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Separations of basic amino acid benzyl esters by pH-zone-refining counter-current chromatography

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Abstract

pH-Zone-refining counter-current chromatography was applied to the separation of basic amino acid benzyl esters. The method uses a retainer base in the stationary phase to retain analytes in the column and an eluent acid to elute the analytes in the decreasing order of pK_a and hydrophilicity. The preparative capability of the method is demonstrated in the separation of a 10-g quantity of the sample.

1. Introduction

pH-Zone-refining counter-current chromatography (CCC) [1–6] is a new preparative CCC method which yields a characteristic elution pattern of analytes comparable to that observed in displacement chromatography [7]. The method uses a retainer acid or base in the stationary phase (or sample solution) to retain analytes which are then eluted with the mobile phase containing the respective counterion. The analytes are eluted as a succession of highly concentrated rectangular peaks with minimum overlap. In the past, applications were limited to the separation of acidic compounds including dinitrophenyl (DNP) amino acids [1–6], various hydroxyxanthene dyes [2,8–12], indole auxins [3], etc.

In this paper pH-zone-refining CCC is applied to basic compounds to illustrate its wider domain. After equilibration of a two-phase solvent system composed of methyl *tert.*-butyl ether and water, triethylamine (retainer base) was added to the upper organic stationary phase and HCl (eluent acid) to the lower aqueous mobile phase. Using a set of amino acid benzyl esters, the separation was obtained by varying the concentrations of both retainer base and eluent acid. The preparative capability is then demonstrated in the separation of a 10-g sample. Its success with these bases suggests its possible application to the separation of natural products such as alkaloids.

2. Experimental

2.1. CCC apparatus

A commercial model (Ito multilayer coil separator/extractor, P.C. Inc., Potomac, MD, USA) of the high-speed CCC centrifuge was

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used throughout the present studies. The detailed design of the apparatus was given elsewhere [13]. 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. long polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) wound around the column holder hub forming 16 layers with 325 ml capacity. Each terminal of the column was connected to a flow tube (0.85 mm I.D. PTFE) (Zeus) 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) was placed at the outlet of the column to stabilize the effluent flow, thus facilitating the recording of elution curves.

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

2.2. Reagents

Methyl *tert.*-butyl ether, methanol and triethylamine were glass-distilled chromatographic grade (Baxter Healthcare, Muskegon, MI, USA). Hydrochloric acid and acetic acid were of reagent grade (Fisher Scientific, Fair Lawn, NJ, USA). Amino acid benzyl esters used in the present studies include: Gly(OBzl)·Tos, Ala(OBzl)·Tos, Leu(OBzl)·Tos, Val(OBzl)·Tos, Glu(OBzl)–OBzl·Tos, Phe(OBzl)·Tos, Asp(OBzl)–OBzl·Tos, Ile(OBzl)·Tos (OBzl = benzyl ester, Tos = *p*-toluenesulfonic acid) (Peninsula Labs., Belmont, CA, USA).

2.3. Preparation of solvent phases and sample solutions

The solvent pair was prepared as follows: methyl *tert.*-butyl ether and distilled water were thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. Triethylamine (2.5–40 m*M*) was added to the upper organic phase which was then used as the stationary phase. The lower aqueous phase was acidified with HCl (5–40 m*M*) and used as the mobile phase.

Sample solutions were prepared by dissolving a set of amino acid benzyl esters *p*-toluenesulfonic acid salts in 10–50 ml solvent, usually consisting of about equal volumes of upper organic phase and water. The solution was neutralized by an addition of triethylamine in an appropriate amount and 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 triethylamine. Then the sample solution was injected through the sample port and the acidified 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 Instruments, Emeryville, CA, USA) while the apparatus rotated. In order to minimize the carryover loss of the stationary phase from the column, the revolution speed was initially set at 600 rpm which was increased to 800 rpm after 20 min of elution. The effluent from the column was continuously monitored by absorbance at 206 nm (Uvicord S; LKB, Bromma/Stockholm, Sweden) and collected at 3.0 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 (80–65%).

