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Short communication

High-performance liquid chromatographic separation of an HIV-1 reverse transcriptase inhibitor and its enantiomer

Tao Wang[a,*], Nelu Grinberg[a], Gary Bicker[a], Patricia Tway[a], Karen Thompson[b]

[a]*Analytical Research Department, Merck Research Laboratories, P.O. Box 2000, R80Y-335, Rahway, NJ 07065-0900, USA*

[b]*Pharmaceutical Research and Development Department, Merck Research Laboratories, P.O. Box 4, WP78-302, Westpoint, PA 19486, USA*

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Abstract

This article describes the direct separation of an HIV-1 reverse transcriptase inhibitor and its enantiomer by HPLC on a silica-bonded polyacrylamide (ChiraSpher) column. The column selection was based on specific interactions between the individual enantiomers and the chiral stationary phase. The influence of some chromatographic conditions, such as concentration of the polar modifier in the mobile phase, column flow-rate and column temperature, on column performance was investigated. The separation was applied to the determination of the minor enantiomer in the bulk drug and as low as 0.3% of minor enantiomer was detectable.

Keywords: Enantiomer separation; HIV-1 reverse transcriptase inhibitor; L-738,372

1. Introduction

Enantiomers of pharmaceutical compounds may display quite different pharmacological behavior [1]. The separation and quantitation of enantiomers present in chiral drugs are important in drug development. High-performance liquid chromatography (HPLC) is a powerful and widely used analytical technique to separate and quantitate enantiomers. A number of reviews pertinent to the subject have recently been published [2–7]. The use of chiral stationary phases (CSP), to directly separate the enantiomers, offers a relatively simple and easy option.

L-738,372 is a drug candidate which controls the reproductive cycle of human immunodeficiency virus by inhibiting reverse transcriptase, the enzyme responsible for transcription of viral RNA onto human DNA [8]. The structure of L-738,372 is presented in Fig. 1. In this article, the direct separation of L-738,372 and its enantiomer on a silica-bonded polyacrylamide (ChiraSpher) column is reported. The influence of some chromatographic conditions, such as concentration of the polar modifier in the mobile phase, column flow-rate and column temperature, on column efficiency, resolution and capacity factor was investigated. The optimized separation was applied to the determination of the minor enantiomer in the

*Corresponding author.

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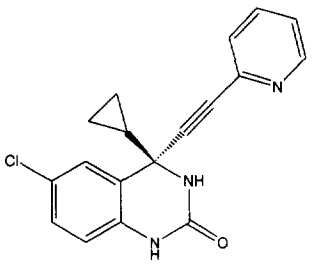

Fig. 1. Structure of L-738,372.

bulk drug and as low as 0.3% of minor enantiomer was detectable.

2. Experimental

2.1. Instrumentation

Chromatography was performed on a Spectra System P4000 HPLC system (Thermal Separation Products, Piscataway, NJ, USA). The stationary phase used was 5 μm silica-bonded polyacrylamide with S-phenylalanine ethyl ester residues. The column (250×4.6 mm) was manufactured by E. Merck (Darmstadt, Germany) under the trade name of ChiraSpher. A Model 7950 column temperature controller (Jones Chromatography, Lakewood, CO, USA) was used to control the column temperature. The samples were introduced into the chromatographic system through a 20-μl sample loop. Detection was performed by measuring UV absorbance at 240 nm. Chromatograms were processed by a PE Nelson data system, equipped with Access*Chrom software (version 1.9) (PE Nelson, Cupertino, CA, USA).

2.2. Materials

All the mobile phase solvents were of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

L-738,372 and its enantiomer were provided by the Process Research and Development Department of Merck Research Laboratories (Rahway, NJ, USA). The synthesis of L-738,372 has been described elsewhere [8,9]. The diluent for preparation of analytical samples was isopropyl alcohol (IPA)–hexane (35:65, v/v).

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of IPA and hexane which was isocratically pump-mixed at specified ratios. The optimum composition of the mobile phase was IPA–hexane (35:65, v/v). The selected flow-rate was 0.4 ml/min. The capacity factor k' of each enantiomer was determined as $k'=(t_R-t_0)/t_0$. The t_0 was determined by injecting pure hexane, which is a weaker solvent than the IPA–hexane mixture, and noting the time of appearance of the hexane peak [10].

3. Results and discussion

3.1. Column selection

One type of CSP is the polyacrylamide. ChiraSpher, which is a silica-bonded polyacrylamide containing S-phenylalanine ethyl ester residues, belongs to this category. The resolution mechanism and applications of polyacrylamide CSP have been reported in the literature [11–13]. Basically, chromatographic resolution is due to the inclusion of the enantiomers into the asymmetric cavities of the three-dimensional network formed from the polymeric chains. The enantiomers exhibit different degrees of fit in the cavities and this determines their average residence time in the stationary phase. Hydrogen bonding between the polar groups of the enantiomers and the -CO-NH- groups of the polymer are believed to be the main adsorbing forces. Therefore, this type of CSP is most suitable for the resolution of compounds with polar functional groups that can form hydrogen bonds. Some examples of compounds separated on polyacrylamide containing S-phenylalanine ethyl ester residues include chlorthalidone, penflutizide, bendroflumethiazide, oxazepam [12] and alkylated lactones [13], all of which have polar functional groups that can form hydrogen bonds with the stationary phase.

