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Separation of peptide derivatives by pH-zone-refining counter-current chromatography

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Abstract

pH-Zone-refining counter-current chromatography was applied to the preparative separation of oligopeptide derivatives containing up to three amino acids. Both acidic benzyloxycarbonyl peptides and basic peptides–β-naphthylamide were successfully separated with two-phase solvent systems composed of methyl *tert.*-butyl ether, 1-butanol, acetonitrile and water using a set of suitable retainer and eluent reagents for each group. The preparative ability of the method was demonstrated in the separation of multigram quantities of analyte with a standard separation column with a total capacity of 325 ml. The effects of important factors such as polarity of the two-phase solvent system, concentrations of the eluent base and the retainer acid were discussed.

1. Introduction

pH-Zone-refining counter-current chromatography (CCC) is a recently developed preparative method which provides important advantages over the conventional CCC method including an over 10-fold increase in sample loading capacity, high concentration of fractions, and concentration of minor impurities. The method uses a retainer acid (or base) in the stationary phase to retain the analytes in the column and an eluent base (or acid) to elute the analytes according to their pK_a values and hydrophobicities [1–15]. It produces a succession of highly concentrated rectangular peaks with minimum overlap similar to those observed in displacement chromatography [16]. The method has been successfully applied to the separation of a variety of compounds including acidic [1,4–7,11] and basic [12] amino acid derivatives, many hydroxyxanthene dyes [2–4,8–10], alkaloids from natural sources [13], geometrical [14], structural [15] and optical [17] isomers from synthetic products, chiral compounds, etc.

In this paper, pH-zone-refining CCC is applied to the separation of peptide derivatives. In order to reduce the complexity of their zwitterionic nature and to enhance the pH effects on their partition coefficient, a set of peptides with protected terminal amino or carboxylic groups was selected. A series of experiments demonstrated the effects of factors such as sample size, polarity of the solvent system, concentration of the retainer acid and eluent base on the separation.

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2. Experimental

2.1. Apparatus

A commercial model (Ito Multilayer Coil Separator/Extractor; P.C. Inc., Potomac, MD, USA) of the high-speed CCC centrifuge was used throughout the present studies. The detailed design of the apparatus was given elsewhere [18]. The apparatus holds a multilayer coil separation column and a counterweight symmetrically at a distance of 10 cm from the central axis of the centrifuge. The column holder is equipped with a plastic planetary gear which is engaged to an identical stationary sun gear mounted around the central axis of the apparatus. This gear arrangement produces the desired planetary motion to the column holder, i.e., rotation about its own axis and revolution around the centrifuge axis in the same direction at the same rate. This planetary motion also prevents the flow tubes from twisting during revolution, thus permitting the elution of the mobile phase through the column without the use of rotary seals.

The separation column consists of a single piece of 160 m × 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) wound around the column holder hub with 16 layers and 325 ml capacity. Each terminal of the column was connected to a flow tube (0.85 mm I.D. PTFE) (Zeus Industrial Products) by the aid of a set of tube connectors (Upchurch Scientific, Oak Harbor, WA, USA) which were rigidly mounted on the holder flange. A narrow-bore PTFE tube (5 m × 0.3 mm I.D.) (Zeus Industrial Products) was placed at the outlet of the column to stabilize the effluent flow, thus reducing noise level in recording the elution curves.

The speed of the apparatus was regulated with a speed controller (Bodine Electric, North Chicago, IL, USA).

2.2. Reagents

Acetonitrile, methanol, trifluoroacetic acid (TFA) and triethylamine (TEA) were glass-distilled chromatographic grade (Baxter Healthcare, Muskegon, MI, USA). Methyl *tert.*-butyl ether and 1-butanol were also HPLC grade while aqueous ammonia, hydrochloric acid and acetic acid were reagent grade (all from Fisher Scientific, Fair Lawn, NJ, USA). Peptide derivatives used in the present studies include (Z = benzyloxycarbonyl): Z–Gly–Asp, Z–Gly–Glu, Z–Gly–Ala, Z–Gly–Val, Z–Gly–Phe, Z–Gly–Leu, Z–Gly–Gly–Gly, Z–Gly–Gly–Val, Z–Gly–Gly–Phe, Z–Gly–Gly–Leu (Novabiochem, La Jolla, CA, USA), Z–Gly–Gly, Z–Ala–Ala, Z–Leu–Ala (Bachem California, Torrance, CA, USA), Z–Ala–Gly–Gly, Z–Gly–Gly–Ala, Z–Gly–Leu–Gly, H–Gly–Gly–βNA (βNA = β-naphthylamide), H–Ala–Ala–βNA, H–Gly–Trp–βNA (Bachem Bioscience, Philadelphia, PA, USA)

