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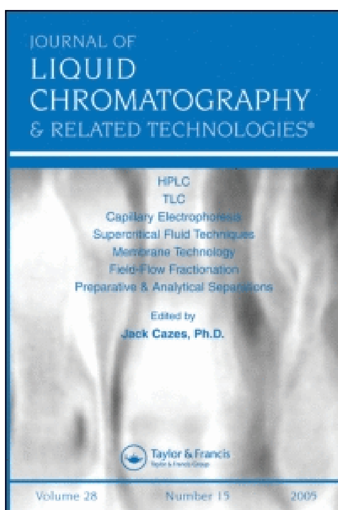

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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ONE-STEP PURIFICATION OF CHOLINESTERASE FROM HUMAN SERUM BY CCC

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Online publication date: 30 June 2001

To cite this Article Shibusawa, Y. , Hosojima, T. , Nakata, M. , Shindo, H. and Ito, Y.(2001) 'ONE-STEP PURIFICATION OF CHOLINESTERASE FROM HUMAN SERUM BY CCC', Journal of Liquid Chromatography & Related Technologies, 24: 11, 1733 — 1744

To link to this Article: DOI: 10.1081/JLC-100104375

URL: http://dx.doi.org/10.1081/JLC-100104375

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J. LIQ. CHROM. & REL. TECHNOL., 24(11&12), 1733–1744 (2001)

EXTRACTIONS AND PURIFICATIONS

ONE-STEP PURIFICATION OF CHOLIN-ESTERASE FROM HUMAN SERUM BY CCC

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ABSTRACT

The type XL cross-axis coil planet centrifuge (CPC) was successfully utilized for purification of cholinesterase (ChE) from human serum by countercurrent chromatography (CCC). Aqueous-aqueous polymer phase systems composed of polyethylene glycol-potassium phosphate buffers were tested at various pHs, ranging from 6.8 to 9.2 for the distribution ratios (D) of ChE, human serum albumin, α- and γ-globulins. The CCC separation was performed with optimized polymer phase systems in two different coiled columns. The best purification was achieved with 16.0% PEG 600-12.5% potassium phosphate at pH 9.2, by eluting the lower phase at 0.5 mL/min through the small capacity column in which ChE was purified directly from human serum within 2h. The ChE fractions confirmed by the enzymatic activity were free of serum proteins by SDS PAGE analysis.

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INTRODUCTION

Albertsson introduced polymer biphasic systems for partitioning a variety of macromolecules and cell particles.[1] Among many types of aqueous-aqueous polymer phase systems available, polyethylene glycol (PEG)-dextran and PEG-potassium phosphate systems have been most commonly used for partition of biological samples.

Using these polymer phase systems, several different approaches have been made for performing purification of biological samples, which include single-step partitioning, repetitive batch extraction, thin-layer countercurrent distribution (CCD),[1] and countercurrent chromatography (CCC).[2-10] Among these techniques, CCC is considered to be most effective in terms of partition efficiency and separation times. CCC is essentially a form of liquid-liquid partition chromatography. Its unique feature, among other chromatographic systems, is derived from the fact that the method uses no solid support, and the stationary phase is retained in the column with the aid of gravity or centrifugal force.

Several types of cross-axis coil planet centrifuge (CPC) have been designed for performing CCC using highly viscous polymer phase systems for preparative scale separations.[2-10] The apparatus, equipped with column holders mounted in the off-center position on the rotary shaft, allows satisfactory retention of the stationary phase of viscous polymer phase systems. This unique capability of the apparatus has been demonstrated in separation and purification of various protein samples, such as histones,[11] serum proteins,[11] recombinant uridine phosphorylase from *E. coli* lysate,[12] human lipoproteins from serum,[13-15] lactic acid dehydrogenase from a crude bovine heart extract,[16] and profilin-actin complex from *Acanthamoeba* extract.[17]

For the small-scale separation, a toroidal-coil centrifuge is mainly used.[18,19] The combined use of aqueous-aqueous polymer phase systems and the cross-axis CPC allows the separation and purification of a variety of enzyme proteins without denaturation, thus, preserving their enzymatic activity.

In the present studies, ChE was purified from human serum using a polymer biphasic system composed of PEG-potassium phosphate using the cross-axis CPC. The careful selection of the solvent system, based on the distribution ratios of ChE and serum proteins, resulted in one-step separation of ChE from the human serum.

