Separation of the Two Enantiomers of Naproxcinod by Chiral Normal-Phase Liquid Chromatography

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Abstract

A normal-phase enantioselective high-performance liquid chromatographic method was developed for the enantiomeric resolution of naproxcinod, the most advanced cyclooxygenase-inhibiting nitric oxide donator of anti-inflammatory drugs designed for treatment of osteoarthritis. The enantiomers of naproxcinod were resolved on a Chiralpak AD-H (250 × 4.6 mm, 5 µm) column using a mobile phase system containing n-hexane and 2-propanol (95:5, v/v). The resolution between the enantiomers was found to be more than 2.0. The limit of detection and limit of quantitation of (*R*)-enantiomer were found to be 5 and 15 ng/mL, respectively, for 20 µL injection volume. The sample solution and mobile phase were found to be stable for at least 48 h. The final optimized method was successfully applied to separate (*R*)-enantiomer from naproxcinod and was proven to be reproducible and accurate for the quantitative determination of (*R*)-enantiomer in bulk drugs.

Introduction

Enantiomers of racemic drugs often show different behaviors in pharmacological action and metabolic process. It is not uncommon for one enantiomer to be active while the other is toxic in biological systems. The pharmaceutical industry has raised its emphasis on the generation of enantiomerically pure compounds before undertaking pharmacokinetic, metabolic, physiological, and toxicological evaluation in the search for drugs with greater therapeutic benefits and low toxicity (1,2). Nowadays, chiral separations are playing more and more important role for the analysis of single enantioemers in the field of pharmaceutical industry (3). However, the development of the methods for the quantitative analysis of chiral compounds and for the assessment of enantiomeric purity is extremely challenging, because the same physical and chemical properties of the two enantiomers make discriminating and separating them very difficult (4). Recently, many liquid chromatographic chiral methods using chiral stationary phases have been reported as ways to separate and thereby individually quantitate the enantiomer of an enantiomeric pair (5–10).

Naproxcinod, 4-(nitrooxy)butyl-(2S)-2-(6-methoxy-2-naphthyl) propanoate, the first in the class of cyclooxygenase-inhibiting nitric oxide donators (CINODS) for the treatment of the signs and symptoms of osteoarthritis, has been evaluated in preclinical and clinical studies (11–12). It was prepared from naproxen as a single isomer [(*S*)-enantiomer], though the (*R*)-enantiomer is the undesired enantiomer, which can be present as a chiral impurity without any pharmacological and toxicological reports by now. So it is essential to find a effective way to analyze the enantiomers of naproxcinod, and this report can be a element task for further researches. The chemical structures of naproxcinod and (*R*)-enantiomer are shown in Figure 1, and (*R*)-enantiomer from naproxcinod may be at low level for little (*R*)-naproxen existing in starting material or racemization in synthesis (13).

Here, the direct enantioseparation of the undesired enantiomer from an active pharmaceutical ingredient, naproxcinod, is reported by normal-phase high-performance liquid chromatography (HPLC) using modified amylose as chiral stationary phases. The aim of this work was to optimize the chromatographic conditions in terms of temperature and mobile phase composition in order to separate and identify the enantiomers of naproxcinod. The developed HPLC method was reproducible and accurate for the quantitative determination of (*R*)-enantiomer in naproxcinod.

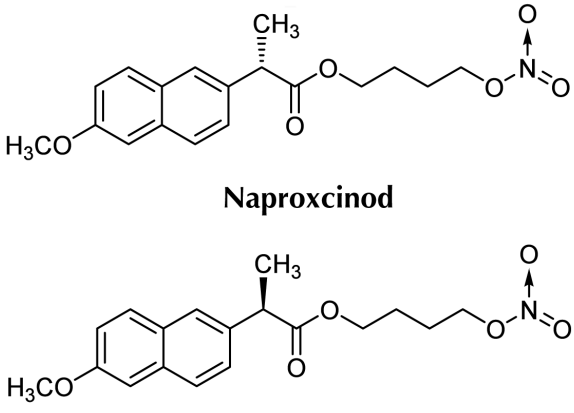

Figure 1. Chemical structures of naproxcinod and (*R*)-enantiomer.

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Experimental

Chemicals

Naproxcinod and (*R*)-enantiomer [the undesired enantiomer (99% ee)] were prepared in the laboratory. HPLC-grade *n*-hexane and 2-propanol were purchased from Tedia (Phoenix, AZ). All other chemicals were of analytical grade.