The selection of the ChiraSpher column for the separation of L-738,372 and its enantiomer was based on the above information about the CSP and

the structure of the enantiomers. L-738,372, whose configuration is S according to the Cahn-Ingold-Prelog convention, has a carbodiamide functionality which can form multiple hydrogen bonds with the amide groups of the ChiraSpher CSP. Therefore, it was expected that resolution could be achieved on a ChiraSpher column. Indeed, separation was obtained as shown in Fig. 2.

3.2. Competitive binding of the solutes and mobile phase modifier to the stationary phase

Muller and Carr [14,15] developed a mathematical model for the characterization of the competitive binding of the solute and mobile phase modifier to a protein-based stationary phase. According to their treatment, a plot of the reciprocal k' vs. the molar concentration of the modifier in the mobile phase should lead to a straight line, if such competition exists. Wainer [16] applied this treatment to characterize the competitive binding of warfarin enantiomers and trichloroacetic acid, which was used as mobile phase modifier, on a bovine serum albumin column. The plots of $1/k'$ of the two enantiomers vs. the molar concentration of the modifier showed straight lines, indicating the existence of competition at the binding sites.

In our case, a similar plot was constructed and the results are presented in Fig. 3. Straight lines were obtained for both enantiomers, indicating that competition exists between the two enantiomers and the polar modifier molecules in the mobile phase at the active sites on the stationary phase.

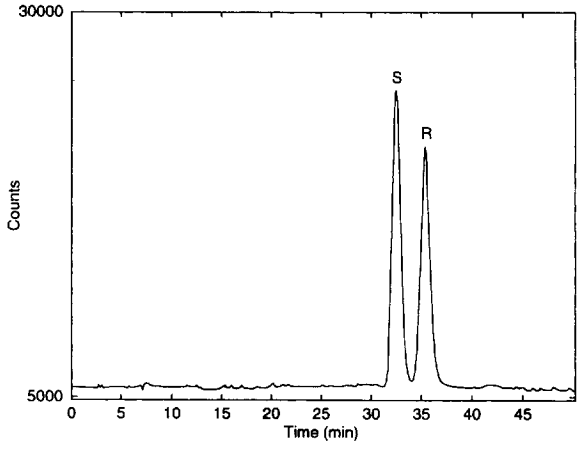

Fig. 2. Chromatographic separation of L-738,372 (*S*) and its enantiomer (*R*). Column: ChiraSpher, 250×4.6 mm, 5 μm; mobile phase, 35:65 (v/v) of IPA–hexane; flow-rate, 0.4 ml/min; detector, UV 240 nm; injection, 20 μl, 5 μg/ml; temperature, 25°C.

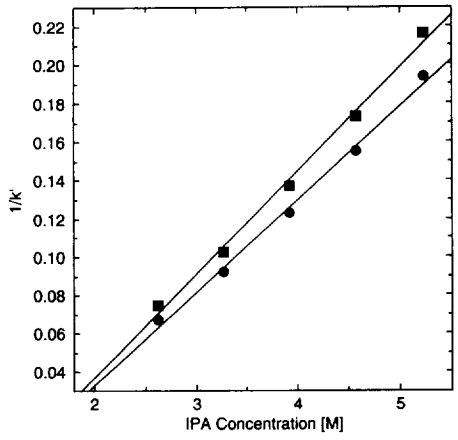

Fig. 3. Influence of the IPA concentration on the reciprocal of the capacity factor ($1/k'$) of the two enantiomers. Flow-rate, 1.0 ml/min; temperature, 25°C. Symbols: ■=*S* enantiomer (R^2= 0.997), ●=*R* enantiomer (R^2=0.997).

3.3. Optimization of experimental parameters

3.3.1. Concentration of polar modifier

To optimize the amount of the polar modifier, the concentration of IPA in the mobile phase was varied from 40 to 15% in 5% increments, with a flow-rate of 1 ml/min and a column temperature of 25°C. As the concentration of IPA decreased from 40 to 15%, the k' of the *S*-enantiomer increased from 4.6 to 20 while the resolution between the two enantiomers improved only slightly, from 1.21 to 1.32. Since improving the resolution through variation of IPA concentration was not very effective, 35% IPA was chosen as the optimum polar modifier concentration, as this gave reasonable retention of the enantiomers with k'=5.8 for the *S*-enantiomer.

