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Study of protein–drug binding using capillary zone electrophoresis

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ABSTRACT

Capillary zone electrophoresis was tested for its suitability for studying protein–drug binding. Three methods were investigated, *viz.*, the Hummel–Dreyer method, the vacancy peak method and frontal analysis. Frontal analysis appeared to be the preferred method.

INTRODUCTION

It is well known that drugs bind reversibly to plasma proteins, particularly albumin and α-acid glycoproteins [1]. As the unbound drug determines the pharmacological activity of the drug, it is necessary to know the ratio of the bound to unbound drug in order to adjust the optimum therapeutic dose of a drug in man. Moreover, for toxic drugs with strong protein binding, additional information about the displacement of the bound drug from the plasma proteins by simultaneously administered exogenous compounds is required. The binding studies involve the determination of the binding parameters, *i.e.*, the binding constants, the maximum number of drug molecules bound to the protein and the classes of binding sites on the protein.

Methods for studying binding phenomena are concerned mainly with the measurement of either the unbound (free) or the bound drug without disturbing the equilibrium. The techniques that meet these requirements are dialysis [2,3], ultrafiltration [4] and size-exclusion chromatography (SEC) [5]. The last technique has nowadays become the method of choice and several variants have been developed, *i.e.*, the Hummel–Dreyer method [5,6], the vacancy peak method [7] and frontal analysis [8,9].

In principle, methods analogous to the SEC methods can be applied in capillary zone electrophoresis (CZE), but so far only flat gel electrophoresis has been used to study protein–drug binding [10]. CZE for studying protein–drug binding looks attractive because it is a simple system with a relatively low surface-to-volume ratio, an extremely large separation power, reasonable speed and good automation prospects. From the point of view of systematic errors and wide applicability, the CZE system appears more promising than other techniques, because of the absence of large surface areas, while the aqueous medium is similar to physiological conditions.

The aim of this investigation was to determine whether CZE can be used to study protein–drug binding and provide an attractive alternative to the aforementioned laborious methods. The study involved a comparison of the Hummel–Dreyer method, the vacancy peak method and frontal analysis using warfarin and bovine serum albumin (BSA) as test compounds.

PRINCIPLE OF CZE METHODS FOR DRUG-BINDING STUDIES

SEC methods exploit the difference in the exclusion of the drug and the protein–drug complex from

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the pores of the packing for the separation of the free drug and the bound drug [11]. In CZE, the drug and the protein–drug complex can be separated by the difference in their electrophoretic mobilities, which depend on the charge and size of the compounds. It is reasonable to assume that the size and charge on the protein are not significantly altered by the presence of adsorbed drug molecules. This means that the protein and protein–drug complex will have the same electrophoretic mobility. Hence, in principle, the same methods for studying protein–drug binding as developed for SEC (*i.e.*, the Hummel–Dreyer method, the vacancy peak method and frontal analysis) can be adapted to CZE, provided that the electrophoretic mobilities of the drug and protein differs. In this study, the electrophoretic mobility of the protein (BSA) is larger then that of the drug (warfarin).

In the Hummel–Dreyer method, the capillary is filled with the buffer containing the drug, which causes a large background detector response. Then a small sample, containing the buffer + drug + protein, is injected into the capillary. The migration of the injection plug after switching on the voltage is illustrated schematically in Fig. 1. The total concentration of the drug in the sample plug is equal to the drug concentration in the buffer, but part of the drug is bound to the protein. As the mobilities of the protein and protein–drug complex are larger than that of the drug, the protein–drug complex migrates from the injection plug, leaving a local deficiency in drug concentration. This deficiency causes a negative peak which moves with the mobility of the drug. The area of the negative peak is a measure of the amount of bound drug. During the migration, the protein–drug complex is always in equilibrium with the free drug in the buffer. Therefore, the protein–drug complex will give a positive peak. When the free protein shows no absorbance at the selected wavelength and the molar absorptivities of the drug and the protein–drug complex are the same, then in principle the areas of the negative and positive peaks must be equal. However, in practice these circumstances are almost never met. It can be seen that in Figs. 1–3 the peaks are represented as ideal blocks but it will be obvious that in practice, owing to the dispersion, the blocks appear as more or less Gaussian peaks.

