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Short Communication

Counter-current chromatography of lipoproteins with a polymer phase system using the cross-axis synchronous coil planet centrifuge

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ABSTRACT

Lipoproteins were separated by counter-current chromatography using the type-XLL coil planet centrifuge. The separation was performed with a polymer phase system composed of 16% (w/w) polyethylene glycol 1000 and 12.5% (w/w) dibasic potassium phosphate by eluting the lower phase at a flow-rate of 0.5 ml/min. About 5 ml of the sample solution containing approximately 150 mg of a lipoprotein mixture were loaded. High- and low-density lipoproteins were resolved within 12 h. Each component was detected by gel electrophoresis with oil red staining.

INTRODUCTION

The chromatographic separations of lipoproteins into three main classes have been reported using several types of colum packings such as Bio-Gel [1,2], Superose [3], TSK GEL [4–6] and hydroxyapatite [7] whereas, to our knowledge, the fractionation of lipoproteins by counter-current chromatography (CCC) has not been published. Since CCC performs separations without the solid support matrix, adsorptive loss and denaturation of proteins at the liquid–solid interface are minimized [8,9].

This paper describes preliminary results of the lipoprotein separation by utilizing the cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC) [10,11]. Recently, the X-axis CPC has been remarkably improved in terms of retention of the stationary phase. The apparatus is designed to accommodate a pair of small column holders each at a lateral location 7.6 cm from the center of the holder shaft. As reported elsewhere [12], this type-XLL X-axis CPC has a unique capability of retaining large amounts of stationary phase of low-interfacial-tension, viscous solvent systems effectively used for separation of polar compounds.

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In the present paper the performance of the type-XLL X-axis CPC is evaluated in separation of high-density (HDLs) and low-density (LDLs) lipoproteins from human plasma using an aqueous–aqueous polymer phase system. The partition coefficients (K) of the HDL and LDL fractions were optimized by choosing a polymer phase system composed of 16.0% (w/w) polyethylene glycol (PEG) 1000 and 12.5% (w/w) anhydrous dibasic potassium phosphate in distilled water. The PEG-rich upper phase was introduced into the column and phosphate-rich lower phase was used for elution of the lipoproteins. The separations were performed in a pair of multilayer coils coaxially mounted around the column holders with 3.8 cm hub diameter. The eluted HDL and LDL fractions were confirmed by agarose gel electrophoresis.

EXPERIMENTAL

Apparatus

Fig. 1 shows a photograph of the X-axis CPC used in the present study. The rotary frame of the apparatus holds a pair of horizontal rotary shafts symmetrically at 7.6 cm from the central axis of the centrifuge. Thus, each rotary shaft forms a cross to the vertical axis of the centrifuge as indicated by the name of the apparatus. A spool-shaped coil holder is mounted on each rotary shaft at a lateral position 15 cm away from its midpoint. Each coil holder measures 3.8 cm in hub diameter and 5 cm in width between the pair of flanges. A separation column was mounted on each holder by winding a 2.6 mm I.D. polytetrafluoroethylene (PTFE) tube (Zeus Industrial Products, Raritan, NJ, USA) directly onto the holder hub making multiple layers of left-handed coils. All coiled layers were connected in series by bridging each neighboring layer across the width of the column with a piece of small-bore PTFE transfer tubing (0.7 mm I.D.) using a short sheath of intermediate-size tubing (1.6 mm I.D.) as an adaptor.

Procedure

The aqueous–aqueous polymer phase system was prepared by dissolving 192 g of PEG 1000 (Sigma,

