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pH-Zone-refining counter-current chromatography: a displacement mode applied to separation of dinitrophenyl amino acids

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Abstract

A new mode of pH-zone-refining counter-current chromatography operates in a manner analogous to displacement chromatography. The method uses a retainer base (acid) in the stationary phase to retain analytes in the column and a displacer acid (base) to elute the analytes in the decreasing order of pK_a and hydrophobicity. The elution produces a train of highly concentrated rectangular solute peaks with minimum overlap. Utility of the method is demonstrated in the separation of dinitrophenyl amino acids in a two-phase solvent system composed of methyl *tert.*-butyl ether and water. Compared with the original mode of pH-zone-refining counter-current chromatography, the present mode is more amenable to ligand-affinity separation which may cover a broader range of analytes including non-ionizable compounds.

1. Introduction

pH-Zone-refining counter-current chromatography (CCC) is a recently developed preparative separation method using a retainer acid (base) to retain acidic analytes and an eluent base (acid) to elute analytes in the order of their pK_a and hydrophobicity [1–5]. The method has been applied to various ionizable compounds such as amino acid derivatives [1–5] and hydroxyxanthene dyes [2–10].

Comparison of this technique to displacement chromatography [11] reveals that the key reagents in these two methods, *i.e.*, retainer in pH-zone-refining CCC and displacer in displacement chromatography, act in an opposite manner: the retainer transfers the solute from the mobile phase to the stationary phase at the front end of the solute bands, whereas the displacer transfers the solute from the stationary phase to the mobile phase at the back of the solute bands. Nevertheless, these two actions produce similar results such as formation of successive rectangular solute peaks and concentration of minor impurities at their boundaries. From this point of view, the "pH-zone-refining CCC" technique reported earlier may be more properly considered as "reverse displacement mode pH-zone-refining CCC".

In this work, pH-zone-refining CCC is carried out in a displacement mode directly comparable to displacement chromatography simply by interchanging the action of the mobile and the stationary phases. Thus, the original eluent base

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becomes a retainer for analytes in the stationary phase, and the original retainer acid becomes a displacer to displace the analytes from the stationary phase to the mobile phase at the back of the solute bands. The results are demonstrated in separations of a set of dinitrophenyl (DNP) amino acids on a two-phase solvent system composed of methyl *tert.*-butyl ether and water using trifluoroacetic acid (TFA) as a displacer and ammonia as a retainer. The preparative capability of the present method is also demonstrated in the separation of gram quantity of samples.

2. Mechanism of displacement pH-zone-refining CCC

A model experiment is initiated by filling the entire column with the stationary aqueous phase which contains a retainer, NH_3. This is followed by injection of a sample solution containing three major components (solutes S_1, S_2 and S_3). The column is then eluted with an organic mobile phase containing a displacer, TFA. As the mobile phase moves through the column by partially displacing the stationary phase, it distributes TFA anions to the stationary phase retained in the column according to its partition coefficient as determined by the pH at a given point in the column. This partition process depletes the TFA from the flowing mobile phase front resulting in formation of a sharp TFA front border due to the concentration-dependent partition behavior of TFA based on its non-linear isotherm. After equilibrium is established, this sharp TFA border travels through the column at a uniform rate lower than that of the mobile phase front.

Fig. 1A schematically illustrates a portion of the separation column which contains the stationary aqueous phase in the lower half (shaded) and the mobile organic phase in the upper half. The sharp TFA border is indicated by a thick line across the column.

In the early stage of the experiment, all solutes present on the right side of the TFA border are exposed to a high pH, mostly deprotonated to

