International Journal of Pharmaceutics, 30 (1986) 123–132
Elsevier

IJP 01003

An automated flow injection-serial dynamic dialysis technique for drug–protein binding studies

P. Macheras [1], M. Koupparis [2] and C. Tsaprounis [1]

Departments of [1] Pharmacy and [2] Chemistry, University of Athens, Athens (Greece)

(Received October 21st, 1985)
(Accepted December 13th, 1985)

Key words: protein binding – dialysis – flow injection analysis – dialytic rates – sulfonamides – automation

Summary

The interface of an automated flow injection analyzer with a dialysis unit to study drug–protein interactions using flow injection serial dynamic dialysis (FISDD) procedure is described. The method is based on the study of the kinetics of dialysis of the ligand in the absence and presence of protein. A study of the binding of sulfamethoxazole, sulfamethizole and sulfisoxazole to bovine serum albumin by means of such an automated system was undertaken to investigate the utility of FISDD technique for protein binding studies. The determination of dialysable sulfonamides was performed automatically by the FIA analyser. The influence of ionic strength and viscosity on the rate of dialysis was investigated. It was found that both variables did not affect the kinetic profile. Binding by the cellophane membrane was not encountered as a problem with the compounds studied. Binding parameters estimated for sulfamethoxazole were found to agree well with those reported in the literature. The Scatchard plots for the binding of sulfamethizole and sulfisoxazole with bovine serum albumin, revealed two classes of binding sites for each sulfonamide. The system was also used for the calculation of the dialytic rate constants. Experimental variables can be readily controlled to yield favoured conditions to study the protein binding phenomenon.

Introduction

During the last years therapeutic drug monitoring has become a general practice in many hospitals. The binding of drugs to plasma proteins can have a marked influence on their pharmakokinetics and pharmacodynamics and, therefore, on therapeutic drug monitoring. Although, evidence suggests that only the unbound or 'free' drug is pharmacologically active, usually total drug concentrations in blood are measured and utilized for dosage regimens designs. However, many drugs exhibit significant intra- and inter-individual differences in their degree of protein binding. For these drugs dosage schedules must be based on determination of free drug concentrations.

The in vitro study of drug–protein interactions is made by a variety of methods including equilibrium dialysis (Klotz et al., 1946; Hunter and Commerfold, 1955; Bush and Alvin, 1973), dynamic dialysis (Meyer and Guttman, 1968; 1970; Colowick and Womack, 1969; El-Rashidy and Niazi, 1978), continuous-flow dynamic dialysis (Sparrow et al., 1982), fluorescence probe technique (Brand et al., 1967; Hsu et al., 1974), ultrafiltration (Feldmann et al., 1950), gel permea-

Correspondence: P. Macheras or M. Koupparis, Department of Pharmacy, Athens University, 104 Solonos St., Athens 10680, Greece.

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tion chromatography (Hummel and Dreyer, 1962), etc. Although the equilibrium dialysis technique is still a favoured method for measuring free drug concentrations, because of its serious drawbacks, it has been replaced by dynamic dialysis methods. These methods offer the advantages of rapidity and the measurement of drug–protein binding over a range of drug concentrations in a single experiment. They are based on the principle that non-diffusible protein–drug complex reaches rapid equilibrium with free protein and diffusible drug in a protein compartment, which is separated from a sink compartment by a semipermeable membrane. The rate of diffusion across the membrane is directly proportional to the free drug concentration in the protein compartment.

The sampling of the dialyzable drug is accomplished by periodical manual removal of a large volume from the external solution which is replaced with fresh buffer solution in order to maintain sink conditions. The measurement is done by UV-spectrophotometry or, when other absorbing species are present, with chemical-visible spectrophotometry. To avoid invasive analytical sampling the continuous dynamic dialysis technique was introduced by Sparrow et al. (1982) where the absorbance of the dialyzable drug is continuously monitored.