2.3. Preparation of solvent phases and sample solutions

The solvent pairs used for CCC separations were prepared as follows: desired volumes of methyl *tert.*-butyl ether, acetonitrile, 1-butanol and distilled water were thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. TFA (5.4–21.6 m*M*) or TEA (5 m*M*) was added to the upper organic phase which was then used as the stationary phase. The mobile phase was prepared by adding aqueous ammonia (2.7–11 m*M*) or HCl (5 m*M*) to the lower aqueous phase.

Sample solutions were made by dissolving a set of peptide derivatives in 10–50 ml of the solvent usually consisting of about equal volumes of each phase. Each solution was sonicated for several minutes before injection into the column.

2.4. Separation procedure

In each separation, the column was first entirely filled with the organic stationary phase containing TFA or TEA. Then the sample solution

was injected through the sample port and the aqueous mobile phase was eluted through the column in the head-to-tail elution mode at a flow-rate of 3.0 ml/min (metering pump from Rainin, Emaryville, CA, USA) while the apparatus was rotated. The revolution speed was initially set at 600 rpm which was increased to 800 rpm after 20 min of elution. This minimized the carryover of the stationary phase after the mobile phase front emerged from the column. The effluent from the column was continuously monitored by absorbance at 206 nm (Uvicord S; LKB, Bromma/Stockholm, Sweden) and collected at 3.3 ml/tube (Ultrorac fraction collector, LKB). After all peaks were eluted, the centrifuge was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line at 80 p.s.i. (1 p.s.i. = 6894.76 Pa). The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

2.5. Continuous UV monitoring

As previously described [12], the elution curve produced by the continuous UV monitoring with an LKB Uvicord S needs further explanation: this monitor uses an interference filter (206 nm) which passes some amount of light in a broad range extending to the visual region. This particular feature helps us monitor the elution curves obtained by pH-zone-refining CCC in that highly concentrated peaks can be recorded at the lower portion of the chart while impurities which absorb different wavelengths can be detected above the main plateau as sharp peaks at the peak boundaries. Consequently, the chromatogram obtained by this monitor shows transmittance as a logarithmic scale, especially near the summit of the peak. Actual absorbance values at the highest portion of the peak (obtained with a Zeiss PM6 spectrophotometer at 206 nm) after diluting the fraction with methanol, were calculated to be about 200 units and obviously could not be directly detected with an ordinary UV monitor. For this reason, we do not indicate absorbance values in the chromatogram.

2.6. Analysis of CCC fractions

The pH value of each fraction was manually determined with a portable pH meter (Accumet portable laboratory; Fisher Scientific, Pittsburgh, PA, USA).

The peptide derivatives were identified by their partition coefficients (K_{std}) in the standard two-phase system composed of *n*-hexane–ethyl acetate–methanol–0.1 *M* NaOH (1:1:1:1, v/v). An aliquot of each fraction (usually 1 ml) was delivered into a test tube and dried under vacuum (Speed Vac concentrator; Savant, Hicksville, NJ, USA). Then, 2 ml of the standard solvent system (1 ml of each phase) were added to each tube and the contents vigorously shaken to equilibrate the solute. An aliquot of each phase (usually 100–200 μl) was diluted with 2 ml of methanol and the absorbance determined at 260 nm. The standard partition coefficient values (K_{std}) were obtained by solute concentration in the upper phase divided by that of the lower phase. The peak fractions were also analyzed by TLC (silica gel 60 F_{254}; EM Separations, Gibbstown, NJ, USA) with a chloroform–methanol–32% acetic acid (16:4:1, v/v) system and detection at 254 nm.

