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Reversed-phase liquid chromatography on a microspherical carbon column at high temperature

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Abstract

The chromatographic conditions for the use of a spherical carbon column at high temperatures up to 160°C are described. The column heating system and the eluate cooling system are also described. Under the conditions studied, it was found that the carbon column at elevated temperature was stable and could be applicable to any compounds that are durable under these conditions. The peptide bonds at this high temperature and at this acidity were still stable enough for analysis, but the indole residues showed poor stability.

Keywords: Temperature effects; Carbon columns; Stationary phases, LC; Peptides; Amino acids

1. Introduction

Temperature is one of the most important parameters in liquid chromatography, even for the separation of peptides, that is, processing at higher temperatures gives a better height equivalent to a theoretical plate (HETP) and better symmetry of the peaks [1–14]. However, owing to the volatile nature of the mobile phase used in reversed-phase liquid chromatography and the poor stability of packing materials such as silica, high temperatures are not necessarily suitable for liquid chromatography. In fact, some reports concerned with high-temperature processing indicated that processing could only be carried out at temperatures lower than 100°C.

Recently, we have studied chromatography using a carbon packing material for liquid chromatography [15–18]. The stability of this packing material at high temperatures, especially with different types of solvents and at various acidities and basicities, was tested through the comparison of chromatograms of peptides before and after heat treatment. The regulation of the column temperature and preheating of the solvent were performed by a block heating system.

The results indicated that processing at high temperatures is applicable to the analysis of strongly adsorbed peptides and to faster chromatography.

2. Experimental

2.1. Amino acids and peptides

Angiotensin I (amino acid sequence INLKALAALAKKIK-NH_2) and substance P

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(amino acid sequence RPKPQQFFGLM-NH_2) were purchased from Protein Research Foundation (Osaka, Japan), glycyl-DL-tryptophan from Nutritional Biochemicals (Cleveland, OH, USA) and L-tryptophan and other amino acids from Wako (Osaka, Japan).

2.2. Materials for chromatography

A prepacked column of Carbonex (TCAS-070510) (100 mm × 4.6 mm I.D.), with average particle size 3.5 μm, specific surface area over 30 m^2/g, specific pore volume 0.35 ml/g, apparent density: 0.57 g/ml, and pore size range 10–700 Å, was obtained from Biotech Research (Saitama, Japan).

Acetonitrile (chromatography grade) was obtained from Merck (Darmstadt, Germany). Water was purified by passage through a mixed-bed ion-exchange resin and using a distillation system (WG-25; Yamato Scientific, Tokyo, Japan), and before use, the deionized, distilled water was purified further with an ultra-pure water system (Milli-Q SPTOC; Millipore, Milford, MA, USA. Trifluoroacetic acid (TFA) (sequencial grade) and other chemicals were of analytical-reagent grade from Wako, unless mentioned otherwise.

2.3. Apparatus and chromatographic procedure

A Yokogawa (Tokyo, Japan) LC-100 HPLC system controlled by a PC-9801 microcomputer (NEC, Tokyo, Japan) was employed. In order to allow automated analysis, this system was attached to an autosample injector (Model 231/401; Gilson, Villiers-le-Bel, France). The stainless steel tubing connecting the modules was changed to thinner tubing (0.10 mm I.D.) than the regular site. Sample amino acids and peptides (2–15 μg) were loaded on to the column and eluted with a linear gradient from 10% to 70% acetonitrile in 0.1% TFA at a flow-rate of 1.0 ml/min [15–17]. The eluate was cooled to the room temperature and monitored by measuring the absorbance at 210 nm. The whole system was controlled by software designated PC-workstation. Under these conditions, the hold-up time (t_0) was determined through the chromatography of acetone.

2.4. Regulation system of column temperature

The regulation of the column temperature and preheating of the solvent were performed by a block heating system which was composed of a steel or aluminium block, with aluminium foil packed into any gaps between the block and column or tubing. The carbon column was placed on the heating pad and bound to the pad with thin copper tape. The column temperature was checked with a thermometer inserted between the tape and the heating pad. The heating system was composed of the heating pad and voltage controller (Fig. 1). In preliminary conditioning, the relationship between voltage and column temperature were determined. The eluate was cooled to the room temperature by passing through the stainless-tubing (0.10 mm I.D.) provided with aluminium foil fins and an enforced air stream. Because the boiling point of the eluent was lower than 100°C (acetonitrile, 81.6°C), back-pressure (about 15 kgf/cm^2) had to be applied at the outlet of the eluent (the pressure limit for this UV detector, 20 kgf/cm^2).

