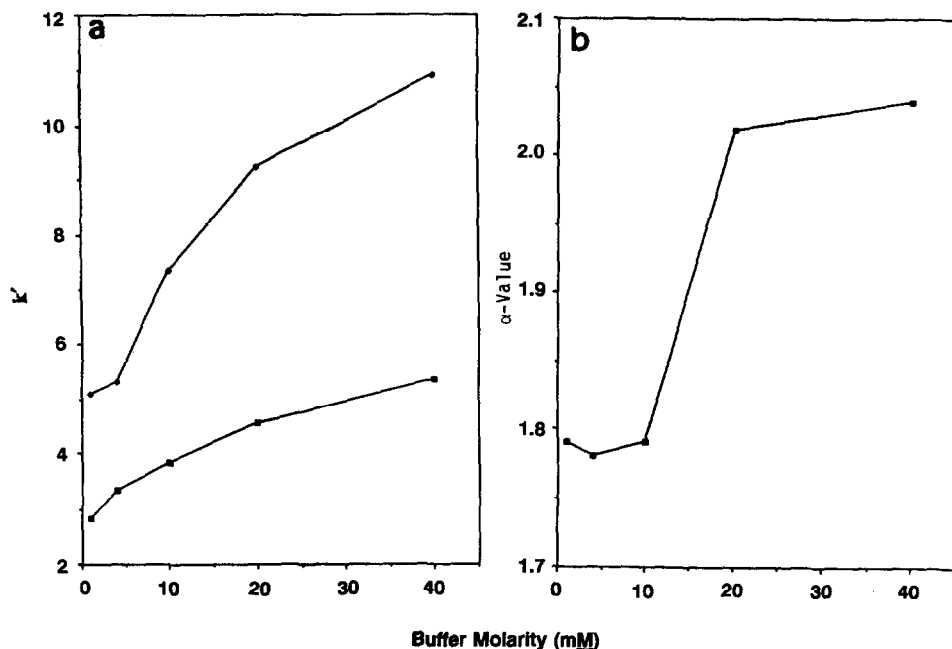
Fig. 4. Effect of buffer concentration on (a) k': $\square$ = R-isomer, $\blacklozenge$ = S-isomer; and (b) resolution of R- and S-naproxen. Chromatographic conditions as in Fig. 3a, except buffer concentrations.

(99.5:0.5, v/v) composition. In addition, at each pH value studied, 10 mM NaCl was added to the mobile phase to evaluate the effect of ionic strength at the respective pH values. The results as shown in Fig. 5 indicate increased resolution with decreasing pH. Retention times for the S-isomer ranged from 5.7 min at pH 7.5 to greater than 3 h at pH 5.0. However, no apparent effect was observed when 10 mM NaCl was present in the mobile phase. Baseline resolution between the enantiomers was achieved throughout the 5.0 to 7.5 pH range.

Effect of temperature
        The chromatography was evaluated at temperatures from 5 to 40°C. The HPLC conditions and mobile phase are those described in Fig. 3. As expected, the resolution increased at the expense of lengthened retention times as well as broadened peak shape when the column temperature was decreased. However, no significant column degradation was observed upon operation at higher temperatures. The retention time of the S-isomer, which was the longer retained enantiomer, was significantly increased at temperatures below 20°C. In the 20–25°C range, the chromatography was not effected by slight changes in temperature. These data are depicted in Fig. 6.

Effect of organic modifier
        Evaluation of the retention characteristics of the enantiomers as a function of the amount of isopropanol in the mobile phase established that the AGP CSP, in addition to chiral recognition, appeared to operate in a reversed-phase mode as well (*i.e.*, the retention times decreased in response to increased levels of isopropanol).
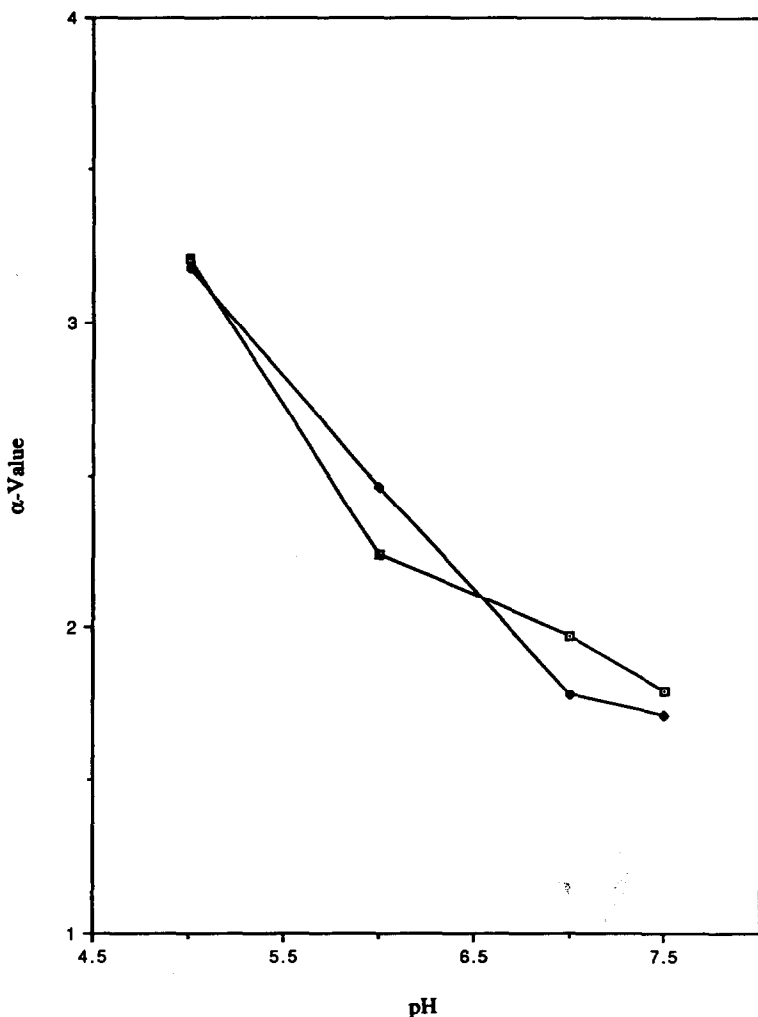
Fig. 5. Effect of pH and ionic strength on resolution of *R*- and *S*-naproxen. Chromatographic conditions as in Fig. 3a, except pH and NaCl concentration. □ = 10 m*M* NaCl; ◆ = no NaCl.

