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Chiral Separation of *rac*-Ornidazole and Detection of the Impurity of (*R*)-Ornidazole in (*S*)-Ornidazole Injection and Raw Material

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ABSTRACT (*S*)-Ornidazole is a subject of research as an antifertility agent in male animals at present. However, there seems to be no relative report on chiral separation for *rac*-Ornidazole, which has been used as an effective medicine for more than 30 years. In this article, the chiral separation of *rac*-Ornidazole on a Chiralcel OB-H column based on normal-phase high-performance liquid chromatography (NP-HPLC) is investigated and the methodology for detection of impurity of (*R*)-Ornidazole in (*S*)-Ornidazole injection and raw material is established. The novel mobile phase is utilized by mixing *n*-hexane, methanol and isopropyl alcohol (95:4:1, v/v/v) instead of the typical mobile phase of *n*-hexane and isopropyl alcohol, although the methanol, which offers a good resolution factor for the enantiomeric separation in this system, is not recommended on the Chiralcel OB-H column according to the instruction supplied by Daicel Chemical Ind., LTD (Japan). *Chirality 18:587–591, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: normal-phase high-performance liquid chromatography; chiral separation; (*S*)-Ornidazole; (*R*)-Ornidazole; impurity detection

rac-Ornidazole [1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole], which was first put on the market by Roche (Switzerland) in the 1970s, can be used in the treatment of hepatic and intestinal amoebiasis, giardiasis, trichomoniasis of the urogenital tract, and bacterial vaginosis. It is also used in the treatment and prophylaxis of susceptible anaerobic infections in dental and gastrointestinal surgery and other mixed aerobic–anaerobic infections. Ornidazole is also advocated in the management of H. pylori duodenal ulcer in combination with other drugs. The antimicrobial activity of *rac*-Ornidazole brings about the loss of the helical structure of DNA and subsequent DNA breakage, thus inhibiting further synthesis and causing degradation of existing DNA.[1,2]

Ornidazole is also a subject of research as an antifertility agent in male animals,[3] possibly due to the release of the chlorinated side-chain during metabolism.[4] It is well known that only the (*S*)-enantiomer of α-chlorohydrin or 3-chlorolactaldehyde, which is able to inhibit the activity of the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase and therefore the synthesis of ATP in sperm, has the capacity of reversible antifertility effects in male animals.[5–8] The inhibition of the enzyme is strongly related to the stereochemistry of the applied compounds. *In vivo*, Ornidazole is at least partly cleaved to its three carbon chloro side-chains,[9] which is probably converted into 3-chlorolactaldehyde. As this is comparable to α-chlorohydrin and similar compounds, the necessity of gaining the pure enantiomer and controlling the enantiomer impurity of Ornidazole is quite obvious, especially if it is being developed as a drug for antifertility.

Chirality is a major concern in the modern pharmaceutical industry. In 1992, the U.S. Food and Drug Administration issued a guideline that for chiral drugs only the therapeutically active isomer can be brought to market. This new trend resulted in an increase in the demand for enantioselective methods for the analysis of drugs in order to check the enantiomeric purity or enantiomeric excess. In many cases, determination of chiral impurities at concentrations below 0.5% is required and all the work depends on analytical methods.[10,11] Chiral HPLC has been proven to be one of the best methods for the direct separation and analysis of enantiomers. Current chiral HPLC methods are either direct, that is, they utilize chiral stationary phases (CSPs) and chiral additives in the mobile phase, or indirect, which involves derivatization of samples. Direct chiral separations using CSPs are more widely used and more predictable, in mechanistic terms, than those using chiral additives in the mobile phase.

In this article, the study of the chiral separation of *rac*-Ornidazole on Chiralcel OB-H is investigated and the methodology for detection of impurity of (*R*)-Ornidazole in (*S*)-Ornidazole injection and raw material is established.

