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A NEW METHOD FOR DETECTION OF DRUG-BINDING PROTEINS USING A PARALLEL-FLOW DIALYSIS TECHNIQUE*

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SUMMARY

A parallel-flow dialysis technique utilizing a Technicon dialyzer and a constant-flow system has been described for the detection of drug-binding proteins. The effect of temperature, flow-rate and drug concentration was investigated by measuring the efficiency of dialysis and detecting the binding of methyl orange to bovine serum albumin. The larger response was shown to be achieved by increasing the efficiency of dialysis or the drug concentration. The present method will enable the continuous monitoring of drug-binding proteins.

INTRODUCTION

Drug binding to various blood proteins and tissue proteins affects the pharmacological activities and blood distribution of drugs. Albumin is the major drug-binding protein in the blood plasma. Binding of albumin to various drugs has been studied by equilibrium dialysis, ultrafiltration, and many other methods [1—4]. Proteins binding organic anions have been found in the 110,000 *g* supernatant of liver homogenate by Levi et al. [5]. They were named Y-protein and Z-protein according to the elution pattern of the organic-anion—protein complex from a Sephadex G-75 column. Some other unknown drug-binding proteins are expected to be detected by a similar chromatography of the drug—tissue homogenate. However, the method is inappropriate when the drug—protein complex dissociates in the column.

To search for unknown drug-binding proteins by column chromatography, detection must be done after elution from the column. However, application of the traditional equilibrium dialysis or ultrafiltration methods to chromatographic fractions is time-consuming and laborious. In such cases, a post-

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column flow detection system is desirable. This paper deals with a parallel-flow dialysis technique which is applicable to the post-column detection of drug-binding proteins.

Concept of the present method

The principle of "parallel-flow dialysis" (PFD) is shown schematically in Fig. 1. Two flow channels in parallel on a dialysis membrane are utilized. The column effluent is directed into one channel, "protein channel", and the drug solution of fixed concentration is directed into the other channel, "drug channel", at the same flow-rate. The concentration of drug in the drug channel will decrease when a drug-binding protein appears in the protein channel. Thus by continuously monitoring the drug concentration at the exit of the drug channel, drug-binding protein can be detected as a decrease in the concentration. The degree of this change may depend upon the concentration of the drug—protein complex and the dialysis rate of the drug.

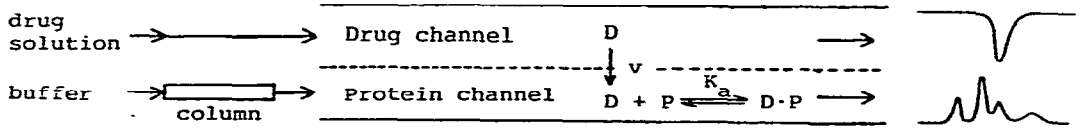

Fig. 1. The principle of the parallel flow dialysis. D = drug; P = protein; D·P = drug—protein complex; K_a = affinity constant; v = dialysis rate.

EXPERIMENTAL

Materials

Crystallized and lyophilized bovine serum albumin (BSA, Product No. A-4378) was purchased from Sigma (St. Louis, MO, U.S.A.). Phenobarbital (PB, Iwaki Seiyaku, Tokyo, Japan), methyl orange (MO), bromphenol blue (BPB, Kanto Chemicals, Tokyo, Japan), phenol red (PR, Tokyo Kasei, Tokyo, Japan) and eosin yellowish (EY, E. Merck, Darmstadt, G.F.R.) were used. Phosphate-buffered saline (PBS) (pH 7.4) was prepared by dissolving 4.03 g of sodium chloride in one liter of 50 m*M* NaH_2PO_4—K_2HPO_4 buffer (pH 7.5). For the dialysis a Technicon Autoanalyzer dialyzer (basic module) fitted with a cellulose membrane (Cuprophan membrane, Technicon Chemicals Co., Belgium) was used. The absorbance of the solutions was measured by a Uvidec-505 spectrophotometer (Jasco, Tokyo, Japan) at 254 nm.

