Journal of Chromatography, 586 (1991) 145–148
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 545

Short Communication

High-performance liquid chromatography of peptides at reduced temperatures: separation of isomers

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(First received January 21st, 1991; revised manuscript received June 6th, 1991)

ABSTRACT

Cholecystokinin analogues containing N-methyl amino acids were studied by reversed-phase high-performance liquid chromatography at reduced temperatures. A reduction in temperature to −17°C led to lower efficiency, but at the same time separations of *cis* and *trans* isomers (and some impurities) were achieved. The velocity constants for *cis–trans* equilibria were calculated.

INTRODUCTION

The effect of increased temperature on the chromatographic separation of peptides has been examined in several papers [1,2]. Usually, increased temperature leads to higher efficiency of the column and the separation of peptides is improved [3]. Lower temperatures generally decrease the efficiency of the column owing to the increased viscosity of the mobile phase, but it can be used for various physico-chemical studies of peptides. Increased energetic barriers allows for the separation of isomers which are otherwis inseparable [4–8]. For example, rapidly interconverting species were observed in the case of muramyldipeptides, and the chromatographic trace was used for calculation of the velocity constants of interconversion [9]. Detailed theoretical studies of this phenomenon were later performed by Melander *et al.* [10]. However, where rapid interconversion of two or more forms of the analyzed compound is likely (*e.g.*, anomers of sugars or glycopeptides or peptide conformers), we can expect to separate these forms only if the interconversion is slowed by decreasing the temperature. Mixtures of isomers often can be observed with peptides containing secondary amino groups (*e.g.*, proline or N-alkylated amino acids). We have applied this thinking to the separation of *cis* and *trans* peptide bond conformers of the cholecystokinin-related pentapeptide Gly–Trp–MeNle–Asp–Phe–NH_2 [11,12]. A decrease in temperature led not only to separation of the expected conformers, but also the separation of impurities present in the sample which were not observed under normal room temperature conditions of chromatography. We were able to show which peaks are conformers by their separation and re-equilibration to the original equilibrium mixture. We could also calculate the velocity constant of the *cis–trans* interconversion [9].

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0021-9673/91/$03.50

EXPERIMENTAL

Materials

The peptide Gly–Trp–MeNle–Asp–Phe–NH_2 was synthesized by solid-phase peptide synthesis using a Boc–benzyl (Bzl) strategy on a *p*-methylbenzhydrylamine polystyrene resin [11,12]. The peptide was purified by high-performance liquid chromatography (HPLC) on a Vydac-C_{18} column (25 × 1 cm I.D.) using a gradient elution with 0.1% trifluoroacetic acid and acetonitrile and/or methanol as the organic modifier.

Methods

Chromatography was performed on a Spectra-Physics SP-8700 instrument using a Spectra-Physics SP-8400 variable-wavelength detector. For the low-temperature experiments, the columns was jacketed in a Tygon tube (40 × 2 cm I.D.) connected to a thermostat adjustable from +60 to −20°C. Two columns were used: a Vydac C_{18} peptides and proteins column (7 μm; 25 × 0.4 cm I.D.) and an EDMA 40 column (10 μm; 8 × 0.8 cm I.D., Tessek, Prague, Czechoslovakia). Velocity constants were calculated according to published methods [9].

RESULTS AND DISCUSSION

Analogs of the C-terminal pentapeptide of cholecystokinin were synthesized in connection with studies of the receptor selectivity and central activities of these compounds [11,12]. The analogues containing N-methylnorleucine such as Gly–Trp–MeNle–Asp–Phe–NH_2 had an interesting range of biological activities and therefore were studied by ^1H NMR spectroscopy [11,12]. These studies have shown that these peptides exist as both *cis* and *trans* conformers about the Trp–MeNle peptide bond [12]. Therefore, we decided to try to separate them by HPLC. Under normal conditions (at room temperature) we observed only a single symmetrical peak (Fig. 1A). A decrease in temperature led to the broadening of this peak and at 0°C four peaks could be detected, although baseline separation was not achieved (Fig. 1B). We initially used acetonitrile as the chromatographic organic solvent, and therefore we could not decrease the temperature further. For this reason, we changed the organic modifier to a mixture of methanol and acetonitrile. In this system we could go down to −20°C, even though under these conditions the flow had to be decreased to 0.8 ml/min owing to the very high back-pressure (260 bar). At −17°C the mixture clearly contained five components (Fig. 1C). We were able to separate in a pure form the three main peaks. The fractions were collected into containers cooled in a bath containing a dry-ice–ethanol mixture. Analytical evaluation at −17°C showed their purity (Fig. 1D–F). When the fractions were heated to room temperature and reinjected into the same column kept at −17°C (Fig. 1G–I), fraction 1 did not change, but fractions 2 and 3 afforded an identical mixture of the two peaks of the peptide conformers. It may be predicted [13] that the fraction containing the less retained compound has the *trans* conformation-containing peptide.