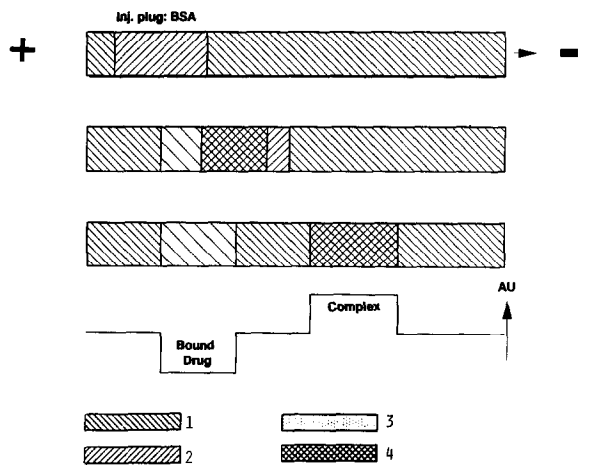

Fig. 1. Schematic representation of the Hummel–Dreyer CZE method. 1 = Drug; 2 = BSA; 3 = buffer; 4 = BSA–drug complex.

In the vacancy peak method, the capillary is filled with buffer containing drug + protein, which causes a large background detector response. Then a small plug of buffer is injected into the capillary and the power supply switched on. The effect of the injected buffer plug on the migration is illustrated schematically in Fig. 2, again assuming that the mobilities of the protein and protein–drug complex are larger than that of the drug. At the front edge of the buffer plug the drug is migrating more slowly than the protein and hence stays behind. At the rear edge of the plug the protein migrates faster than the drug. This continues until the two fronts reach other. In this middle region the protein again

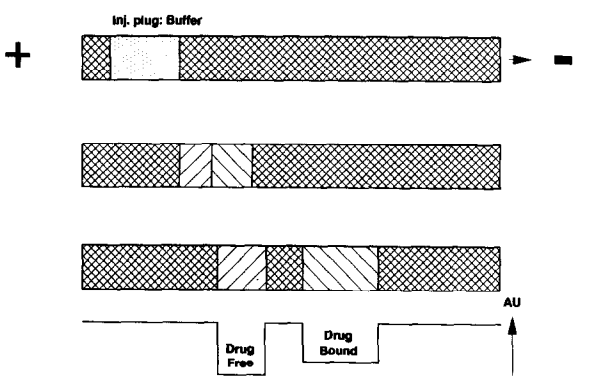

Fig. 2. Schematic representation of the vacancy peak CZE method.

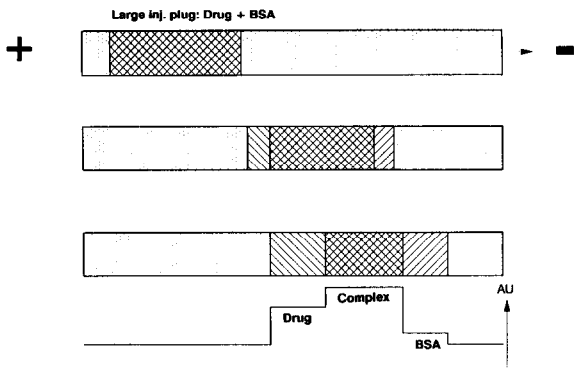

Fig. 3. Schematic representation of the frontal analysis CZE method.

adsorbs drug molecules until an equilibrium is attained, reconcentrating the drug to the original concentration. From that point on a steady state is reached, resulting in two negative bands (peaks) in the electropherogram. The first peak reflects the bound drug and the second peak the free drug concentration. The evidence that the negative peaks reflect the bound and free drug is given in ref. 7.