3. Results and discussion

Fig. 1 shows a typical chromatogram of Z-dipeptides obtained by the present method. The separation was performed with the solvent system methyl *tert.*-butyl ether–acetonitrile–water (2:2:3, v/v) in which 16 m*M* TFA (retainer acid) was added to the organic stationary phase and 5.5 m*M* ammonia (eluent base) to the aqueous mobile phase. A set of 8 different Z-dipeptides each in 100 mg amount was eluted as a broad rectangular peak. TLC analysis and partition

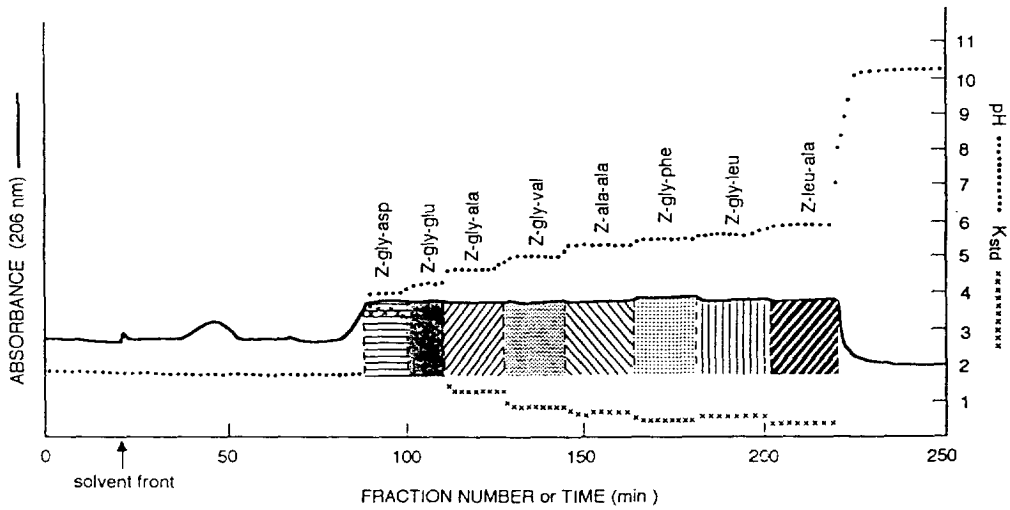

Fig. 1. Separation of eight Z-dipeptides by pH-zone-refining CCC. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; solvent system: methyl *tert.*-butyl ether–acetonitrile–water (2:2:3, v/v), 16 m*M* TFA in organic stationary phase (pH 1.83) and 5.5 m*M* NH_3 in aqueous mobile phase (pH 10.62); samples: eight Z-dipeptides as indicated in the chromatogram, each 100 mg dissolved in 50 ml solvent (25 ml each phase); flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm (600 rpm until 66 ml of mobile phase were eluted); stationary phase retention: 65.1% of the total column capacity.

coefficient measurements of each fraction revealed that each component was separated as a rectangular peak with minimum overlap as illustrated in the chromatogram. Sharp peak boundaries drawn in the chromatogram each contained no more than several milliliters of the mixing zone. The pH of the collected fractions formed a successive series of plateaus running in an upward staircase fashion (dotted line), each plateau corresponding to the specific analyte zone. These results indicate that the eight peptide derivatives with similar structures were efficiently separated.

Fig. 2 is a similar chromatogram of five Z-tripeptides in which only the terminal amino acid is different. The separation was performed with a more hydrophilic solvent system composed of methyl *tert.*-butyl ether–1-butanol–acetonitrile–water (2:2:1:5, v/v), where the organic stationary phase contained TFA (retainer acid) at 16 m*M* and the aqueous mobile phase contained ammonia (eluent base) at 2.7 m*M*. Again, each analyte was separated as a rectangular peak forming its own pH plateau as indicated by the dotted line in the diagram. TLC and partition coefficient analyses revealed that each pH plateau corresponds to the Z-tripeptide shown and that they elute in order of increasing hydrophobicity. As in the separation of Z-dipeptides (Fig. 1), each boundary between the neighboring peaks contained several milliliters of the mixing zone.

The present method also can be applied to the separation of basic analytes. Fig. 3 shows a typical separation of three free-amino-terminal naphthylamides (β-NA-dipeptides) using a solvent system methyl *tert.*-butyl ether–acetonitrile–water (2:2:3, v/v). The separation of these basic peptides was achieved by adding TEA (retainer base) to the organic stationary phase and eluting with an aqueous mobile phase containing HCl (eluent acid) at 5 m*M*. The TLC analyses of the eluted fractions indicated that the β-NA derivatives of three dipeptides were well resolved yielding three to seven pure fractions vs. one overlapping fraction and eluted in in-