Columns

Preliminary column screening involved modified cellulose columns, namely: Chiralcel OD-RH (150 × 4.6 mm), Chiralcel OJ-RH (150 × 4.6 mm) of Daicel (Fort Lee, NJ), and then Chiral AGP (150 × 4.0 mm), Chiral HSA (150 × 4.0 mm), Chiral CBH (150 × 4.0 mm) of Chromtech (Apple Valley, MN) were also employed. The column used in the major method development activities was a modified amylose based chiral column: Chiralpak AD-H (250 × 4.6 mm, 5 µm, Daicel) column.

Chromatography

Chromatography was carried out by using Agilent Technologies 1200 series instrument (Palo Alto, CA) equipped with column oven, UV detector, and the adata was processed using a computer program (Chemstation, Dayton, OH). The chromatographic conditions were optimized using a chiral stationary phase, Chiralpak AD-H column (250 × 4.6 mm, 5 µm, Daicel, Japan). The isocratic mobile phase composition was a mixture of *n*-hexane and 2-propanol (95:5, v/v), which was pumped at a flow rate of 0.8 mL/min. The temperature of the column was maintained at 20°C, and the eluant was monitored at a wavelength of 232 nm. The injection volume was 20 µL.

Sample preparation

Stock solutions of naproxcinod (1 mg/mL) and (*R*)-enantiomer (1 mg/mL) were prepared by dissolving the appropriate amount of the substances in 2-propanol. The analyte concentration of naproxcinod was fixed as 15 µg/mL. Naproxcinod solutions spiked with low levels of (*R*)-enantiomer were prepared by transferring calculated amount of undesired enantiomer stock solution with pipette into the calculated amount of naproxcinod stock solution, and then the solution was added to volume with mobile phase and mixed well.

Validation of the method

Method reproducibility was determined by measuring repeatability and intermediate precision of retention times and peak areas for each enantiomer. The repeatability of the method was determined by analyzing six replicate injections containing naproxcinod (15 µg/mL) spiked with (*R*)-enantiomer (1%, 150 ng/mL). The intermediate precision was determined over 3 days by performing six successive injections each day.

The limit of detection (LOD) and limit of quantitation (LOQ) were achieved by injecting a series of dilute solutions of (*R*)-enantiomer. The precision of the developed chiral method for (*R*)-enantiomer was checked by analyzing six test solutions of (*R*)-enantiomer prepared at the LOQ level and calculating the percentage relative standard deviation of area.

Detector response linearity was assessed by preparing six calibration sample solutions of (*R*)-enantiomer covering from 15 ng/mL (LOQ) to 200 ng/mL (15, 30, 45, 60, 100, and 200 ng/mL) in mobile phase. The regression curve was obtained by plotting peak area versus concentration, using the least squares method. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve was calculated. Standard addition and recovery experiments were conducted to determine the present method for the quantitation of (*R*)-enantiomer in bulk drug samples. The study was carried out in triplicate at 0.6%, 0.8%, and 1.0% of the naproxcinod target analyte concentration. The recovery of (*R*)-enantiomer was calculated from the slope and Y-intercept of the calibration curve obtained.

To determine robustness of the method, flow rate was changed at the pace of 0.05 units from 0.7 to 0.9 mL/min. The effect of change in the percent 2-propanol (± 1%), and column temperature at 15 and 25°C instead of 20°C were studied, and the other chromatographic conditions were held constant as stated previously.

The stability of naproxcinod in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. The content of (*R*)-enantiomer was checked at 6 h interval up to the study period. Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in naproxcinod sample solutions prepared freshly at 6 h interval for 2 days. The same mobile phase was used during the study period.

Results and Discussion

Optimization of chromatographic conditions

Racemic mixture solution of naproxcinod and (*R*)-enantiomer (10 µg/mL each) prepared in mobile phase was used in the method development. To develop a rugged and suitable HPLC method for the separation of the two enantiomers, different stationary phases and mobile phases were employed. Initial screening of chiral column was carried out by several chiral HPLC column suppliers. Various chiral columns, namely: Chiralcel OJ-RH of Diacel, Chiral AGP, Chiral HSA, and Chiral CBH of Chromtech were employed. All these columns failed to provide selectivity between naproxcinod peak and the undesired enantiomer peak using different possible mobile phases. In the following method development activities, a Chiralcel OD-RH column (150 × 4.6 mm, 5 µm) with mobile phase consisting of

Table I. Effect of Mobile Phase Composition on Retention Time and Resolution of Naproxcinod and (*R*)-enantiomer using Chiralpak AD-H Column

Hexane (%)	2-Propanol (%)	Retention time (min)		Rs	α
		N*	E†		
70	30	6.75	7.02	0.88	1.04
80	20	7.79	8.15	1.12	1.05
90	10	10.54	11.18	1.57	1.06
95	5	18.42	19.84	2.32	1.08

* N = Naproxcinod
† E = Undesired enantiomer

water and methanol (80:20, v/v) was used, but all that was obtained was a defective separation of the two enantiomers with a very low resolution.