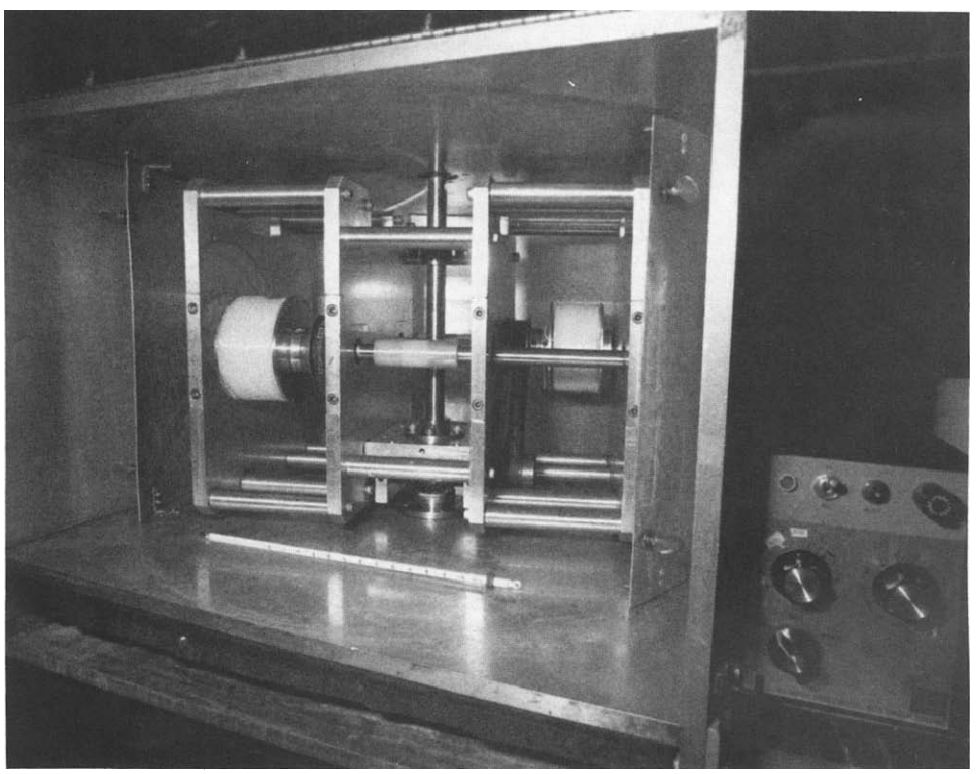

Fig. 1. Photograph of type-XLL cross-axis coil planet centrifuge.

St. Louis, MO, USA) and 150 g of anhydrous dibasic potassium phosphate (J.T. Baker, Phillipsburg, NJ, USA) in 858 g of distilled water. This composition yielded approximately equal volumes of upper and lower phases. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The partition coefficients of the HDL annd LDL fractions and of human serum albumin and α- and γ-globulins (Sigma) were determined in the two-phase solvent system composed of PEG 1000 and dibasic potassium phosphate dissolved at various concentrations in distilled water. Human HDL and LDL fractions were isolated by ultracentrifugation [13]. A lipoprotein suspension, 0.4 ml containing 5.29 mg/ml protein for the HDL fraction and 0.76 mg/ml of protein for the LDL fraction, was partitioned in 3 ml of the aqueous polymer two-phase system (1.5 ml each of the upper and lower phases). An 0.8-ml aliquot of each phase was diluted with 2 ml of distilled water and the absorbance was measured at 280 nm with a Zeiss (Hanover, MD, USA) PM6 spectrophotometer. The partition coefficient values ($K = C_L/C_U$, where C_L and C_U are the solute concentrations in the lower and upper phases, respectively) were calculated by dividing the absorbance in the lower phase with that in the upper phase. The K values of the other plasma proteins were determined by adding about 2 mg of each sample to the solvent system.

The sample solution was prepared by adding 1 g of PEG 1000 and 0.9g of anhydrous dibasic potassium phosphate to the mixture of 3 ml HDL fraction (44.2 mg/ml protein) and 2 ml LDL fraction (11.4 mg/ml protein). Each experiment was initiated by filling the entire column with the stationary upper phase. This was followed by sample injection through the sample port. Then the apparatus was rotated at 750 rpm while the mobile lower phase was pumped into the column at a flow-rate of 0.5 ml/min in the proper elution mode [12]. The effluent from the outlet of the column was continuously monitored with an LKB (Stockholm, Sweden) Uvicord S at 280 nm and then collected into test tubes with an LKB Ultrorac fraction collector. An aliquot of each fraction was diluted with distilled water and the absorbance was determined at 280 nm with the Zeiss PM6 spectrophotometer. The peak fractions (20–50 ml) were placed into dialysis tubing (Spectro/Por, molecular weight cutoff 6000–8000; Spectrum Medical Industries, Los Angeles, CA, USA) which was immersed into an aqueous 30% (w/v) PEG 8000 solution. After 5–6 h dialysis, the fraction was concentrated to 0.2–0.3 ml. The lipoproteins in each fraction were confirmed by 0.6% agarose gel electrophoresis (Helena Labs., Beaumont, TX, USA) [14].

RESULTS AND DISCUSSION

CCC is a liquid–liquid partition method where the separation is based on the difference in the partition coefficient of solutes. For achieving efficient separation of lipoproteins from other serum proteins, it is essential to optimize the partition coefficient of each component by selecting a suitable composition of the polymer phase system. We found that a two-phase solvent system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate provides desired K values for the HDL fraction (3.8) and the LDL fraction (1.8) which are substantially different from those of other plasma proteins such as albumin (0.12), α-globulin (0.05) and γ-globulin (0.02).