2.5. Studies on effects of eluent acid and retainer base concentrations

A series of experiments was carried out with a set of three amino acid benzyl esters *p*-toluenesulfonic acid salts (monobenzyl esters of glycine and leucine and dibenzyl ester of glutamic acid) to study the effects of the eluent acid (HCl) and retainer base (triethylamine) on the separation. In the first series the effect of the eluent acid was studied by varying the HCl concentration in the aqueous mobile phase from 5 to 40 m*M* while the concentration of triethylamine in the organic stationary phase was kept constant at 5 m*M*. In the second series, the effect of the retainer base was investigated by varying the concentration of triethylamine in the organic stationary phase from 2.5 to 40 m*M* while the HCl concentration in the mobile phase was fixed at 10 m*M*.

In each separation, the column was first filled with the organic stationary phase followed by injection of a sample mixture containing 0.5 mmol of each component in 10 ml solvent (5 ml upper phase and 5 ml water). In order to neutralize the sample solution, 200 μl of triethylamine was added. Then the column was rotated at 600 rpm and eluted with the acidified organic phase at a flow-rate of 3.0 ml/min. As mentioned earlier, the revolution speed was increased to 800 rpm after 20 fractions were collected (20 min). The effluent from the column was continuously monitored with a UV monitor at 206 nm. The average solute concentration in each fraction was estimated from the width of each peak. The partition coefficient of the retainer base was also computed from its retention volume and the volume of the stationary phase retained in the column [6].

2.6. Continuous UV monitoring

As previously described [3], 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 actually passes some light in a broad range, even extending to the visual region. This particular feature allows us to monitor elution curves at several wavelengths. Thus the lower portion of the absorbance scale correctly reflects the 206 nm absorption when this is the major chromophore present. At the higher sample concentrations found in pH-zone-refining CCC, the sample may be opaque to 206 nm light but the detection will still respond to absorption of other frequencies. Thus, impurities absorbing at different wavelengths can be detected above the main plateau as sharp peaks at the zone boundaries. (In the present study no impurities were detected in this fashion.) The actual absorbance values at the tops of the peaks (not shown) were determined using a Zeiss PM6 spectrophotometer at 206 nm after diluting the fraction with methanol. These values were about 200 absorbance units and obviously could not be directly monitored by an ordinary UV monitor. For this reason, we do not indicate absorbance values in the chromatogram.

2.7. 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 amino acid benzyl esters were identified by their partition coefficients, K_{std}, in a standard two-phase system composed of *n*-hexane–ethyl acetate–MeOH–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 Instruments, 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, K_{std}(U/L) or K_{std}, was expressed as solute concentration in the upper

phase (U) divided by that in the lower phase (L). The peak fractions were also analyzed by TLC (Kieselgel 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 amino acid benzyl esters obtained by the present method. Seven components (each 0.5 mmol) were eluted together as a broad rectangular peak (Fig. 1A) due to detector saturation (see above). Partition coefficient measurement, K_{std}(U/L), revealed that it consisted of a series of narrow rectangular peaks of the individual components with minimum overlap in the same fashion as observed in separation of acidic analytes [1–3]. Although the chromatogram in Fig. 1A indicates sharp boundaries between neighboring peaks, the TLC analyses (Fig. 1B) of fractions show that each boundary actually consists of several milliliters of the mixing zone. Each component formed a pH plateau in a downward staircase fashion. Considerable irregularity of the pH plots at some peaks was caused by the carryover of organic stationary phase which, however, did not affect the peak resolution. The results indicate that basic amino acids with similar structures can be separated in decreasing order of their pK_a and hydrophilicity since these two parameters determine the pH of the eluted solute zone [3].