In frontal analysis, the capillary is filled with the buffer, then a very large sample plug, containing buffer + protein + drug, is injected into the capillary. As the mobilities of the protein and drug differ, the free drug leaks out of the plug at the rear edge (as occurs in the vacancy method) and a plateau is formed, as represented schematically in Fig. 3. Finally, the elution profile consists of three parts: at the front edge a plateau related to the free protein, in the middle region a plateau related to the protein–drug complex + free drug and at the rear edge a plateau due to the unbound (free) drug. Depending on the selected wavelength, the first plateau (the free protein) is often not detected. The height of the drug plateau reflects the free drug concentration.

EXPERIMENTAL

Apparatus

The CZE system was constructed from separate parts and consisted of a UV detector (Model 757, Kratos, Ramsey, NJ, USA) with a modified cell arrangement for on-column detection, a 0–35 kV d.c. high-power supply (HCN 35-35000; FUG, Rosenheim, Germany) and platinum electrodes used for connection of the supply with the buffer reservoirs at both ends of the capillary. The detection was carried out at the cathodic side. The whole set-up (except the power supply) was placed in a Plexiglas box. For safety, an automatic shut-off switch was mounted in the door. The temperature in the box was kept at 25°C.

In one experiment, a commercial CZE system [Model 270 A-HT; Applied Biosystems Inc. (ABI), CA, USA] was used. The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 50–60 cm × 50 μm I.D. The distance between the injection and detectors was about 35 cm. Before starting the measurements, the capillary was flushed daily for 5 min each with 1 *M* KOH, ethanol and buffer. The voltage was usually set to 200 V/cm. Sample injection was done by means of electromigration (2.5–60 s at 10 kV). The wavelength of detector was adjusted to 315 nm. At very high warfarin concentrations the wavelength was sometimes set at 340 nm.

Chemicals

The 0.067 *M* phosphate buffer (pH 7.4) was prepared by mixing of 0.53 g of KH_2PO_4 and 2.94 g of $K_2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany) in 250 ml of doubly distilled water. The buffer solution was prepared daily and filtered through a Millipore filter (Type HV, 0.45 μm) before use. Bovine serum albumin (BSA) was obtained from Merck and warfarin from Sigma (St. Louis, MO, USA). The sample solutions were daily prepared from stock solutions stored at −20°C.

Procedures

In order to calculate properly the binding parameters of warfarin, 15–20 points of the warfarin–BSA isotherm are desired. In all studies the BSA concentration was kept constant at $4 \cdot 10^{-5}$ *M* and the warfarin concentration varied according to the following scheme: 3, 6, 9, 12, 15, 18, 30, 60, 90, 120, 200, 300, 400, 600 and $900 \cdot 10^{-6}$ *M*. For the Hummel–Dreyer and vacancy peak methods a small sample plug was injected (2.5 at 10 kV) and for frontal analysis a large sample plug (60 s at 10 kV). The experimental data were fitted according to a bi-Langmuir isotherm using a non-linear least-squares curve fitting program [12]. The experimental data were also visualized in a Scatchard plot [13] to show the spread of the data points.

For the calculation of the bound fraction with the Hummel–Dreyer method, the simplified method as given by Pinkerton and Koeplinger [14] was applied. The procedure involves two injections at a given warfarin concentration: the sample (consisting of buffer + protein) and the blank buffer. From the peak areas the bound drug concentration, $[D]_b$, can be calculated according to

$$[D]_b = \left(\frac{A_p - A_e}{A_e}\right) C_D \quad (1)$$

where A_p is the area of the sample peak, A_e is the area of the buffer peak and C_D the drug concentration in the buffer.

In the vacancy peak method, the free drug concentration was determined by internal calibration. For this purpose the buffer was mixed with increasing amounts of warfarin and then injected. By plotting the added warfarin *versus* the peak area, the free concentration can be determined by interpolating to zero absorbance (the concentration needed to fill up the vacancy peak).