Flow dialysis system

The flow diagram of the system is illustrated in Fig. 2. Continuous flow in the protein and drug channels was produced by two plunger-type pumps, KSD-16 and KHD-16 (Kyowa Seimitsu, Tokyo, Japan). In place of the column, a 240-μl loop injector (Toyo Soda, Tokyo, Japan) was used for in-stream injection of protein samples. The dialyzer and the buffer reservoir were placed in a water bath to maintain a constant temperature. Concentrations of BSA and drugs were monitored by measuring absorbance at 254 nm with a Uvigraph

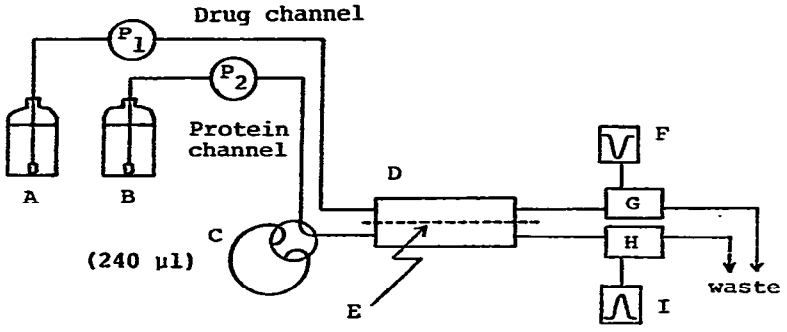

Fig. 2. A schematic diagram of the parallel-flow dialysis system. A = reservoir of PBS; B = reservoir of the drug solution in PBS; C = loop injector; D = dialyzer of the Technicon Autoanalyzer basic module; E = cellulose Cuprophan membrane; F = TOA EPR-100A recorder; G = LDC SF-1205 detector; H = Uvigraph LC-1 detector; I = Shimadzu R-101 recorder; P_1 = KHD-16 plunger pump; P_2 = KSD-16 plunger pump. Teflon tubes of outer diameter 2 mm and inner diameter 1 mm were used for connection with the partial aid of silicone tubes of outer diameter 3 mm and inner diameter 1 mm.

LC-1 monitor (Iatron Laboratories, Tokyo, Japan) and an LDC SF-1205 UV monitor (Atto Corporation, Tokyo, Japan), respectively.

Efficiency of dialysis

Efficiency of dialysis was adopted as an index for the dialysis rate of the drug from the drug channel into the protein channel. The effluents from both channels were collected under steady-state conditions. Then the concentration of the drug in the protein channel (D_p) and in the drug channel (D_d) was estimated by measuring the absorbance of each solution at 254 nm. Efficiency of dialysis was defined as

$$\frac{2D_p}{D_d + D_p} \times 100 \quad (\%)$$

Under this definition, the efficiency is 100% when the equilibrium is completed.

RESULTS

Response of the PFD system

In order to verify the concept of the PFD system, detection of the binding of MO to BSA was tested by the system shown in Fig. 2. A 100-μM MO solution was pumped through the drug channel at 0.35 ml/min while the solvent (PBS) was pumped through the protein channel at the same flow-rate. By maintaining these conditions a steady-state baseline was obtained in each channel. Then a BSA solution (10 mg/ml) was injected into the protein channel. The response in each channel is shown in Fig. 3. As expected, a transient decrease in the recorder response of the drug channel appeared as an inverse peak corresponding to the elution of BSA.