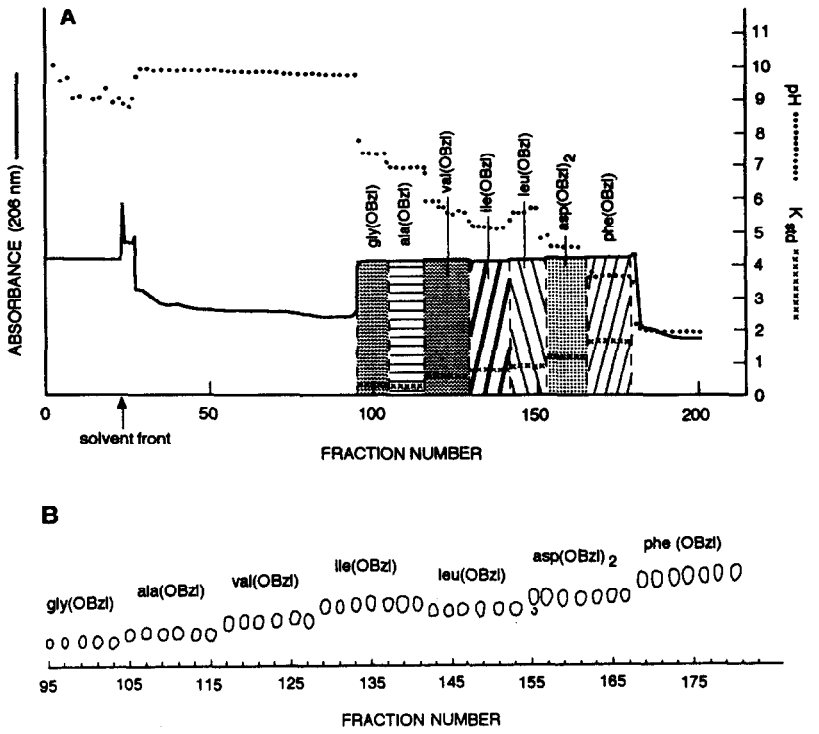

Fig. 1. Separation of seven amino acid benzyl esters by pH-zone-refining CCC. (A) Elution profile. Experimental conditions: apparatus: high-speed CCC centrifuge; column: semipreparative multilayer coil, 160 m × 1.6 mm I.D., 325 ml capacity; solvent system: methyl *tert.*-butyl ether, 10 m*M* triethylamine in upper organic stationary phase and 10 m*M* hydrochloric acid in lower aqueous mobile phase; sample: a synthetic mixture of 7 amino acid benzyl esters as labelled in the chromatogram, each 100 mg dissolved in 20 ml solvent; flow-rate: 3 ml/min; revolution: 800 rpm; retention of stationary phase: 71.2%. (B) TLC analysis of peak fractions. TLC plate: Kieselgel 60 F_{254}, EM Separations; solvent: chloroform–methanol–32% acetic acid (16:4:1, v/v).

A series of experiments was performed to investigate the effects of concentration of the eluent acid and retainer base on those of the eluted analytes. The effects of the eluent acid concentration are shown in Fig. 2A where molar concentrations of three analytes are plotted against those of HCl (eluent acid) in the mobile phase. As the HCl concentration is increased from 5 to 40 m*M*, all analytes increase their concentrations at a nearly 1:1 molar ratio suggesting that the eluent acid acts as the counterion to effectively determine the analyte concentration in the mobile phase. On the other hand, the increased HCl concentration causes a sharp decrease of the partition coefficient (K) of the retainer base (triethylamine) resulting in the reduced retention time of the analyte peaks.

Fig. 2B similarly shows the effects of the retainer base (triethylamine) concentration in the stationary phase while the concentration of the eluent acid (HCl) in the mobile phase is fixed at 10 m*M*. In this case, the concentrations of all analytes were almost unaltered with the increased triethylamine concentration by maintaining a near 1:1 molar ratio to the eluent acid that serves as the counterion mentioned above. The increase in concentration of the retainer base, however, causes the increase of the partition coefficient (K), thus enhancing the retention of the analytes. As described elsewhere [3], the partition coefficient (K) of the retainer is equal to those for all analytes within the succeeding pH zones. This fact indicates that the increased retainer base concentration increases the analyte concentration in the stationary phase while it produces no change in that in the mobile phase.

In short, the eluent acid determines the analyte concentration in the mobile phase while the retainer base modifies that in the stationary phase. The retention time of the analyte is counterbalanced by concentrations of the retainer base and the eluent acid. A short retention time may result in incomplete resolution between the early-eluting zones. On the other hand, an excessively long retention time may cause decomposition of the analyte which follows immediately behind the retainer base by hydrolysis of these benzyl esters by the exposure to high pH. This complication may be avoided by using a suitable spacer which occupies the column space between the retainer base and the analytes.

