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Short communication

Hydrophilic interaction chromatography on amino-silica phases complements reversed-phase high-performance liquid chromatography and capillary electrophoresis for peptide analysis

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Abstract

Hydrophilic interaction chromatography (HILIC) on amine bonded-phase silica columns provides separations of peptides that are complementary to those obtained with reversed-phase HPLC and free solution capillary electrophoresis. This is illustrated with the peptide drug atosiban and nine diastereomers. Moreover, one of the HILIC methods was suitable for coupling with electrospray mass spectrometry.

Keywords: Hydrophilic interaction chromatography; Capillary electrophoresis; Liquid chromatography–mass spectrometry; Stationary phases, LC; Diastereomer separation; Peptides; Atosiban

1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) are often used as complementary techniques for the separation of peptides [1]. Hydrophilic interaction chromatography (HILIC) is another technique that is complementary to RP-HPLC and CE [2–4]. HILIC is of general utility for polar analytes such as carbohydrates, nucleic acids, amino acids, and peptides [2–11]. HILIC separations have been conducted on a variety of bonded stationary phases with mobile phases containing more than 70% acetonitrile. HILIC chromatography is related to normal-phase chromatography since retention of solutes increases with increasing solute polarity and decreases with increasing mobile phase polarity [2]. Alpert has described HILIC as "partitioning between the (hydrophobic) mobile phase and a layer of mobile phase enriched with water and partially immobilized on the stationary phase" [2].

We became interested in HILIC during development work on atosiban [**1**, 1-(3-mercaptopropanoic acid)-2-(O-ethyl-D-tyrosine)-4-L-threonine-8-L-ornithine-oxytocin, see Fig. 1], an oxytocin antagonist that may be used for the treatment of preterm uterine activity [12]. We required a detailed knowledge of the synthesis-related impurities and degradation products. Complementary analytical methodologies were needed to ensure that all relevant peptides were detected in matrices such as drug substances, reaction product mixtures, and therapeutic formulations. We found that HILIC offered a unique selec-

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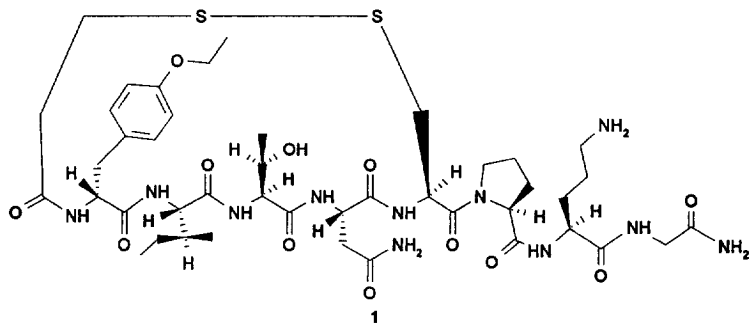

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Fig. 1. Molecular structure of atosiban.

tivity for separating a number of potential impurities and degradation products with structures that were closely related to **1**. This selectivity will be illustrated below for atosiban and a series of diastereomeric peptides that differ only by the configuration at one chiral center (i.e. epimers; Table 1).

Typically, peptides have been separated by HILIC on a negatively charged cation-exchange stationary phase (polysulfoethyl aspartamide) or on a polar neutral stationary phase (polyhydroxyethyl aspartamide) [2–5]. We have also found that peptides can be separated by HILIC on amine bonded-phases which have been used for the separation of carbohydrates [2–9], but seldom for peptides [10]. Use of such positively charged stationary phases with our positively charged peptides was feasible, since the differences between charged and uncharged stationary phases decrease with increasing levels of organic solvents in the mobile phase [2].