In frontal analysis, the free drug concentration, $[D]_f$, can be calculated from the height of the sample plateau and the height of the plateau obtained on injecting a plug of buffer + the same warfarin concentration, C_D, as used in the sample according to

$$[D]_f = \left(\frac{\text{sample height}}{\text{warfarin height}}\right) C_D \quad (2)$$

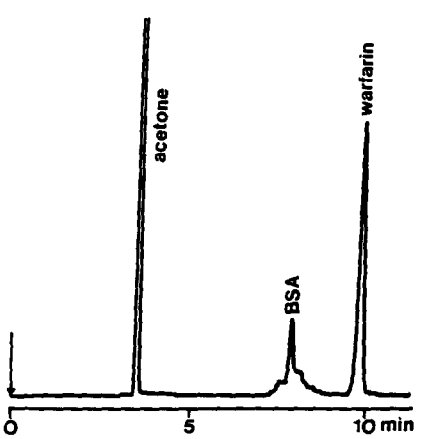

Fig. 4. Electropherogram of the test solutes. Electrophoretic mobilities ($m^2/V \cdot s$): acetone (neutral compound), $\mu_0 = +3.70 \cdot 10^{-8}$; $\mu_{BSA} = -1.67 \cdot 10^{-8}$; $\mu_{warfarin} = -2.00 \cdot 10^{-8}$.

The electropherogram of the test solutes in 0.067 *M* phosphate buffer is shown in Fig. 4. It can be seen that the BSA is not completely homogeneous.

RESULTS AND DISCUSSION

During the explorative experiments to adapt the liquid chromatographic drug-binding techniques to our laboratory-made CZE system, we were faced with unexpected experimental difficulties, such as blockage of the capillary, noisy detector signals and large variations in the electropherograms, particularly in the Hummel–Dreyer and vacancy peak methods. By systematically investigating these problems, it became clear that acceptable data could be produced provided that flushing of the capillary, the preparation of the solutions and the measurements were performed according to a stringent protocol. For instance, a daily pretreatment of the capillary, freshly prepared buffer and sample solutions and the analysis of a series of samples in a standardized time scheme were found to be of paramount importance for obtaining reliable isotherms. Fig. 5 shows typical electropherograms obtained with (A) the Hummel–Dreyer method, (B) the vacancy peak method and (C) frontal analysis.

Effect of protein concentration

The reversible binding of a drug to a protein is governed by the multiple equilibria theory [15,16], expressed by the following equation:

$$r = \frac{[D]_b}{[P]} = \sum_{i=1}^{m} \frac{n_i K_i [D]_f}{1 + K_i [D]_f} \quad (3)$$

where r is the mean number of drug molecules adsorbed per protein molecule, m is the number of classes of independent adsorption sites, n_i is the number of sites in a class i with an association constant of K_i and $[D]_f$ is the concentration of the unbound (free) drug; r is equal to the ratio of the concentration of bound drug, $[D]_b$, and the total protein concentration, [P]. For the BSA–warfarin combination two binding constants and two classes of binding sites are assumed. According to eqn. 3, r, the number of drug molecules adsorbed to a protein molecule, is independent of the protein concentration. However, it has been found that the equilibria