3. Results and discussion

3.1. Elution behavior in high-temperature chromatography on a carbon column

A standard solution of L-tryptophan (5.0 μg per 5.0 μl in distilled water) was loaded on the column at room temperature and eluted with standard gradient elution. The chromatogram is shown in Fig. 2a. The column temperature was raised to 46, 86, 94, 127 and 160°C and the eluate was cooled to room temperature before passing to the UV detector. The chromatograms are shown in Fig. 2b, c, d, e and f, respectively. The results indicated that the cooling fins attached to the stainless-steel tubing were adequate for cooling the column to room temperature. The re-

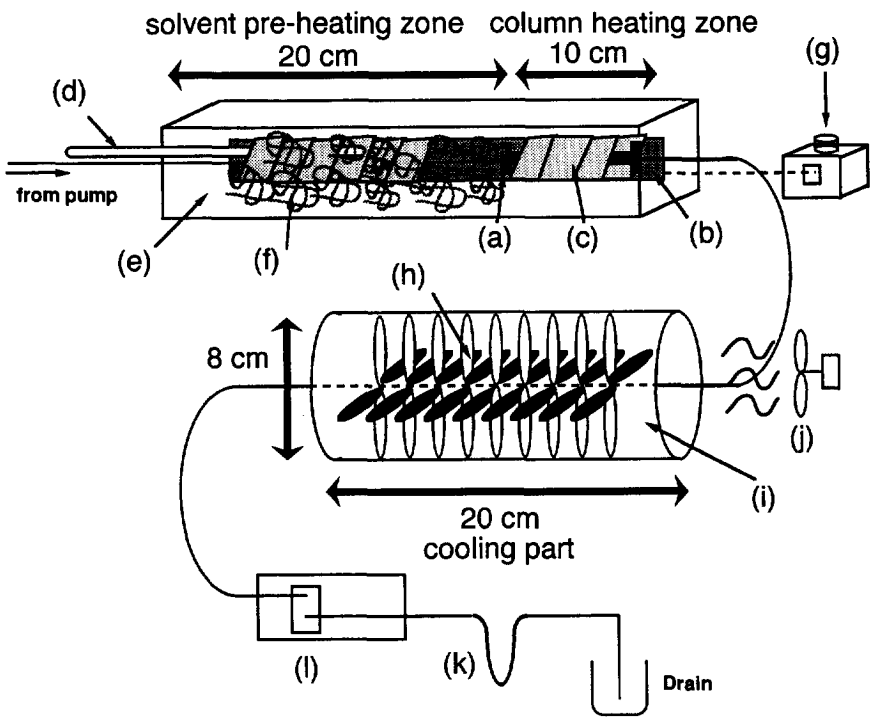

Fig. 1. Construction of the temperature regulation system on the carbon column. Carbon column (a) was placed on a heating pad (b) and bound to the pad with a thin copper tape (c). A thermometer or thermocouple (d) was inserted between the tape and the heating pad. The column with heating pad was placed in a steel block (e) and aluminium foil (f) was packed into any gaps between the block and column. Thus, the section for preheating the solvent was about 20 cm long and that for column heating was about 10 cm long. The temperature of the column was controlled with a voltage controller (g). The eluate was introduced into a tube with ten pairs of aluminium foil fins (h) with an air cooling duct (i) and an enforced air stream with a cooling fan (j). Teflon tubing with sharp turns (k) to give the required back pressure was attached at the outlet of the eluent. (a) Carbon column (100 mm × 4.6 mm I.D.); (b) heating pad (275 × 18 × 4 mm, 100 V, 100 W); (c) thin copper tape (0.1 mm thick, 20 mm width); (d) mercury thermometer (scale limit 300°C); (e) steel block (40 × 40 × 300 mm), sometimes covered with polystyrene foam sheet; (f) aluminium foil packing; (g) voltage controller (100 V, 500 W); (h) aluminium foil fin (20 mm width, 10 cm length); (i) air cooling duct (20 cm × 8 cm I.D.); (j) cooling fan (100 V, 6 W; diameter of fan, 70 mm); (k) Teflon tubing (0.25 mm I.D.) with sharp turns; (l) UV detector.

tention of L-tryptophan decreased with increase in the column temperature. At 160°C, the retention time had decreased to about one third of that at room temperature and the peak width was smaller, although some leading was observed. This may indicate some degradation of tryptophan under these acidic and high-temperature conditions. It is known that the degradation can be prevented by the addition of a reducing agent to the elution solvent.