Table 1
Atosiban diastereomers

Compound	Structure
1	Atosiban
2	L-Tyr(Et)^2 diastereomer
3	L-*allo*-Ile^3 diastereomer
4	D-*allo*-Ile^3 diastereomer
5	L-*allo*-Thr^4 diastereomer
6	D-*allo*-Thr^4 diastereomer
7	D-Asn^5 diastereomer
8	D-Cys^6 diastereomer
9	D-Pro^7 diastereomer
10	D-Orn^8 diastereomer

2. Experimental

2.1. Chemicals

Atosiban and its diastereomers were obtained from Ferring Peptide Production (Malmö, Sweden). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Trifluoroacetic acid was from Pierce (Rockford, IL, USA). Ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). γ-Cyclodextrin and lithium hydroxide were obtained from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Fisher (Springfield, NJ, USA).

2.2. Liquid chromatographic apparatus, columns, and mobile phases

The HPLC systems consisted of Series 4 solvent delivery systems and ISS-100 or 200 autosamplers (Perkin-Elmer, Norwalk, CT, USA), column ovens (Waters, Milford, MA, USA), and diode-array detectors (Hewlett-Packard, Palo Alto, CA, USA).

All HILIC separations required the use of mobile phase as the injection solvent to obtain satisfactory results.

HILIC separations were conducted on a new Spherisorb NH_2 column (Alltech, Deerfield, IL, USA; 250×4.6 mm, 5-μm particles, 80-Å pores, 220 m^2/g surface area) which was flushed with 2-propanol at 60°C (to reduce pressure) to remove the hexane shipping solvent and aqueous trifluoroacetic acid (pH 2.0, 75°C) to protonate the amino groups. The use of lower temperatures for the latter process

gave poorer chromatographic results. Ammonium acetate (0.77 g) and sodium perchlorate (140.46 g) were dissolved in 4 l of water (pH 6.36). Acetonitrile (3694 ml) was then added to this solution to give the mobile phase (Fig. 2).

HILIC was conducted on a YMC-Pack Polyamine II column (YMC, Wilmington, NC, USA; 250×4.6 mm, 5-μm particles, 120-Å pores, 300 m^2/g surface area) without pre-treatment. Ammonium acetate (1.54 g) was dissolved in water (330 ml) to provide a solution of pH 6.50. Acetonitrile (3670 ml) was added to this solution to give the mobile phase (Fig. 3).

HILIC was conducted on a new polyhydroxyethyl aspartamide column (PolyLC, Columbia, MD, USA; 200×4.6 mm, 5-μm particles, 300-Å pores) which was conditioned before use by flushing with aqueous 0.4 *M* ammonium formate solution (pH 5.0). A stock triethylammonium phosphate (TEAP) solution was prepared by adding 125 ml of 2 *M* phosphoric acid (67.8 ml of 85% phosphoric acid diluted to 500 ml with water) to 200 ml of water, adjusting the pH to 5.3 with triethylamine, and diluting the resulting solution with water to 500 ml. Solution A was prepared by mixing 40 ml of the stock TEAP solution, 160 ml of water, and 1800 ml of acetonitrile. Solution B was prepared by mixing 20 ml of stock TEAP solution, 480 ml of water, and 500 ml of 2-propanol. The mobile phase was prepared by mixing 1940 ml of solution A and 60 ml of solution B (Fig. 4).

RP-HPLC was conducted on Inertsil ODS-2 columns (MetaChem, Torrance, CA, USA; 250×4.6 mm, 5-μm particles, 100-Å pores). The aqueous component of the mobile phase was obtained by adjusting the pH of water to 3.3 with trifluoroacetic acid. This solution (750 ml), methanol (100 ml), and acetonitrile (150 ml) were mixed together to give the mobile phase (Fig. 5).

2.3. Capillary electrophoresis apparatus and conditions

Capillary electrophoresis was performed on a Model 270A-HT instrument (Perkin-Elmer, Norwalk, CT, USA) with bare fused-silica capillaries that were obtained from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were flushed with 1 *M* NaOH before use. Water was used as the injection solvent.

A 10-ml volume of 1 *M* phosphoric acid was diluted to 90 ml with water and the pH was adjusted to 2.5 with 2 *M* lithium hydroxide. The resulting solution was diluted to 100 ml with water (pH 2.5). The electrophoretic buffer (Fig. 6) was prepared by dissolving γ-cyclodextrin (162.7 mg) in 25 ml of phosphate buffer.

