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Short communication

pH-Zone refining counter-current chromatography of polar catecholamines using di-(2-ethylhexyl)phosphoric acid as a ligand

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Abstract

The use of di-(2-ethylhexyl)phosphoric acid (DEHPA) as a ligand in the stationary phase effectively increased the partition coefficient of polar catecholamines. pH-Zone refining counter-current chromatography of six components, i.e. five catecholamines and one amino acid (DOPA), was successfully performed using a two-phase solvent system composed of methyl *tert.*-butyl ether and water by adding DEHPA (20%) and ammonium acetate (200 m*M*) to the organic phase and HCl (50 m*M*) to the aqueous mobile phase. DOPA was eluted first as a normal peak followed by the five catecholamines which formed a succession of highly concentrated rectangular peaks associated with sharp impurity peaks at their borders (UV tracing at 280 nm). Both pH and standard partition coefficient of collected fractions indicated minimum overlap between the main peaks. Each component was identified by NMR analysis.

Keywords: pH-Zone refining counter-current chromatography; Counter-current chromatography; Catecholamines; Di-(2-ethylhexyl)phosphoric acid

1. Introduction

A recently developed preparative technique, pH-zone refining counter-current chromatography (CCC) [1–5], has various advantages over conventional CCC in terms of sample loading capacity, purity of fractions and separation time. It produces a succession of highly concentrated rectangular peaks with minimum overlapping as observed in displacement chromatography with which it shares a common modality. The method has been successfully applied to a variety of compounds including acidic and basic derivatives of amino acids [1–3,5,6] and peptides [5,7]; hydroxyxanthene dyes [1,3,8–12]; alkaloids [5,13]; indole auxins [3]; structural [14], geometrical [15], and optical [5,16] isomers, etc. In the present paper pH-zone refining CCC is applied to polar catecholamines using di-(2-ethylhexyl)phosphoric acid [17] as a ligand.

Catecholamines with two to three hydroxyl groups are highly hydrophilic and predominantly partition in the aqueous phase of the conventional organic/aqueous two-phase solvent systems, hence a suitable partition coefficient is only obtained by saturating a polar butanol–water solvent system with a salt such as $BaCl_2$ [18]. The use of basic solvent systems is impractical since these analytes are extremely unstable under alkaline conditions.

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We have found that introduction of a lipid soluble acid such as di-(2-ethylhexyl)phosphoric acid in the stationary phase acts as a ligand toward bases and improves retention of these analytes in the column even at a neutral pH. The partition coefficient of each species can be adjusted by choosing a pH from the neutral to acidic ranges. pH-Zone refining CCC of six components, i.e. five catecholamines and one amino acid (DOPA), was successfully performed using a solvent system consisting of methyl *tert.*-butyl ether and water by adding di-(2-ethylhexyl)phosphoric acid and ammonium acetate to the organic stationary phase and dilute HCl to the aqueous mobile phase.

2. Experimental

2.1. 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 basic design of the apparatus was given elsewhere [19].

The separation column was prepared in our laboratory by winding a single piece of 1.6 mm I.D., 160 m long Tefzel tubing (Zeus Industrial Products, Orangeburg, SC, USA) around the column holder hub making 16 layers with a 315-ml capacity.

The speed of the apparatus was regulated with a speed controller (Bodine Electric, North Chicago, IL, USA). An optimum speed of 800 rpm was used in the present studies.

2.2. Reagents

Methyl *tert.*-butyl ether (HPLC grade), ammonium acetate (reagent grade) and hydrochloric acid (reagent grade) were purchased from Fisher (Fair Lawn, NJ, USA). Di-(2-ethylhexyl)phosphate (DEHPA) was obtained from Sigma (St. Louis, MO, USA). The analytes including DL-DOPA (97%), epinephrine (95%), norepinephrine hydrochloride (99%), normetanephrine hydrochloride (99%), hydroxytyramine hydrochloride (98%) and tyramine hydrochloride (98%) were all from Aldrich (Milwaukee, WI, USA).

2.3. Preparation of solvent phases and sample solutions

The solvent pairs were prepared as follows. About equal volumes of methyl *tert.*-butyl ether and distilled water were thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated. DEHPA (20%, v/v) and ammonium acetate (200 m*M*) were added to the upper organic stationary phase. The lower aqueous phase was acidified with hydrochloric acid (50 m*M*) and used as the mobile phase.

The sample solution was prepared by dissolving a sample mixture (each component 500 mg) in 40 ml of the aqueous phase where 4 ml of 1 *M* HCl was added.

