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Short Communication

Direct isomeric separation of a 3-hydroxyproline-containing prodrug, L-693 989, by high-performance liquid chromatography with a porous graphitic carbon column

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Abstract

The use of high-performance liquid chromatography (HPLC) with a porous graphitic carbon (Hypercarb) column for the direct separation of a positional isomer of L-693 989, a 3-hydroxyproline-containing prodrug, is described. The isomer peak was isolated by HPLC and analyzed by mass spectrometry and proton nuclear magnetic resonance spectroscopy. An authentic sample of the isomer was also synthesized for chromatographic comparison. The results confirm that the peak in question is a 4-hydroxyproline isomer which is difficult to separate from L-693 989 compound with the silica-based reversed-phase columns. The observed chromatographic elution supports the retention mechanism based on the unique electronic donor–acceptor interaction between the lone-pair electrons of the analyte (donor) and the delocalized electron conduction bands on the graphitized carbon stationary phase (acceptor).

1. Introduction

L-693 989 is a 3-hydroxyproline-containing, semi-synthetic phosphate prodrug that possesses potent antifungal and antipneumocystis activity (Fig. 1) [1–3]. In the initial development stage, the synthesized bulk drug contained a small amount (*ca.* 7%) of the 4-hydroxyproline isomer (L-702 303). The first batches of L-693 989 were prepared from the fermentation product, L-688 786 [1], which contained *ca.* 7% of the 4-hydroxyproline isomer (L-700 098) as a minor fermentation by-product. Although the amount of the 4-hydroxyproline isomer in the starting material could be quantitated by high-performance liquid chromatography (HPLC), we were unable to resolve the isomer at the prodrug stage using a variety of reversed-phase columns such as the Nova-Pak C_{18}, Vydac (C_{18} and C_4) and Zorbax RX (C_8 and C_{18}). With all of these columns, the 4-hydroxyproline isomer co-eluted with L-693 989. The amount of the 4-hydroxyproline isomer in the bulk drug was monitored by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy in a fashion similar to that observed for the L-688 786/L-700 098 natural products [4]. A more quantitative method for determining the amount of the isomer, however, was needed in order to ensure the purity of the bulk drug, and to demonstrate the selectivity of a

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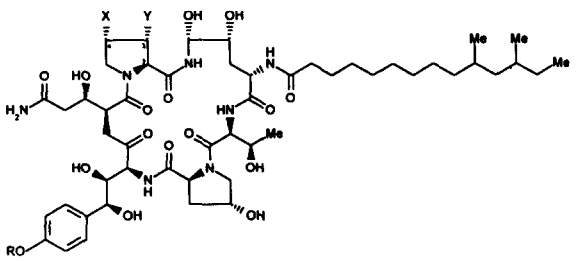

Compound	X	Y	R
L-693 989	H	OH	$-P-(OH)_2$
L-688 786	H	OH	H
L-702 303	OH	H	$-P-(OH)_2$
L-700 098	OH	H	H

Fig. 1. Molecular structures of L-693989 and related compounds.

quantitative assay for L-693 989 in dosage forms. Our research on a different type of HPLC column, the porous graphitic carbon (PGC), demonstrated a direct separation of the isomer from L-693 989.

PGC is a newly developed column packing material, recently made available for HPLC [5–9], with retention properties resembling that of a classical silica-based reversed-phase system. It is considered a "pure" reversed-phase material because it contains a strongly hydrophobic adsorbent with a flat surface consisting of hexagonal layers of covalently bonded carbon atoms and no unreacted silanol groups [6]. The advantages of PGC include unique selectivity, column stability in the pH range of 0 to 14, solvent compatibility (it does not swell with organic solvent), hardness to withstand high pressures (mechanical stability), surface homogeneity and reproducible performance. The pharmaceutical and biomedical applications of PGC have several literature precedents [10–15]; however, the number of literature applications with the PGC column is still very sparse. Resolution of chiral compounds using a PGC column with mobile phase additives has also been illustrated [16,17]. One of the significant features of PGC is the capability for the direct separation of positional isomers due to its unique possession of conduction bands of delocalized electrons as described by Bassler and co-workers [18,19]. In these studies, the retention mechanism on the PGC stationary phase for the separation of positional isomers of cresol and some natural products has been shown via an electronic donor–acceptor interaction between the electron-rich solutes (donor) and the delocalized electron-conduction bands of PGC (acceptor). This unique retention mechanism has inspired us to utilize the PGC column for a direct separation of L-693 989, which contains several electron-rich substituents, and its 4-hydroxy positional isomer. The isolation and data to support the identification of the isomer peak by mass spectrometry (MS) and ^1H NMR are presented in this study. The retention mechanism on the PGC column for isomeric separation is also proposed.