EXPERIMENTAL

Apparatus

The CCC purification of cholinesterase (ChE) from human serum was performed using type-XL cross axis coil planet centrifuge (CPC) (Fig. 1). The appa-

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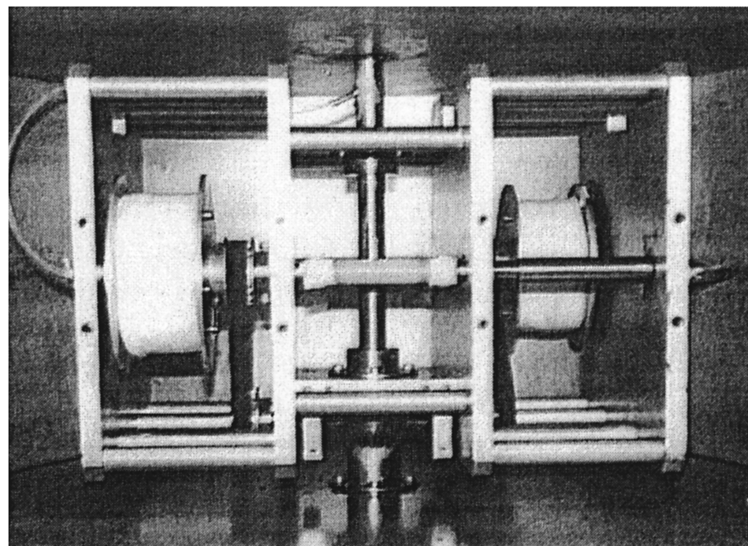

Figure 1. Photograph of type XL cross-axis coil planet centrifuge.

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ratus holds a pair of horizontal rotary shafts symmetrically, one on each side of the rotary frame, at a distance of 10 cm from the central axis of the centrifuge. A spool-shaped column holder is mounted on each rotary shaft at a lateral location 10 cm away from the midpoint.

The small capacity separation column was prepared from 3.0 mm-I.D. and 5.7 m long polytetrafluoroethylene (PTFE) tubing (Tokyo Rikakikai Co. Ltd., Tokyo, Japan) by winding it onto a 5 cm-diameter holder hub, making a single layer of left-handed coils between a pair of flanges spaced 5 cm ($\beta = 0.5$). The large column was similarly made by winding 2.6 mm-I.D., 37 m long PTFE tubing (Zeus Industrial Products, Raritan, NJ, USA), making three layers of left-handed coils ($\beta = 0.5$-0.6). Each number of tubing turns per spool is 6 turns for small capacity column and is 15 turns for large one. A pair of columns mounted on the rotary frame was connected in series to provide a total capacity of 40 mL for the small column and 165 mL for the large column. The revolution speed of the apparatus is regulated at 400 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

Reagents

Polyethylene glycol (PEG) 400, 600, and 1000 (average molecular mass, Mr =400, 600, and 1000), and monobasic and dibasic potassium phosphate for

the preparation of aqueous polymer two-phase systems, were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cholinesterase (ChE) from horse serum (100 U/mg), used as the standard enzyme for the measurement of the distribution ratio of ChE, and Cholinesterase B test Wako for the measurement of the ChE enzyme activities were obtained from Wako. Other chemicals were all of analytical reagent grade.

Preparation of CCC Sample Solution

Human peripheral blood (*ca.* 10 mL) was collected from healthy male volunteers by venipuncture after 12-16 h of fasting. The blood was allowed to stand at room temperature until agglutination was completed. The serum was collected after centrifugation at 1000 g at room temperature for 15 min. The sample solution for CCC was then prepared by adding 1.6 g of PEG and 1.25 g of potassium phosphate to about 7.15 g human serum, to bring the phase composition similar to that of the solvent system used for the separation.

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Preparation of PEG-Potassium Phosphate Aqueous Two-Phase Systems

Two-phase solvent systems, composed of 12.5-25.0% (w/w) PEG and 12.5 %(w/w) potassium phosphate aqueous solutions, were prepared by dissolving 250-500 g of PEG and 250 g of potassium phosphate (a mixture of monobasic and dibasic potassium phosphates) in 1250-1500 g of distilled water. The pH values of the solvent systems were adjusted to 6.8, 7.3, 8.0, and 9.2 by changing the molar ratio between monobasic and dibasic potassium phosphates in the two-phase system.

