Reversed-Phase High-Performance Liquid Chromatographic Separation of LY309887 (Thienyl-5,10-Dideazatetrahydrofolate) Stereoisomers Using β-Cyclodextrin as a Mobile Phase Additive

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Abstract

The separation of the four stereoisomers of LY309887 (thienyl-5,10-dideazatetrahydrofolate) is studied here using commercially available high-performance liquid chromatography column technology. The selectivity needed to resolve the four stereoisomers is provided by an achiral reversed-phase column used with β-cyclodextrin as a mobile phase additive. The separation is dependent on triethylamine and β-cyclodextrin concentration in the buffer portion of the eluent, eluent strength, and buffer pH.

Introduction

The growing number of chiral drug candidates in the pharmaceutical industry is increasing the need for chiral-selective analytical methods. A reversed-phase chiral high-performance liquid chromatographic (HPLC) method for the separation of the four stereoisomers of thienyl-5,10-dideazatetrahydrofolate (LY309887) is reported here using β-cyclodextrin as a mobile phase additive. A series of commercially available reversed-phase columns offering different selectivities were examined. The only column that successfully separated the four stereoisomers was the Dupont (Wilmington, DE) Zorbax SB-Phenyl column (25 cm × 4.6-mm i.d.). The effects of mobile phase pH, β-cyclodextrin concentration, mobile phase strength, and other mobile phase additives on the separation were examined.

LY309887 is a thienyl analog of 5,10-dideazatetrahydrofolate (DDATHF) that inhibits the glycinamide–ribonucleotide formyltransferase (GAR FTase) enzyme involved in purine biosynthesis (1,2). Thienyl-DDATHF is of interest as an antitumor agent, and it is currently being examined in phase 1 clinical trials. LY309887 contains two totally independent stereogenic centers (Figure 1). The principal stereochemical impurity in LY309887 is isomer 2 (C_6 epimer). The L-glutamic acid portion of the molecule is introduced with high chiral purity, but the possible epimerization of the L-Glu center in subsequent synthetic steps is a concern. Chiral inclusion complex HPLC methods have been developed for the separation of the C_6-diastereomers of DDATHF and its analogs (3). However, an analytical method was needed to ensure that epimerization was not occurring at the L-Glu center.

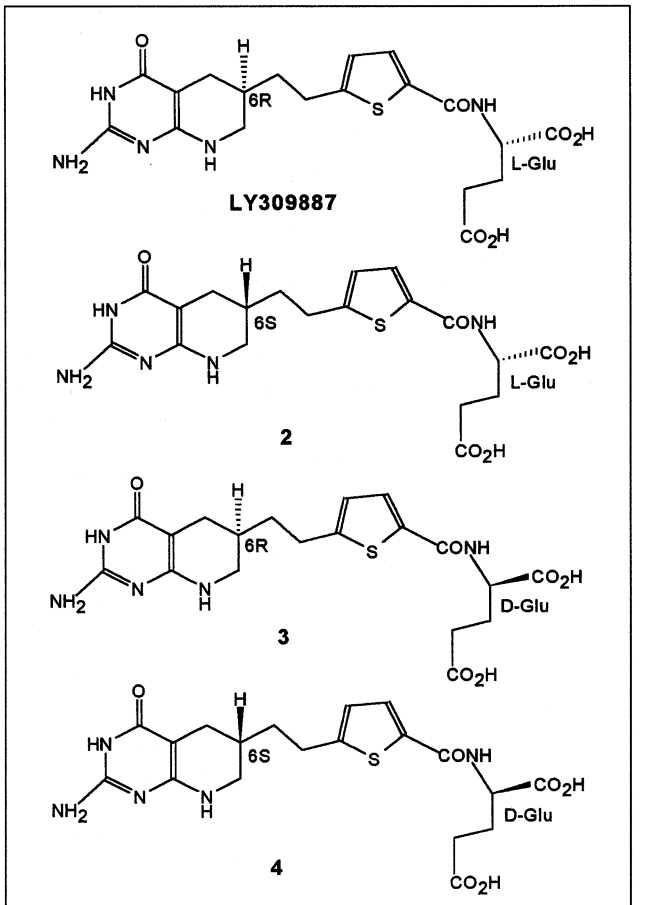

Figure 1. LY309887 and its stereoisomers.