Albeit that dynamic dialysis techniques have shortened the experiment time to 4–6 h, this time is still rather long and a further decrease using a novel technique is desirable.

The present paper describes a new automated method for protein binding studies based on the principle of dynamic dialysis in conjunction with the novel and versatile analytical technique Flow Injection Analysis (FIA). FIA has already been used by Abdullahi et al. (1983) in drug protein binding studies by adaptation of the fluorescence probe technique, but only the percentage displacement of the probe has been estimated.

In this novel method, a conventional dialyzer unit used in clinical air-segmented analyzers, with two identical compartments of 1.0 ml volume capacity, is connected to an automated FIA analyzer. The receiving compartment has become the 'sample loop' of the FIA analyzer, in which carrier solution is enclosed to receive the dialyzable drug of the 'protein compartment' through which the protein–drug solution is continuously circulated. Periodically this 'sample zone' is injected in the analytical manifold to be measured and fresh solution is enclosed in the sample loop. The change of drug concentration in the 'protein compartment' is achieved by successive manual additions of small volumes of standard drug solution.

The new technique described here, which will be called hereafter Flow Injection Serial Dynamic Dialysis (FISDD), provides automation of dialysis, sampling and analysis processes. The features of the new method are explored by the binding study of three sulfonamides with bovine serum albumin (BSA).

Experimental

Apparatus

The FISDD system, shown in Fig. 1, was developed by interfacing of a homemade flow injection photometric analyzer (Koupparis and Anagnostopoulou, 1984) with a conventional 4-slot dialysis unit [1]. All other parts of this analyzer were identical to those previously described by Koupparis et al. (1984).

The dialyzer unit, constructed from plexiglas, has a dialysis surface of 670 mm^2 with two identical chambers of 1.0 ml volume each. The two chambers were separated by semipermeable membrane [2] with 20 μm pores, hydrated for a short time before placement. The lower chamber, connected to ports 1 and 4 of the rotary valve (the 'sample loop' of the FIA analyzer) serves as the 'receiving chamber'. The upper chamber was connected with the sample pump which circulates the protein–drug solution from the thermostated at 25 ± 0.5°C cell of 50 ml volume.

The analytical manifold for the automated determination of sulfonamides based on the classical Bratton and Marshall (1939) reaction, is shown in Fig. 2. The reagent pump can be automatically stopped during dialysis to avoid waste of the

[1] Technicon Instruments Corporation, U.S.A.
[2] Cuprophan membranes, Technicon Chem.Co.

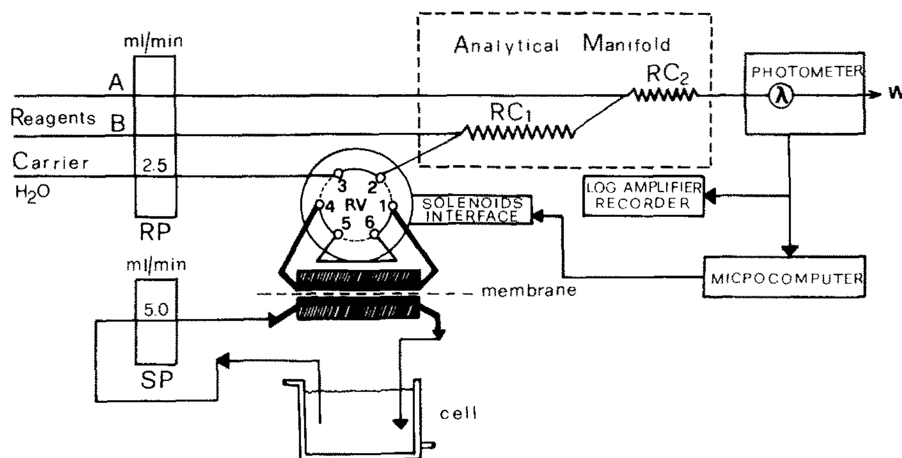


Fig. 1. Schematic diagram of Flow Injection Serial Dynamic Dialysis (FISDD) system. RP, reagents pump; SP, sample pump; RC1, RC2, reaction coils; W, waste; RV, rotary valve.

analytical reagents. All experiments were carried out at constant temperature, pH, flow-rates, stirring, etc.