Measurement of Distribution Ratio of Cholinesterase and Standard Serum Proteins

The distribution ratios of ChE and standard protein samples were determined spectrophotometrically by a simple test tube procedure. About 1.0 mL of each phase of the equilibrated aqueous-aqueous two-phase solvent system was delivered in a test tube, to which about 25 µL of ChE solution (500 U/mL) or 1 mg of the standard protein was added. The contents were thoroughly mixed and allowed to settle at room temperature. After two clear layers were formed, the enzyme activities in the 100 µL of the aliquots of the upper and lower phases were determined with the method described by Gomi.[20] The distribution ratios of the standard serum proteins were determined as follows: An aliquot (usually 0.5

mL) of each phase was pipetted and diluted with 1.0 mL of distilled water to determine the absorbance at 280 nm using a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). The distribution ratios ($D=C_U/C_L$) of ChE were obtained by dividing the enzyme activities of the upper phase by those of the lower phase. The distribution ratios of human serum albumin (HSA), α- and γ-globulin were also determined by dividing the absorbance of the upper phase by that of the lower phase.

CCC Fractionation of Cholinesterase from Human Serum

In each experiment, the CCC column was first entirely filled with the PEG-rich upper stationary phase, and the CCC sample solution (usually 10 g) was injected into the column using an EYELA type SV-6010 sample injector (Tokyo Rikakikai, Tokyo, Japan). Then, the potassium phosphate-rich lower mobile phase was eluted through the column at a flow rate of 0.5 mL, while the apparatus was rotated at 400 rpm. The effluent from the outlet of the column was continuously monitored with an EYELA UV-9000 absorbance monitor (Tokyo Rikakikai) at 280 nm, and fractionated into a Bio-Rad Model 2110 fraction collector (Bio-Rad, Richmond, CA, USA). After the desired peaks were collected, the column was eluted with the PEG-rich upper phase (initially used as the stationary phase) in the opposite direction without stopping the centrifuge run. This reversed elution mode was continued until all retained analytes were eluted from the column.

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Measurement of ChE Enzyme Activity

ChE in the CCC fractions was determined by the benzoylcholine substrate method[20] using Cholinesterase B test Wako reagent kit as follows: benzoylcholine, the substrate of ChE, was decomposed into choline and benzoic acid by ChE in each CCC fraction. Choline was then oxidized by cholineoxidase to produce hydrogen peroxide. Then, phenol was coupled with 4-amino antipiline by the action of hydrogen peroxide, resulting in formation of red quinone compounds. The red color of this product was measured by absorbance at 505 nm. The ChE enzymatic activities in the CCC fractions were determined using the calibration curves constructed from the standard ChE solutions.

Analysis of CCC Fractions

An aliquot of each fraction was diluted with distilled water by three times its volume, and the absorbance was measured at 280 nm with a Shimadzu UV-1200 spectrophotometer. CCC fractions, containing ChE and serum proteins,

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were concentrated with an ultrafiltration membrane YM 10 (Millipore, Bedford, MA, USA) for the characterization of the ChE and serum proteins by SDS slab gel electrophoresis (SDS-PAGE), according to the method of Laemmli.[21] Gels containing 3%(w/v) (stacking gel) and 10 % (w/v) (separation gel) acrylamide were prepared from a stock solution of 30 % (w/v) acrylamide and 0.8 % (w/v) N, N'-methylene-bis acrylamide. A 5.5 x 10 cm separation gel and a 1.0 x 10 cm stacking gel, each 0.75 mm thick were prepared between glass plates. A 5 µL-volume of eluate was mixed with 95 µL of sample solution [a mixture of 0.025M tris (hydroxymethyl) aminomethane, 2%(w/v) sodium dodecyl sulfate (SDS), 5% (w/v) 2-mercaptoethanol, 4% (w/v) glycerol, and 0.01% (w/v) bromophenol blue (BPB)], and 10-20 µL of the sample solution was loaded over the stacking gel.

Electrophoresis proceeded at a current of 10 mA until the BPB marker reached the stacking gel. Thereafter, the current was increased to 20 mA and the electrophoresis continued until the BPB marker reached the bottom of the separation gel. The migrated proteins were stained for 5 min at room temperature with a staining solution composed of 0.25% (w/v) Coomassie brilliant blue, 50% (v/v) methanol, and 10% (v/v) acetic acid. The gel was unstained by washing in a mixture of 7.5% (v/v) acetic acid and 2.5% (v/v) methanol.