2.4. Electrospray mass spectrometric instrument and conditions

Mass spectral analyses were carried out with a Finnigan MAT 900 (Bremen, Germany) double-focusing instrument equipped with a Finnigan MAT electrospray ionization interface.

3. Results and discussion

The conditions for the HILIC chromatography of atosiban and nine diastereomers (Table 1) were optimized on an aminopropylsilane column (Spherisorb NH_2; Fig. 2). This method separated all ten diastereomers and only one diastereomer eluted in the vicinity of atosiban. Fortunately, this latter diastereomer (**3**) was well separated by CE (Fig. 6).

Analyses times were long but reduced times could be obtained at higher flow-rates with some sacrifice of resolution (Fig. 2). High concentrations of salt (250 m*M* sodium perchlorate) were required to obtain acceptable peak shape. Sodium perchlorate was chosen because this salt is transparent in the 200–210 nm UV region monitored (for adequate sensitivity for impurities) and soluble in mobile phases with high acetonitrile content [2]. Retention of analytes was very sensitive to the water content of the mobile phase. For example, increasing the water content from 7.65% to 9% or 12% reduced the atosiban retention time to 65% or 25% of the original value, respectively. Use of the 12% water level caused peaks to merge.

Although HILIC on the Spherisorb NH_2 column separated all of the diastereomers, the method (Fig. 2) was not compatible with electrospray LC–MS because of the non-volatile salts in the mobile phase

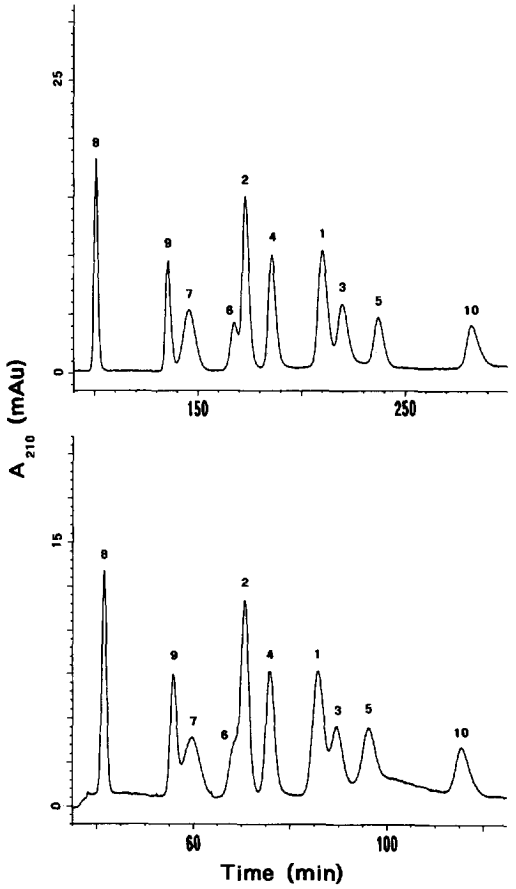

Fig. 2. HILIC on Spherisorb NH_2 column (40°C). Mobile phase (isocratic), 2.5 m*M* ammonium acetate and 250 m*M* sodium perchlorate in CH_3CN–H_2O (92.35:7.65%, v/v); detection, 210 nm; flow-rate, 0.5 ml/min (top), 1.2 ml/min (bottom); sample, 0.07–0.11 mg/ml of each diastereomer; 20-μl injection.