Reagents

Concentrated stock solutions of sulfonamides were prepared in 0.05 M NaOH at concentrations of 0.04 M for sulfamethoxazole [3] and sulfisoxazole [3], and 0.05 M for sulfamethizole [3]. A working solution of 5.0×10^{-4} M of BSA [4] fraction V (powder) was prepared in phosphate buffer 0.05 M, pH 7.4. The analytical reagents used, i.e. HCl 0.80 M, $NaNO_2$, 2.0×10^{-4} M and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) [4] 0.1% (w/v), were prepared from analytical grade reagents. Deionized distilled water was used.

[3] Cyanamid, U.S.A.
[4] Sigma, U.S.A.

System set-up and operation

The appropriate 'dialysis' program is loaded in the microcomputer memory and the operator is prompted to provide information which includes number of measurements, dialysis, injection and mixing-washing times. Then the reagents pump is started, the appropriate wavelength is set on the photometer (540 nm for sulfonamides) and the 100% transmittance and the recorder baseline are calibrated. At this point 50.0 ml of buffer or buffered protein solution are pipetted into the cell and the sample pump is started. In execution of the program the rotating valve is turned to the 'injection' position and the operator is prompted

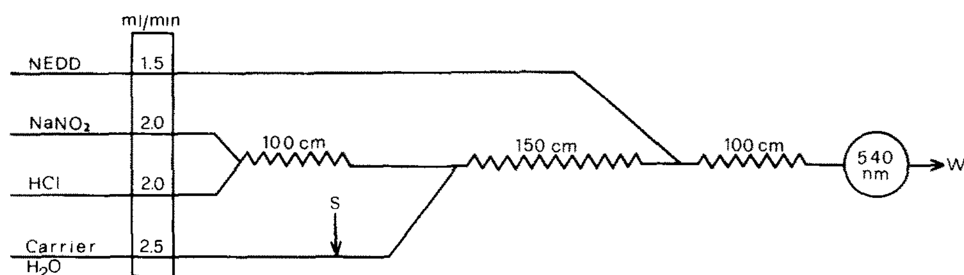


Fig. 2. Analytical manifold for FIA determination of sulfonamides. NEDD, N-(1-naphthyl)ethylenediamine dihydrochloride, 0.10% (w/v); $NaNO_2$ 2.0×10^{-4} M; HCl 0.80 M. S represents the dialysate transferred by the carier into the reagents stream.

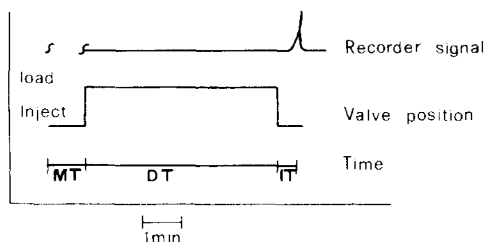


Fig. 3. Timing diagram of operation of FISDD system. MT, mixing time; DT, dialysis time; IT, injection time.

to add the first portion of the standard stock solution of the drug under test, using a microsyringe [5]. The valve remains in this position, during which the carrier solution (H_2O) flows through the receiving chamber, for the preset mixing-washing time (1 min) in order to ensure adequate mixing and transfering of the protein solution. Then the computer turns the valve to the 'load' position during which the receiving solution is stopped. When the preset 'dialysis' time is reached, the valve is turned to the 'injection' position and the carrier solution transfers the dialysate to the analytical manifold to be measured. The absorbance peak, corresponding to the concentration of the dialyzed drug, is recorded and its value is read by the microcomputer. After the injection time (30 s) has elapsed, the operator is prompted to add the next portion of the standard stock solution of the drug and the cycle is repeated. The timing diagram of the operation is shown in Fig. 3. During the mixing-washing time the recorder pen is inactivated by the computer to avoid signals from the limited dialysis of the drug, since the valve is at the 'injection' position.