Fig. 3 shows a comparison among the chromatograms of substance P obtained at (a) room temperature, 25°C, (b) 127°C and (c) 152°C. In these cases also, decreases in retention time at higher temperatures were observed, but not as large as in the case of tryptophan. Also, at 152°C, the peak height was smaller and the peak width was greater. This tendency was noticed with angiotensin I also, but not with the general peptides. The chromatogram showed approximately a single peak, and the degradation or hydrolysis of the peptide bond under these conditions was not plausible, but might be on the C-terminal amide.

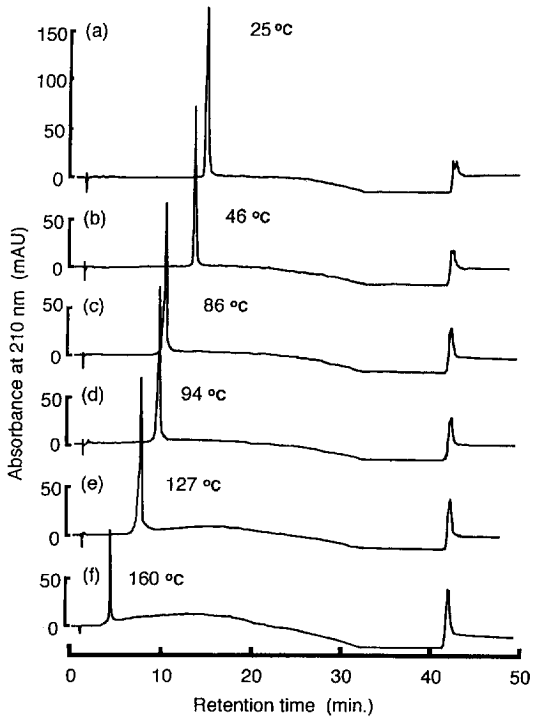

Fig. 2. Elution behavior in high-temperature chromatography on the carbon column. L-Tryptophan (5 μg) was loaded on the carbon column and eluted with a linear 30-min gradient from 10 to 70% (v/v) acetonitrile in 0.1% TFA at a flow-rate of 1.0 ml/min. Temperature of column: (a) room temperature (25°C), (b) 46°C, (c) 86°C, (d) 94°C, (e) 127°C and (f) 160°C.

3.2. Correlation between temperature and pressure drops of carbon column

At various temperatures, the pressure drops of the carbon column were estimated at the initiation of the gradient and at the end of the gradient. These column pressures were determined at the pump head at a flow-rate of 1.0 ml/min in isocratic elution. The results are shown in Fig. 4. Generally, the pressure drops were larger at low temperature and smaller at high temperature. And higher temperatures, the pressure drops became smaller. At temperatures higher than 100°C, no differences in the pressure drops were observed between the initial and final stages. The pressure drop at 160°C is about 20% of that at room temperature, so we can apply about a five times high pressure on the column.

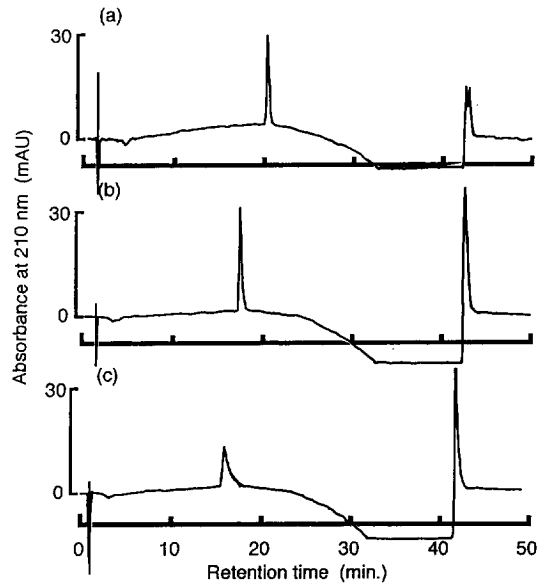

Fig. 3. Elution profiles in high-temperature chromatography of substance P on the carbon column. A 20-μl volume of substance P solution (100 μg/ml) was loaded on to the carbon column. Chromatographic conditions as in Fig. 2. Temperature of column: (a) room temperature (25°C), (b) 127°C and (c) 152°C.