2. Experimental

2.1. Chemicals and reagents

L-693 989 ($C_{50}H_{80}N_8O_{20}PK$) was manufactured by Merck Research Labs. (Rahway, NJ, USA) [20]. Acetonitrile (HPLC grade), potassium phosphate and ammonium acetate (reagent grade) were purchased from Fisher Scientific (Philadelphia, PA, USA). All solvents and reagents were used as received without further purification. Deionized water with at least 18 MΩ purified by a Milli-Q system (Millipore, Bedford, MA, USA) was used for mobile phase and standard preparations.

2.2. Instruments and chromatographic conditions

Separation

A Hewlett-Packard (Avondale, PA, USA) 1090 LC system connected to a variable-wavelength UV detector (Applied Biosystems 783A, Foster City, CA, USA) was used for the chromatographic separation and isolation. A Shandon Hypercarb (Keystone Scientific, State College, PA, USA) analytical column (5 μm particle size, 100 mm × 4.6 mm I.D.) with a mobile phase

of 0.02 *M* potassium phosphate, pH 6.8–acetonitrile (55:45) delivered at a flow-rate of 1.5 ml/min was used to separate the 4-hydroxyproline isomer from L-693 989. The separation was carried out at ambient temperature with 10-μl injections of 0.3 mg L-693 989/ml in water. The wavelength was set at 220 nm for UV detection.

Isolation and identification

The isomeric fractions were collected by HPLC for identification with a more volatile mobile phase (0.02 *M* ammonium acetate, pH 6.8–acetonitrile; 55:45) and a larger injection volume (50 μl). The collected fractions of L-693 989 and its isomer were evaporated to remove the solvent with a rotary evaporator. Ammonium acetate solid was further removed by vacuum. The resulting samples were analyzed by MS and NMR spectroscopy. MS analyses were performed on a ZAB mass spectrometer with both fast atom bombardment (FAB) positive ion and negative ion modes using glycerol as a FAB matrix. ^{1}H NMR spectra were generated with a Bruker 400 MHz spectrometer using $C^{2}H_{3}O^{2}H$ as the solvent.

3. Results and discussion

3.1. Separation of the isomer

Because of the similar physical and chemical properties (such as hydrophobicity) of L-693 989 (3-hydroxyproline) and L-702 303 (4-hydroxyproline), direct separation of the two isomers by HPLC with the reversed-phase columns is difficult. Several reversed-phase columns, including Nova-Pak C_{18}, Vydac C_{18}, Vydac C_4, Zorbax RX-C_8 and Zorbax RX-C_{18} were investigated to support the determination of the 4-hydroxyproline isomer. The 4-hydroxyproline isomer co-eluted with L-693 989 in all the above instances. The concept of using the PGC column in this study is based on its unique retention mechanism which is dominated by the electronic interaction between the solute and the stationary phase rather than the hydrophobicity of the solute which normally is the key factor for reversed-phase chromatography. The difference in electronic properties influenced by the surrounding group for the title compound (mainly on the 3-hydroxy group) and its 4-hydroxyproline isomer renders the direct separation of the isomers on the PGC column feasible (see section 3.3).

Under the optimized chromatographic conditions described in the Experimental section, the two isomers were well separated with L-693 989 eluting at *ca.* 6 min followed by the isomer (L-702 303) at *ca.* 10 min. A typical chromatogram is shown in Fig. 2. L-693 989 exhibited shorter retention than the isomer counterpart indicating that the electronic donor–acceptor interaction was weaker for L-693 989. This result was in agreement with the hypothetical retention mechanism (see below). Two small peaks eluting before L-693 989, which have not been identified, will not be discussed in this study.

Prior to the isolation and identification of the

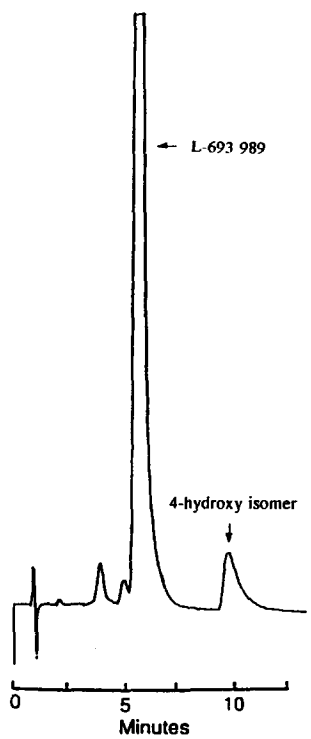

Fig. 2. Typical chromatogram of the resolved 4-hydroxy isomer from L-693989 on a porous graphitic carbon column. Chromatographic conditions are described in the Experimental section.