While isopropanol acted as an effective regulator of retention times, resolution of enantiomers could be manipulated by varying the isopropanol content in that the longer retained *S*-isomer showed more response in its retention time towards the organic modifier. Specifically, a total loss of resolution between the naproxen enantiomers was observed by increasing the isopropanol content to amounts greater than 2.0% (v/v).

From the above evaluation, the optimal chromatographic conditions for the direct resolution of the optical isomers of naproxen using the Chiral AGP column were defined as follows: a mobile phase composed of 99.5% 4 m*M* phosphate at pH 7.0 and 0.5% isopropanol operating at a column temperature of 25°C, at a flow-rate
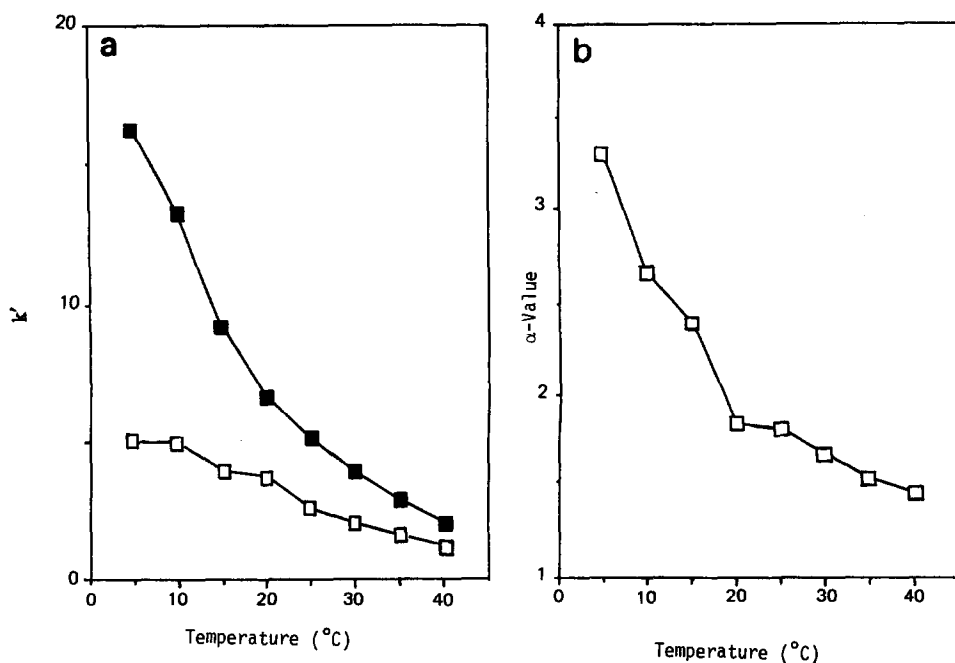
Fig. 6. Effect of temperature on (a) k': $\square$ = R-isomer, $\blacksquare$ = S-isomer; and (b) resolution of R- and S-naproxen. Chromatographic conditions as in Fig. 3a, except temperature.

of 0.8 ml/min. Typically, UV detection at 263 nm and a sample loading of 1.0 μg should be used.