Theory

In the absence of protein, the passage of small molecules through the dialysis membrane follows first-order diffusion kinetics. The permeation rate can be described by the following equation:

$$dD_i/dt = K_d(D_t - D_i) \qquad (1)$$

[5] Hamilton, U.S.A.

where D_i is the instantaneous drug concentration in the receiving compartment, D_t is the concentration in the protein compartment and K_d the apparent permeability rate constant. When protein is included in the solution undergoing dialysis, small molecules bound to the protein are non-diffusible. Therefore, the rate of passage is related not to the total concentration, but to the free or unbound concentration D_f, and the kinetics for each dialysis run is described by the equation:

$$dD_i/dt = K_d(D_f - D_i) \qquad (2)$$

assuming that the dialytic rate constant is unaffected by the presence of protein. Integration of Eqn. 2 gives:

$$D_i = D_{fo}(1 - e^{-K_d t}) \qquad (3)$$

where D_{fo} is the initial free drug concentration in the protein compartment after equilibrium has been reached and before dialysis is initiated. In reality, Eqn. 3 describes the time profile of the dialysate concentration in the receiving compartment during a dialysis run. Since the protein solution has a large volume (50 ml), in comparison with the volume of the receiving solution (1.0 ml), D_f remains practically constant and can be considered as equal to D_{fo}. As it is implicit from Eqn. 3, using constant experimental conditions and dialysis time, D_i is linearly related to D_{fo}.

Using the appropriate analytical manifold in the FIA analyzer, in conjunction with a variety of detectors (photometers, fluorometers, electrochemical detectors, etc.), an analytical parameter P_i is measured as a peak, which is related to D_i in a specific analytical range. Therefore, by choosing an appropriate value for the dialysis time the analytical parameter measured, P_i, is directly related to D_{fo} in a specific range of concentrations. For the FIA analyzer used in this study, with a photometer detection, a calibration curve of absorbance peaks vs free drug concentration can be easily constructed by performing a series of drug additions and the consequent dialysis runs, in a buffer solution in the absence of protein. By repeating the procedure with buffered protein solution and measuring the absorbance peak of

each successive drug addition–dialysis run, the free drug concentration, D_{f_0}, can be estimated from the calibration curve. Since the total drug concentrations, D_t, are known from the successive drug additions, calculation of the binding parameters can be made by standard procedures (Scatchard, 1949).

The experimental dialytic rate constant can be easily calculated from Eqn. 3. At first, a limited number of standard solutions of the drug studied, are introduced in the receiving compartment through the ports 5 and 6 of the rotary valve. Their absorbance peaks are measured and a calibration curve, absorbance peak vs D_i, is constructed. Then a buffered solution of the drug is pipetted into the cell and a special program is run which increases the dialysis time in a preset step. Thus, the dialysis–time profile of the drug is obtained. The calculation of K_d can be based on Eqn. 4:

$$\ln(D_f - D_i/D_f) = -K_d t \qquad (4)$$

This equation is obtained from Eqn. 3 after rearrangement and logarithmic transformation. In addition, Eqn. 4 can provide the dialysis time required for a specified percent of dialysis ($\%D_i$),

$$t = -\frac{1}{K_d}\ln\left(1 - \frac{\%D_i}{100}\right) \qquad (5)$$

For example, by assigning the value 10% to $\%D_i$, one can find how long sink conditions prevail. Solving Eqn. 5 in terms of $\%D_i$, the percent of dialysis for a specified dialysis time can be obtained:

$$\%D_i = 100(1 - e^{-K_d t}) \qquad (6)$$

Hence, optimization of the experimental conditions of dialysis can be based on Eqns. 5 and 6.