Cyclodextrins are chiral cyclic oligosaccharides obtained from starch through enzymatic degradation. Their structures are composed of α-(1,4)-linked d-glucose units arranged toroidally (4). The three main types of cyclodextrins are α-,β-,

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and γ-cyclodextrin, which each contain six, seven, and eight glucose units, respectively. The structure of β-cyclodextrin is shown in Figure 2. The toroidal cyclodextrin geometry gives them a truncated cone appearance (Figure 3) with an internal cavity lined by glycosidic oxygen bridges and C–H groups (5). The size of the internal cavity is determined by the number of glucose units present. The glycosidic oxygen lining gives the internal cavity hydrophobic character, whereas the primary and secondary hydroxyl linings of the external cone faces are relatively hydrophilic (6). Cyclodextrins are unique because they are able to form inclusion complexes with a wide variety of guest molecules. The guest must only have a spatial geometry that allows partial inclusion into the internal cyclodextrin cavity.

The uses of cyclodextrins in analytical chemistry have been described in several review articles (5,7,8). Cyclodextrins have traditionally been used in LC as unique enantioselective stationary phases (9,10) and as mobile phase additives in reversed-phase chiral LC separations (3,6,11,12). Chiral recognition may only occur when the enantiomers partially fit into the cyclodextrin cavity and when interactions occur between the secondary 2- and 3-hydroxyl groups (located on the wide

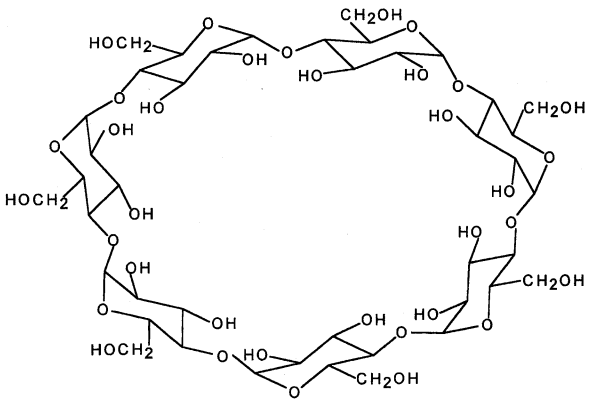

Figure 2. Chemical structure of β-cyclodextrin.

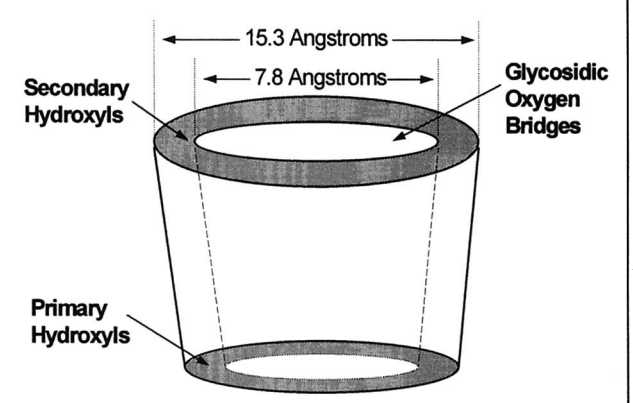

Figure 3. Geometric shape of the β-cyclodextrin molecule.

