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IMPROVEMENT OF THE PARALLEL FLOW DIALYSIS TECHNIQUE FOR THE DETECTION OF DRUG-BINDING PROTEINS IN COLUMN EFFLUENTS

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SUMMARY

The parallel flow dialysis technique was improved for application to the detection of drug-binding proteins in a column chromatographic effluent. To prevent the baseline drift, the pressures of both protein and drug channels were maintained equal during chromatography, and Brij-35 was added to the solvents. The improved method was successfully applied to the detection of methyl orange-binding proteins in human serum and bromophenol blue-binding proteins in rat liver homogenate.

INTRODUCTION

We have recently reported a method for the detection of drug-binding proteins by using a parallel flow dialysis (PFD) technique[1]. Preliminary application of the system to the detection of drug-binding proteins in a gel filtration column effluent was unsuccessful owing to baseline drift during chromatography.

In this work, various factors associated with the drift were studied in order to set up the system and to improve the technique for the chromatographic detection of drug-binding proteins, using methyl orange (MO) and bromophenol blue (BPB) as models of drugs.

EXPERIMENTAL

Materials

MO, BPB and sodium azide were purchased from Kanto Chemicals (Tokyo, Japan). Crystalline bovine serum albumin (BSA, product number A-4378) was purchased from Sigma (St. Louis, MO, U.S.A.) and antisera to human albumin and human IgG from Behring Institute (Hoechst, Frankfurt/M, G.F.R.). Brij-35 was obtained from Nakarai Chemicals (Kyoto, Japan). Phosphate-buffered saline (PBS) was prepared as described previously[1]. Sephacryl S-300 and Pharmacia K16/100 columns were purchased from Pharmacia (Uppsala, Sweden). A loop injector (Toyo Soda, Tokyo, Japan) with a 675-μl volume was used for the in-stream injection of BSA solutions. A Technicon dialyser for the AutoAnalyzer (basic module) fitted with

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a cellulose membrane (Cuprophan membrane, Technicon Chemicals, Orcq, Belgium) was used. Two detectors [LDC SF-1205 (Atto Corp., Tokyo, Japan) and Uvigraph LC-1 (Iatron Labs., Tokyo, Japan)] equipped with a low-pressure mercury vapour lamp, and Hitachi Model 634 (Hitachi Seisakusho, Tokyo, Japan) and Shimadzu SPD-1 (Shimadzu Seisakusho, Kyoto, Japan) variable wavelength-detectors were used. The signals were recorded on a TOA EPR-100A or a TOA EPR-2TC recorder (TOA Electronics, Tokyo, Japan).

Preparation of protein samples

Ten millilitres of venous blood were taken from a healthy male (aged 29), allowed to stand for 30 min at room temperature and centrifuged at 1500 *g* for 10 min. The upper layer was taken as the human serum sample and used immediately or kept in a refrigerator (4°C) until required for use.

Rat liver homogenate was prepared according to the method of Arias and coworkers[2–4] with a slight modification. Liver of male Wistar rats (300–400 g) was homogenized with PBS containing 0.001 % of Brij-35.

Identification of human albumin and human IgG in chromatographic fractions by the immunoprecipitation method

A 10-μl aliquot of the fractions obtained with the Sephacryl S-300 column was mixed with 200 μl of the antiserum to human albumin or human IgG and allowed to stand for 60 min at room temperature. The turbidity of each sample due to the formation of an immuno-complex was measured with a Behring laser–nepherometer (Hoechst).

Measurement of pressure by an air damper-type pressure gauge

A pressure gauge was devised for determining the generated pressure without interfering with the solvent flow. It was made of a glass tube, one end of which was closed (28 cm × 2 mm I.D.) and connected by a PTFE tube to a model flow system. The pressure was calculated by measuring the volume of air in the glass tube and fitting it to Boyle's law. The flow-rate was maintained at 0.2 ml/min throughout the experiment.