The flow-rate would also increase fivefold, that is, to 5 ml/min, and one cycle of analysis would be finished within 6 min.

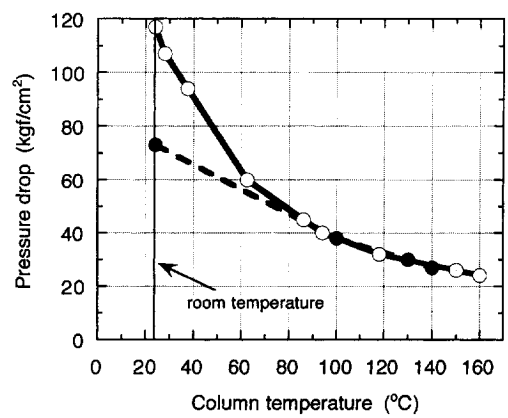

Fig. 4. Correlation between temperature and pressure drops of carbon column. On the abscissa, the temperatures of the carbon column and on the ordinate, the pressure drops of the column are indicated. ○ = 10% acetonitrile in 0.1% TFA; ● = 70% acetonitrile in 0.1% TFA.

3.3. Influence of temperature on the capacity factor

Figs. 2 and 3 show the effect of temperature on the capacity factor, k'. The hold-up time was determined with acetone to be 1.13 min. The effects of temperature were therefore observed with various substances more precisely. The results are shown in Fig. 5. The largest effect was observed in the case of tryptophan, that is, the interaction of indole groups with the graphitic carbon was the most heat-sensitive.

3.4. Influence of temperature on the peak width

At various temperatures, the peak widths were estimated with various compounds. The results are shown in Fig. 6. The peak width was calculated at the peak half-height using a function of the system software. The rate of decrease of the peak width was larger with tryptophan and indole-containing compounds. However, these compounds also showed a decrease in the capacity factor k', so the change in plate number was not large (not shown). For the peptides angiotensin I and substance P, better results were obtained but at 160°C the peak width increased 2–3-fold. The reason is not known, but it might be due to the hydrolysis of the C-terminal amide.

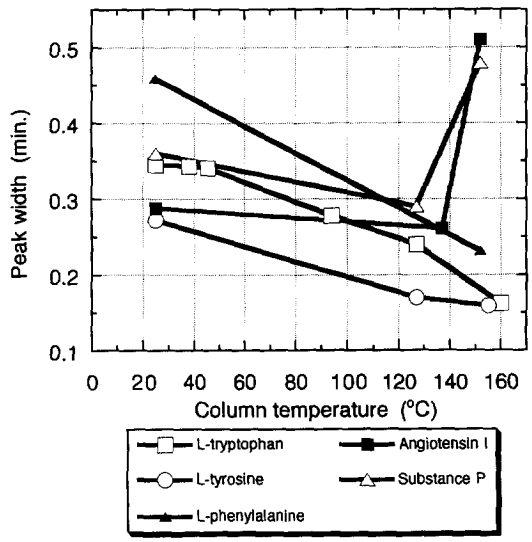

Fig. 6. Influence of temperature on peak width. Chromatography conditions as in Fig. 2. Sample and symbols as in Fig. 5.