In order to demonstrate the capability of the X-axis CPC for the separation of lipoproteins from other plasma proteins, a mixture of LDL and HDL fractions from human plasma was eluted using the polymer phase system composed of 16.0% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate. As shown in Fig. 2A, the HDL and LDL fractions were eluted from the column in the order of their partition coefficient values and also partially separated from other plasma proteins. The separation was completed within 12 h and the volume of the upper stationary phase retained in the column was 45.0% of the total column capacity (250 ml). Fig. 2B shows the agarose gel electrophoretic patterns of each peak. The lipid moiety of the lipoproteins was stained by oil red 7B. The fractions 43–50 and 60–70, corresponding to center cuts of the first and second peaks in the chromatogram, contained HDLs and LDLs, which migrated to the respective positions in lanes 1 and 2. The third peak in the chromatogram may represent plasma protein, because the fraction showed no lipid staining. No lipoprotein was detected in either the upper or

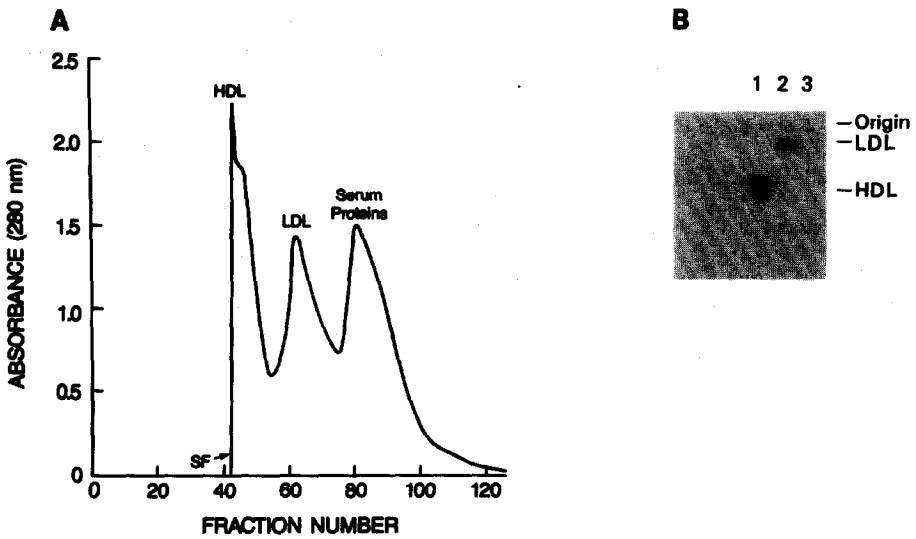

Fig. 2. Separation of lipoproteins by the X-axis CPC (A) and 0.6% agarose gel electrophoretic profile of the fractions (B). Columns: A pair of multilayer coils connected in series, 2.6 mm I.D. and 250 ml capacity; solvent system: 16.0% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase: phosphate-rich lower phase; flow-rate: 0.5 ml/min; revolution speed: 750 rpm; maximum column pressure: 20 p.s.i. SF (A) = Solvent front. Lanes (B): 1 = CCC fractions 43–50; 2 = CCC fractions 60–70; 3 = CCC fractions 80–90.

lower phase collected from the column after the completion of the separation.

From the obtained chromatogram, the partition coefficient of each peak was computed using the classical equation [8,9]. The results yielded 17.8 for the first peak, 1.8 for the second peak, and 0.86 for the third peak. Among these, the K value of the second peak agreed with the measured K value of LDLs while that of the first peak was over four times the measured K value of HDLs (3.8). As mentioned earlier, the lipoprotein sample used for determination of K values was prepared by ultracentrifugation (flotation method). Consequently, the HDL fraction contained some amounts of serum protein while the LDL fraction had much higher purity. Since the K value was determined by measuring UV absorbance, the presence of substantial amounts of serum protein in the HDL fraction resulted in an error in the K value. As of yet, we have no reasonable explanation for the discrepancy in K values between the third peak and serum protein.

The aqueous–aqueous polymer systems are useful for the partition of macromolecules including proteins, nucleic acids, polysaccharides, etc. [15]. However, high viscosity and low interfacial tension between the two phases tends to delay the phase separation, thus requiring long separation times. Although various types of counter-current chromatographs [16–19] have been introduced to overcome this problem, the amount of the stationary phase retained in the column is extremely limited unless the flow-rate of the mobile phase is reduced to a few tenths of a milliliter per minute. The type-XLL X-axis CPC provides a satisfactory retention of the stationary phase at relatively high flow-rates of 0.5–1 ml/min, thus facilitating the use of these solvent systems for the separation of various biopolymers.

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