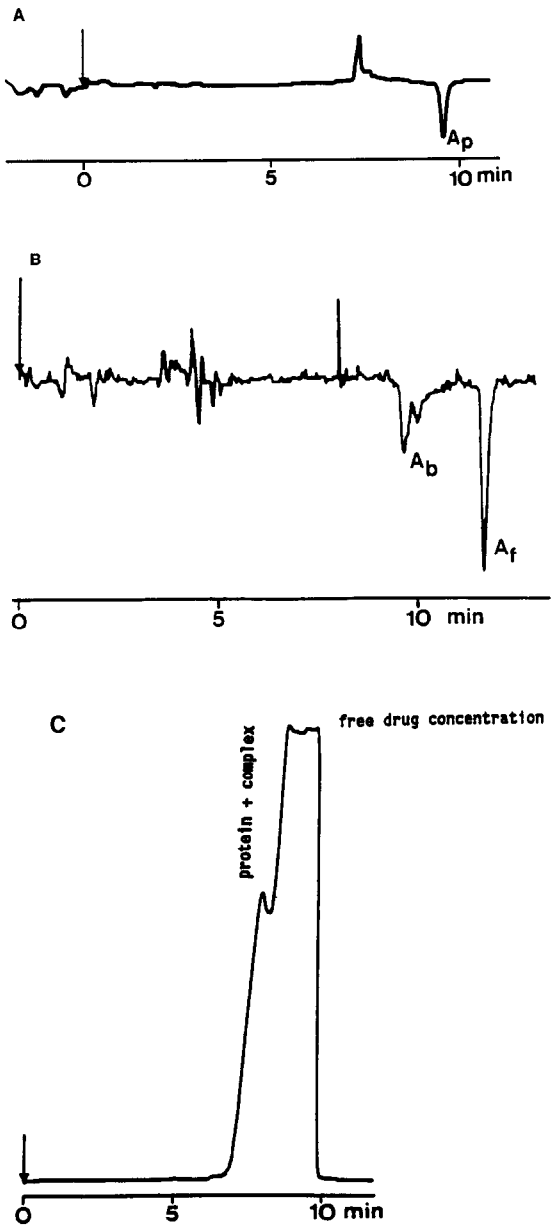

Fig. 5. Typical electropherograms obtained with (A) the Hummel–Dreyer method, (B) the vacancy peak method and (C) frontal analysis.

are disturbed at higher protein concentrations [17] and this can affect r. In order to determine the protein concentration where r is constant, different BSA concentrations were injected at a constant warfarin concentration and the r values were calculated. The results are given in Table I. As can be seen, the r value tends to become constant with decreasing BSA concentration. In all subsequent measurements, the BSA concentration was kept below 50 μM.

Hummel–Dreyer method

Fig. 6 shows a typical adsorption isotherm and the corresponding Scatchard plot as measured with the Hummel–Dreyer method. The data points are the means of duplicate measurements. As can be seen, the isotherm looks reasonable but the points in the Scatchard plot are scattered significantly at lower r values. The scattering at low warfarin concentration is due to the imprecision in the determination of the small peak heights at the highest sensitivity setting of the detector. The peak heights of duplicates vary by about 10% and this has a large effect on the calculation of r. At medium and higher warfarin concentrations the spread of duplicate measurements is smaller (5% and 2.5% respectively). Owing to the relatively large spread at lower warfarin concentrations, meaningful data points for the fitting can be obtained provided that averages of replicate experiments are used. The relatively large spreading can be partly attributed to the manual operation on our laboratory-made CZE system and better results can be expected with an automated CZE system.

The binding parameters of warfarin with BSA, by fitting the data points as reflected in Fig. 6, are given in Table II, including parameters given in the literature. The values of k_1 and n_1 are smaller than published values obtained using SEC. As the dilution of the bands in CZE is significantly smaller than in HPLC, the difference might be attributed to the