Fig. 3 shows the separations of three amino acid benzyl esters in three different sample sizes of 0.6 g (A), 3 g (B) and 6 g (C) under otherwise similar experimental conditions. Separations

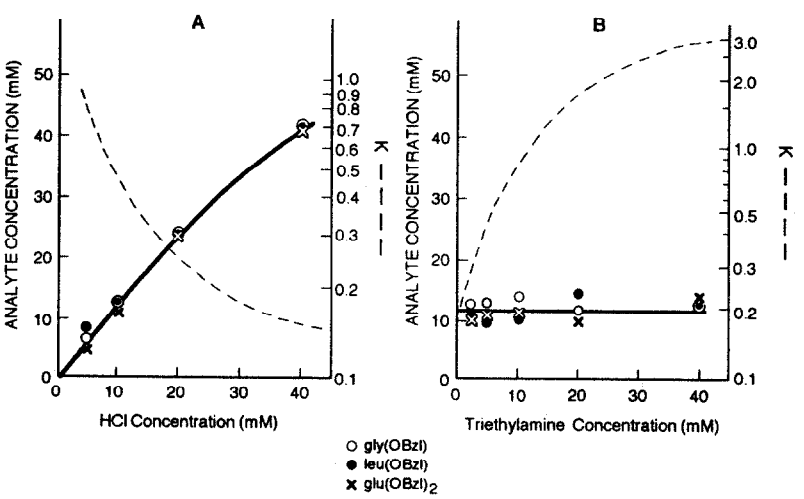

Fig. 2. Effects of eluent acid (A) and retainer base (B) on analyte concentrations in the mobile phase. (A) Analyte concentration in the mobile phase was plotted against the concentration of the eluent acid from 5 to 40 m*M* while the concentration of the retainer base was fixed at 10 m*M*. (B) Analyte concentration in the mobile phase was plotted against the concentration of the retainer acid from 5 to 40 m*M* while the eluent acid concentration was fixed at 10 m*M*.

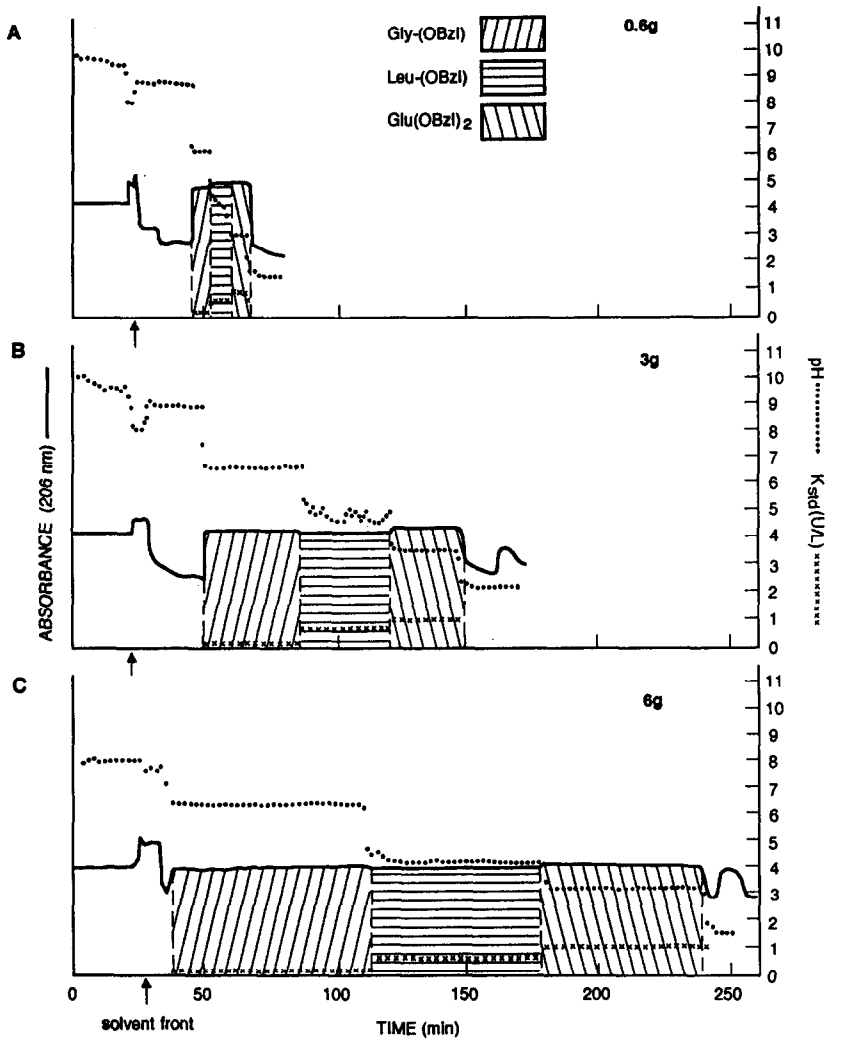

Fig. 3. Separation of three amino acid benzyl esters by pH-zone-refining CCC. Experimental conditions: apparatus and columns as in Fig. 1; solvent system: methyl *tert.*-butyl ether, 5 m*M* triethylamine in the upper organic stationary phase and 20 m*M* hydrochloric acid in the lower aqueous mobile phase; sample: Gly(OBzl)·Tos, Leu(OBzl)·Tos and $Glu(OBzl)_2$·Tos, each 0.2 g (A), 1 g (B) and 2 g (C); flow-rate: 3 ml/min; revolution: 800 rpm; retention of stationary phase: 76.5% (A), 68.3% (B) and 77.8% (C).