2.4. Separation procedure

The column was first entirely filled with the organic phase containing 20% DEHPA and 200 m*M* ammonium acetate (retainer salt). This was followed by sample injection through the sample port. Then, the acidified aqueous phase containing HCl (eluent acid) at 50 m*M* was pumped into the inlet of the column at a flow-rate of 3.3 ml/min in the head-to-tail elution mode, while the column was rotated at 800 rpm. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 280 nm and collected into test tubes at 2-min intervals (6.6 ml/tube) using a fraction collector (Ultrorac, LKB Instruments). After the desired peaks were eluted, the apparatus 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. (552 kPa). 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. Analysis of CCC fractions

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

The catecholamines were identified by their parti-

tion coefficient, K_{std}, in a standard two-phase system composed of methyl *tert.*-butyl ether–DEHPA–aqueous solution containing ammonium acetate (0.1 *M*) and HCl (0.05 *M*) (9:1:10) (solvent system VII in Table 1). An aliquot of each fraction (0.1 ml) was delivered into a test tube. Then 2 ml of the standard solvent system (1 ml of each phase) was added to each tube and the contents vigorously shaken to equilibrate the solute. After the two layers were formed, 0.1 ml of each phase was diluted with 2 ml of methanol and the absorbance determined at 430 nm. The standard partition coefficient (K_{std}) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

The analytes in each pH zone were also identified by NMR spectra using respective standards.

2.6. Results and discussion

pH-Zone refining CCC has been successfully applied to the separation of ionizable compounds. For the present application with basic components, the following two requirements must be fulfilled: (1) the analytes should partition predominantly into the organic stationary phase containing the retainer (ammonium acetate) but free of the eluent acid (hydrochloric acid); and (2) they should partition predominantly into the aqueous mobile phase containing the eluent acid but free of the retainer.

In the present studies, however, all analytes predominantly partition into the aqueous phase even in the hydrophilic solvent systems such as *n*-butanol–water and *n*-butanol–0.1 *M* ammonium acetate (1:1) (solvents I and II in Table 1). Because these catecholamines are unstable under basic conditions, basic reagents such as ammonia and triethylamine cannot be used as a retainer in the solvent system. We have found that this problem is overcome by a combined use of DEHPA (ligand) and ammonium acetate (retainer) in the organic stationary phase. Ammonium acetate at a 0.2 *M* concentration in the stationary phase maintains a subacidic condition to retain the analytes while further raising the pH to a neutral range would result in an extensive carryover of the stationary phase due to emulsification of the solvent phases. Total elimination of this retainer salt, on the other hand, would cause elution of all the analytes immediately after the solvent front without separation even under the presence of the ligand in the stationary phase.

Table 1 lists partition coefficients for the six analytes in seven different two-phase solvent systems. As mentioned earlier, *n*-butanol systems (I and II) failed to produce satisfactory partition coefficients for all the analytes. In ligand-free solvent systems III (MBE–water) and IV (MBE–ammonium acetate), the analytes favorably partitioned into the aqueous phase throughout the applied pH range. With the presence of the ligand, however, all five catecholamines predominantly partitioned into the stationary phase under neutral conditions (solvent system V) and into the aqueous phase under acidic conditions (solvent system VI), while the ligand was almost unilaterally distributed into the organic phase because of its high *K* value (>100). Since this ligand has such a high partition coefficient in the present solvent system, its contamination in the eluted fractions is almost negligible. As described earlier, this complication may be completely eliminated by placing a small volume of ligand-free stationary phase at the end of the column so that a ligand present in the flowing mobile phase is constantly absorbed by the stationary phase [16].

In brief, the introduction of the ligand in the stationary phase provided an ideal solvent system for pH-zone refining CCC of polar catecholamines by using ammonium acetate as the retainer salt and hydrochloric acid as the eluent acid.

Fig. 1 shows a pH-zone refining CCC separation of the six analytes: one amino acid and five catecholamines (each 500 mg). In the UV tracing (solid line), all catecholamines were eluted as highly concentrated fused rectangular peaks associated with sharp impurity peaks at their borders. The pH-curve of the fractions (dotted line) reveals a succession of plateaus in a downward staircase fashion, where each zone shows a specific pH that may be determined by both pK_a and hydrophobicity of the analyte [3,5]. The $\log K_{std}$ line (crosses) further indicates sharp borders of each zone with minimum overlap. DOPA was eluted much earlier than the major peaks because it is an amino acid (zwitterion) with an extremely small *K* value as indicated in Table 1.