TABLE I

EFFECT OF THE BSA CONCENTRATION ON THE r VALUE WITH $5 \cdot 10^{-5}$ M WARFARIN

BSA (10^{-4} M)	r
1.52	1.16
1.25	1.40
1.01	1.59
0.75	1.68
0.48	1.73

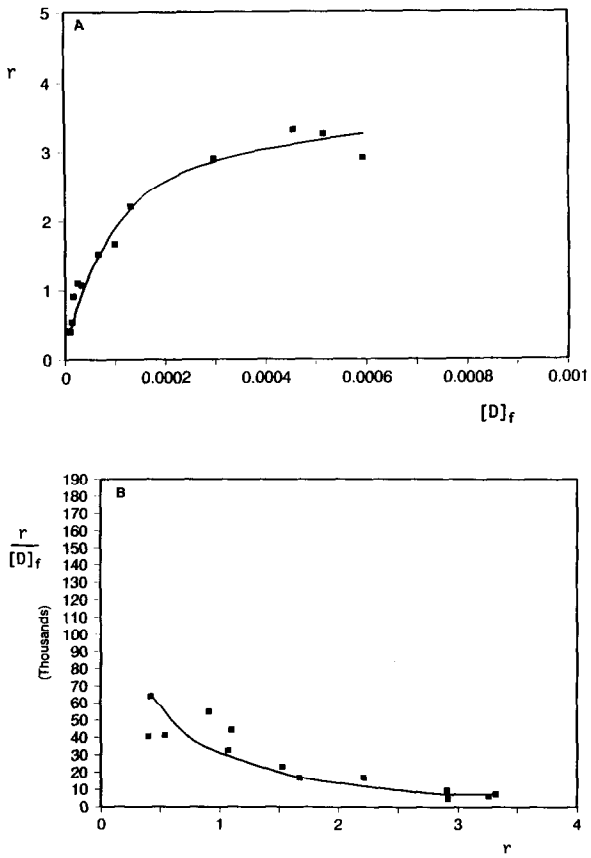

Fig. 6. (A) BSA–warfarin isotherm and (B) Scatchard plot measured with the simplified Hummel–Dreyer method.

effect of the actual protein concentration on r. This aspect is being investigated in more detail.

Vacancy peak method

Fig. 7 shows the isotherm and Scatchard plot obtained with the vacancy peak method. Both curves looks better than those obtained with the Hummel–Dreyer method. However, also with the vacancy peak method the scatter of the data points in the Scatchard plot at smaller r values is large. The binding parameters deviate from those in the literature, probably because of the mentioned effect of the protein concentration on r. The vacancy peak method can be easily applied but requires a large number of measurements owing to the calibration procedure.

Frontal analysis

The BSA–warfarin isotherm and Scatchard plot obtained with the frontal analysis method are shown in Fig. 8. In contrast to the aforementioned methods, with this method smooth curves are obtained over the whole warfarin concentration range investigated. The curves are very reproducible and the binding constants fit well with those reported in the literature, as can be seen from Table II. Owing to the simple internal calibration, a complete curve can be recorded within 8 h.

Frontal analysis was tested with an automated CZE apparatus (ABI) made available for demon-

TABLE II

COMPARISON OF PROTEIN BINDING PARAMETERS FOR BSA–WARFARIN IN 0.067 *M* PHOSPHATE BUFFER (pH 7.4)

Method	Apparatus	K_1 ($\times 10^5$)	n_1	K_2 ($\times 10^3$)	n_2
CZE, Hummel–Dreyer	Laboratory-made	1.00	0.67	6.7	3.24
CZE, vacancy	Laboratory-made	0.70	1.98	1.4	2.74
CZE, frontal	Laboratory-made I	1.30	1.33	2.1	2.67
	Laboratory-made II	1.53	1.16	2.9	3.17
	Laboratory-made III	1.58	1.22	4.3	2.70
	ABI, hydrodynamic injection	1.51	1.31	6.9	1.63
	ABI, Electromigration injection	1.61	1.24	9.2	1.41
Ref. 18		2.41	1.46	5.6	2.42
Ref. 19		2.31	0.95	5.9	3.69
Ref. 20		2.03	1.38	2.2	3.73
Ref. 6		2.10	1.16	–	–
Ref. 6		2.18	1.31	4.2	3.75