Table I. Resolution of LY309887 from Isomer 3 Using Commercial Columns and Various Eluent Systems

	Free acid form		Di-ester form	
Columns examined	**Eluent system**	**Resolution**	**Eluent system**	**Resolution**
Zorbax SB-C18	3–8% acetonitrile/ β-cyclodextrin buffer* (pH = 3)	minimal	same	none
Zorbax RX-C8	5–10% acetonitrile/ β-cyclodextrin buffer (pH = 7,4, and 2.5)	minimal	same	none
Zorbax SB-CN	2% acetonitrile/ 98% β-cyclodextrin buffer (pH = 3)	r_s = 0.7	same	none
Zorbax SB-Phenyl	3% acetonitrile/ 97% β-cyclodextrin buffer (pH = 4.93)	r_s = 2.0		
Astec Cyclobond I	5–15% acetonitrile/ 25mM NaH_2PO_4–H_2O, 0.1% TEA, pH = 2.5	minimal	same	none
S,S-Whelk-01	5–20% acetonitrile/ 25mM NaH_2PO_4–H_2O (pH = 7,4, and 2.5)	none	same	none
S,S-Whelk-01	2% MeOH/ 5% CH_2Cl_2/ 93% hexane	none	same	none
ULTRON ES-OVM	3–15% acetonitrile/ 25mM NaH_2PO_4–H_2O (pH = 7,4, and 2.5)	none	same	none
Resolvosil BSA-7	1–5% acetonitrile/ 25mM NaH_2PO_4–H_2O, (pH = 7,4, and 2.5)	minimal	same	minimal

* 25mM NaH_2PO_4–H_2O, 0.1% (v/v) TEA, β-cyclodextrin (0.007M).

rim of the cyclodextrin cone) and the enantiomers. Chiral resolution results from stability constant differences between the inclusion complexes formed by the cyclodextrin with each enantiomer (4).

Although commercially available cyclodextrin bonded-phase LC columns have become more robust in recent years, cyclodextrin used as a mobile phase additive has become the more versatile technique for reversed-phase chiral LC separations. Inclusion complex stability constants have been reported to be related to cyclodextrin concentration (13,14). If enough cyclodextrin is not present, the inclusion complex is not completely formed, and resolution suffers. This observation has proven to be a limitation of cyclodextrin-bonded columns that contain a preloaded unadjustable amount of cyclodextrin.

Experimental

Chemicals

Phosphoric acid (85%, analytical reagent grade) and sodium hydroxide solution (50% [w/w], analytical reagent grade) were both obtained from Mallinckrodt (Paris, KY). Triethylamine (TEA) (99%) was obtained from Aldrich Chemical (Milwaukee, WI). Acetonitrile (HPLC grade) was obtained from Burdick & Jackson (Muskegon, MI). β-Cyclodextrin was obtained from Advanced Separation Technologies (Whippany, NJ). Sodium monobasic phosphate (monohydrate) was obtained from EM Science (Gibbstown, NJ).

Di(ethylene glycol) ethyl ether (99%) and Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, 99%) were obtained from Aldrich Chemical. Diethyl ether (anhydrous) and potassium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ). A Diazald kit (#Z10,025-0) was also obtained from Aldrich Chemical.

LY309887 and its stereoisomers were synthesized in our laboratory. A mixture of LY309887 and isomer 3 was made by the racemization of LY309887. Racemization of a mixture of LY309887 and isomer 2 produced a mixture of all four stereoisomers.

Instrumentation

The instrumentation used in this study included a Spectra-Physics (Thermo Separation Products, San Jose, CA) SP8800 ternary HPLC pump, an SP8880 autosampler, an SP8500 dynamic mixer, an SP8790 column heater, an ABI Spectroflow 773 absorbance detector (ABI Applied Biosystems, Foster City, CA), and a Perkin-Elmer-Nelson (Norwalk, CT) Turbochrome (version 4.0) system for integration and data management.

The following Dupont Zorbax reversed-phase HPLC columns were used: the SB-C18 (25 cm × 4.6-mm i.d.), RX-C8 (25 cm × 4.6-mm i.d.), SB-CN (25 cm × 4.6-mm i.d.), and the SB-Phenyl (25 cm × 4.6-mm i.d.). The following chiral columns were examined: Astec Cyclobond I (β-cyclodextrin phase) (25 cm × 4.6-mm i.d.), the S,S-Whelk-01 (25 cm × 4.6-mm i.d.), the Ultron ES-OVM (protein-based phase) (15 cm × 4.6-mm i.d.), and the Resolvosil BSA-7 (bovine serum albumin-based phase) (15 cm × 4.0-mm i.d.). All Dupont Zorbax columns and the Ultron ES-OVM column were obtained from MAC-MOD Analytical (Chadds Ford, PA). The Astec Cyclobond I column was obtained from Astec (Whippany, NJ). The S,S-Whelk-01 column was obtained from Regis Chemical (Morton Grove, IL). The Resolvosil BSA-7 column was obtained from Alltech (Deerfield, IL).