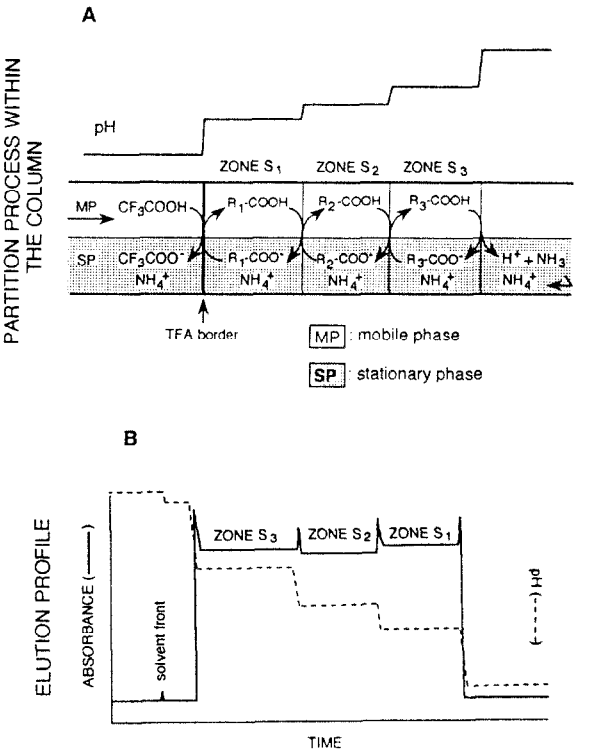

Fig. 1. Mechanism of displacement mode of pH-zone-refining CCC. (A) Partition process within the separation column, (B) elution profile.

become a hydrophilic form ($R-COO^-$) and partitioned into the aqueous stationary phase. As the TFA front advances, these compounds are exposed to a low pH, protonated into a hydrophobic form (R–COOH) and transferred into the flowing organic phase, *i.e.*, TFA in the mobile phase gradually displaces all solute molecules present in the stationary phase in a manner analogous to the action of the displacer in displacement chromatography.

As this process continues, the solute concentration at the front of the TFA border increases causing the pH to fall. In this situation, solute S_1 with the lowest pK_a and hydrophobicity among the three components will act as a displacer of the other two solutes to occupy the column space immediately after the sharp TFA border. It forms the first zone (zone S_1) by making a sharp front border. The competition continues among other two solutes in which solute S_2 with a lower

pK_a and hydrophobicity will form the second zone (zone S_2) which is in turn preceded by the third zone (zone S_3) consisting of solute S_3.

When this partition process is completed, a train of solute zones is formed in front of the TFA border as shown in Fig. 1A. As in displacement chromatography, each zone consists of a single species, is equipped with self-sharpening boundaries, and has the solute partition coefficient (K_s) (the solute concentration in the stationary phase divided by that in the mobile phase) equal to that of TFA (K) in the succeeding zone. As indicated earlier, all zones are arranged in a decreasing order of pK_a and hydrophobicity and move together at the same rate determined by that of the succeeding TFA border.

As indicated by curved arrows, proton transfer and displacement of the solute molecule takes place between the two neighboring species at each zone boundary. Ammonium ion created at the far front border (solute S_3) remains permanently in the stationary phase and serves as the counterion for all species. Charged impurities present in each solute zone are quickly eliminated toward the zone boundaries of either side where they accumulate to form narrow bands as seen in displacement chromatography. Consequently the solutes are eluted as successive rectangular peaks with minimum overlap and with sharp impurity peaks at their boundaries as shown in Fig. 1B. Each zone shows a distinct pH plateau in a downward staircase fashion as indicated by a dotted line. As described elsewhere [3,5], net pH values of these plateaus may be predicted from three parameters, *i.e.*, pK_a, δ (intrinsic partition coefficient) and K_s (apparent partition coefficient) of each solute.

3. Experimental

3.1. CCC apparatus

A commercial model (Ito Multilayer Coil Separator/Extractor, Potomac, MD, USA) of the high-speed CCC centrifuge was used throughout the present studies. The detailed design of the apparatus was given elsewhere [12]. Briefly, 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 gear which is engaged to an identical stationary gear mounted around the central axis of the apparatus. This gear arrangement produces the desired planetary motion to the column holder, *i.e.*, rotation and revolution of the holder 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 rotating 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, Raritan, NJ, USA) wound around the column holder hub with 16 layers and 325 ml capacity. Both terminals of the column were 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) 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 restrict the flow, thus preventing formation of negative pressure at the inlet of the column which may cause suction of excess solvent from the reservoir through the check valves of the pump.

The speed of the apparatus was regulated with a speed controller (Bodine Electric Co., North Chicago, IL, USA). An optimum speed of 600 rpm was used throughout the present studies.