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MATERIAL AND METHODS

Reagents and Chemicals

Ethyl acetate of analytical quality was provided by Beijing Chemical Reagents Company (Beijing, China). *n*-Hexane of HPLC grade was purchased from Tianjin Chemical Reagents Company (Tianjin, China). Methanol (Tedia, USA) and isopropyl alcohol (Tedia, USA) were also HPLC grade. The raw material and reference of (*S*)-Ornidazole and reference of (*R*)-Ornidazole were obtained from the Institute of Medicine in Changsha (Hunan Province, China). The pharmaceutical preparation (for experimental use only) of (*S*)-Ornidazole injection was prepared by Department of Pharmaceutical Preparation, Beijing Hospital.

Standard Preparation

The reference of (*S*)-Ornidazole (50 mg, accurately weighed) was dissolved in isopropyl alcohol in a 50 ml volumetric flask and then diluted with the mobile phase to volume as a stock solution. This stock solution of 1.00 mL was transferred into a 100 ml volumetric flask and then diluted with the mobile phase to obtain a standard solution of 10 μg per mL. The standard solutions with the concentration of 1 mg · mL^{-1} and 10 μg · mL^{-1} of (*R*)-Ornidazole were prepared separately with the same method. Then stock solution of (*R*)-Ornidazole 1.00 mL and stock solution of (*S*)-Ornidazole 1.00 mL were transferred into a 100 ml volumetric flask and diluted with the mobile phase to volume, and mixed (Solution I). The second solution was prepared with mixing stock solution of (*R*)-Ornidazole 0.05 mL and stock solution of (*S*)-Ornidazole 9.95 mL as a standard (Solution II) to evaluate the impurity of (*R*)-Ornidazole existing in the injection and raw material of (*S*)-Ornidazole.

Sample Preparation

The raw material of (*S*)-Ornidazole (50 mg, accurately weighed) was dissolved in isopropyl alcohol in a 50 ml volumetric flask and then diluted with the mobile phase to volume for detecting the enantiomer impurity in the raw material.

The (*S*)-Ornidazole injection (20 mL, equivalent to about 50 mg of (*S*)-Ornidazole) was extracted with three 20 mL portions of ethyl acetate, which were combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved with isopropyl alcohol and transferred into a 50 mL volumetric flask completely, then diluted with the mobile phase to volume for detecting the enantiomer impurity in the injection.

Calibration Curves

For the construction of the calibration curves, working solutions of (*R*)-Ornidazole were prepared at concentrations of 0.5, 1, 2, 5, 10, 20, and 50 μg · mL^{-1} by appropriate dilution of the stock solution of (*R*)-Ornidazole with the mobile phase. Calibration curves were prepared by plotting the peak area *A* against the concentration *C* of (*R*)-Ornidazole and the linear regression was performed. The acceptance criterion for the correlation coefficient *r* was $r \geq 0.999$.

Apparatus and Chromatographic Conditions

Chromatographic analyses was performed on a Hewlett-Packard Series 1100 system (Palo Alto, CA, USA) equipped with a pump, an autosampler, a column oven, and a UV detector, connected to HP ChemStation Software (Hewlett-Packard, Palo Alto, CA, USA). Separation was performed on a Daicel Chiralcel OB-H column (5 μm, 4.6 × 250 mm) from Daicel Chemical Ind., LTD (Japan) with a guard column from the same company. The mobile phase was prepared by mixing *n*-hexane, methanol, and isopropyl alcohol (95:4:1, v/v/v) and filtering through a 0.22 μm membrane filter and degassed by sonication for 10 min before using. The column temperature was controlled at 30°C and the eluent was monitored at 311 nm. The flow rate was 0.8 mL · min^{-1} and the injection volume was 5 μL.

RESULTS AND DISCUSSION

Standard and Sample Preparation

Isopropyl alcohol was utilized as the first solvent in the preparation of standards and samples, because Ornidazole cannot be dissolved in the mobile phase directly.

Due to the normal phase system in the separation, and because water was the chief solvent in the pharmaceutical preparation of (*S*)-Ornidazole injection, ethyl acetate was utilized as the extracting solvent. Considering the (*S*)-Ornidazole as a basic sample, two methods for extraction, whether or not the processing of alkalization with 1 mol · L^{-1} of NaOH was included, were evaluated. The extraction percentages were calculated by comparing the peak area of the extraction solution with the peak area obtained by injecting equivalent amounts of the stock solution of (*S*)-Ornidazole. The recoveries were scarcely affected by the alkalinized procedure; the values of recovery are listed in Table 1 for both methods. The results demonstrate that abandoning the alkaline extract procedure, which could make the sample preparation easier and reduce the chance of contamination, would not influence the routine impurity detection in this experiment.