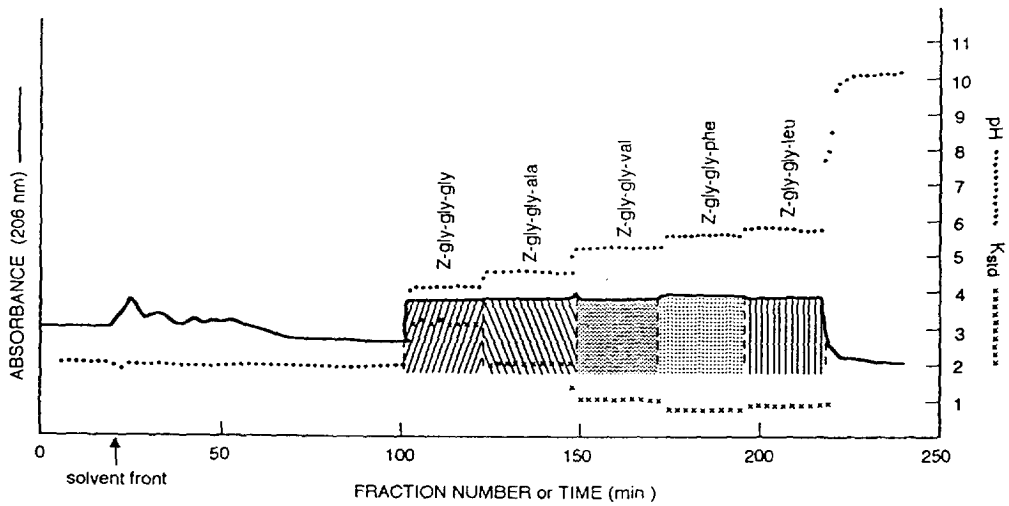

Fig. 2. Separation of five Z-tripeptides by pH-zone-refining CCC. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; solvent system: methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5, v/v), 16 m*M* TFA in organic stationary phase (pH 2.00), 2.7 m*M* NH_3 in aqueous mobile phase (pH 10.35); samples: five Z-tripeptides as indicated in the chromatogram, each 100 mg dissolved in 50 ml of solvent (25 ml each phase); flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm (600 rpm until 66 ml of mobile phase were eluted); stationary phase retention: 59.4% of the total column capacity.

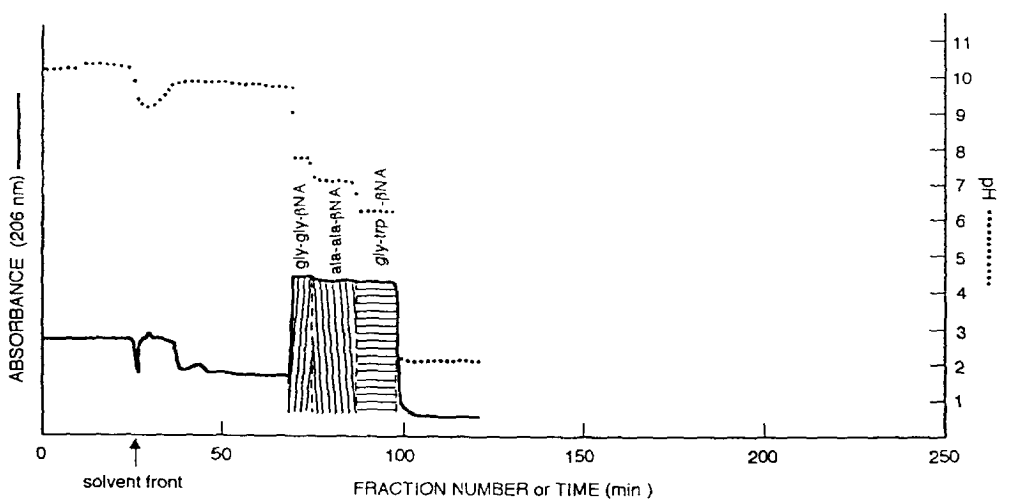

Fig. 3. Separation of three dipeptide βNA derivatives by the present method. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; solvent system: methyl *tert.*-butyl ether–acetonitrile–water (2:2:3, v/v), 5 m*M* TEA in the organic stationary phase (pH 10.18) and 5 m*M* HCl in aqueous mobile phase (pH 2.08); samples: three dipeptide βNA derivatives as indicated in the chromatogram, each 100 mg dissolved in 30 ml solvent (15 ml each phase); flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm (600 rpm until 66 ml of mobile phase were eluted); stationary phase retention: 67.0% of the total column capacity.

creasing order of their hydrophobicities. The percentage overlap will be reduced as the sample size is increased. As indicated by the dotted line in the chromatogram, pH plateaus display a downward staircase pattern, each corresponding to the respective analyte.