RESULTS AND DISCUSSION

Distribution Ratio of Standard Proteins

CCC uses a two-phase solvent system where the separation is based on the difference in distribution ratios of solutes between the two phases. In order to achieve efficient separation of ChE from human serum, it is essential to optimize the distribution ratio of proteins in the solvent system, so that ChE is well resolved from serum proteins, such as serum albumin, α - and γ-globulin.

Table 1 shows the distribution ratios ($D=C_U/C_L$) of ChE in PEG 400, 600, and 1000-potassium phosphate solvent systems each at 6.8, 7.3, 8.0, and 9.2. In the PEG 400 system, the D ratios of ChE are greater than 3.7. In the two-phase solvent systems containing PEG 600, the D ratios of ChE are reduced to 2.1 at 20% while the D ratios of less than 1.0 are obtained in the 16.0% PEG 600-12.5% potassium phosphate at pH 8.0 and 9.2. In the solvent systems containing PEG 1000, the D ratios of ChE were less than 1.0 except for the high PEG concentration of 25.0%. In order to separate the ChE from human serum proteins, the distribution ratios of HSA, α- and γ-globulins, were measured in the solvent system composed of several concentrations of PEG 600 or 1000-12.5% potassium phosphate buffer at pH 9.2, in which the D ratios of ChE were smaller than 0.20.

Table 1. Distribution Ratio of Cholinesterase in Several PEG-Potassium Phosphate Buffer Two-Phase Systems

Concentration % w/w	pH 6.8	pH 7.3	pH 9.2
PEG 400			
22.5%	21.3	3.79	4.29
25.0%			5.75
PEG 600			
16.0%	—	—	0.2
20.0%	4.1	4.2	2.1
22.5%	6.8	4.3	2.0
25.0%	16.1	11.2	4.4
PEG 1000			
12.5%	—	—	0.01
16.0%	0.10	0.09	0.02
20.0%	0.07	0.18	0.08
22.5%	0.22	0.30	0.14
25.0%	3.77	3.70	1.62

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In Fig. 2A, the D ratios of cholinesterase, three standards for human serum proteins, are plotted on a logarithmic scale against the PEG concentration of the PEG 600-potassium phosphate system at pH 9.2. The D ratio of ChE increases as the PEG concentration is increased from 16.0 to 22.5%. The maximum D ratios of HSA, α- and γ-globulin around 100 are obtained at 20.0% PEG concentration, whereas, differences in D ratios between ChE and three human serum proteins are greatest in the 16.0% PEG 600-12.5% potassium phosphate buffer at pH 9.2.

In Fig. 2B, the D ratios of ChE and three serum proteins are plotted on a logarithmic scale against the PEG concentration of the PEG 1000-potassium phosphate buffer systems at pH 9.2. The D ratios of ChE, HSA and α-globulin increase with the concentration of PEG 1000, while the D ratio of γ-globulin shows an opposite trend, decreasing as the concentration of PEG 1000 is increased. Large differences in D ratios between ChE and human serum proteins, especially HSA, are observed in the 12.5% PEG 1000-12.5% potassium phosphate buffer at pH 9.2.

CCC Purification of Cholinesterase from Human Serum

From the results of the partition experiment described above, two polymer systems composed of 16% PEG 600-12.5% potassium phosphate buffer (pH 9.2)

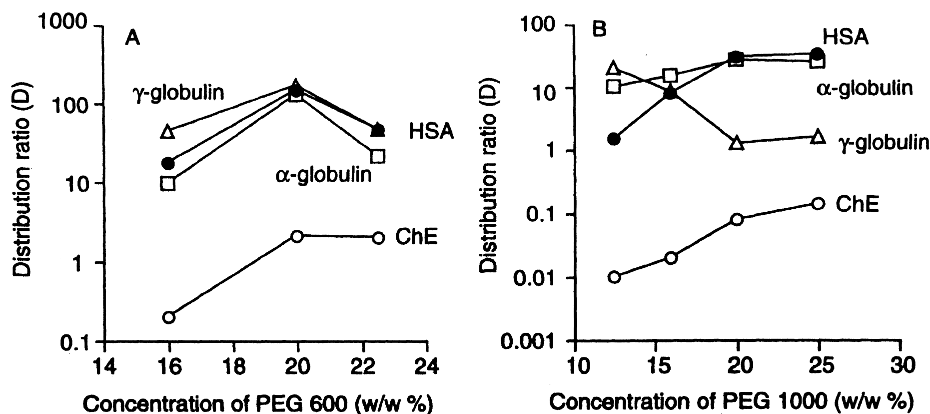

Figure 2. Effect of the concentration of polyethylene glycol 600 (A) and 1000 (B) on the distribution ratios of cholinesterase, HSA, α- and γ-globulins.

and 12.5% PEG 1000-12.5% potassium phosphate buffer (pH 9.2), were selected for the CCC purification of ChE from human serum.