Stability of the Solutions

The stability of solutions I and II and 1 mg · mL^{-1} of (*S*)-Ornidazole, which were stored under room temperature, was assessed by measuring the peak area of each

TABLE 1. Extraction percentage of (*S*)-Ornidazole in injection with ethyl acetate compared with the average area of 1 mg · mL^{-1} of (*S*)-Ornidazole

With alkalinized procedure	R.S.D.	Without alkalinized procedure	R.S.D.
96.21%		95.43%	
96.35%		95.24%	
96.51%	0.19%	95.24%	0.16%
95.98%		95.62%	
96.24%		95.47%	

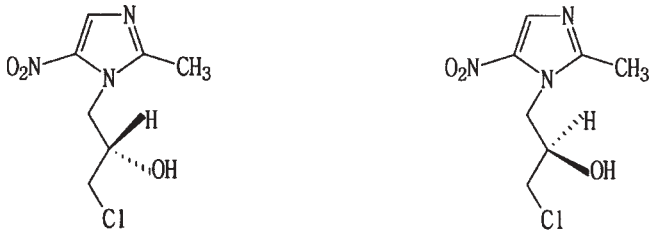

Fig. 1. Stereochemistry of the enantiomers of Ornidazole.

solution after sample injections at 0 h, 2 h, 4 h, 8 h, 16 h, 24 h, 2 d, 4 d, and 8 d. The results showed that all the solutions were stable under this condition and suitable for impurity detection.

Linearity

The method exhibited good linear response over the selected concentration range by linear regression analysis. Standard curves were constructed on five different days. The correlation coefficient r for the concentration range 0.5–50 $\mu g \cdot mL^{-1}$ were >0.9999. The mean standard curve was typically described as follows: $A = 20.2572C - 1.4237$, $r = 0.99997$.

Precision of the Method

To assess the precision of the procedure, reproducibility for both day-to-day and within-day variations were determined. The relative standard deviations (R.S.D.) for 1 $mg \cdot mL^{-1}$ of (*S*)-Ornidazole and 5 $\mu g \cdot mL^{-1}$ of (*R*)-Ornidazole in the within-day and day-to-day studies were 0.12% and 0.57% respectively. The R.S.D. for resolution factors of solutions I and II in the within-day and day-to-day studies were 0.69% and 0.94% respectively.

LLOD and LLOQ

Based on the concentration of 1 $\mu g \cdot mL^{-1}$ and injection volume of 5 μL corresponding to a signal-to-noise ratio of 3:1, the LLOD was lower than 0.2 $\mu g \cdot mL^{-1}$ and LLOQ was 0.5 $\mu g \cdot mL^{-1}$ for both (*S*)-Ornidazole and (*R*)-Ornidazole (Figs. 1 and 2). The result demonstrated that the amount of (*R*)-Ornidazole in the assay preparation can be detected to the percentage of 0.05% at least, much lower than 0.5%, if the concentration of sample was 1 $mg \cdot mL^{-1}$ with 5 μL of injection volume.

Selection of Chromatographic Conditions

The selection of optimal mobile phase for the chiral separation of (*S*)-Ornidazole and (*R*)-Ornidazole is very important. Several different proportions of mobile phase are summarized in Table 2. The use of the Daicel Chiralcel OB-H column, which employed a simple mobile phase in application, has been reported for the successful chiral separation.[12]

Suitable mobile phase were hexane/2-propanol (100/0∼0/100 v/v) and hexane/ethanol (100/0–0/100 v/v), and the no. 5 chromatographic condition (Table 2) (hexane/2-propanol, 90/10 v/v) is recommended as the typical mobile phase used on the Chiralcel OB-H column. However, the resolution factor was so low that the chromatographic condition is not suitable for chiral separation of *rac*-Ornidazole, no matter what the proportion of hexane/2-propanol or hexane/ethanol is.