It was continued to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. There was an indication of separation on Chiralpak AD-H (250 × 4.6 mm, 5 µm) column using a mobile phase consisting of *n*-hexane and 2-propanol (70:30, v/v). The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. The results of resolution factor (Rs) and selectivity factor (α) are summarized in Table I. Based on the data obtained from method development and optimization activities, Chiralpak AD-H (250 × 4.6 mm, 5 µm) column with mobile phase of *n*-hexane and 2-propanol (95:5, v/v) was selected for the final method. The flow rate of the final method was 0.8 mL/min with an injection volume of 20 µL. The column temperature was 20°C, and the detection wavelength was 232 nm. Under these conditions, the two enantiomers were separated well and the peak of (*R*)-enantiomer eluted after the peak of naproxcinod. In the optimized method, the typical retention times of naproxcinod and (*R*)-enantiomer were approximately 18.4 and 19.8 min, respectively. Baseline separation of naproxcinod and (*R*)-enantiomer was obtained with a total run time of 30 min. The separation of an approximately 1:1 (wt/wt) mixture solution (in mobile phase) of the two enantiomers is shown in Figure 2. A HPLC chromatogram of naproxcinod bulk drug sample (15 µg/mL) spiked with (*R*)-enantiomer (1%) was shown in Figure 3.

An amylose based chiral stationary phase contained five chiral centers per unit, and naproxcinod had only one chiral center close to the carbonyl group in the structure. The stereoelectronic interactions between the enantiomers and the chiral stationary phase generated enantioselectivity, thus causing significant differences in the migration of the enantiomers inside the column. Having the right amount of 2-propanol in the mobile phase also played an important role in affecting the steric environment of the chiral cavities or channels of the stationary phase and contributes to enantioselectivity. However, an excessive amount of 2-propanol was likely to cut down the resolution by taking up chiral centers of the chiral stationary phase or forming hydrogen bondings with the enantiomers instead of the hydrogen bondings between the enantiomers and the stationary phase (14). Other important interactions between the enantiomers and the stationary phase such as π–π bonding, vander Waals forces, dipole induced dipole attractions, and steric effects can also achieve better resolution on Chiralpak AD-H column.

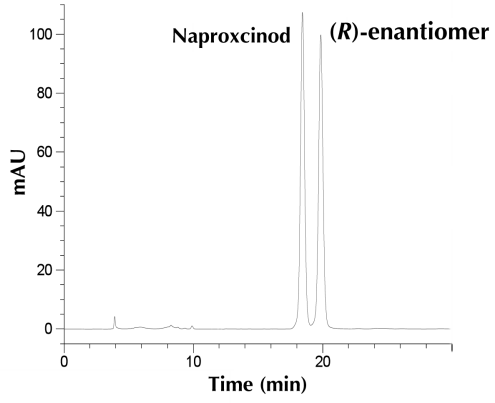

Figure 2. Enantiomeric resolution of Naproxcinod and (*R*)-enantiomer (10 µg/mL each) on Chiralpak AD-H column.

Validation results of the method

The HPLC condition of the final method was evaluated for its precision, LOD, LOQ, linearity, recovery, and robustness. The repeatability and intermediate precision were expressed as relative standard deviation (RSD). For this study, solution of naproxcinod (15 µg/mL) spiked with (*R*)-enantiomer (1%, 150 ng/mL) was analyzed in six replicates to establish repeatability. The RSD values were better than 0.5% for the retention times of both the enantiomers, 1.0% for naproxcinod peak area and 3.0% for (*R*)-enantiomer peak area (Table II). In the intermediate precision study, results showed that RSD values were in the same order of magnitude than those obtained for repeatability studies (Table III). All these values indicated that the method was precise.