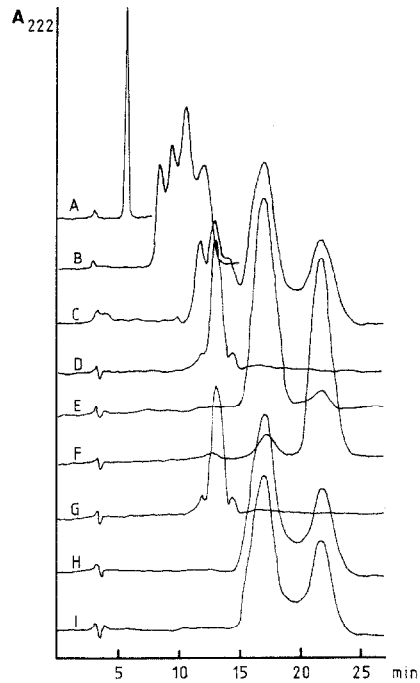

Fig. 1. Chromatography of an HPLC-purified sample of Gly–Trp–MeNle–Asp–Phe–NH_2 at various temperatures. Conditions: column, Vydac C_{18} peptides and proteins (25 × 0.4 cm I.D.); eluent, methanol–acetonitrile–0.1% trifluoroacetic acid (50:13:37); flow-rate 1 ml/min; detection at 222 nm. (A) 23°C; (B) 0°C; (C) −17°C; (D) fraction one (from C) kept frozen, analyzed at −17°C; (E) fraction two kept frozen, analyzed at −17°C; (F) fraction three kept frozen, analyzed at −17°C; (G) fraction one heated to room temperature, analyzed at −17°C; (H) fraction two heated to room temperature, analyzed at −17°C; (I) fraction three heated to room temperature, analyzed at −17°C.

On closer inspection, the low-temperature HPLC of the conformational mixture reveals (Fig. 2) that the trace contains a plateau between peaks B and A. This is similar to previous observations [4–9,13] and provides the possibility of calculating the velocity constant for interconversion of the *cis* and *trans* conformations of the peptide. In the present situation, however, it is not feasible to determine exactly the equilibrium constant because faster elution could not be achieved owing to the high back-pressures. The determination of the areas A, B and C [9] was also complicated by the fact that the peaks were not sharp. Therefore, we performed approximate calculations with the best data we could obtain. Two values of K were obtained; the first did not include areas A′ and B′ with the area C, and the second (in our opinion the more correct, because it reflects in part the computer-simulated situation) included these areas (for the theory of the calculation [9]). The value of K was roughly estimated to be 0.47. According to our calculation, the velocity constant of *cis–trans* interconversion at −17°C is between $1.7 \cdot 10^{-2}$ and $2.9 \cdot 10^{-2}$ min^{-1}. Even though our calculation is much simpler than that suggested by Melander *et al.* [10], the results are comparable.

The peaks eluted earlier from the Vydac C_{18} column at −17°C are probably impurities that were not observed or separated in the runs performed at room temperature. We decided to test different chromatographic materials to see if the separation of these impurities could be achieved. An alternative hydrophobic material used for reversed-phase HPLC is a modified polystyrene in which the polystyrene is coated with ethylene dimethylacrylate (EDMA 40). This column was made available to us in the form of a small (8 × 0.8 cm I.D.) experimental column, courtesy of Tessek. This column clearly separates the impurities from the main product, but a decrease in temperature does not lead to an improvement in the separation. On the contrary, the efficiency of the column is decreased and at −6°C unacceptably broad peaks with very high retention times were obtained. Hence, no complete separation of the conformers was achieved, and only shoulders on the broad peak of the main component of the mixture were observed (Fig. 3). However, it is important to note that this column provides an alternative to the usual C_{18} and other hydrocarbon-modified supports for reversed phase chromatography.