A column temperature of 40°C was maintained during all injections for retention time reproducibility. The injection volume was 20 µL for all injections. A constant flow rate of 1.00 mL/min was used throughout method development. The ultraviolet (UV) maximum of LY309887 (280 nm) was used for detection.

Solutions

Samples of LY309887 were dissolved in 25mM NaH_2PO_4–H_2O (pH = 6.6). Disodium salt forms of the acid (LSN 329201) were dissolved in water. Acidic and neutral pH NaH_2PO_4–H_2O buffers were prepared with varying amounts of β-cyclodextrin and TEA. Buffer pH was adjusted after the addition of all additives using phosphoric acid and sodium hydroxide solution. All buffers were filtered with Nylon-66 membrane filter paper (0.45-µm pore size) prior to use.

Derivatization

An alcohol-free, ether solution of diazomethane (CH_2N_2) was prepared using an Aldrich Diazald kit (15). Potassium hydroxide (3 g, 0.054 mol) was added to a reaction flask containing water

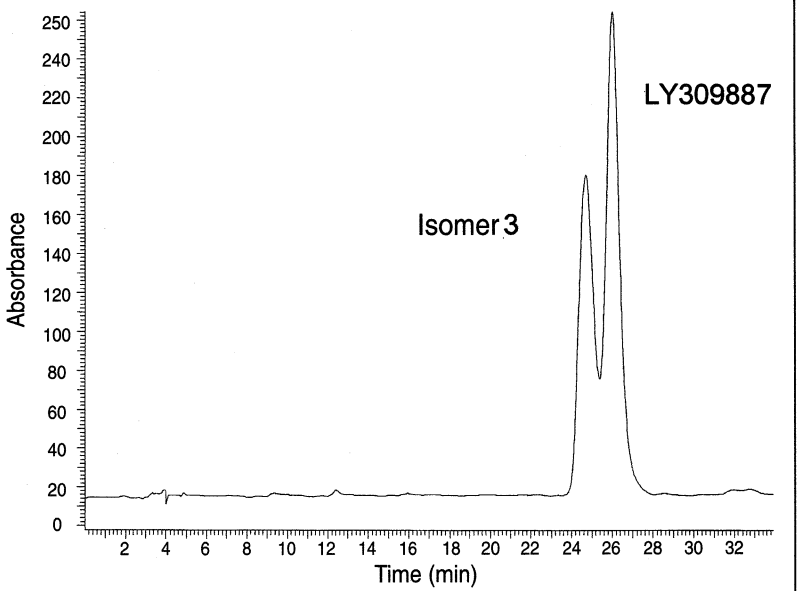

Figure 4. Partial resolution of isomer 3 and LY309887. Conditions: Dupont Zorbax SB-Phenyl column (25 cm × 4.6-mm i.d.); 5% acetonitrile, 95% buffer (25mM NaH_2PO_4–H_2O, 0.1% TEA, β-cyclodextrin [8 g/L], pH = 2.97) mobile phase.

(15 mL), di(ethylene glycol) ethyl ether (20 mL), and diethyl ether (10 mL). The reaction flask was equipped with an addition funnel and a condenser for distillation and heated to 70°C in a water bath. A solution of Diazald (11 g, 0.051 mol) in diethyl ether (70 mL) was then slowly added over 20 min with agitation. A distilled ether solution of diazomethane was collected in a receiving flask cooled with an ice bath.

The dimethyl-ester forms of the stereoisomers were generated in situ by CH_2N_2 derivatization in EtOH–EtOAc (1:1). The dimethyl-ester formation was verified by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy.