isomer peak, there were two indicators which supported the proposed peak identity of L-702 303. First, the HPLC area percent of the peak in question correlated well with the percent of 4-hydroxyproline isomer present in different lots of bulk drug (between 2 and 9%) as determined by ^{1}H NMR spectroscopy. Second, an experiment was done by adding a 0.1 *M* NaOH solution to the bulk drug solution to investigate the degradation behavior of L-693 989 and the peak of interest. Both peaks decreased in peak size and formed two base-induced degradates [1,4] eluting prior to the major drug peak (relative retention times were 0.57 and 0.70). These observations suggested that the peak of interest is chemically closely related to L-693 989. Based upon these observations, it is reasonable to suggest that the peak in question is the 4-hydroxyproline isomer of L-693 989. Although the peak could be proposed as the 4-hydroxy isomer, it was preferable to isolate the fractions for further characterization to gain additional support of the peak identity before an L-702 303 authentic sample was synthesized for chromatographic comparison.

3.2. Identification of the isomer peak

Fractions were collected by HPLC to provide samples for MS and ^{1}H NMR identification (see Experimental). Results of the MS analysis showed that both the front (L-693 989) and end (isomer) fractions exhibited an identical parent m/z of 1183 for the positive ion (K^+ salt) mode and 1143 for the negative ion (anion, without K^+ salt) mode. These two sets of mass spectra confirmed that the two components were isomers. Identification of the isomer was determined by ^{1}H NMR [4]. The structure of the isomer was confirmed by an authentic sample of the 4-hydroxyproline isomer which was later synthesized.

After the MS and ^{1}H NMR studies, the chromatographic behavior of the authentic sample was also compared. It was found that the peak in question matched perfectly with the authentic sample in terms of chromatographic elution. This observation further supports the proposed identity of the isomer peak.

3.3. Retention mechanism

The retention of the isomers on the PGC column observed in this study could be explained based on the electronic donor–acceptor interaction between the solute (donor) and stationary phase (acceptor) similar to that described by Bassler and co-workers [18,19]. The lone-pair electrons on the solute and the unique feature of delocalized electron conduction bands of the graphitized carbon play a key role in the retention of the PGC column. In our study, the substituent groups near the active–OH group (electron donor) on the proline ring, which affected the electronic activity due to the steric hindrance, governed the isomeric separation and the elution order of the two isomers.

L-693 989, the 3-hydroxyproline derivative, exhibited the electron donating group (–OH) next to a bulky substituent on the 2-position of the proline derivative leading to a large steric hindrance to the electronic interaction between the 3-hydroxyproline isomer and the graphite stationary phase. On the other hand, the 4-hydroxyproline isomer (–OH away from the bulky group) encountered less steric effect than the 3-hydroxy counterpart. Consequently, L-693 989 should elute faster than the 4-hydroxyproline isomer L-702 303 because of less electronic donor–acceptor interaction. The observed chromatography was in good agreement with the proposed retention mechanism. It was also consistent with the retention order reported for the separation of positional isomers of substituted phenols [18].

Alternatively, instead of the bulky steric effect, it was thought that the possibility of hydrogen bonding formation between the 3-hydroxy group and the neighboring–OH group on the ornithine residue could also explain the observed elution sequence. Because the lone-pair electrons on the 3-hydroxy (L-693 989) would become less available due to the hydrogen bonding interaction, there was less electronic donor–acceptor interaction between L-693 989 and the

graphite stationary phase, resulting in shorter retention. The 4-hydroxyproline isomer was less likely subject to the hydrogen bonding because of the unfavorable location of the lone-pair electrons on the active 4-OH group. Thus, it would possess more electronic donor–acceptor interaction and thereby a longer retention time than the 3-hydroxyproline isomer. Although speculative, both phenomena of steric hindrance and hydrogen bonding supported the retention sequence of the isomeric separation observed in this study.

4. Conclusions

The unique electronic feature of the porous graphitic carbon column enables the direct isomeric separation of L-693 989. This successful isomeric separation has exploited further the application of the relatively newly developed PGC column. Chromatographic data as well as the instrumental analysis by MS and ^{1}H NMR spectroscopy have supported the hypothesis that the peak in question is the 4-hydroxyproline isomer, a semi-synthetic fermentation minor. The retention mechanism on the graphite column is based on the electronic donor–acceptor interaction between the compound of interest and the graphite's delocalized electron conduction bands. Steric hindrance and/or hydrogen bonding formation from the neighboring group on the ornithine residue affects the electronic property of the active lone-pair electrons which plays an important role in the separation of the positional isomers.

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