The quantitative analysis can be performed under the resolution factor of $R_s \geq 1.5$ usually. And the adequate resolution factor ($R_s = 1.52$) was achieved for the separation of *rac*-Ornidazole using the no. 1 chromatographic condition (Table 2). It took 35 min to finish the whole assay. Unfortunately, this proportions of the mobile phase were not suitable for the separation of (*R*)-Ornidazole as an impurity from the 1 $mg \cdot mL^{-1}$ of (*S*)-Ornidazole because of the lower resolution factor ($R_s = 1.21$). In order to eliminate this problem, the no. 2 chromatographic condition was utilized, and it took less than 80 minutes to complete the whole analysis with the resolution factor of $R_s = 1.58$ for the separation of (*R*)-Ornidazole as an impurity from the 1 $mg \cdot mL^{-1}$ of (*S*)-Ornidazole and $R_s = 1.87$ for *rac*-Ornidazole. With the hope of a better resolution factor for the separation of enantiomers, the no. 4 chromatographic condition was used in our previous experiment, but it took more than 120 min for the analysis and with the similar resolution factor of the no. 2 chromatographic condition. The 0.5 $mL \cdot min^{-1}$ flow rate was used in the abovementioned experimental condition at first. In order to save time and take into consideration the column pressure limit, the 0.8 $mL \cdot min^{-1}$ flow rate was utilized in our separation condition (the no. 3 chromatographic condition); the resolution factor of various chromatographic conditions is summarized in Table 2. Considering the efficiency of the analysis, the no. 3 chromatographic condition was chosen as the proper chromatographic condition in our experiment with $R_s = 1.56$ for solution II, since it can reduce the

TABLE 2. Resolution of solutions I and II under the different chromatographic conditions

No. of condition	*n*-Hexane/ Methanol/ Isopropyl alcohol	Analysis time	Rt of (*S*)-Ornidazole	Resolution factor of solution II	Resolution factor of solution I
1	90:8:2	35 min	29.759 min	1.21 (0.5 $mL \cdot min^{-1}$)	1.52 (0.5 $mL \cdot min^{-1}$)
2	95:4:1	80 min	73.851 min	1.58 (0.5 $mL \cdot min^{-1}$)	1.87 (0.5 $mL \cdot min^{-1}$)
3	95:4:1	60 min	54.052 min	1.56 (0.8 $mL \cdot min^{-1}$)	1.86 (0.8 $mL \cdot min^{-1}$)
4	145:4:1	>120 min	118.327 min	1.60 (0.5 $mL \cdot min^{-1}$)	1.91 (0.5 $mL \cdot min^{-1}$)
5	90:0:10	20 min	17.062 min	0.76 (0.5 $mL \cdot min^{-1}$)	1.03 (0.5 $mL \cdot min^{-1}$)