The overall results of the present studies indicate that the use of a ligand in the stationary phase will

Table 1
Partition coefficient of catecholamines in seven different solvent systems

	I BuOH[a]–H_2O	II BuOH –0.1 *M* NH_4OAc[b] (1:1)	III MBE[c]–H_2O	IV MBE– 0.1 *M* NH_4Ac (1:1)	V MBE–DEHPA[d]– 0.1 *M* NH_4OAc (9:1:10)	VI MBE–DEHPA –0.05 *M* HCl (9:1:10)	VII MBE–DEHPA– 0.1 *M* NH_4OAc (9:1:10) plus 0.05 *M* HCl
DOPA	0.21	0.06	<0.01	<0.01	0.15	0.02	0.12
Epinephrine	0.24	0.13	0.06	0.16	5.83	0.01	1.42
Norepinephrine	0.20	0.10	0.04	<0.01	6.12	0.01	1.80
Normetanephrine	0.27	0.18	<0.01	0.02	13.0	0.02	2.84
Dopamine	0.44	0.18	0.08	0.01	19.3	0.03	7.19
Tyramine	0.18	0.39	<0.01	0.02	21.3	0.08	9.76

Note that solvent VII was used as a standard system to measure K_{std} (Fig. 1).
[a] *n*-Butanol.
[b] Ammonium acetate.
[c] Methyl *tert.*-butyl ether.
[d] Di-(2-ethylhexyl)phosphoric acid.

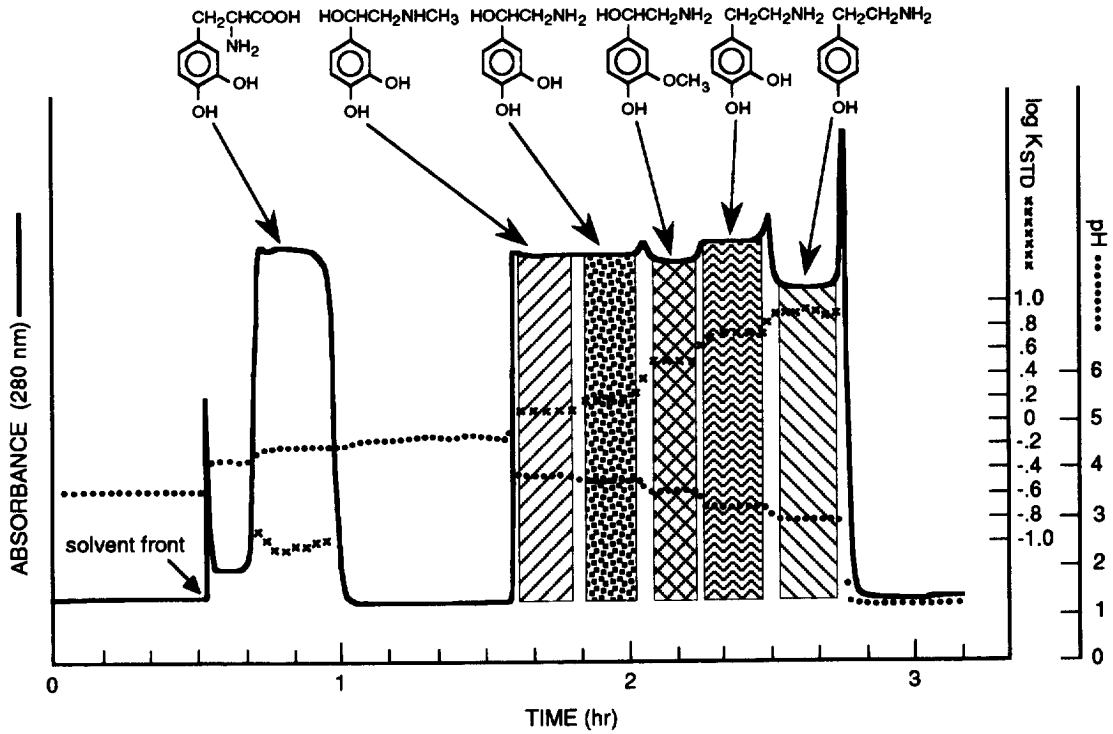

Fig. 1. Separation of the six analytes by pH-zone refining CCC. Experimental conditions are as follows. Apparatus: high-speed CCC centrifuge equipped with a multilayer coil prepared from 1.6 mm I.D., 160-m long Tefzel tubing with a 315-ml capacity; sample: six analytes as indicated in the chromatogram, each 500 mg dissolved in 40 ml of the aqueous phase to which 4 ml of HCl was added; solvent system: methyl *tert.*-butyl ether–water; stationary phase: upper organic phase containing 20% (v/v) DEHPA and 200 m*M* ammonium acetate; mobile phase: lower aqueous phase containing 50 m*M* HCl; flow-rate: 3.3 ml/min; revolution: 800 rpm; detection: 280 nm; retention of the stationary phase: 70% of the total column capacity.

extend the domain of applications for pH-zone refining CCC.

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