3.3.2. Flow-rate

Columns used for the separation of enantiomers are usually characterized by high selectivity for a certain set of optimum conditions. However, the efficiency of such systems is often poor. Optimization of the efficiency may be achieved by varying

the mobile phase flow-rate. Since a slow velocity of the mobile phase will increase the analysis time, despite giving a high number of theoretical plates, a compromise must be made between the analysis time and the efficiency of the system [17]. Optimization of the flow-rate of a mobile phase consisting of 35% IPA in hexane was examined between 0.4 and 1.4 ml/min with increments of 0.2 ml/min. The influence of flow velocity on the height of the theoretical plate (H) is shown in Fig. 4. The lowest plate height was obtained at a flow velocity of 0.83 mm/sec, corresponding to a flow-rate of 0.4 ml/min. The change of the resolution between the two enantiomers with the flow-rate was also studied (Fig. 5). The resolution improved as the flow-rate decreased and the improvement was more significant at the lower flow-rate region. The highest resolution was obtained also at 0.4 ml/min. Under this condition, the retention time of the later-eluting enantiomer was approximately 35 min and an almost baseline separation was achieved (Fig. 2).

3.3.3. Column temperature

The influence of column temperature on resolution and column efficiency was studied at the selected mobile phase composition of 35:65 (v/v) of IPA–hexane and with a flow-rate of 0.4 ml/min. While heating the column above room temperature caused a decrease in the resolution, cooling the column below

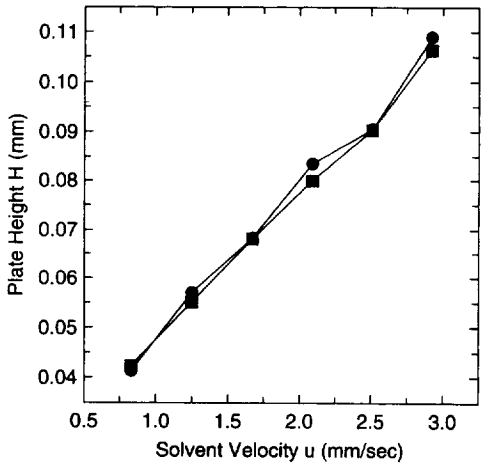

Fig. 4. Influence of flow velocity (u) on the height of theoretical plate (H). Mobile phase, 35:65 (v/v) of IPA–hexane; temperature, 25°C. Symbols: ■=S enantiomer, ●=R enantiomer.

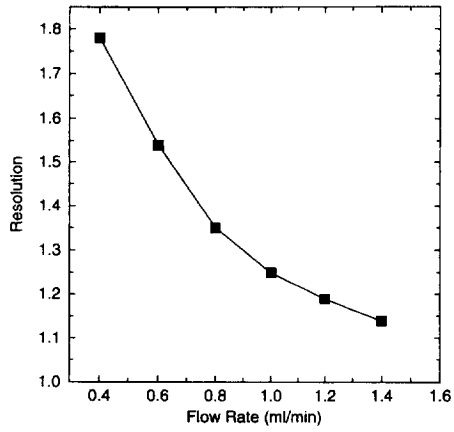

Fig. 5. Influence of flow-rate on resolution. Mobile phase, 35:65 (v/v) of IPA–hexane; temperature, 25°C.

room temperature improved the resolution only slightly, from 1.78 at 25°C to 1.89 at 8°C. However, this slight improvement of resolution was at the expense of loosing almost two-fold of column efficiency (plate height of R-enantiomer changed from 0.042 mm to 0.080 mm) and lengthening the analysis time from 36 min to 58 min. Therefore, keeping the column at room temperature is considered to be the optimum.

3.4. Application

The final application of this separation is to determine trace amounts of minor enantiomer present in the bulk drug of L-738,372. Therefore, it is necessary to determine the minimum detectable level of minor enantiomer. This was determined by spiking different amounts of minor enantiomer into a sample of pure drug substance. With the concentration of L-738,372 being 0.05 mg/ml, it was possible to detect 0.3% of minor enantiomer (Fig. 6).

4. Conclusion

The polyacrylamide CSP ChiraSpher was successfully applied to the separation of L-738,372 and its enantiomer. Under the selected chromatographic conditions, 0.3% of minor enantiomer is detectable.

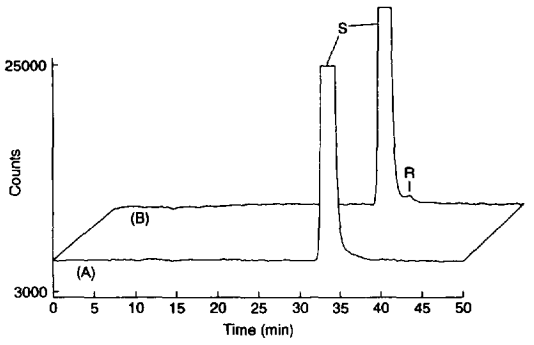

Fig. 6. Chromatograms of (A) pure drug substance of L-738,372 (*S*) and (B) pure drug substance of L-738,372 (*S*) spiked with 0.3% of the minor enantiomer (*R*). Conditions used were the same as in Fig. 2. The concentration of L-738,372 was 0.05 mg/ml.

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