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Note

High-performance liquid chromatographic separation of α- and β-aspartyl peptides

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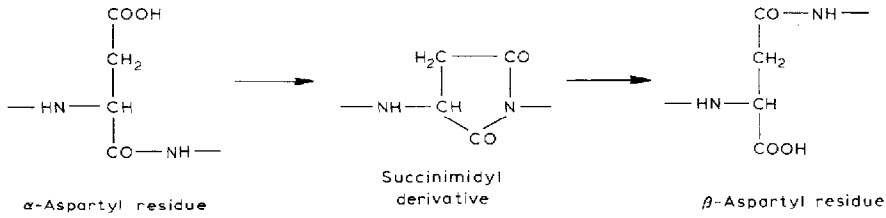
The aspartyl bonds, in particular environments and circumstances, can undergo $\alpha \rightarrow \beta$ rearrangements through a succinimidyl intermediate:



Schaaper *et al.*[1] published a method for the separation of [β-Asp[3]]-secretin from secretin by high-performance liquid chromatography (HPLC) using an ion-pair reagent, *i.e.*, fluorooctanoic acid. O'Hare and Nice[2] and Lewis *et al.*[3] reported the effect of a low pH on the HPLC separation of peptides and proteins.

The consideration of the different pK values for the α- and β-carboxyl groups in aspartic acid led us to investigate the effect of pH on the separation of synthetic peptides related to ceruletide** and containing α- and β-aspartyl residues, by HPLC in an isocratic mode.

EXPERIMENTAL

Chromatography was carrried out at constant pressure, using a DuPont Model 841 high-performance liquid chromatograph equipped with a detector operating at a fixed wavelength of 254 nm. The organic solvents used were HPLC grade (E. Merck, Darmstadt, F.R.G.). The peptides tested were obtained from our Peptide Department. All the other materials were of analytical grade. Samples for chromatography were freshly dissolved in the mobile phase and injected via a Rheodyne septumless valve with 50-μl loop onto a μBondapak C_{18} column (30 cm × 3.9 mm) (Waters

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** Ceruletide and caerulein are synonymous: ceruletide is the international non-proprietary name proposed by the World Health Organization.

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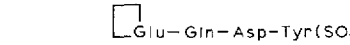
Assoc., Hartford, U.K.). All separations were performed isocratically, at room temperature (*ca.* 25°C); using a flow-rate of 1 ml/min and an inlet pressure of 800 p.s.i.

The organic solvents chosen for this study were those often used in reversed-phase HPLC because of their miscibility with water and low UV cut-off. Before use, all mobile phases were degassed by sonication and filtered through Millipore filters.

RESULTS AND DISCUSSION

A series of mobile phases was prepared with 40% methanol in aqueous 0.02 *M* acetic acid solution buffered at different pH values with ammonium hydroxide.

Ceruletide, which has the following structure[4]



and its [β-Asp^3]isomer were eluted without separation at pH 6–8. Both the retention time and the separation of the two compounds were increased with decreasing pH. A relatively low resolution was observed at pH 5 and 4, but complete separation was obtained only from pH 3.5 (retention time: 18.1 min for [β-Asp^3]-ceruletide *versus* 22.8 min for ceruletide)*.

The aspartic acid carboxyl group in the β-peptidic linkage is almost completely ionized (about 95%) at pH 3.5. Consequently the [β-Asp^3] isomer was more polar than ceruletide at this pH value and it was eluted sooner.

The resolution of these two products in RP-HPLC could probably be obtained also under more acidic conditions. However, we have chosen the pH 3.5 for the following reasons: (i) ceruletide contains in its structure a tyrosine-O-sulphate residue that is unstable under strongly acidic conditions; (ii) the prolonged use of mobile phases buffered at lower pH values could damage the chromatographic column.

In order to reduce the elution time and to optimize the analysis conditions, we modified the composition of the mobile phase. It should be noted that the presence of 10% acetonitrile in the eluent drastically reduced the retention time and resulted in improved peak shape and resolution. The best results were achieved using the following mobile phase: 30% methanol, 10% acetonitrile in 0.02 *M* ammonium acetate buffer at pH 3.5. Under these conditions we have analyzed ceruletide, [β-Asp^3]-ceruletide, [β-Asp^9]-ceruletide and [β-Asp^3, β-Asp^9]-ceruletide, as can be seen in Fig. 1.

The analogue with both aspartyl residues in the β-form was eluted before the other peptides, probably because of the contribution of two carboxyl groups in the dissociated form. The [β-Asp^9]-ceruletide and [β-Asp^3]-ceruletide had different elution times, perhaps as a consequence of their different molecular conformations. In ceruletide the N-terminal region up to approximately position 6 has been predicted by the method of Chou and Fasman[5] to be predominantly in a β-turn: the C-terminal

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* In all experiments carried out isocratically at pH 6 with different amounts of methanol and with different ratios of methanol: acetonitrile, the two peptides co-eluted. In paper electrophoresis at pH 2.7 in pyridine–glacial acetic acid–water (1:100:899, v/v/v) a good resolution between ceruletide and its [β-Asp^3] isomer was obtained only after enzymatic desulphatation.

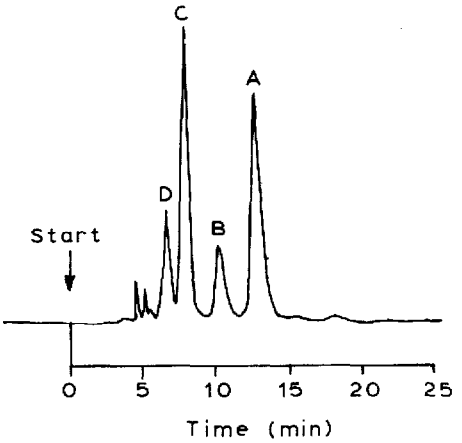

Fig. 1. Isocratic separation of known ceruletide analogues in a single chromatographic run, using a μBondapak C_{18} column. Start indicates the injection point. Peaks: A = ceruletide; B = [β-Asp³]-ceruletide; C = [β-Asp⁹]-ceruletide; D = [β-Asp³, β-Asp⁹]-ceruletide. Sample size: *ca.* 5 μg of each peptide (0.04 a.u.f.s.). For details, see text.

section of the molecule is present in a coil conformation. These predictions, similar to those obtained by other methods[6], remain to be tested by experimental analyses (X-ray crystallography, laser Raman spectroscopy, etc.), because one might have some reservations for applying those rules to small size peptides.

We consider this method to be of interest for preparative purposes, because the solvent is easily removed *in vacuo* or by lyophilization, without the risk of the individual compounds undergoing a change in structure, as confirmed by thin-layer chromatography of the collected fractions.

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