The preparative capability of the present method was demonstrated by a multigram separation of Z-dipeptides. Fig. 4 shows a chromatogram obtained from 3 g of a sample mixture containing Z–Gly–Gly, Z–Gly–Ala and Z–Gly–Leu each in 1 g. All components were resolved and eluted as highly concentrated successive rectangular peaks in 4.5 h. Impurities present in the sample solution were mostly accumulated at the boundaries of the third peak. A sharp boundary between the first and second peaks consisted of no more than several milliliters of the mixing zone while the boundary between the second and third peaks contained about 20 ml of the mixing zone including a sharp impurity peak. The above finding suggests that an increase in sample size nearly proportionally increases the width of the zone plateau while sharp peak boundaries are maintained, thus providing an even higher percentage yield of pure compound.

In the present method, the minimum width of the mixing zone is determined mainly by the partition efficiency of the column regardless of the applied sample size within a reasonable limit. Consequently, the percentage yield of pure fractions is improved by increasing the sample size as shown above. The method also provides a high recovery rate of the analyte since the separation column contains no solid support matrix which would cause adsorptive sample loss and deactivation.

In pH-zone-refining CCC, the concentration of the analyte in the eluted fractions is determined by and becomes close to the concentration of the eluent base (counterion) in the mobile phase, regardless of the initial analyte concentration in the sample solution [5,6]. Thus, the system is capable of concentrating the analytes from a dilute sample solution. In practice the sample loading capacity of the present system is limited by the solubility of the analytes in the organic stationary phase. When the concentration of the analyte in the stationary phase exceeds the solubility limit, solid precipitates are formed in the column leading to a detrimental loss of the stationary phase and finally plugging the tubing.

We found that the choice of solvent system has a great effect on the separation. Among the

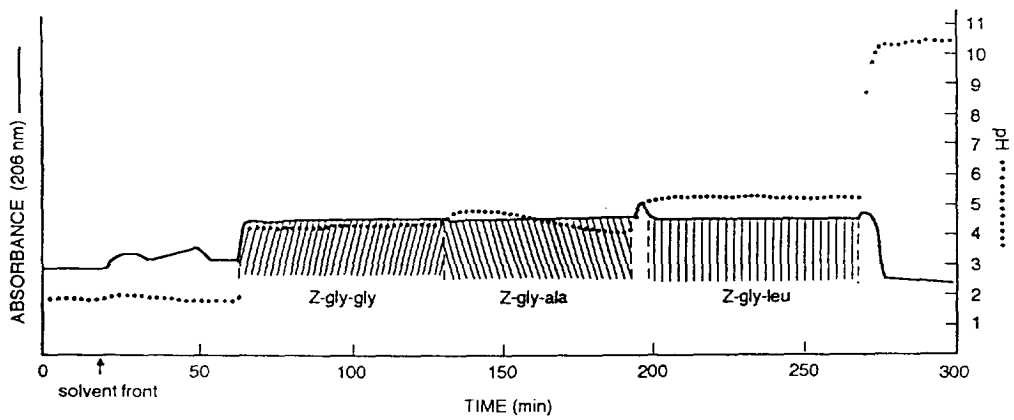

Fig. 4. Preparative-scale separation of Z-peptides by pH-zone-refining CCC. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; solvent system: methyl *tert.*-butyl ether–acetonitrile–water (2:2:3, v/v), 16 m*M* TFA in organic stationary phase (pH 2.00), 5.5 m*M* NH_3 in aqueous mobile phase (pH 10.62); samples: three Z-dipeptides as indicated in the chromatogram, each 1000 mg dissolved in 100 ml of solvent (50 ml each phase); flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm (600 rpm until 66 ml of mobile phase were eluted); stationary phase retention: 59.4% of the total column capacity.

Z-dipeptides studied, Z-Gly–Phe and Z–Gly–Leu were incompletely resolved in a hydrophobic binary solvent system methyl *tert.*-butyl ether–water. In this solvent system, the separation performed under optimum conditions using 21 m*M* TFA in the organic stationary phase and 2.5 m*M* ammonia in the aqueous mobile phase resulted in more than 60% overlap between the two peaks as shown in Fig. 5A. When a slightly more hydrophilic solvent system composed of methyl *tert.*-butyl ether–acetonitrile–water (4:1:5, v/v) was applied under otherwise identical experimental conditions, the mixing zone between the second and third peaks was reduced to 30% (Fig. 5B). Further improvement in the resolution between these two peaks was achieved by using a still more polar solvent system composed of methyl *tert.*-butyl ether–1-butanol–acetonitrile–water (2:2:1:5, v/v) as shown in Fig. 5C where the mixing zone between these two peaks was no more than several milliliters.