To observe the influence of reduced temperature on the separation of peptides having similar retention times, we tested the separation of oxytocin analogues containing D- and L-tetrahydroisoquinolinecarboxylic acid in position 2 [14]. In this instance, the separation was completely lost at decreased temperature owing to the poorer efficiency of the column (the situation was the same using either a Vydac C_{18} or a EDMA-40 column; the latter result

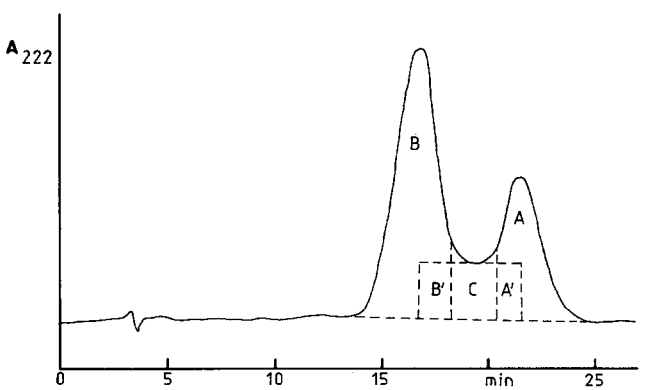

Fig. 2. Chromatographic trace of re-equilibrated mixture of *cis* and *trans* conformers of Gly–Trp–MeNle–Asp–Phe–NH_2 used for the calculation of velocity constants. For details see ref. 9. Conditions as in Fig. 1; temperature, −17°C.

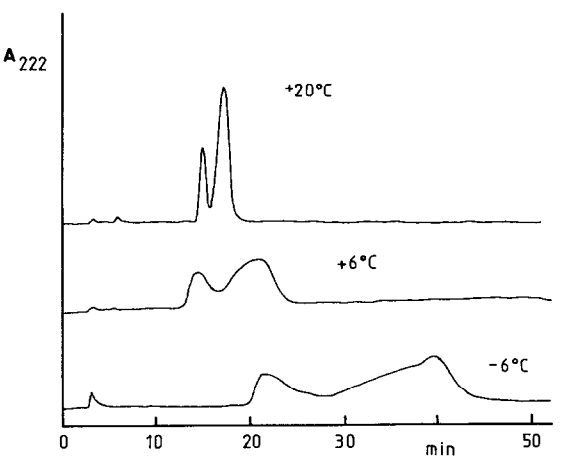

Fig. 3. Chromatography of HPLC-purified sample of Gly–Trp–MeNle–Asp–Phe–NH_2 at various temperatures. Conditions: columns, EDMA 40 (8 × 0.8 cm I.D.); eluent, methanol–acetonitrile–0.1% trifluoroacetic acid (50:3:47); flow-rate 1 ml/min; detection at 222 nm.

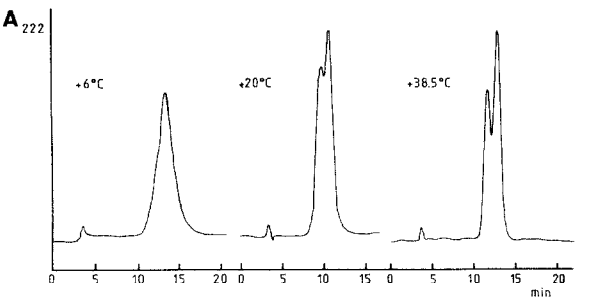

Fig. 4. Influence of temperature on the separation of [2-(D- and L-tetrahydroisoquinolinecarboxylic acid)]oxytocin. Conditions: column, EDMA 40 (8 × 0.8 cm I.D.); eluent, methanol–acetonitrile–0.1% trifluoroacetic acid (50:3:47); flow-rate 1 ml/min; detection at 222 nm.

is illustrated in Fig. 4), but was improved by heating the column.

In conclusion, it is hard to predict *a priori* the influence of lowering the temperature on the separation of peptides. Decreased efficiency and increased retention times may be expected. However, in some instances, as in the separation of isomers that interconvert at room temperature and thus require reduced temperature to slow the rate of interconversion, separation can only be achieved by lowering the temperature. The use of alternative solid supports for reversed-phase HPLC is strongly recommended for verification of the purity of synthetic peptides.

ACKNOWLEDGEMENTS

This work was supported by US Public Health Grant DK 36289 and the National Science Foundation.

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