Results and Discussion

A typical dialysis profile of a sulfonamide obtained using the FISDD system is shown in Fig. 4. Dialysis time was increased stepwise by 1.0 min

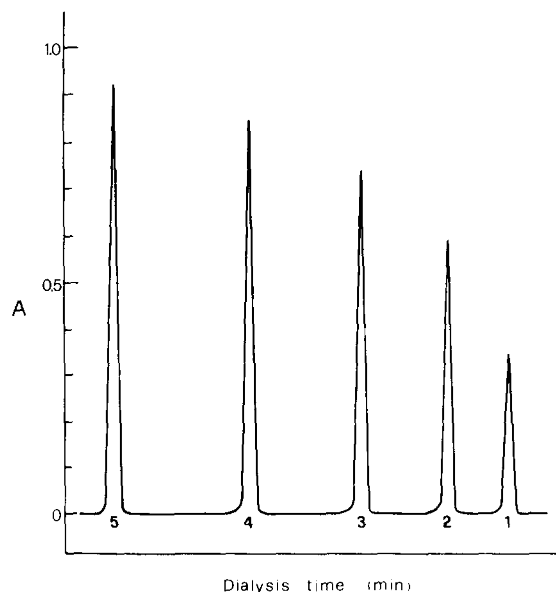


Fig. 4. Typical dialysis profile of sulfamethoxazole 2.5×10^{-4} M for K_d calculation.

by the computer program. The calculated dialysis rate constants for sulfisoxazole, sulfamethizole and sulfamethoxazole at pH 7.4 are shown in Table 1 along with the statistical treatment of data. The good linearity of the plots in conjunction with the precision of the results show the validity of the technique for the rapid evaluation of dialysis rate constants. The time required for a calibration curve (absorbance peaks vs D_i) of three standards

TABLE 1

CALCULATION OF DIALYSIS RATE CONSTANTS OF SULFONAMIDES AT pH 7.4 and 25°C

Drug (Concentration, M)	K_d (±S.D.) [a] (min^{-1})	r [b]
Sulfamethizole (4×10^{-4})	0.095 ± 0.011	0.997
Sulfamethoxazole (2.5×10^{-4})	0.117 ± 0.011	0.990
Sulfisoxazole (3×10^{-4})	0.138 ± 0.018	0.991

[a] Standard deviation of 5 runs.

[b] Correlation coefficient of Eqn. 4.

TABLE 2

PRECISION OF FISDD SYSTEM

Dialysis run [a]	Absorbance peak	
	Absence of protein	With protein
1	0.822	0.305
2	0.825	0.305
3	0.820	0.302
4	0.808	0.298
5	0.798	0.300
$\bar{x}$	0.814	0.302
Standard deviation	0.011	0.003
%R.S.D. [b]	1.4	1.0

[a] Dialysis time 5.0 min, injection time 0.5 min and washing time 1.0 min. Total time of dialyses 32.5 min.

[b] Percent relative standard deviation.

and the dialysis runs (7 points) is only 20 min.

The precision of the system (dialysis and analytical measurement) was studied by performing series of 5 runs using 2.0×10^{-4} M sulfamethoxazole buffered solutions in the presence and absence of protein. The results are shown in Table 2. The excellent reproducibility obtained is the result of the precise timing control of the microcomputer and the stability of the flow rates of the pumps. After half-an-hour of dialyses the difference of absorbance peaks is negligible showing that the drug concentration remains practically constant.

In order to study the effect of ionic strength and viscosity on the dialysis, experiments were carried out with standard sulfomethoxazole solutions containing various amounts of NaCl and sucrose. The latter was chosen for the study of the viscosity effect since it is not expected to bind sulfonamides and also, if it is dialyzable, does not interfere with the analytical procedure. The results shown in Table 3 demonstrate that the increased ionic strength and viscosity do not affect the rate of dialysis. Similar conclusions have been obtained by Meyer and Gutman (1970) for their dynamic dialysis system.