Fig. 3 shows the chromatogram of human serum obtained from the optimized PEG 1000 solvent system using a large capacity column (165 mL). The

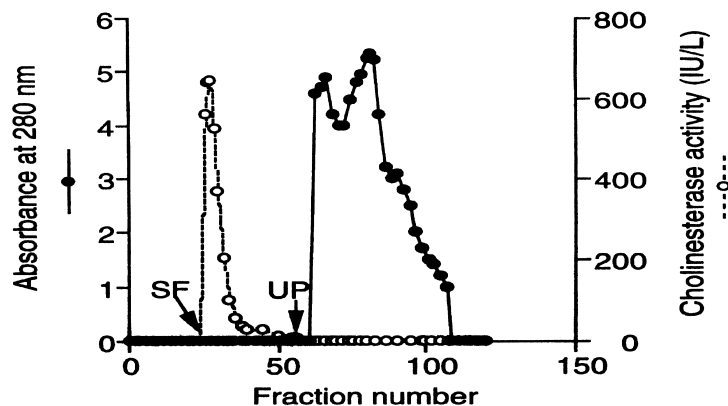

Figure 3. Purification of cholinesterase from human serum by CCC. Experimental conditions: column: a pair of 2.6 mm-ID Teflon multilayer coils with a total capacity of 165 mL; sample: a mixture of 1.6 g PEG 1000, 1.25 g potassium phosphate and 7.15 g human serum; solvent system: 12.5 % PEG 1000 and 12.5 % potassium phosphate at pH 9.2; mobile phase: phosphate-rich lower phase; flow rate: 0.5 mL/min; revolution: 400 rpm; SF: solvent front; UP: upper phase eluted in the reversed direction.

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sample solution consisted of 1.6 g of PEG 1000, 1.25 g of dibasic potassium phosphate, and 7.15 g human serum, which gave a phase composition similar to that of the solvent system used for CCC separation. The separation was performed at 400 rpm at a flow-rate of 0.5 mL/min, using the lower phase as the mobile phase. The cholinesterase (D=0.01) was eluted within 3.3 h. After the elution of the enzyme, PEG 1000-rich upper phase was pumped into the column, in the reversed direction, to facilitate rapid elution of the serum proteins (HSA, α- and γ-globulin with D=1.5, 10.1 and 20.5, respectively) still remaining in the column. The retention of the stationary phase is estimated at 52.7% of the total column capacity at the time when the elution mode was reversed. Fig. 4 shows the 10% slab SDS-PAGE analysis of the CCC fractions. The first peak corresponding to fractions 26-30 contained ChE, in which no protein bands was detected. The second peak obtained by the reversed elution, consisted mostly of human serum proteins, such as HSA, α- and γ- globulins. The results clearly indicate that ChE was completely resolved from the serum proteins.

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Similar separation was performed with the second solvent system composed of 16.0% PEG 600-12.5% potassium phosphate buffer at pH 9.2, in which the D ratio of ChE is small (D=0.2). Fig. 5 shows a chromatogram of human serum with the above PEG 600 solvent system using a large multilayer coil (165

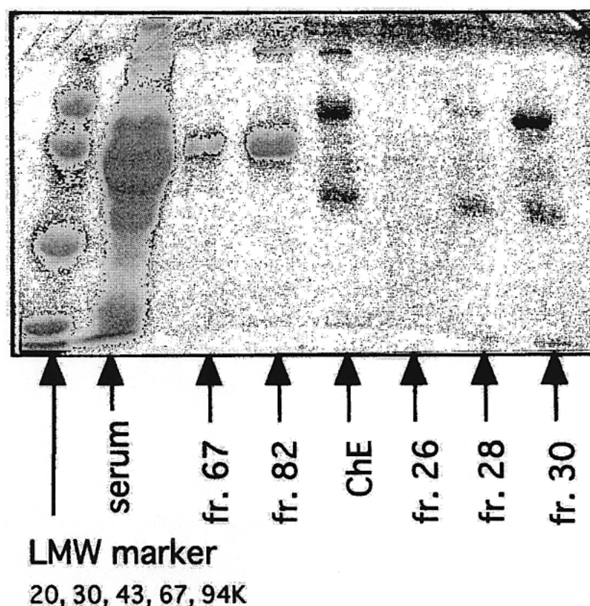

Figure 4. SDS 10% polyacryl amide gel electrophoresis of CCC fractions.