Flow dialysis system

The flow diagram of the improved PFD system is illustrated in Fig. 1. The elution solvent for the protein channel consisted of 0.02 % sodium azide and 0.001 % Brij-35 in PBS. Continuous flow in the protein channel and the drug channel was effected with two plunger-type pumps, KSD-16 and KHD-16 (Kyowa Seimitsu, Tokyo, Japan). Two types of PTFE tubes (9 m × 1 mm I.D. and 7 m × 0.1 mm I.D.) were connected in series to form a damper. A gel filtration column packed with Sephacryl S-300 (Superfine, 100 cm × 16 mm I.D.) was used for the separation of proteins. The absorbance of the effluent from the dialyzer in the protein channel was monitored at 280 nm with a Hitachi Model 634 detector and that of the drug channel was monitored with a Shimadzu SPD-1 detector at 490 nm with MO or at 590 nm with BPB. To equalize the resistance of the two channels, a PTFE tube (8.5 m × 0.5 mm I.D.) was connected to the Hitachi Model 634 detector. The products from the two channels were led to a bottle filled with PBS, keeping its surface at a constant level by means of an overflow.

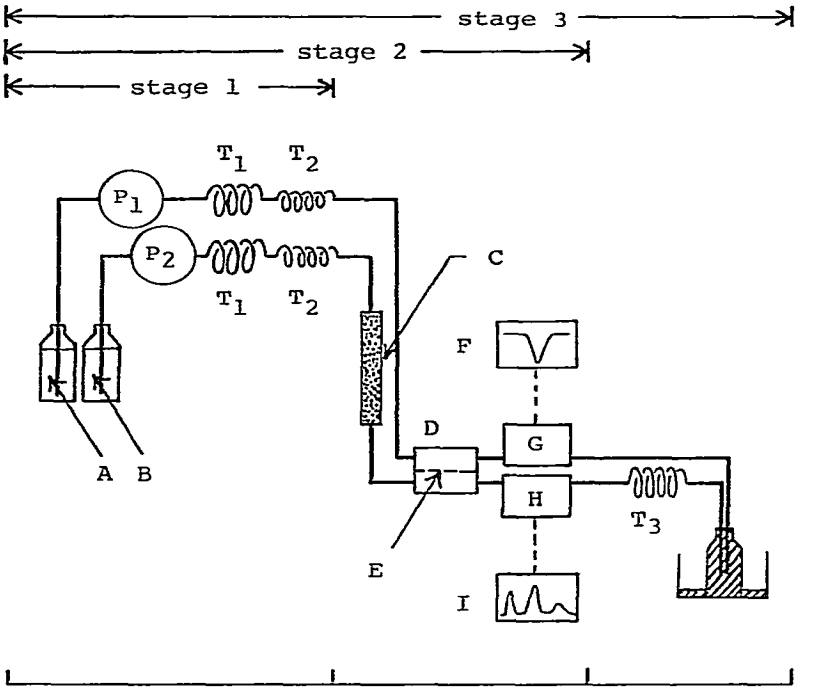

Fig. 1. Schematic representation of the improved parallel flow dialysis system as a detector for column chromatography. A, 0.02% NaN_3–0.001% Brij-35–PBS; B, MO or BPB solution in solvent A; C, Sephacryl S-300 (superfine) column (100 cm × 16 mm I.D.); D, dialyzer of the Technicon AutoAnalyzer basic module; E, Cuprophan cellulose membrane; F, TOA EPR-100A recorder; G, Shimadzu SPD-1 detector; H, Hitachi Model 634 detector; I, TOA EPR-2TC recorder; P_1, KHD-16 plunger pump; P_2, KSD-16 plunger pump; T_1, PTFE tube (9 m × 1 mm I.D.); T_2, PTFE tube (7 m × 0.1 mm I.D.); T_3, PTFE tube (8.5 m × 0.5 mm I.D.).