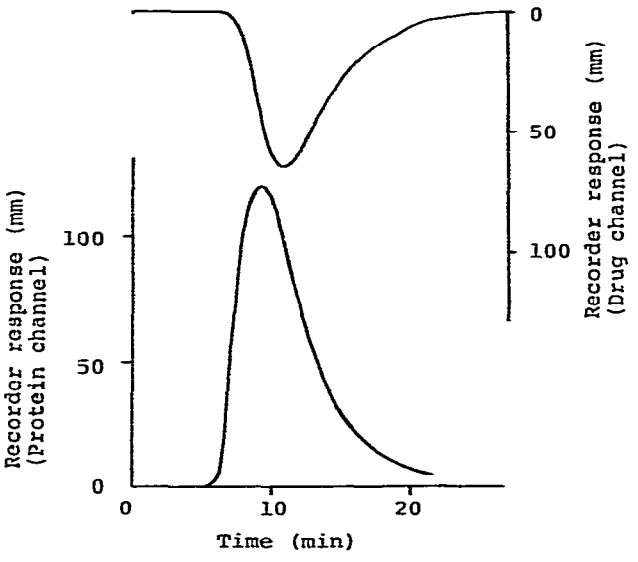

Fig. 3. The recorder responses of the system caused by injection of 2.4 mg of BSA. Recorder range of the protein channel was 0.64 a.u.f.s. (250 mm full scale) and that of the drug channel was 0.04 a.u.f.s. (150 mm full scale).

Factors affecting response

Various factors such as temperature, flow-rate and drug concentration affect the response of the drug channel. As shown in Fig. 4, the peak height of the response and the efficiency of dialysis increased with increasing temperature. However, when the temperature was higher than 32°C, the appearance of small bubbles in the dialyzer interfered with detection. Therefore, a temperature of 27°C was selected for further experiments. As shown in Fig. 5, increasing the flow-rate resulted in a decrease in peak height and ef-

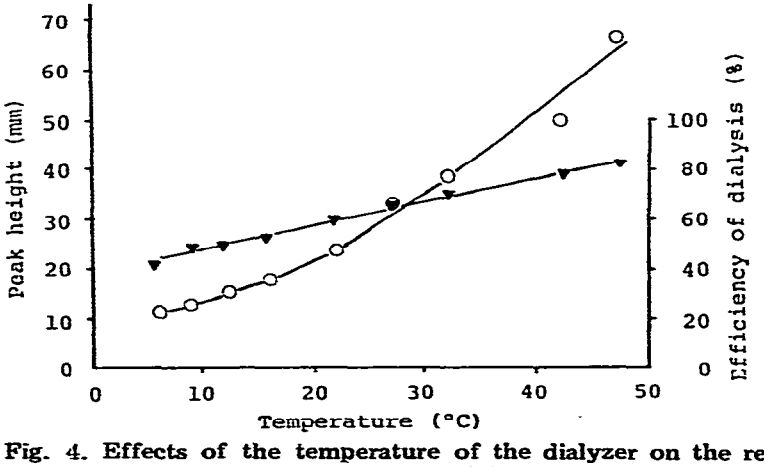

Fig. 4. Effects of the temperature of the dialyzer on the response of the drug channel (o) and on the efficiency of dialysis (▾). A 100-μM MO solution was passed through the drug channel at a flow-rate of 0.35 ml/min. A 240-μl volume BSA solution (5 mg/ml) was injected through the loop injector.

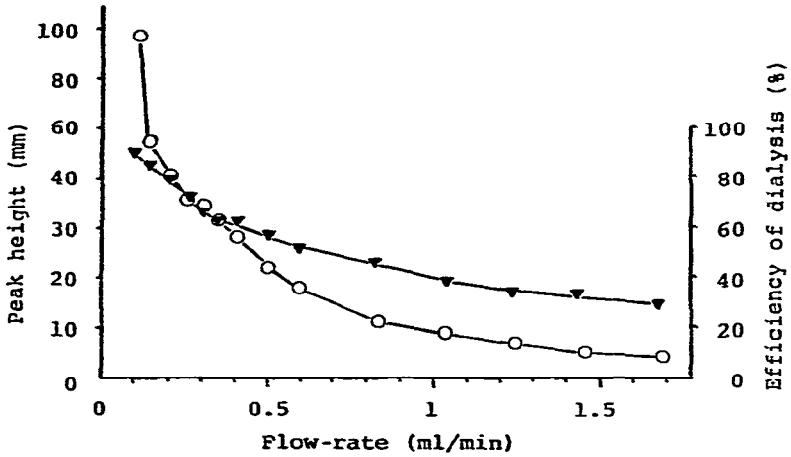

Fig. 5. Effects of the flow-rate on the response of the drug channel (○) and on the efficiency of dialysis (▼). A 100-μM MO solution was passed through the drug channel, the temperature of the dialyzer being kept constant at 27°C. A 240-μl volume of BSA solution (5 mg/ml) was injected through the loop injector.

ficiency of dialysis. In the concentration range $2 \cdot 10^{-5}$ M to $2 \cdot 10^{-4}$ M of MO solution applied to the drug channel, the peak height of the drug channel was almost proportional to MO concentration (Fig. 6) even if the efficiency of dialysis was constant.