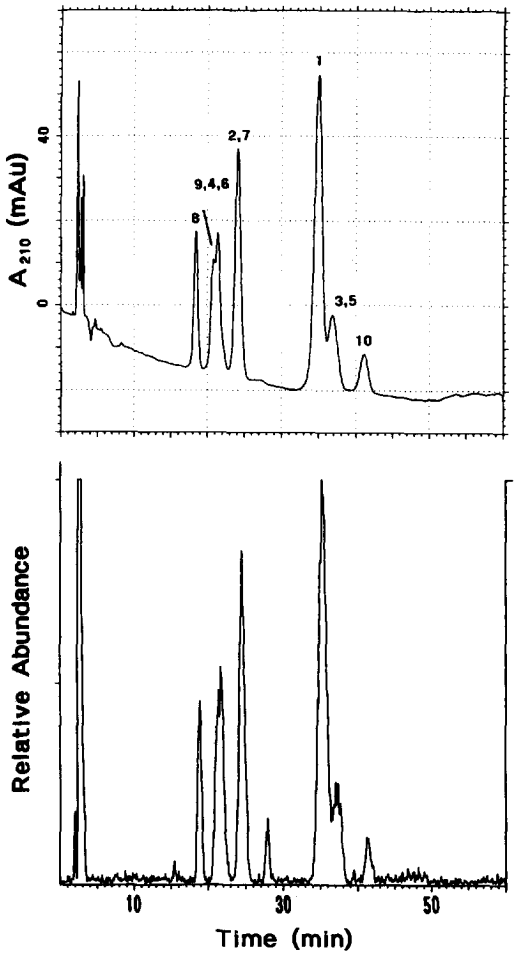

Fig. 3. HILIC on Polyamine II column (60°C). Mobile phase (isocratic), 5.0 m*M* ammonium acetate in CH_3CN–H_2O (91.8:8.2%, v/v); UV detection (top), 210 nm; flow-rate, 1.2 ml/min. The electrospray mass spectra, from which the total-ion chromatogram (bottom) was derived, show single intense signals at *m/z* 994 ($[M+H]^+$). Sample, 0.01–0.02 mg/ml of each diastereomer; 100-μl injection. The peak at 28 min is a low-molecular-mass impurity.

and the long analysis times. A second HILIC method Fig. 3 was developed for LC–MS on a proprietary Polyamine II column which contained secondary and tertiary (rather than primary) amino groups. This stationary phase permitted HILIC separations with only 5 m*M* ammonium acetate in the mobile phase. The selectivity was slightly different from that of the Spherisorb NH_2 method Fig. 2 and fewer diastereomers were separated. Nevertheless, the Polyamine II method proved highly successful for electrospray LC–MS (Fig. 3).

The HILIC separation of the diastereomer mixture on a polyhydroxyethyl aspartamide column (Fig. 4) was performed for comparison purposes since this column has been used for HILIC peptide separations [2]. The conditions were typical of those reported [2] for this column, but they were not fully optimized for the specific components of this test mixture. Selectivity was somewhat similar to the HILIC methods on the amine columns but this method was not as useful since three diastereomers eluted near to atosiban.

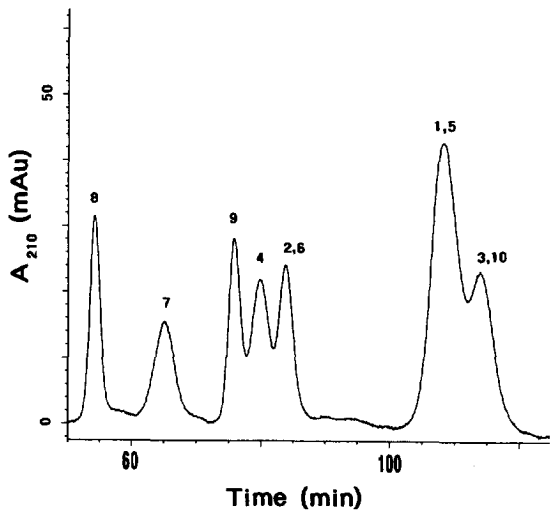

Fig. 4. HILIC on polyhydroxyethyl aspartamide column (35°C). Mobile phase (isocratic), 10 m*M* triethylammonium phosphate in CH_3CN–$(CH_3)_2CHOH$–H_2O (88.6:0.8:10.6%, v/v); flow-rate, 1.5 ml/min; detection, 210 nm; sample, 0.1–0.29 mg/ml of each diastereomer; 5-μl injection.