RESULTS

Setting up the system

The recorders used were stable for over 10 h when tested by connecting a 15-kΩ resistor to the input terminals. When 10^{-4} *M* MO solution was kept in the flow cell for over 10 h, the Shimadzu SPD-1 and the Hitachi Model 634 detectors maintained steady baselines at 490 and 280 nm, respectively, whereas the Uvigraph LC-1 and LDC SF-1205 detectors showed unsteady baselines. When measured at the open exits of the PTFE tube dampers (stage 1 in Fig. 1), the plunger-type pumps used delivered constantly a steady flow (0.20 ml/min) for over 10 h. Then, after connecting the column, the dialyzer and the detectors (stage 2, Fig. 1), the flow-rates were adjusted so as to be identical (0.20 ml/min) at the exits of the detectors by regulating the pumps. However, baseline drift was observed over a long period (10 h). This drift seemed to be due to the connection of detectors with different flow cells and tubing (see Fig. 2), which produced different pressures in the dialyzer. Therefore, in order to adjust the pressures generated by different detectors used in each channel, a PTFE tube (0.5 mm I.D.) was connected at the exit of the Hitachi Model 634 detector. Moreover, to

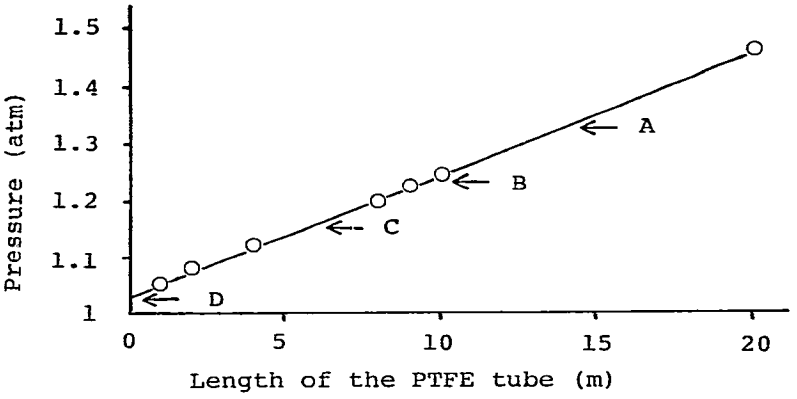

Fig. 2. Generation of pressure by various length of PTFE tubing (0.5 mm I.D.) and various detectors. The pressures generated by various detector cells were shown by arrows. A, Sample cell of Hitachi Model 634; B, sample cell of Shimadzu SPD-1; C, reference cell of Shimadzu SPD-1; D, reference cell of Hitachi Model 634, sample cell of Uvigraph LC-1 and both cells of LDC SF-1205.

maintain the pressures of the exits identical and constant, the exits were immersed in the waste reservoir, which was filled with the solvent (stage 3, Fig. 1). After setting up the system to stage 3 in Fig. 1, a steady baseline was maintained for over 10 h.

Removal of interference with protein

When albumin was applied to the column, the baseline in the drug channel started to drift immediately after the elution of albumin. The drift always occurred in one direction such that the absorbance in the drug channel increased, which suggested a decrease in membrane permeability, probably caused by adsorption of albumin. Addition of a detergent to the solvent to prevent the baseline drift was examined. Brij-35, a non-ionic detergent, was selected for this purpose because its solution does not show absorbance in either the ultraviolet or the visible region. The experiment was performed with a modified PFD system in which a loop injector was used instead of the column. The baseline drift was prevented by the addition of as little as 0.0001 % of Brij-35 (Fig. 3). However, the peak height decreased at Brij-35 concentrations higher than 0.1 %, where the efficiency of dialysis decreased (Table I). Therefore, the concentration of Brij-35 selected was 0.001 %.

Application of the improved PFD technique

The separation and detection of MO-binding proteins in human serum by the improved PFD technique were studied (Fig. 4). The response of the protein channel showed distinct peaks of IgG at 9.1 h and albumin at 10 h. The identification of these proteins was carried out by immuno-precipitation methods using antiserum to human albumin and antiserum to human IgG. The response of the drug channel showed a predominant peak at 10.1 h corresponding to albumin, together with peaks at 6.5 and 8.8 h, probably due to β-lipoprotein and IgG, respectively. The peaks at 6.5 and 10.1 h appeared reproducibly and increased in size with increasing sample volume.