The choice of solvent system requires consideration of the hydrophobicity of the analytes to be separated. A decrease of hydrophobicity of the solvent system usually improves the resolution of the polar analytes. In related studies, we have found it necessary to use several different solvent systems to cover a relatively broad range of hydrophobicity. For example, a binary solvent system composed of methyl *tert.*-butyl ether and water was successfully used for the separation of amino acid derivatives such as dinitrophenyl amino acids [5,11] and amino acid benzyl esters [12]; a more hydrophilic ternary solvent system methyl *tert.*-butyl ether–acetonitrile–water (2:2:3, v/v) for the separation of Z-dipeptides (Fig. 1); and the polar quaternary solvent system methyl *tert.*-butyl ether–1-butanol–acetonitrile–water (2:2:1:5, v/v) for the separation of hydrophilic Z-tripeptides (Fig. 2).

As reported elsewhere [5], the pH of the equilibrated analyte zone (pH_z) in the present method is expressed using pK_a and both intrinsic (δ_s) and apparent (K_s) partition coefficients of the analyte according to the following equation:

$$pH_z = pK_{a\text{-}s} + \log [(\delta_s / K_s) - 1] \quad (1)$$

where the intrinsic partition coefficient (δ_s) represents the relationship between the hydrophobicity of the analyte and that of the two-phase solvent system used for separation. It appears that the difference in pH_z between the analytes may play an important role in determining the degree of peak resolution. In fact, the polar solvent system (Fig. 5C) gives a substantial difference in pH between the second and the third zones.

A set of four similar Z-tripeptides (Fig. 6) were chosen to investigate the effect of eluent base concentration in the two-phase solvent system methyl *tert.*-butyl ether–1-butanol–acetonitrile–water (2:2:1:5, v/v) where TFA (retainer acid) was added to the organic stationary phase at 16 m*M*. In the first experiment using ammonia (eluent base) at 2.7 m*M* in the aqueous mobile phase, the isomeric Z–Ala–Gly–Gly and Z–Gly–Gly–Ala peaks overlapped more than 33% (Fig. 6A). When the concentration of ammonia was increased to 5.5 m*M* under otherwise identical conditions, the resolution of the two peaks was slightly improved to about 25% overlap (Fig. 6B). Finally, when the concentration of the eluent base was further increased to 11 m*M*, complete resolution of the four Z-tripeptides was obtained. However, this approach must be used cautiously since an excessively high concentration of the eluent base in the mobile phase may precipitate analytes in the column leading to carryover of stationary phase and loss of peak resolution.

The concentration of the retainer acid also affects the resolution of the analytes. In our studies the separation of Z–Gly–Phe and Z–Gly–Leu was substantially improved by increasing the concentration of TFA (retainer acid) from 5.4 to 21.6 m*M* in the organic stationary phase while keeping the concentration of ammonia (eluent base) constant at 2.7 m*M* in the aqueous mobile phase. Increased concentration of the retainer acid does not significantly affect the concentration of analyte in the mobile phase but raises its concentration in the stationary phase. The improved peak resolution described above may be caused by the combined effects of a longer retention time and a shortened band width in the column. The overall results of the