were performed with a methyl *tert.*-butyl ether–water system by adding triethylamine (retainer base) to the upper organic stationary phase at 5 m*M* and hydrochloric acid (eluent acid) to the lower aqueous mobile phase at 20 m*M*. In order to normalize the retention time of the analytes, the sample solution was neutralized with triethylamine (0.2, 1.0 and 2.0 ml were added for A, B and C, respectively). Under a flow-rate of 3.0 ml/min and a revolution speed of 800 rpm, the separations were completed in about 1.1, 2.5 and 4.0 h for the sample sizes of 0.6 g, 3 g and 6 g, respectively. In all separations, three components were eluted together as fused rectangular peaks with sharp boundaries as indicated by the abrupt transition of the partition coefficient (K_{std}) and pH levels (dotted line) in the diagrams. The results show that with a given

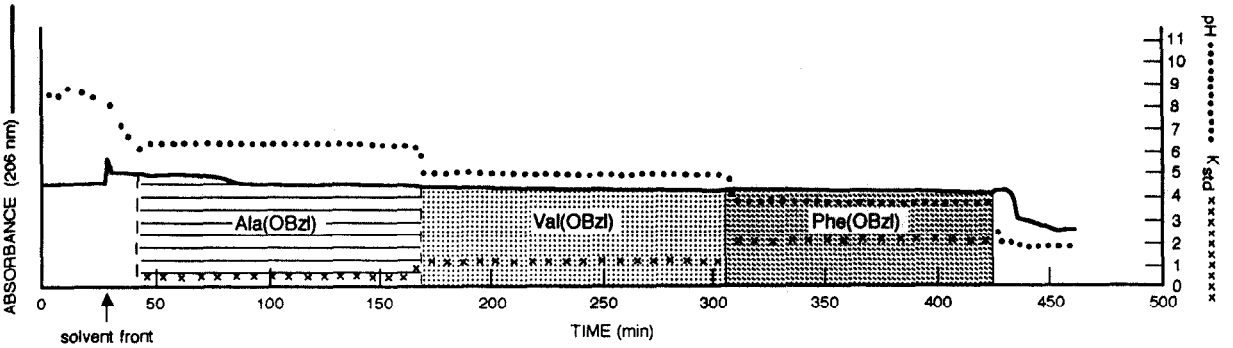

Fig. 4. Preparative separation of three amino acid benzyl esters by pH-zone-refining CCC. Sample: Ala(OBzl)·Tos, Val(OBzl)·Tos, and Phe(OBzl)·Tos each 3.3 g in 100 ml solvent. Other experimental conditions as in Fig. 3.

concentration of the retainer base and eluent acid the analyte concentrations stay similar regardless of the sample size. Consequently the length of each peak increases nearly proportional to the applied sample size while the widths of the mixing zones between the neighboring peaks are not significantly altered. This allows one to harvest a greater overall yield of pure product by increasing the sample size.

Fig. 4 shows a chromatogram of three amino acid benzyl esters at a sample size of 10 g by the present method. Each component is well resolved and eluted in slightly over 7 h. The stable pH plateau in each analyte zone indicates that no carryover of the stationary phase occurred. The partition coefficient measurement, K_{std}, revealed that the mixing zone at each peak boundary amounts no more than several milliliters. This separation is the first case in our laboratory where a sample size of 10 g was successfully separated using a semipreparative multilayer coil with a 325-ml total capacity.

4. Conclusions

The overall result of our studies indicates that pH-zone-refining CCC can be successfully applied to basic amino acid benzyl esters. The present method provides more than a 10-fold increase in sample capacity for a given column. Separation of three components from a 10 g sample size was achieved using a 325 ml capacity column in about 7 h. The method should be applicable to other basic compounds such as alkaloids from the natural products.

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