3.2. Reagents

Methyl *tert.*-butyl ether, methanol and TFA were glass-distilled chromatographic grade (Burdick & Jackson, Muskegon, MI, USA). Ammonium hydroxide, hydrochloric acid, acetic acid, propionic acid and *n*-butyric acid were of reagent grade (Fisher Scientific, Fair Lawn, NJ, USA). DNP-Amino acids used in the present studies include N-2,4-DNP-L-aspartic acid (DNP-Asp), N-2,4-DNP-DL-glutamic acid (DNP-Glu),

N,N′-2,4-diDNP-L-cystine [diDNP-$(Cys)_2$], N-2,4-DNP-L-alanine (DNP-Ala), N-2,4-DNP-L-proline (DNP-Pro), N-2,4-DNP-L-valine (DNP-Val) and N-2,4-DNP-L-leucine (DNP-Leu) (Sigma, St. Louis, MO, USA).

3.3. Preparation of solvent phases and sample solutions

The solvent pairs were prepared as follows: methyl *tert.*-butyl ether and distilled water were thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated. The upper organic phase was acidified with TFA (displacer) at 5–20 m*M* and used as the mobile phase. Spacer acids such as propionic acid and *n*-butyric acid were sometimes added to the stationary phase in arbitrary amounts. Aqueous ammonia (retainer) was added to the lower aqueous phase used as the stationary phase.

Solutions were prepared by dissolving 2,4-DNP derivatives in 5–40 ml of stationary upper phase. A small amount of methyl *tert.*-butyl ether was added to form two phases and spacer acids were occasionally added directly to the sample solution. The 2,4-DNP derivatives were only partially soluble and it was necessary to sonicat the solution for several minutes to disperse undissolved particles before injection into the column.

3.4. Separation procedure

In each separation, the column was first entirely filled with the aqueous ammoniacal stationary phase. The sample solution was injected through the sample port and the organic mobile phase eluted through the column in the tail to head elution mode at a flow-rate of 3.3 ml/min (metering pump: Rainin, Emeryville, CA, USA) while the apparatus rotated at 600 rpm. 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 or 1 min/tube (Ultrorac fraction collector: LKB). After the peaks eluted, the apparatus was stopped and the column contents 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.

3.5. Studies on concentrations of displacer and retainer

Series of experiments were carried out with a set of three components, DNP-Leu, DNP-Ala and DNP-Asp to study the effects of the displacer (TFA) and retainer (NH_3) on the separation. In the first series, the effect of the displacer (TFA) was studied by varying its concentration in the organic mobile phase from 0.02% (2.6 m*M*) to 0.16% (20.5 m*M*) while the concentration of the retainer (NH_3) in the aqueous stationary phase was kept constant at 0.1% (11 m*M*). In the second series, the effect of the retainer (NH_3) was investigated by varying its concentration in the aqueous stationary phase from 5.5 to 44 m*M* while the displacer (TFA) concentration in the mobile phase was fixed at 0.4% (10.8 m*M*).

In each separation, the column was first filled with the aqueous stationary phase followed by injection of a sample mixture consisting of 100 mg each component in 5 ml solvent (4 ml lower phase and 1 ml methyl *tert.*-butyl ether). Then, the column was rotated and eluted with an acidified organic phase at a flow-rate of 3.3 ml/min. The effluent from the column was continuously monitored with a UV monitor at 206 nm. From the resulting chromatogram, the average solute concentration was estimated from the width of each peak. The partition coefficient of the displacer TFA was also computed from its retention volume and the volume of the stationary phase retained in the column.

3.6. Analysis of fractions

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

DNP-Amino acids were identified by their partition coefficients K_{std} (U/L) in a standard two-phase system composed of chloroform–acetic acid–0.1 *M* HCl (2:2:1); U and L stand for upper and lower phase, respectively. 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 then diluted with 2 ml of methanol and the absorbance determined at 430 nm.

4. Results and discussion

Fig. 2 shows a typical chromatogram of DNP-amino acids obtained by the present method. A 100-mg amount of each of seven DNP-amino acids was eluted as a broad rectangular peak.