Results and Discussion

The long, elliptical shapes of LY309887 and similar antifolates make them ideal candidates for reversed-phase inclusion complex chiral LC. Method development was performed on a mixture of LY309887 and isomer 3. A summary of the commercial columns and eluent systems examined along with their resulting resolutions is shown in Table I.

Several generalizations concerning column performance may be made based on the results shown in Table I. Of the eight commercially available columns examined, only the protein-based Ultron ES-OVM and the S,S-Whelk-01 failed to partially resolve the diastereomers in either the free acid or di-ester forms. The chemically bonded β-cyclodextrin phase could not form inclusion complexes of sufficiently differing strengths to resolve the diastereomers. Although the bovine serum albumin-based phase is known to effectively resolve optical isomers of amino acids, it could not resolve the stereoisomers. All four of the Dupont Zorbax reversed-phase columns were able to provide some resolution when used with β-cyclodextrin as a mobile phase additive. However, the SB-CN and the SB-Phenyl columns clearly provided the type of selectivity needed to resolve the antifolate when used with β-cyclodextrin as a mobile phase additive. The π-systems capping the aliphatic chains offered the interactions necessary to produce diastereomeric resolution.

Carboxylic acids are often derivatized prior to chiral LC analysis to avoid severe hydrogen bonding effects, which counteract chiral selectivity. Producing the dimethylester forms of the stereoisomers resulted in the loss of chiral selectivity in the β-cyclodextrin systems examined. The carboxylic acids at the glutamate end of the molecule must play a crucial role in the inclusion complex stabilities of the stereoisomers.

Stability constants of inclusion complexes have been reported to be dependent on cyclodextrin concentration (13,14). Maximum resolution of the antifolate diastereomers was achieved at a β-cyclodextrin concentration of 7mM in the buffer portion of the eluent. Resolution could not be improved by increasing the amount of β-cyclodextrin used. However, resolution was sacrificed when the β-cyclodextrin concentration was cut in half.

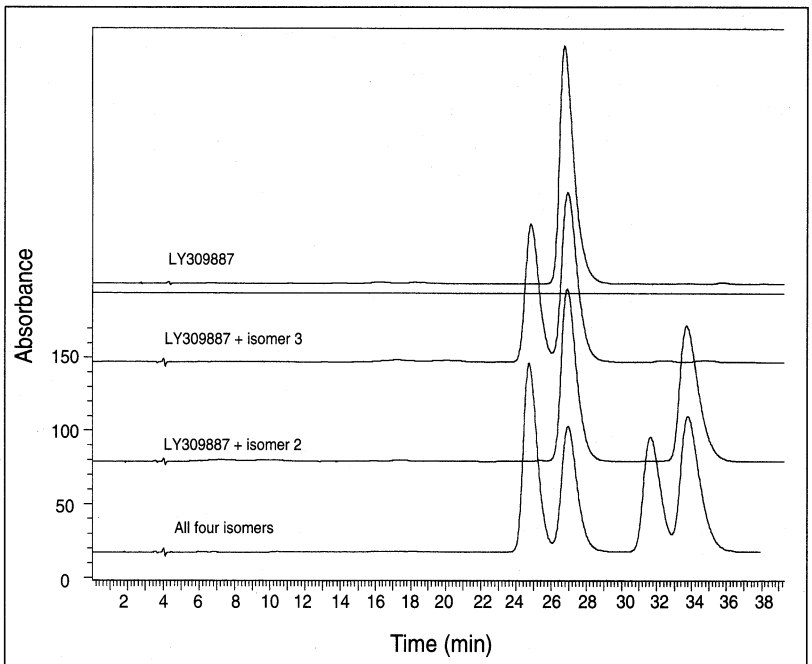

Figure 5. Typical chromatograms showing the separation of LY309887 from its stereoisomers. Conditions: Dupont Zorbax SB-Phenyl column (25 cm × 4.6-mm i.d.), 3% acetonitrile, 97% buffer (25mM NaH_2PO_4–H_2O, 0.1% TEA, β-cyclodextrin [8 g/L], pH = 4.93) mobile phase.