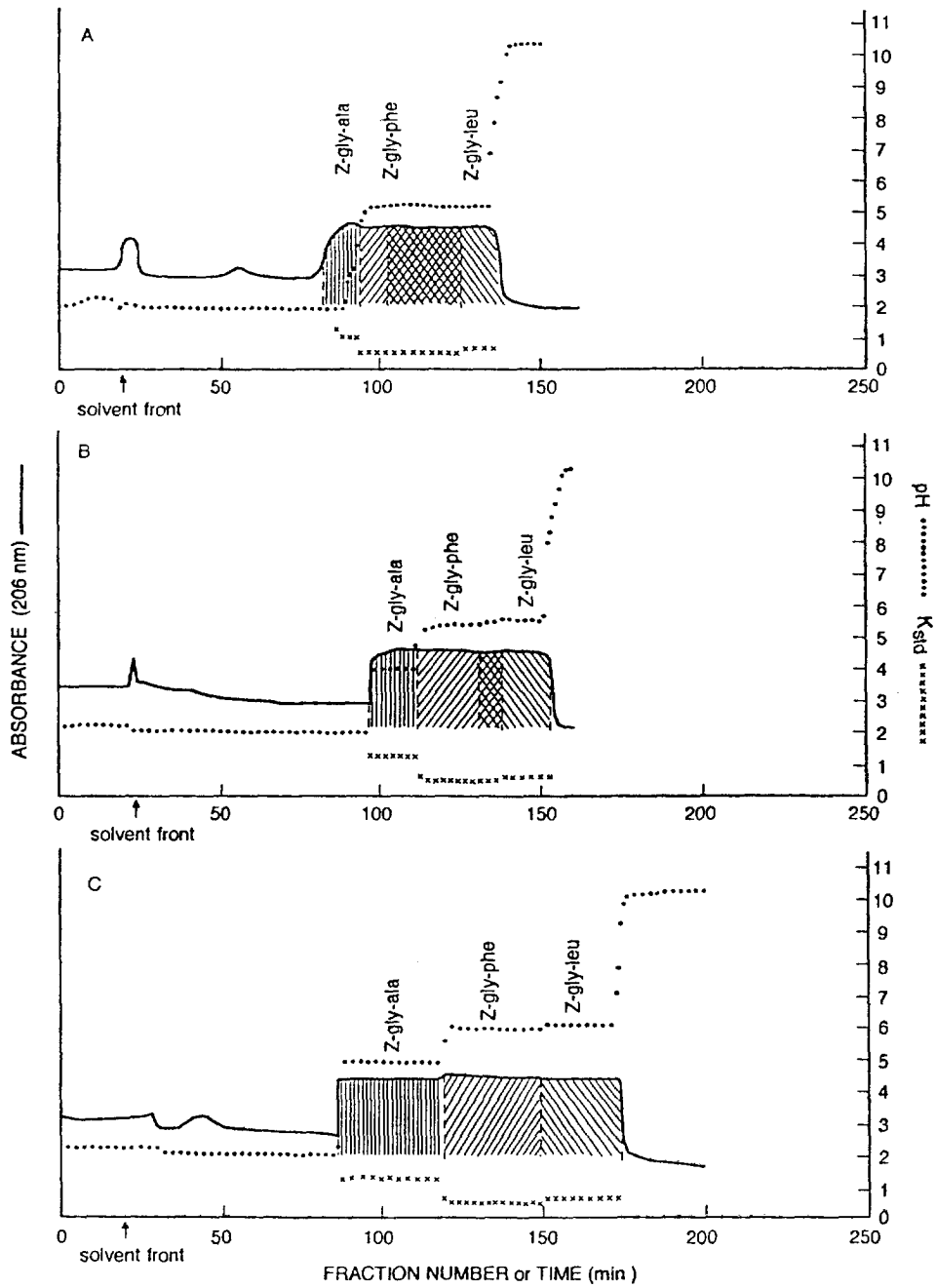

Fig. 5. Effects of hydrophobicity of solvent system on the separation of Z-dipeptides: Experimental conditions: **apparatus:** multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; solvent system: **(A)** methyl *tert.*-butyl ether–water; (B) methyl *tert.*-butyl ether–acetonitrile–water (4:1:5, v/v); and (C) methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5), 21 m*M* TFA in organic stationary phase and 2.7 m*M* NH_3 in aqueous mobile phase; samples: three Z-dipeptides as indicated in the chromatogram, each 100 mg dissolved in 6 ml of each phase; flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm (600 rpm until 66 ml of mobile phase were eluted); stationary phase retention: (A) 73.6%, (B) 71.8% and (C) 57.3% of the total column capacity.