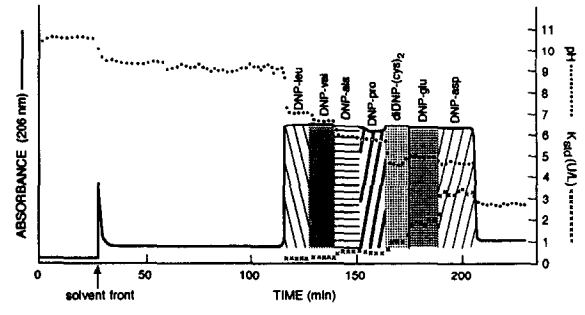

Fig. 2. Separation of seven DNP-amino acids by the present method. An abrupt change of the pH at each peak boundary suggests minimum overlap between neighboring zones while the partition coefficient values (K_{std}) indicate that each mixing zone is no more then several milliliters. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 325 ml capacity; sample: seven DNP-amino acids as indicated in the chromatogram, each 100 mg dissolved in 10 ml of solvent consisting of 8 ml lower phase and 2 ml methyl *tert.*-butyl ether; solvent system: methyl *tert.*-butyl ether–water, 22 m*M* NH_3 in aqueous stationary phase (pH 10.53) and 10.8 m*M* TFA in organic mobile phase (pH 2.68); flow: 3.3 ml/min, tail to head elution mode; revolution: 600 rpm; stationary phase retention: 76.2% of total column capacity.

Partition coefficients of each fraction, however, revealed that it consisted of a series of narrow rectangular peaks with minimum overlap as observed in the reverse mode pH-zone-refining CCC [1–4]. Each component formed a pH plateau in a downward staircase fashion. Some irregularity of the pH level at the diDNP-$(Cys)_2$ peak may be due to its significantly lower pH in the organic mobile phase than that in the aqueous stationary phase.

A series of experiments has been performed to investigate effects of concentrations of displacer acid and retainer base on those of the eluted analytes. The effects of the TFA (displacer) concentration are shown in Fig. 3A where concentrations of three analytes (DNP-Leu, DNP-Ala and DNP-Asp) are plotted against those of the displacer acid (TFA) in the mobile phase. Concentrations of all analytes increase rather sharply as the TFA concentration increases. Concentrations of DNP-Leu and DNP-Ala are

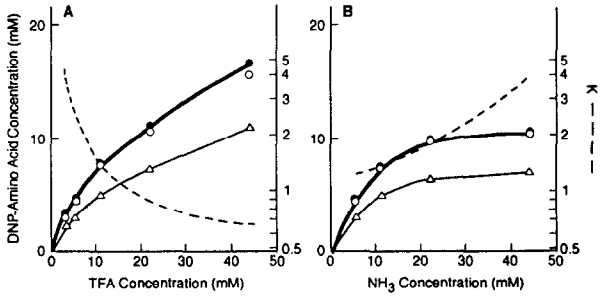

Fig. 3. Effects of displacer (TFA) (A) and retainer (NH_3) (B) on the concentration of DNP-amino acids and the partition coefficient (K) for TFA. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge; column: 1.6 mm I.D. PTFE multilayer coil with 325 ml capacity; sample: DNP-Leu (○), DNP-Ala (●) and DNP-Asp (△), each 100 mg in 5 ml solvent consisting of 4 ml aqueous stationary phase and 1 ml methyl *tert.*-butyl ether; solvent system: methyl *tert.*-butyl ether–water, (A): NH_3 11 m*M* in aqueous stationary phase and TFA 2.7–43 m*M* in organic mobile phase; (B) TFA 10.8 m*M* in organic mobile phase and NH_3 5.5–44 m*M* in aqueous stationary phase; flow: 3.3 ml/min in tail to head elution mode; revolution: 600 rpm. K was determined from each chromatogram using the conventional formula: $K = (V - V_m)/V_s$ where V is the retention volume of the TFA border and V_m and V_s are the the volumes of the mobile and stationary phases in the column, respectively. Note that K values are equal for TFA and all solutes in each experiment (see text for more details).

almost identical while that of the divalent DNP-Asp is much less than those of the above monovalent acids. Fig. 3B similarly shows the effects of NH_3 (retainer) concentration in the stationary phase. Concentrations of all analytes sharply rise with the increased NH_3 concentration up to 22 mM where they show a tendency to saturate. Here again, concentrations of divalent DNP-Asp are much lower than those of other two monovalent acids.