The separation and detection of BPB binding proteins in rat liver homogenate

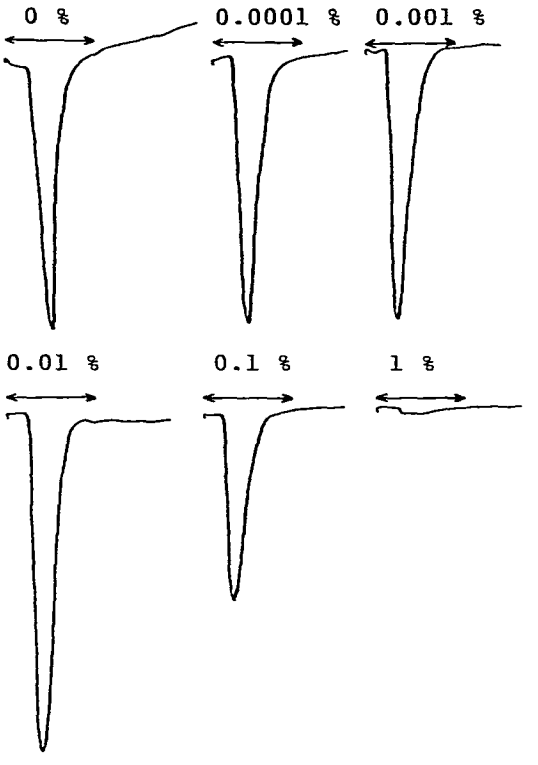

Fig. 3. Effect of Brij-35 concentration on the response in the drug channel. Solutions of various Brij-35 concentration were applied to the protein channel and 10^{-4} *M* MO solutions of the same Brij-35 concentrations were applied to the drug channel. A BSA solution of 5 mg/ml was injected into the same system through a loop injector (675 μl) at a flow-rate of 0.2 ml/min. The drug channel was monitored at 490 nm with a Shimadzu SPD-1 detector at 0.04 a.u.f.s. The arrows show 1 h of retention time.

TABLE I

EFFECT OF ADDITION OF BRIJ-35 TO THE SOLVENT ON THE EFFICIENCY OF DIALYSIS

Concentration of Brij-35 (%)	*Efficiency of dialysis* (%)*
0	72
0.0001	72
0.001	72
0.01	72
0.1	54
1	18

* The concentrations of the drug in the dialyzates from the protein channel (D_p) and the drug channel (D_d) were determined by measuring the absorbance of each solution at 254 nm. Efficiency of dialysis was defined as $[2D_p/(D_d + D_p)] \cdot 100$ (%). With this definition, the efficiency is 100% when the equilibrium is completed.

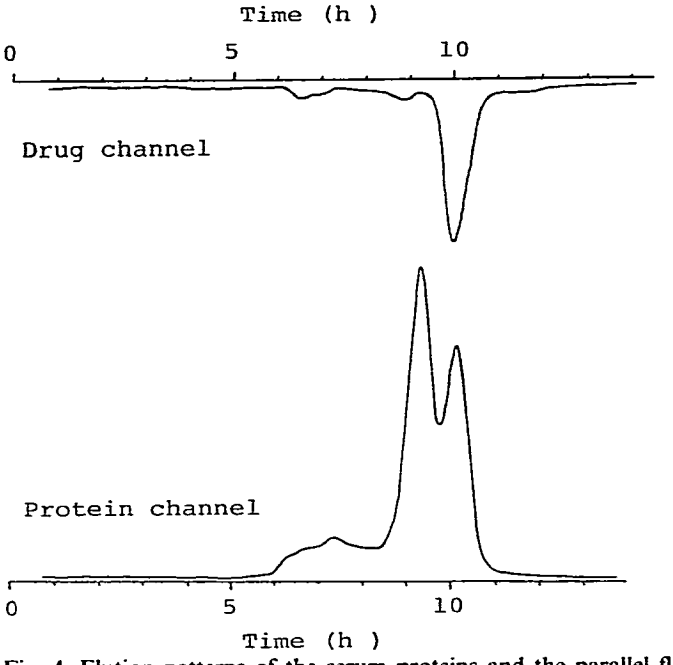

Fig. 4. Elution patterns of the serum proteins and the parallel flow dialysis response. A 10^{-4} *M* MO solution of the elution solvent was applied to the drug channel at a flow-rate of 0.20 ml/min. Room temperature was 24°C. The sensitivity of the drug channel detector was 0.08 a.u.f.s. and that of the protein channel was 0.64 a.u.f.s. A 200-μl aliquot of human serum was used as the protein sample.