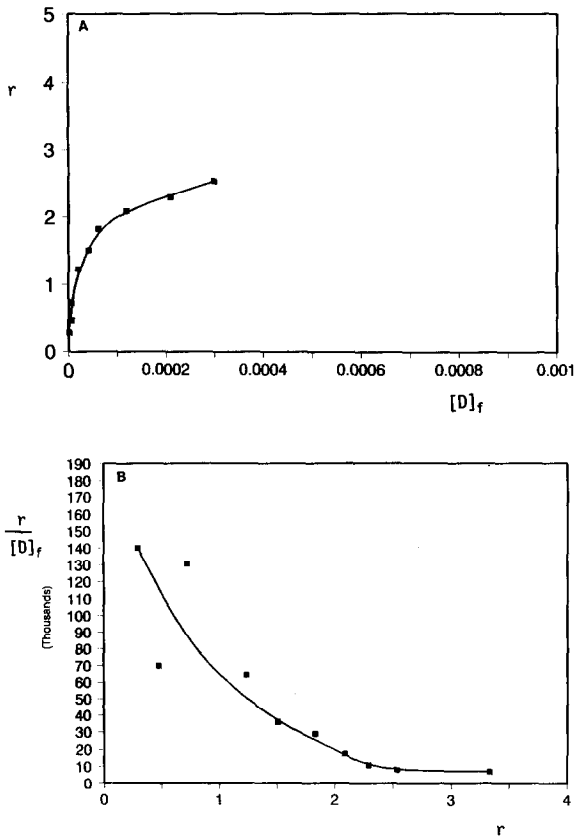

Fig. 7. (A) BSA–warfarin isotherm and (B) Scatchard plot measured with the vacancy peak method.

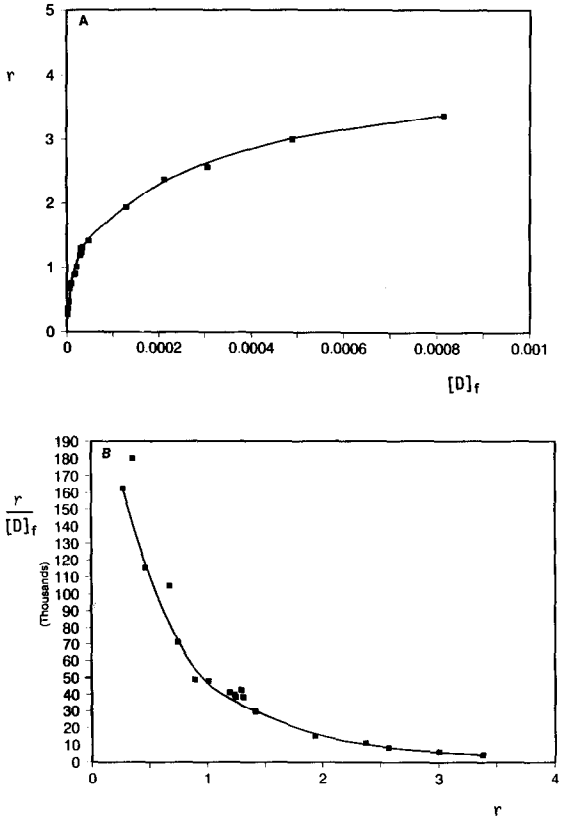

Fig. 8. (A) BSA–warfarin isotherm and (B) Scatchard plot measured with frontal analysis.

strations by the supplier. Two injection modes were applied: electromigration and hydrodynamic. The system was programmed in such a way that after each analysis a washing step (1 *M* KOH) was applied. The isotherm obtained is given in Fig. 9 and shows that Frontal analysis can be well automated.

From Table II, it can be seen that the number of binding sites at high warfarin concentration (n_2) appears smaller with the ABI system than with our laboratory-made CZE system, in which the KOH flushing between each analysis was omitted. The reasons for this difference are not yet clear but is might be attributed to reversible adsorption of BSA on the surface of the capillary or to an effect of the length and type of the applied capillaries, which differed in the two systems. These aspects are under investigation.

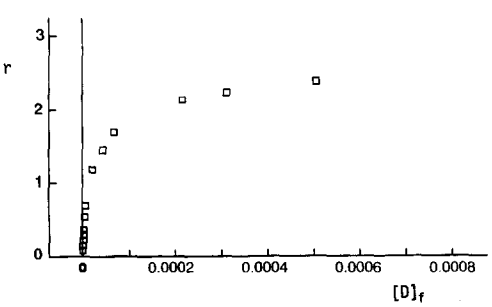

Fig. 9. (A) BSA–warfarin isotherm and (B) Scatchard plot measured with frontal analysis on an ABI CZE system. Injection mode: electromigration.

CONCLUSIONS

The results obtained so far have shown that CZE is a suitable technique for studying protein–drug binding, provided that an automated CZE system is used. Of the methods investigated, frontal analysis appears to be the most attractive.

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