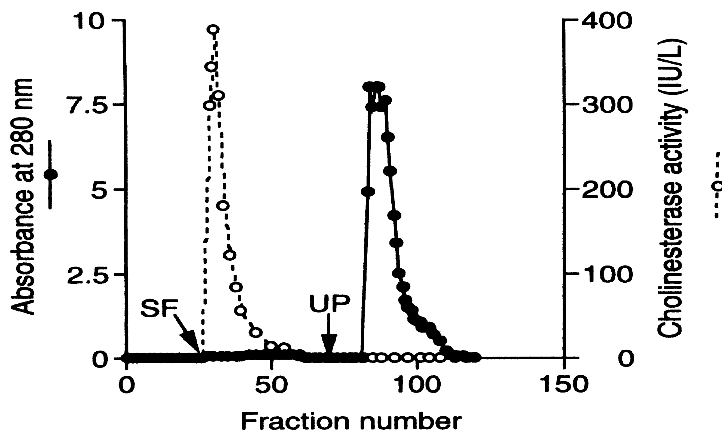

Figure 5. Purification of cholinesterase from human serum by polymer phase CCC. Experimental conditions: solvent system: 16 % PEG 600 and 12.5 % potassium phosphate at pH 9.2; other conditions are described in the Fig. 3 caption. SF: solvent front; UP: upper phase eluted in the reversed direction.

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mL). ChE was eluted within 5.0 h, and the elution mode was reversed to elute the serum proteins. The retention of the stationary PEG 600-rich upper phase is estimated to be 49.0% of the total column capacity. The SDS-PAGE analysis (data not shown) showed no serum proteins present in the fractions 25-50, which exhibited the ChE activities.

In order to reduce the separation time of ChE from the human serum, the small capacity column (40 mL) was tested. Fig. 6 shows a similar separation performed using the solvent system composed of 16.0% PEG 600-12.5% potassium phosphate buffer at pH 9.2. The sample solution containing 1.6 g PEG 600, 1.25 g of dibasic potassium phosphate and 7.15 g human serum, was injected into the column. ChE was eluted within 2h as a sharp peak, and the elution mode was reversed to elute the serum proteins. Using the small capacity column, the separation time of ChE was successfully reduced to less than 2h without affecting the yield of ChE. The retention of the stationary upper phase is estimated to be 32.5% of the total column capacity.

The results of the above studies, indicated that the combined use of 16.0% PEG 600-12.5% potassium phosphate buffer (pH 9.2) and the cross-axis CPC enable the one step purification of ChE from human serum in a relatively short elution time. The yield of the ChE from the column is estimated about 90-100% by calculation from the enzyme activities. The 10 g sample solution containing 7.15 g of human serum was loaded on the small capacity column, and ChE was separated from the serum proteins. It must be shown, that the large capacity col-

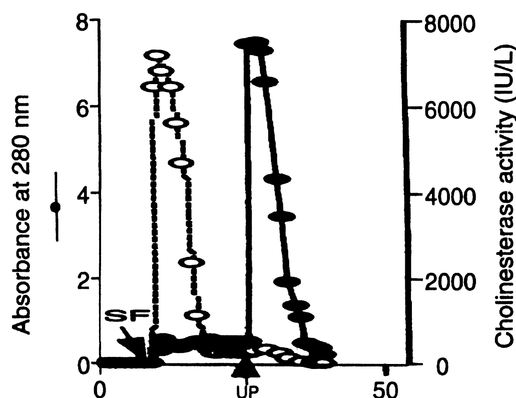

Figure 6. Rapid purification of cholinesterase from human serum by CCC with a small-capacity column. Experimental conditions: column: a pair of 3.0 mm-ID Teflon single layer coil with a total capacity of 40 mL; other experimental conditions are described in the Fig. 5 caption. SF: solvent front; UP: upper phase eluted in the reversed direction.

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umn enables separation of a large amount of human serum, and to purify ChE by only a one step operation.

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Received August 9, 2000
Accepted October 10, 2000
Author's Revision October 2, 2000
Manuscript 5484

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