The method was examined for precision, linearity and limits of detection under the above conditions. The precision of the method was evaluated by performing six replicate injections of a solution of S-naproxen containing ca. 1.3% R-naproxen. The mean value for S-naproxen was 98.71% and the standard deviation was 0.1% indicating that the method is precise. The intended application of this chiral separation method is to quantitate low levels of R-naproxen present in the S-naproxen product. Therefore, linearity of the method was determined by analyzing spiked solutions of 0.2, 0.5, 1.0, 2.0, 4.0 and 5.0% of R-naproxen in the presence of S-naproxen. Fig. 7 depicts the experimentally determined linearity plot from these spiked samples. This method exhibits good linearity over the range tested, following the derived linear equation of $y = 0.00198 + 0.998778x$, where y = observed response and x = theoretical response of % R-naproxen. The average deviation from a theoretical calibration line having a slope of 1.00, expressed as the standard error of estimate, is 0.05%. The coefficient of correlation is 0.9998. The method is, therefore considered to be linear in the examined range of concentration. The limit of detection was determined to be ca. 0.1% of either enantiomer in the presence of its enantiomeric counterpart.

Finally, for comparison, four resolved S-naproxen samples of varying optical purities were separately subjected to the two optical purity determination methods described in this work. The results (Table I) showed good correlation between the
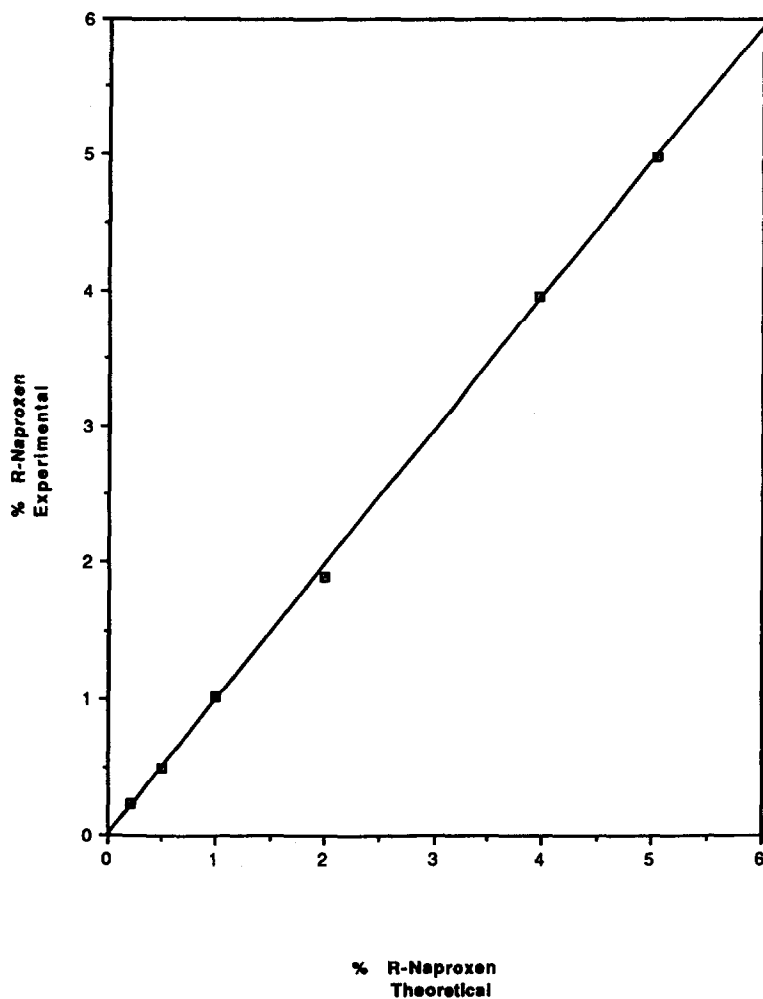
Fig. 7. Linearity plot for *R*-naproxen spiked in *S*-naproxen.

TABLE I

COMPARISON OF CHROMATOGRAPHIC RESULTS OF THE ENANTIOMERIC PURITY OF NAPROXEN USING THE PIRKLE CSP AND CHIRAL AGP CSP

Sample	(%S-Naproxen)	
	Chiral AGP	Pirkle CSP
A	99.87	99.55
B	99.55	99.06
C	98.65	98.50
D	95.82	95.62

methods using the Pirkle (R)-N-(2-naphthyl)alanine column and the Chiral AGP column.

Other chiral stationary phases

Seven other commercially available chiral stationary phases were evaluated for their ability to separate the optical isomers of naproxen. These included β-cyclodextrin, bovine serum albumin, Chiralpak OT($+$) and four cellulose based CSPs, namely, Chiralcel OB, OD, OJ, and CA-1. In all cases, the attempted separations were performed on naproxen using mobile phases suggested by the respective column manufacturer. Chiral separations of derivatives of naproxen were not attempted with these columns. No separation of the optical isomers of naproxen was observed with any of these seven columnms.

CONCLUSIONS

Two chromatographic methods for the separation of the optical isomers of naproxen were presented. Both methods offer advantages over existing literature methods in both separation factors and ease of use. In particular, the Chiral AGP CSP gave direct resolution of the optical isomers of naproxen in less than 5 min. This CSP has been shown to be superior to the Enantiopak AGP CSP with regard to column stability, peak shape and separation efficiency. The methods reported in this manuscript may prove applicable to other α-methylarylacetic acid NSAIDs since these compounds all share common functional groups and often share similar chromatographic characteristics towards chiral separations.

ACKNOWLEDGEMENT

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