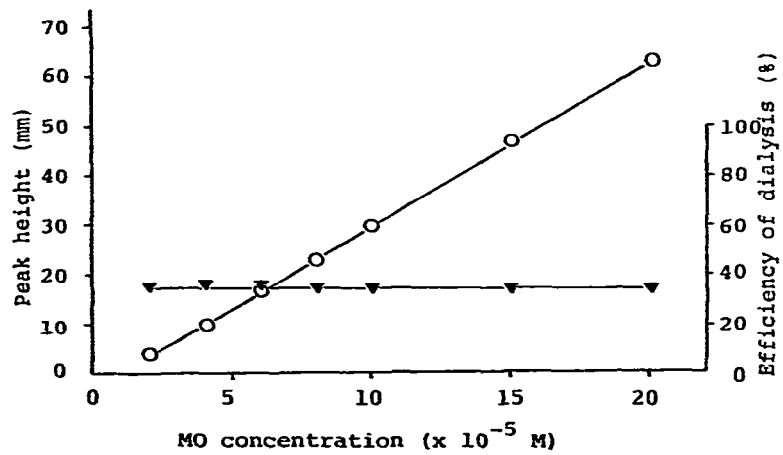

Fig. 6. Effects of MO concentration on the response of the drug channel (○) and on the efficiency of dialysis (▼). Various concentrations of MO were applied to the drug channel. The temperature of the dialyzer was 27°C and the flow-rate was 0.35 ml/min. A 240-μl volume of BSA solution (5 mg/ml) was injected through the loop injector.

Application of various compounds to PFD

A further experiment was planned to clarify the limit and scope of the PFD system by using various compounds of different affinity constants to BSA (Table I). In order to compare the results of different compounds, the peak height of the drug channel was converted to absorbance change by using

TABLE I

AFFINITY CONSTANTS OF VARIOUS COMPOUNDS TO BSA

Compound	n_1	K_1	n_2	K_2	Method	Reference
Phenobarbital	1	2500	—	—	Dynamic dialysis	6
Methyl orange	22	2080	—	—	Dynamic dialysis	7
Phenol red	1	174,000	6	1970	Dynamic dialysis	7
Bromphenol blue	5	242,000	—	—	Equilibrium dialysis	8
Eosine yellowish	3.9	5,620,000	5.8	231,000	Ultracentrifugation	9

working curves for the protein channel (Fig. 7) and the drug channel (Fig. 8). Zero adjustment of the protein channel recorder was made against solutions of 0.21 and 0.43 absorbance, which represent the actual experimental conditions. Also the drug channel detector response was studied by introducing solutions of 0.34 and 1.16 absorbance into the reference cell. The ratio of

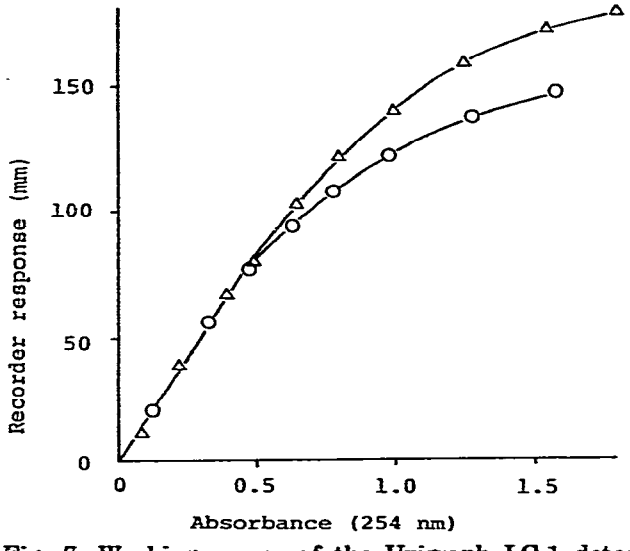

Fig. 7. Working curves of the Uvigraph LC-1 detector (protein channel). Recorder range was set at 0.64 a.u.f.s. (250 mm full scale). Zero adjustment was made against MO solutions with absorbances of 0.21 (△) and 0.43 (○) at 254 nm.