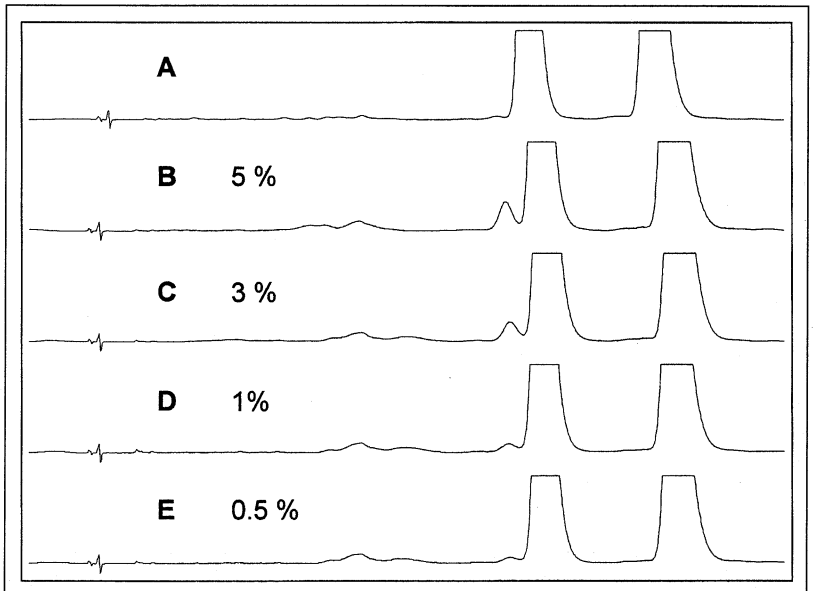

Figure 6. Chromatograms showing the detection limit of isomer 3 from LY309887. (A) A mixture of LY309887 and 2. (B–E) Varying weight percentages of isomer 3 spiked into solutions of the mixture shown in A. Conditions were the same as in Figure 5.

Resolution was improved by using TEA as a buffer additive. The resolution enhancement seen with the addition of TEA to the buffer portion of the eluent reached a maximum at 0.1% (v/v), and the addition of more TEA could not improve the separation.

Resolution was most dramatically affected by buffer pH and eluent strength. At a buffer pH of 2.97 and an eluent composition of 5% acetonitrile and 95% buffer, LY309887 and isomer 3 were partially resolved on the Dupont Zorbax SB-Phenyl column (Figure 4). However, adjusting the buffer pH to 4.93 and the mobile phase composition to 3% acetonitrile, 97% buffer resulted in not only the complete resolution of the 6R-diastereomers ($R_s = 2.0$), but also the resolution of the other two stereoisomers (Figure 5). Slightly acidic or basic variations in buffer pH resulted in loss of resolution.

The elution order of the four stereoisomers was established by analyzing mixtures of stereoisomers (Figure 5). The detection limit of isomer 3 from LY309887 was established at concentrations greater than or equal to 0.5% (w/w), as shown in Figure 6.

Conclusion

Chiral and cyclodextrin bonded phase HPLC columns failed to offer the selectivity needed to resolve the stereoisomers of LY309887. Reversed-phase Dupont Zorbax stablebond columns with π-system capping groups used with β-cyclodextrin as a mobile phase additive provided the selectivity needed for partial resolution. The separation was found to be dependent on TEA and β-cyclodextrin concentration in the buffer portion of the eluent, eluent strength (acetonitrile content), and buffer pH. The four stereoisomers were successfully resolved only on the Dupont Zorbax SB-Phenyl column using β-cyclodextrin as a mobile phase additive. The detection limit of isomer 3 from LY309887 was established as greater than or equal to 0.5% (w/w).

Acknowledgments

The author gratefully acknowledges R. Brian Scherer and Joseph H. Kennedy for numerous technical conversations which proved to be very helpful while completing this work. Special thanks to Dr. Charles J. Barnett, Thomas M. Wilson, Michael E. Kobierski, William D. Miller, and the entire project team for supplying LY309887 and its stereoisomers.

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Manuscript accepted November 26, 1996.