In each diagram, concentrations of the monovalent acids (DNP-Leu and DNP-Ala) are approximated by a thick solid line which represents the ratio of the NH_3 concentration in the stationary phase to the partition coefficient of TFA (K) indicated by a dotted line. Since the partition coefficient of each analyte (K_s) is equal to that of the retainer TFA (K) as described elsewhere [3,4], the above result indicates that the concentration of each analyte in the retained stationary phase is mainly determined by the concentration of its ammonium counterion. The above result suggests that the concentration of a divalent acid may become one half the concentration of the monovalent acids to yield the 1:1 charge ratio to the ammonium counterion. The actual plots of the divalent acid (DNP-Asp), however, fall at about 2/3 times those of the monovalent acids as shown by thin solid lines. Similar results were also obtained in other divalent amino acids such as DNP-Glu and diDNP-$(Cys)_2$ indicating that the concentration of these divalent acids in the stationary phase can significantly exceed the 1:1 charge ratio to that of the ammonium counterion due to their relatively high polarities.

As in displacement chromatography and isotachophoresis, the spacers may be used to isolate particular components in pH-zone-refining CCC. In the present method, spacers can be introduced either in the sample solution or in the mobile phase. In the examples shown below, the two spacer organic acids, propionic acid and n-butyric acid, were chosen for separating DNP-Leu, DNP-Ala and DNP-Asp. Selection of the spacer acids was based on the results of preliminary experiments where various organic acids and DNP-amino acids were cochromatographed to investigate the order of their elution.

Fig. 4 shows separations of the three DNP-

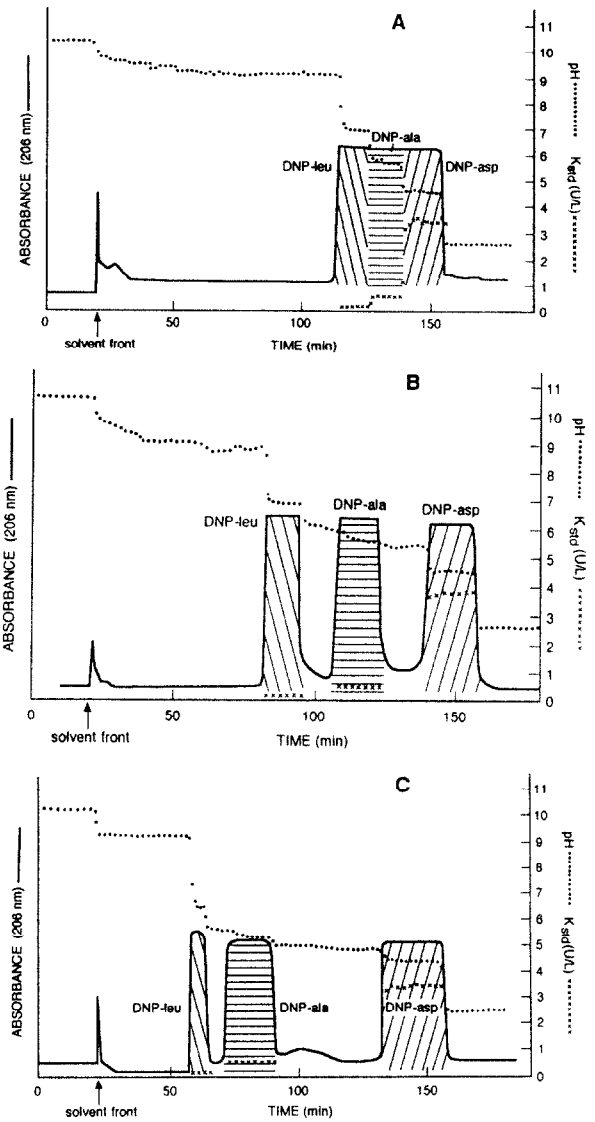

Fig. 4. Separation of DNP-amino acids with spacer acids. (A) Chromatogram obtained from the control run without spacer acids, (B) chromatogram obtained by adding the spacer acids in the sample solution, (C) chromatogram obtained by adding the spacer acid in the mobile phase. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge; separation column: 1.6 mm I.D., 325 ml capacity; sample: DNP-Leu, DNP-Ala and DNP-Asp each 100 mg in 5 ml solvent (4 ml aqueous stationary phase and 1 ml methyl *tert.*-butyl ether); spacer acid: (A) none, (B) propionic acid and n-butyric acid each 50 μl in the sample solution, (C) propionic acid and n-butyric acid each 0.08% in the organic mobile phase; solvent system: methyl *tert.*-butyl ether, NH_3 22 mM in aqueous stationary phase, TFA 10.8 mM in organic mobile phase; flow: 3.3 ml/min, tail to head elution mode; revolution: 600 rpm.