the response to the absorbance change was constant under 1.5 absorbance by the LDC SF-1205 detector. For experimental convenience, the concentrations of the five compounds were chosen so that their absorbance at 254 nm was below 1.5. From the working curves of the drug channel (Fig. 8) and the molar extinction coefficient, the concentration change corresponding to a 100-mm recorder response was calculated (Table II). The relative change of the concentration in the drug channel was used to represent the response of the drug channel. This was obtained by first converting the peak height to the concentration change (ΔD_d) using the conversion coefficient in Table

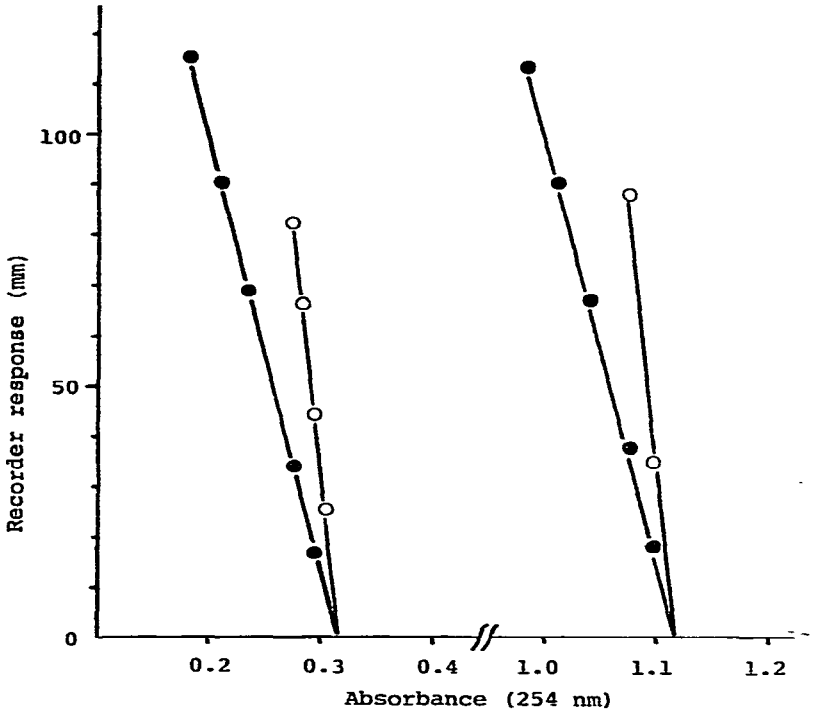

Fig. 8. Working curves of the LDC SF-1205 detector (drug channel). Recorder range was 0.04 a.u.f.s. (•) and 0.01 a.u.f.s. (○) at 150 mm full scale. Zero adjustment was made against a PR solution with an absorbance of 0.34 at 254 nm or an EY solution of absorbance 1.16 at 254 nm.

TABLE II

RELATION OF CONCENTRATION, ABSORBANCE OF DRUG AND RECORDER RESPONSE

Compound	ϵ (254 nm)	Conc. (M)	Absorbance (254 nm)	Conc. corresponding to a 100-mm recorder response (M)
Phenobarbital	$2.6 \cdot 10^3$	$5 \cdot 10^{-4}$	1.3	$2.0 \cdot 10^{-5}$
Methyl orange	$7.7 \cdot 10^2$	$1 \cdot 10^{-4}$	0.76	$7.0 \cdot 10^{-6}$
Phenol red	$8.1 \cdot 10^3$	$1 \cdot 10^{-4}$	0.81	$6.7 \cdot 10^{-6}$
Bromphenol blue	$6.4 \cdot 10^3$	$1 \cdot 10^{-4}$	0.64	$8.4 \cdot 10^{-6}$
Eosin yellowish	$3.0 \cdot 10^4$	$2 \cdot 10^{-5}$	0.59	$1.8 \cdot 10^{-6}$