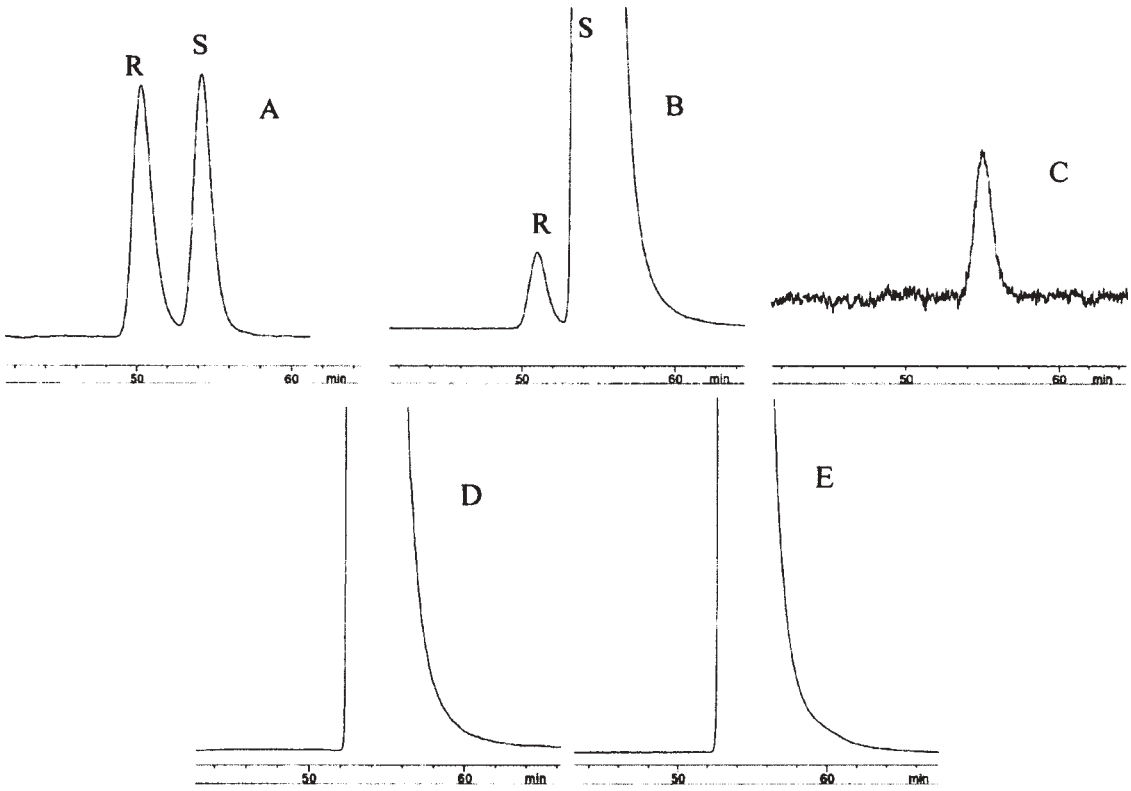

Fig. 2. The chromatogram of *rac*-Ornidazole (A), solution II (B), 1 μg · mL^{-1} of (*S*)-Ornidazole (C), (*S*)-Ornidazole raw material (D) and (*S*)-Ornidazole injection (E).

analytical time and supply an acceptable resolution factor for impurity detection.

Chiral Separation of rac*-Ornidazole*

Figure 2 shows the chromatogram, which was obtained under the condition depicted as an item of "Apparatus and Chromatographic Conditions" for solution I. The results indicated that the separation of *rac*-Ornidazole was successful.

*Detection for Impurity of (*R*)-Ornidazole*

Solution II and all other samples were assayed under the condition depicted as the item of "Apparatus and Chromatographic Conditions" and the chromatogram of solution II is shown in Figure 2. The results demonstrated that the resolution factor fit the separation and detection of impurity exiting in (*S*)-Ornidazole and all the samples were impurity test-passed according to solution II, containing 0.5% impurity of (*R*)-Ornidazole. In fact, the impurity of (*R*)-Ornidazole was not detected in this experiment and the (*S*)-Ornidazole injection and raw material were valid (Fig. 2).

CONCLUSIONS

Chiralcel OB-H column can be employed for the separation of many compounds containing group of amide, carbonyl, nitro, cyano, hydroxyl, aryl, sulfuryl, and so on (the specifications were supplied by Daicel Chemical Ind., LTD, Japan). Chiral separation based on CSP (cellulose tribenzoate) has also been evaluated for the separation and determination of Ornidazole enantiomers in this article. The impurity of (*R*)-Ornidazole in (*S*)-Ornidazole injection and raw material was detected with an adequate resolution factor for quantitative analysis and no impurity of (*R*)-Ornidazole was detected under this chromatographic condition. The present results confirm that the proposed chiral HPLC method is well suited for the enantioselective analysis of Ornidazole. There seems to be no relative report on chiral separation for *rac*-Ornidazole, which has been used as an effective medicine for more than 30 years. Although methanol (which offers a good resolution factor for the enantiomeric separation) is not recommended as a typical solvent for mobile phase on this Chiralcel OB-H column according to the instruction supplied by Daicel Chemical Ind., LTD, the no. 3 chromatographic condition allowed the chiral separation of *rac*-Ornidazole with high plates of 8000 and 11,000 for (*R*)-Ornidazole and (*S*)-Ornidazole, respectively.

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