amino acids without spacer acids (Fig. 4A), with the spacer acids in the sample solution (Fig. 4B) and with the spacer acids in the mobile phase (Fig. 4C). In Fig. 4A, three components were eluted as a single rectangular peak as shown in Fig. 2. When the spacer acids (each 50 μl) were introduced into the sample solution, DNP-amino acids were completely resolved into three rectangular peaks each corresponding to the single species as labeled in the chromatogram (Fig. 4B). In this case, the two spacers show similar widths and the original peak width for each component is preserved. When the same spacer acids were introduced into the mobile phase (each 0.08%), however, the width and the retention time of each peak were altered in such a way that the first peak became narrower and eluted much earlier while the third peak became much broader compared with those in Fig. 4A. The peak spacing also became different: the width between the first and the second peaks is much narrower than that between the second and the third peaks. These changes were mainly produced by a steady supply of the spacer acids through the mobile phase which increased the traveling rate of the front border of each spacer acid and the preceding solute band.

The above results indicate that for uniform spacing and preservation of the original peak width of each component the spacers should be introduced locally in the sample solution.

Fig. 5 shows the separation of three DNP-amino acids, each 0.5 g, obtained by the present method. The experiment was performed by methyl *tert.*-butyl ether–water system by adding NH_3 at 0.4% (44 m*M*) to the aqueous stationary phase and TFA at 0.8% (10.8 m*M*) to the organic mobile phase. The sample volume was 40 ml consisting of 30 ml acidified aqueous phase and 10 ml methyl *tert.*-butyl ether. A solution containing undissolved sample was thoroughly sonicated into a uniform suspension and introduced into the column without filtration. Under a flow-rate of 3.3 ml/min and a revolution speed of 600 rpm, the separation was completed in about 4 h. Three components were eluted as broad rectangular peaks with sharp boundaries as indicated by the abrupt transition of the partition coefficient (K) and pH levels plotted in the diagram.

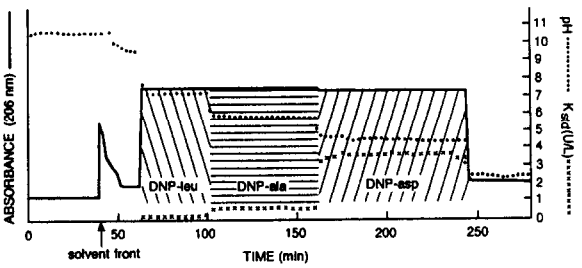

Fig. 5. Preparative separation of DNP-amino acids by the present method. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge; column: 1.6 mm I.D. multilayer coil with 325 ml capacity; sample: DNP-Leu, DNP-Ala and DNP-Asp, each 0.5 g in 25 ml of solvent (20 ml aqueous stationary phase and 5 ml methyl *tert.*-butyl ether); solvent system: methyl *tert.*-butyl ether, NH_3 44 m*M* in aqueous mobile phase (pH 10.68), TFA 10.8 m*M* in organic stationary phase (pH 2.62); flow: 3.3 ml/min, tail to head elution mode; revolution: 600 rpm; retention of stationary phase: 51.3%.

5. Conclusions

The technique of pH-zone-refining CCC can be efficiently performed in the displacement mode to separate gram quantities of DNP-amino acids in a few hours. As in the reverse displacement mode, the method produces concentrated rectangular elution peaks with minimum overlap. As observed in displacement chromatography, a better yield and purity is realized as the sample size is increased [2]. The method can be used for separation of organic bases by adding a retainer acid such as HCl to the aqueous stationary phase and a displacer base such as triethylamine to the organic mobile phase.

Compared to the original reverse displacement mode of pH-zone-refining CCC, the displacement mode of operation results in salt-free organic fractions which are easily evaporated. The most important advantage of the displacement mode, however, derives from the fact that the retainer is permanently located within the column instead of being eluted. Consequently, the displacement mode is better adapted to a ligand-affinity separation. In this way, applica-

tions may be extended to cover the separation of non-ionizable compounds.

We believe that the displacement mode of the pH-zone-refining CCC technique will open a rich domain of applications in the preparative-scale separation.

6. Acknowledgement

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