II, and then dividing by the steady-state concentration (D_d) in the drug channel. The relative change was defined as

$$\frac{\Delta D_d}{D_d} \times 100 \quad (\%)$$

As shown in Fig. 9, the responses of PB, PR and MO were in proportion to

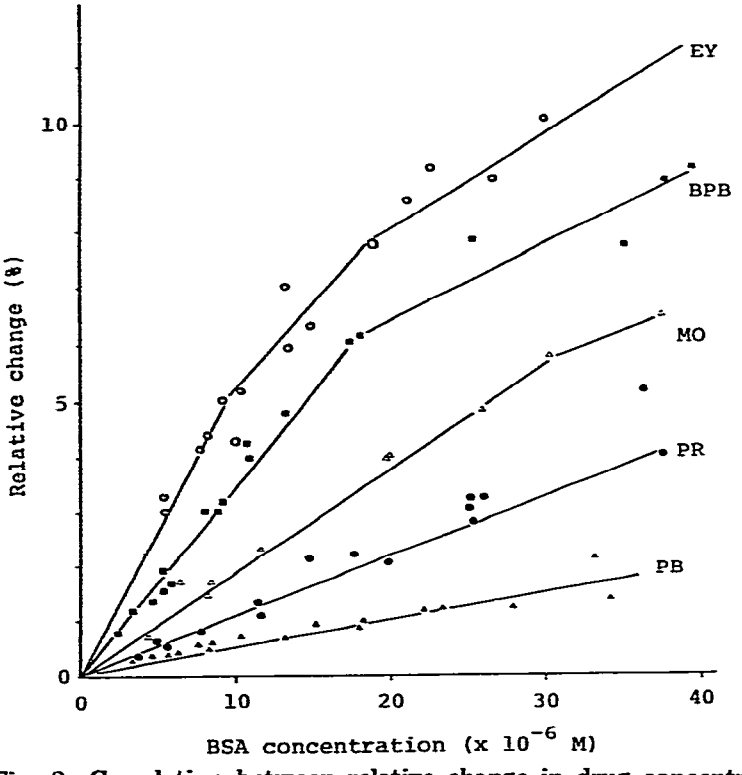

Fig. 9. Correlation between relative change in drug concentration and the concentration of BSA. The temperature of the dialyzer was 27°C and the flow-rate was 0.35 ml/min.

BSA concentration up to $30 \cdot 10^{-6}$ *M*, while BPB and EY gave saturation curves. At the same concentration of BSA, compounds with higher affinity constants tended to show a higher relative change. The efficiency of dialysis for each compound was: PB, 66%; PR, 52%; MO, 60%; BPB, 44%; and EY, 48%.

DISCUSSION

A PFD technique was developed by which binding of BSA to five compounds was detected. In this system, a larger response can be obtained with higher dialysis efficiency (higher temperature or lower flow-rate). In the case of MO, the efficiency of dialysis reaches 90% by 22 minutes of residence in the dialyzer at 27°C (Fig. 5), indicating effective dialysis of the drug in the present co-current flow system compared to a static equilibrium dialysis system. The use of a higher drug concentration in the PFD system also resulted in a larger response. However, the detector response lost linearity at higher concentrations of drug that gave an absorbance of more than 1.5 at 254 nm with the LDC SF-1205 detector. To overcome this defect, the use of a variable-wavelength detector is recommended.

As illustrated in Fig. 9, the response of PB, PR and MO were almost proportional to the concentration of BSA. On the other hand, the responses show a curved line in the cases of EY and BPB which have strong affinity to

BSA. This phenomenon suggests that the concentration of the compound to BSA is saturated in the protein channel. Furthermore, Fig. 9 conversely suggests that proteins with larger affinity constants to the drug show a larger response. Thus, the PFD system will be useful for the continuous detection of unknown drug-binding proteins in a column effluent. The following paper [10] will describe the detection of drug-binding proteins of human serum or rat liver homogenate in column effluent.

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