Dialysis experiments for the estimation of binding parameters of sulfonamides are shown in Fig. 5. The calibration curves (absorbance peaks vs D_f) obtained using buffer solutions in the absence of protein are shown along with the dialysis runs obtained in the presence of protein (5×10^{-4} M). The statistical treatment of data for calibration curves are shown in Table 4. The differences of the slopes of the calibration curves are caused by the differences of K_d and also of the molar absorptivity of the Bratton-Marshall reaction product of each sulfonamide.

The dialysis time chosen for these experiments was 5 min in order to increase the sensitivity of the analytical measurement. Using this dialysis time, the detection limit of the determinations was about 5×10^{-6} M (concentration corresponding to absorbance peak equal to twice the standard deviation of the most dilute standard). The per-

TABLE 3

EFFECT OF IONIC STRENGTH AND VISCOSITY ON DIALYSIS [a]

A: Effect of ionic strength

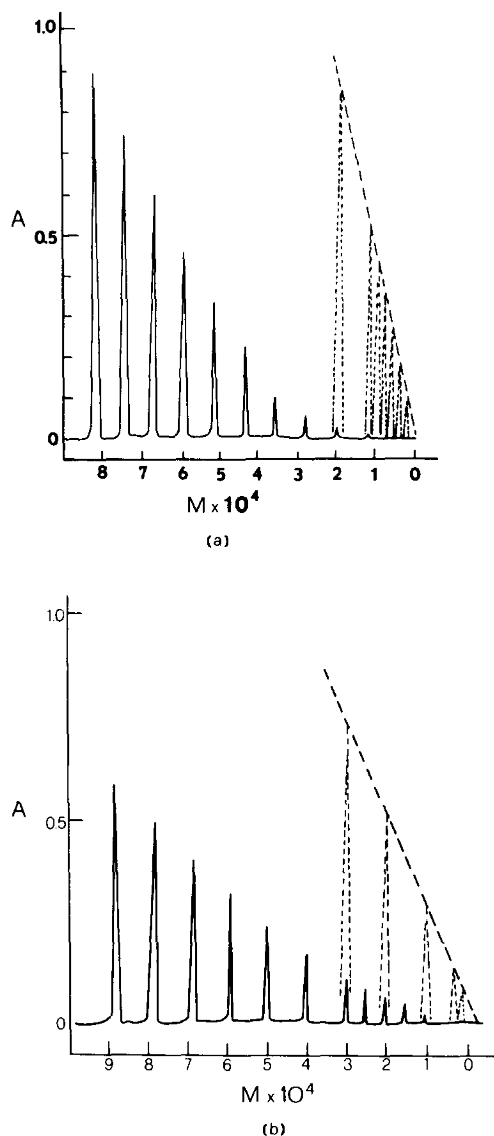
	Ionic strength	Absorbance peak	% Relative difference
(1) buffer [b]	0.11	0.598	–
(2) + 1% NaCl	0.28	0.588	−1.7
(3) + 10% NaCl	1.82	0.591	−1.2

B: Effect of viscosity

	Rel. viscosity	Absorbance peak	% Relative difference
(1) buffer	1.000	0.491	–
(2) + 1% sucrose	1.026	0.498	+1.4
(3) + 10% sucrose	1.333	0.466	−5.0

[a] Dialysis time 3 min.

[b] Phosphate 0.050 M, pH 7.4.



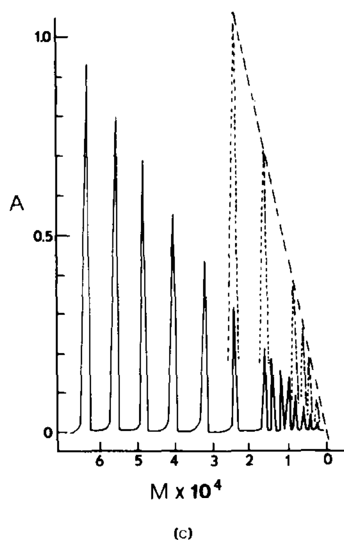


Fig. 5. Typical dialysis experiments for binding study of: (a) sulfisoxazole; (b) sulfamethizole; (c) sulfamethoxazole. Peaks for calibration curve in dashed line. Protein concentration 5.0×10^{-4} M. Dialysis time 5 min.