The LOD and LOQ concentrations were estimated to be 5 and 15 ng/mL for (*R*)-enantiomer, respectively, when signal-to-noise (S/N) ratios of 3 and 10 were used as the criteria. The method precision for (*R*)-enantiomer at LOQ was 4.3% RSD. Therefore,

Table II. Repeatability and Intermediate Precision Results of the Developed Chiral HPLC Method

Parameter	Results
Repeatability (*n* = 6, % RSD)	
Retention time (*S*-enantiomer)	0.3
Retention time (*R*-enantiomer)	0.4
Area (*S*-enantiomer)	0.8
Area (R-enantiomer)	2.9
Repeatability (*n* = 18, % RSD)	
Retentiontime (*S*-enantiomer)	0.4
Retention time (*R*-enantiomer)	0.6
Area (*S*-enantiomer)	1.2
Area (*R*-enantiomer)	3.5

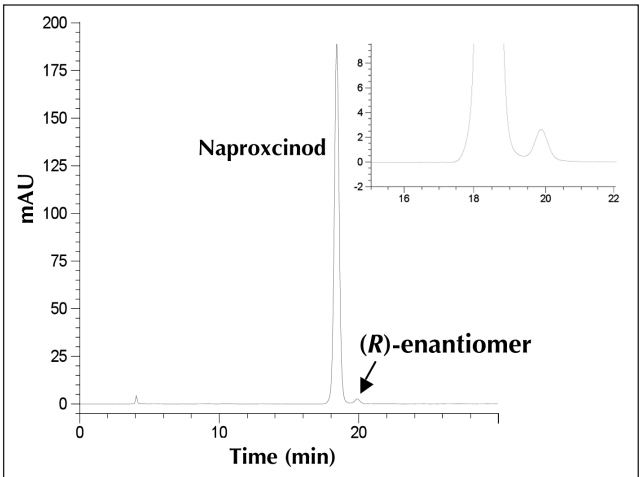

Figure 3. Enantiomeric resolution of Naproxcinod solution (15 µg/mL) spiked with approximately 1% (*R*)-enantiomer on Chiralpak AD-H column.

this method had adequate sensitivity for the detection and estimation of (*R*)-enantiomer in naproxcinod.

Good linearity of (*R*)-enantiomer was evaluated over six levels of (*R*)-enantiomer solutions from 15 to 200 ng/mL, with the linear regression equation $y = 0.247x + 0.517$ (correlation coefficient $r = 0.9997$), where x is the concentration of the undesired enantiomer in ng/mL, and y is the corresponding peak area of the undesired enantiomer in mV/s. Linearity was checked for (*R*)-enantiomer over the same concentration range for three consecutive days. The RSD of the slope and Y-intercept of the calibration curve were 2.7% and 13.6%, respectively.

The standard addition and recovery experiments were conducted for (*R*)-enantiomer in bulk samples in triplicate at 0.6% (90 ng/mL), 0.8% (120 ng/mL), and 1.0% (150 ng/mL) of analyte concentration. The recovery was calculated from the slope and Y-intercept of the calibration curve obtained linearity study and percentage recovery was ranged from 91.3% to 98.4% (Table III). The method was proved to be accurate in estimating the amount of the undesired enantiomer of naproxcinod between 15–200 ng/mL.

The method robustness studies were demonstrated by adjusting flow rate, column temperature and mobile phase composition variations. The chromatographic resolution of naproxcinod and (*R*)-enantiomer peaks was used to evaluate the method robustness. The resolution between naproxcinod and (*R*)-enantiomer was more than 2.0 under all separation conditions testeded (Table IV), demonstrating sufficient robustness.

The stability of the solutions and mobile phase used in this method was tested over a long time. No significant change in (*R*)-enantiomer content was observed in naproxcinod sample during solution stability and mobile phase stability experiments, and the RSD values were less than 4.0% for (*R*)-enantiomer peak area. Hence, the naproxcinod sample solution and the mobile phase were stable for at least 48 h.

Table III. Recovery Results of (*R*)-enantiomer in Bulk Drugs (n =3)

Added (ng/mL)	Recovered (ng/mL)	Recovery (%)	RSD (%)
90.0	82.2	91.3	4.7
120.0	112.6	93.8	3.2
150.0	147.6	98.4	2.8

Table IV. Robustness Results of the Chiral HPLC Method

Parameter	Resolution between Naproxcinod and (*R*)-enantiomer
Flow rate (mL/min)	
0.70	2.47
0.75	2.39
0.80	2.32
0.85	2.24
0.90	2.15
Column temperature (°C)	
15	2.45
20	2.32
25	2.20
2-Propanol percentage in mobile phase	
4	2.64
5	2.32
6	2.16

Conclusion

A simple, rapid, and accurate normal-phase enantioselective HPLC method was successfully developed, which was capable of separating the undesired enantiomer from naproxcinod. Amylose based chiral column Chiralpak AD-H column was found to be selective for the enantiomers of naproxcinod. The developed and validated method can be used for the chiral purity testing of naproxcinod. The developed method is also stable and can be used for the quantitative determination of chiral impurity in bulk materials.

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