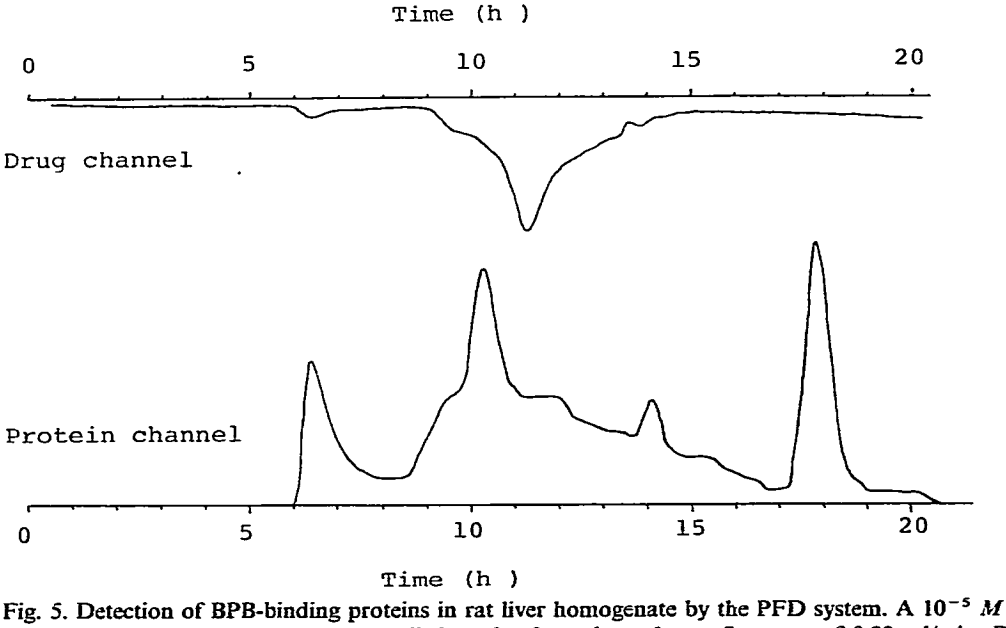

Fig. 5. Detection of BPB-binding proteins in rat liver homogenate by the PFD system. A 10^{-5} *M* BPB solution of the elution solvent was applied to the drug channel at a flow-rate of 0.22 ml/min. Room temperature was 24°C. The sensitivity of the drug channel detector was 0.08 a.u.f.s. and that of the protein channel was 0.64 a.u.f.s. A 2-ml aliquot of 100,000 *g* supernatant of the rat liver homogenate was used as the protein sample.

was similarly performed (Fig. 5). Various peaks were observed in the drug channel, especially at 6.5, 9.5 and 11.2 h.

DISCUSSION

We believe that the present method is the first for the continuous and selective *in situ* detection of drug-binding proteins in column effluents. Adjustment of the resistance of each channels to equalize the pressures is essential for obtaining a steady baseline. The successful effect of Brij-35 supports our assumption that the detergent may prevent proteins from adsorbing on to the dialysis membrane. After these improvements, this method can be used reliably for overnight operation.

The method was shown to be useful in the detection of two MO-binding proteins, albumin[5] (mol. wt. $6.5 \cdot 10^4$)[6] and β-lipoprotein[7] (mol. wt. $3.16 \cdot 10^6$)[8], in serum. Further, the method was shown to permit the detection of BPB-binding proteins in rat liver homogenate. The broad peak of BPB-binding activity (centred at around 11.2 h in Fig. 5) seems to correspond not only to Y-protein (mol. wt. $4.4 \cdot 10^4$)[3] and Z-protein (mol. wt. $1.2 \cdot 10^4$)[9], which are known to bind to BPB[10], but also to some other unknown proteins.

Similarly, the method can be applied to the detection of drug-binding proteins and other hydrophilic macromolecules. A combination of high-speed aqueous gel permeation chromatography and a micro-dialyzer will lead to increased sensitivity and shorter analysis times.

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