cent dialysis (%D_i) achieved with 5 min dialysis time, ranged from 37.8 to 50% for the three sulfonamides, but the loss of drug from the protein compartment is only 0.8–1.0%. Since the drug concentration is increased before each successive dialysis, the total loss is negligible. In addition to that, the calibration curve serves to correct any loss of this kind. Although sink conditions were not maintained, linear calibration curves (absorbance peaks vs D_f) were obtained showing the validity of Eqn. 3 under constant experimental conditions.

The binding parameters estimated by a computer program in BASIC based on non-linear regression analysis are shown in Table 5. The results are also presented in Fig. 6 in the form of Scatchard plots. Sulfisoxazole and sulfamethizole can be classified as highly bound with affinities higher than 5×10^3 M^{-1} while sulfamethoxazole is moderately bound. The parameters found were compared with values from bibliography (Table 5). As can be seen from the values listed, the estimates for sulfamethoxazole are in good agreement with those reported in literature. For sulfamethizole and sulfisoxazole the present study revealed two groups of binding sites. It is interesting to note, however, that the estimates of the secondary binding sites are similar to the estimates for the unique binding sites of the published data (Table 5). Moreover, Cruze and Meyer (1976) have also reported two classes of binding sites for the interaction of the structurally relevant sulfathiazole with BSA using a dynamic dialysis technique.

Differences in the estimated parameters of

TABLE 4

CALIBRATION CURVES FOR DIALYSIS EXPERIMENTS USED IN SULFONAMIDE BINDING STUDIES

Drug	Calibration equation [a]	S.E.E. [b]	r [c]	Detection limit (M)
Sulfamethizole	$A = 0.021(\pm 0.012) + 2415(\pm 71)C$	0.011	0.999	9.1×10^{-6}
Sulfamethoxazole	$A = 0.052(\pm 0.017) + 3858(\pm 143)C$	0.025	0.998	5.7×10^{-6}
Sulfisoxazole	$A = 0.016(\pm 0.013) + 4347(\pm 120)C$	0.016	0.999	5.1 ± 10^{-6}

[a] Absorbance peak height vs drug concentration in moles · l^{-1}; 6 standards.
[b] Standard error of the estimate.
[c] Correlation coefficient.

binding could be caused by technique parameters, temperature and buffer composition. It is well recognized, however, that the elucidation of the exact nature of drug-protein interaction requires: (i) a reliable and reproducible technique; (ii) a precise and accurate method of analysis; (iii) studies at low drug-to-protein ratio; and (iv) absence or correction of membrane binding. Otherwise, the detailed interaction is overmasked and simplified and the real situation is not revealed. The proposed dynamic technique fulfils the prerequisites mentioned. The reproducibility was ensured by the microcomputer. The sensitivity of analytical methods was enhanced by the appropriate choice of the experimental conditions while the calibration curves were constructed under identical conditions and within an hour of the actual experiments. Thus, the absence of membrane binding in the present study, justified by the linearity of the calibration curves at low drug concentrations, enabled the experiments to be carried out at low drug-to-protein ratios. Overall, this work was undertaken under experimental conditions which are optimal for studying the protein binding phenomenon. Consequently, the existence of two classes of binding sites found for sulfamethizole and sulfisoxazole is most likely to be real and not due to artefacts.