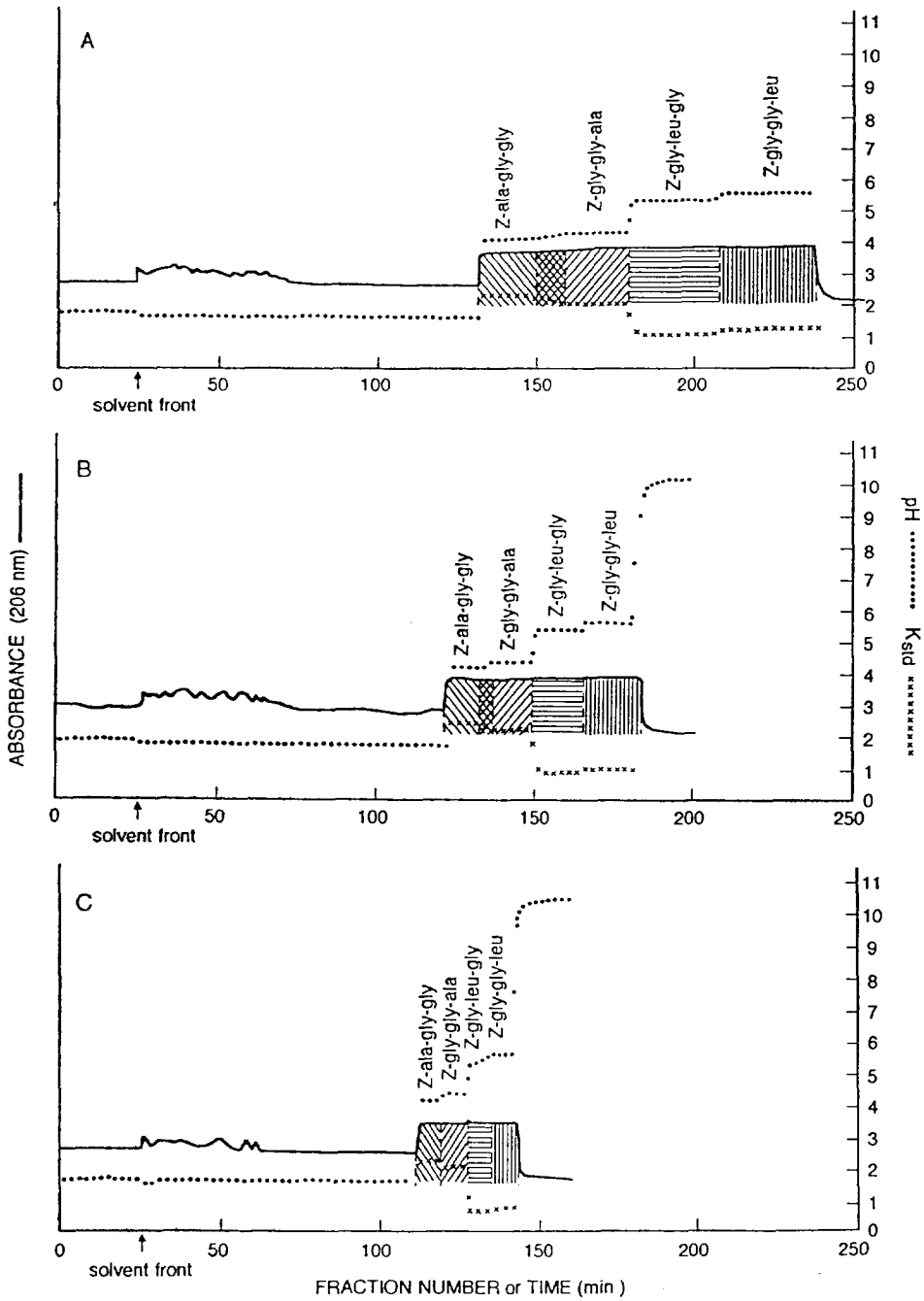

Fig. 6. Effects of eluent base concentration on the separation of Z-tripeptides by pH-zone-refining CCC. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; solvent system: methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5, v/v), 32 m*M* TFA in organic stationary phase (pH 2.00), (A) 2.7 m*M* NH_3 (pH 10.27); (B) 5.5 m*M* NH_3 (pH 10.48); and (C) 11 m*M* NH_3 (pH 10.64) in aqueous mobile phase; samples: four Z-tripeptides as indicated in the chromatogram, each 100 mg dissolved in 40 ml solvent (20 ml of each phase); flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm (600 rpm until 66 ml of mobile phase were eluted); stationary phase retention: (A) 59.8%; (B) 56.2%; and (C) 56.9% of the total column capacity.

present studies indicate that pH-zone-refining CCC is useful for the separation of oligopeptide derivatives containing either acid or basic groups. The two-phase solvent systems composed of methyl *tert.*-butyl ether–1-butanol–acetonitrile–water at various volume ratios are universally used for separations of analytes in a broad range of hydrophobicities. In all separations, TFA (retainer acid) and ammonia (eluent base) are used for acidic peptides and triethylamine (retainer base) and HCl (eluent acid) for basic peptides. We are currently examining extension of the method to derivatives of longer peptides.

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