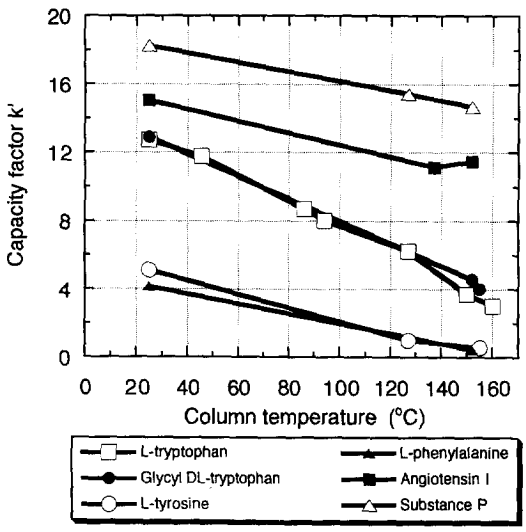

Fig. 5. Influence of temperature on capacity factors k', of the sample amino acids and peptides. Chromatographic conditions as in Fig. 2. □ = L-tryptophan (15 μg per 15 μl); ○ = L-tyrosine (15 μg per 15 μl); ● = glycyl-DL-tryptophan (15 μg per 15 μl); ■ = angiotensin I (2 μg per 20 μl); △ = substance P (2 μg per 20 μl); ▲ = L-phenylalanine (30 μg per 15 μl).

3.5. Durability of carbon column

The carbon column could be used for up to ca. 100 cycles at elevated temperature. The boiling points of acetonitrile and water are 81.6 and 100°C, respectively. At 160°C or at higher temperatures it still works normally in this system. The stainless-steel column tubing would be affected under acidic conditions and at high temperatures, but the carbon packing material is very durable under these conditions. This column can be treated with very alkaline solutions, e.g., up to 4% sodium hydroxide, to clean up any scum on the column. Hence the chromatographic system could be operated even at temperatures higher than 180°C, but under these conditions trypotophan was oxidized and degraded easily and there were possibility of damage to the

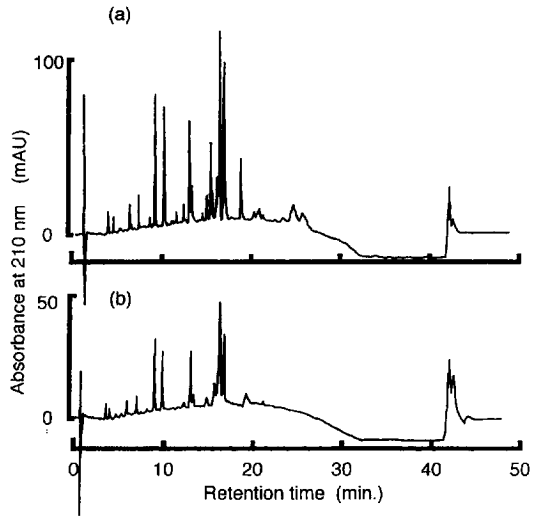

Fig. 7. Comparison of chromatograms of tryptic peptides of calmodulin on the carbon column before and after high-temperature chromatography. (a) Tryptic hydrolysate of calmodulin (about 37 μg) was applied to the carbon column under standard conditions. These peptides were eluted with linear 30-min gradient from 10 to 70% (v/v) acetonitrile in 0.1% TFA at a flow-rate of 1.0 ml/min at room temperature, before high-temperature chromatography. (b) after 50 cycles of high-temperature chromatography, the hydrolysate of calmodulin (about 20 μg) was loaded on the column. Other chromatographic conditions as in (a).

column and its accessories. Therefore, procedures to prevent the degradation, e.g., the addition of a reducing agent, need to be studied as in the case of regular amino acid analysis. The durability of the carbon column, however, was further indicated by the comparison of chromatograms between the first cycle and 50 cycles. The chromatograms of calmodulin hydrolysate at room temperature are shown in Fig. 7. Virtually no deterioration of the column was observed. Hence the durability of the column seems to be good and these high temperatures and alkaline treatment conditions correspond to the sterilization procedure for columns used in food or pharmaceutical processing.

4. Conclusions

Under high-temperature conditions, the peak shape of amino acids and peptides was good, that is, with small leading or tailing and a smaller peak width, especially for peptides with aromatic and indole residues. The chromatographic pattern of the hydrolysate of calmodulin did not change after many cycles of high-temperature chromatography, indicating that microspherical carbon packings show good stability in this high-temperature procedure with the possibility of rapid analysis under these conditions. The results indicate that one can apply high temperatures in carbon column chromatography, provided that the sample remains durable in rapid separation without a previous sterilization procedure. This suggests future applications in the food industry, e.g., for the very rapid separation of oligopeptides on a large-scale preparative column without any previous sterilization.

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