Contrary to the other protein binding methods, the FISDD technique has the advantages of rapidity and automation. Therefore, it can be anticipated that the described technique can also be applied in general practice using serum samples of patients. Obviously, temperature control, i.e. 37°C, is easy to achieve. It requires the described conditions to be met and the development of suitable

TABLE 5

BINDING DATA FOR THE STUDIED SULFONAMIDES TO BOVINE SERUM ALBUMIN

Compound	Binding data [a]		Reference
Sulfamethoxazole	$n = 2.0 \pm 0.1$	$K = 2.9 \pm 0.3 \times 10^3\ M^{-1}$	This study
	$n = 2.9$	$K = 1.6 \times 10^3\ M^{-1}$	Hsu et al. (1974)
Sulfamethizole	$n_1 = 0.43 \pm 0.06$	$K_1 = 9 \pm 2 \times 10^5\ M^{-1}$	This study
	$n_2 = 2.7 \pm 0.3$	$K_2 = 2.0 \pm 0.5 \times 10^3\ M^{-1}$	This study
	$n = 2.0$	$K = 2.0 \times 10^4\ M^{-1}$	Moriguchi et al. (1968)
	$n = 2.9$	$K = 5.2 \times 10^3\ M^{-1}$	Hsu et al. (1974)
Sulfisoxazole	$n_1 = 0.80 \pm 0.02$	$K_1 = 1.3 \pm 0.2 \times 10^6\ M^{-1}$	This study
	$n_2 = 4.1 \pm 1.7$	$K_2 = 5.5 \pm 2.8 \times 10^3\ M^{-1}$	This study
	$n = 2.0$	$K = 1 \times 10^5\ M^{-1}$	Moriguchi et al. (1968)
	$n = 2.5$	$K = 1.47 \times 10^4\ M^{-1}$	Nakagaki et al. (1964)
	$n = 2.9$	$K = 9.6 \times 10^3\ M^{-1}$	Hsu et al. (1974)

[a] n, n_1, n_2 = number of binding sites; K, K_1 and K_2 = binding constants.

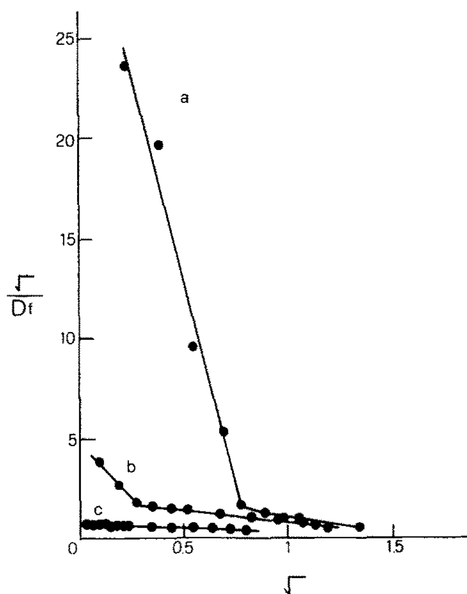


Fig. 6. Typical Scatchard plots for sulfonamides binding studies using data obtained with FISDD. a, sulfisoxazole; b, sulfamethizole; c, sulfamethoxazole. Dialysis experiments at pH 7.4, 25°C, protein 5×10^{-4} M.

analytical manifolds for drugs calling for careful monitoring of free blood levels in their routine use. Care should be exercised though with the variety of the possible interfering small-sized molecules present in serum.

In conclusion, flow injection analysis brings to the study of drug–protein binding its usual advantages of flexibility, speed, precise timing control, and automation of sampling-measurement processes. Although the present study was limited to bovine serum albumin-containing systems, there is no reason why the method could not be used in studies of binding by natural macromolecules, synthetic polymers, adsorbents and micelle-forming materials. The proposed system can be also utilized for permeation studies. The dialysis unit can accomodate various types of membranes for a rapid estimation of the dialytic rate constants. Furthermore, by developing specific analytical manifolds (like that proposed for sulfonamides) competitive binding studies, where mixtures of ligands interact with macromolecules, can be also accurately performed. In our laboratory further experiments are in progress to study the interaction of milk proteins with drugs.

Acknowledgements

The authors would like to thank Mr. T. Christopoulos for help in computing the binding parameters.

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