Reversed-phase HPLC of the diastereomers was likewise conducted for comparison purposes with a method that was not fully optimized for only these peptides (Fig. 5). All but three diastereomers eluted in the vicinity of atosiban. Selectivity was different from that of the HILIC methods.

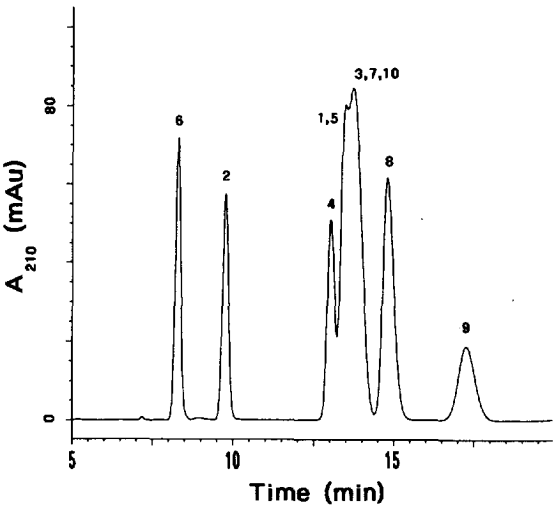

Fig. 5. RP-HPLC on Inertsil ODS-2 column (60°C). Mobile phase (isocratic), H_2O (pH 3.3 with CF_3COOH)–CH_3OH–CH_3CN (75:10:15%, v/v); flow-rate, 1.2 ml/min; detection, 210 nm; sample, 0.2–0.35 mg/ml of each diastereomer; 5-μl injection.

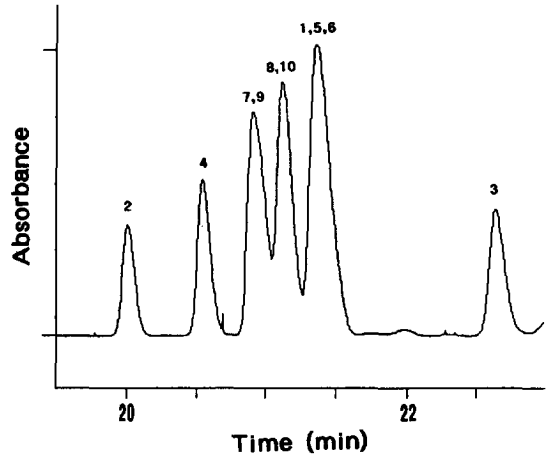

Fig. 6. Capillary electrophoresis with bare fused-silica capillary (30°C, 80 cm×75 μm, 60 cm to detector). Electrophoretic buffer, 100 m*M* phosphate (pH 2.5) with 5 m*M* γ-cyclodextrin; Voltage, 26 kV; detection, 195 nm; sample, 0.1 mg/ml of each diastereomer; 10-nl injection.

Free solution capillary electrophoresis of the diastereomer mixture was conducted with an electrophoretic buffer of 100 m*M* phosphate with 5 m*M* γ-cyclodextrin (Fig. 6). A pH of 2.5 was chosen to protonate the ornithine groups and reduce electroosmotic flow. Little separation was obtained in the absence of cyclodextrin because the charge to mass ratios of the diastereomers were nearly identical. The added cyclodextrin molecules probably formed inclusion complexes with the diastereomers and altered their mobilities [13,14]. Higher concentrations of γ-cyclodextrin did not improve resolution. Capillary electrophoresis separated fewer diastereomers than HILIC, but, in contrast to HILIC, the diastereomer **3** was well separated from atosiban and the other diastereomers in the mixture.

Hindered rotation about the Cys–Pro bond of oxytocin and related peptides has been suggested to lead to chromatographic band broadening [15]. This phenomenon may have produced some band broadening in the methods that are described here.

4. Conclusions

The results reported here suggest that the utility of HILIC may be comparable to that of RP-HPLC and

CE, at least when structurally similar peptides are involved. Amino columns appear to be alternatives to columns that have previously been reported for HILIC peptide analyses [2].

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