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AN AUTOMATED FLOW-INJECTION SERIAL DYNAMIC DIALYSIS TECHNIQUE FOR DRUG-PROTEIN BINDING STUDIES

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SUMMARY

An automated flow-injection analyzer is interfaced with a dialysis unit to study drug-protein binding interactions. The binding of some sulfonamides to bovine serum albumin is studied by means of the automated system and gives results similar to those obtained by other procedures. A usual time for a complete run is 100 min, including calibration. The dialysable sulfonamides are quantified spectrophotometrically by using a modified Bratton-Marshall method. The system is also used for the calculation of dialysis rate constants.

Studies on drug-protein interaction have become important because of their relevance to pharmacokinetics. Such studies in vitro have been done by various methods including equilibrium dialysis [1—3], dynamic dialysis [4—7], continuous-flow dynamic dialysis [8], fluorescence probe techniques [9, 10] and ultrafiltration [11]. Although the classical equilibrium dialysis technique is still often favoured, it has serious drawbacks and is increasingly 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 a nondiffusible 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 dialysable drug is sampled by periodic manual removal of a certain volume from the external solution, which is replaced with fresh buffer solution in order to maintain sink conditions. The measurement is done by u.v. spectrophotometry or, when other absorbing species are present, by visible spectrophotometry after derivatization. To avoid invasive sampling, the continuous dynamic dialysis technique [8] was introduced; in this method the absorbance of the dialysable drug is continuously monitored. Although dynamic dialysis techniques have shortened the time needed for an experiment to 4—6 h, any further decrease would be useful.

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The present paper describes an automated method for protein-binding studies based on the principle of dynamic dialysis in conjunction with the versatile techniques of flow injection analysis (f.i.a.). Flow-injection techniques have already been used in drug-protein binding studies with a fluorescence probe [12], but only the percentage displacement of the probe was estimated. In the proposed method, a conventional dialyzer unit, as used in clinical air-segmented analyzers, with two identical compartments of 1.0-ml capacity, is connected to an automated flow-injection analyzer. The receiving compartment becomes the "sample loop" of the analyzer, in which carrier solution is enclosed to receive the dialysable drug of the "protein compartment" through which the protein-drug solution is continuously circulated. Periodically, this "sample zone" is injected into the spectrophotometric manifold and fresh solution is enclosed in the sample loop. The drug concentration in the "protein compartment" is changed by successive manual additions of small volumes of standard drug solution.

The technique described here, which is called hereafter flow-injection serial dynamic dialysis, provides automation of dialysis, sampling and spectrophotometric determination. The features of the method are explored by a binding study of three sulfonamides with bovine serum albumin (BSA). The manifold is based on the classical Marshall-Bratton reaction adapted for f.i.a. [13].

EXPERIMENTAL

Apparatus and reagents

The system, shown in Fig. 1, was developed by interfacing a homemade flow-injection photometric analyzer [14] with a conventional 4-slot dialysis unit (Technicon). This analyzer consists of reagent (RP) and sample (SP) peristaltic pumps, a rotating valve (RV, Rheodyne Type 50) operated with a pneumatic two-position actuator (Rheodyne Model 5001), an appropriate manifold of teflon tubing, a conventional photometer equipped with a flow-

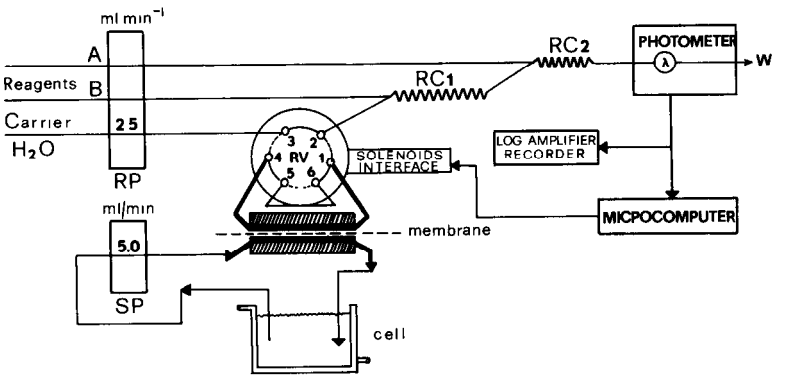


Fig. 1. Schematic diagram of the flow-injection serial dynamic dialysis system: RP, reagent pump; SP, sample pump; RC1 and RC2, reaction coils; W, waste; RV, rotary valve.

cell (Hellma Model 172.12; 18-μl volume), a strip-chart recorder with a logarithmic amplifier, and a Rockwell AIM-65 microcomputer to control the whole system.

The dialyser 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 are separated by a semipermeable membrane (Cuprophan membranes; Technicon) with 20-μm pores, hydrated for a short time before being placed in position. The upper chamber, connected to ports 1 and 4 of the rotary valve (the "sample loop" of the analyzer) serves as the "receiving chamber". The lower chamber is connected to the sample pump which circulates the protein-drug solution from the 50-ml cell thermostatted at 25 ± 0.5°C.

The manifold for the automated determination of sulfonamides is shown in Fig. 2. The reagent pump can be automatically stopped during dialysis to avoid waste of the reagents. All experiments were done at constant temperature, pH, flow rates, stirring, etc.

The sulfonamides used were obtained commercially. Concentrated stock solutions were prepared in 0.05 M sodium hydroxide at concentrations of 0.04 M for sulfamethoxazole and sulfisoxazole, and 0.050 M for sulfamethizole. Bovine serum albumin (fraction V, powder; Sigma) was used to prepare a 5×10^{-4} M working solution in 0.050 M phosphate buffer, pH 7.4. Analytical-grade reagents were used to prepare 0.080 M HCl, 2.0×10^{-4} M sodium nitrite and 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD). Deionized/distilled water was used.

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 reagent pump is started, the appropriate wavelength is set on the photometer (540 nm for sulfonamides) and the 100% transmittance and recorder baseline are calibrated. Buffer or buffered protein solution (50.0 ml) are pipetted into the cell and the sample pump is started. In execution of the program, the rotating valve is turned at the injection position and the operator is

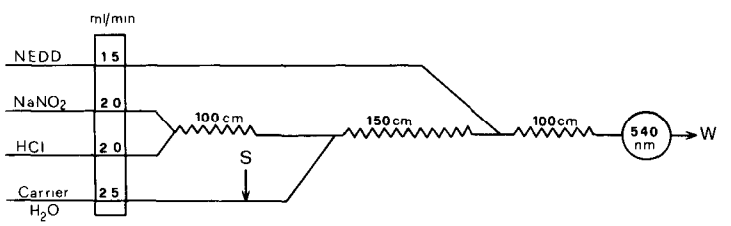


Fig. 2. Manifold for the determination of sulfonamides. NEDD, *N*-(1-naphthyl)ethylenediamine dihydrochloride, 0.10% (w/v); sodium nitrite, 2.0×10^{-4} M; hydrochloric acid, 0.80 M. S represents the dialysate transferred by the carrier into the reagent stream.

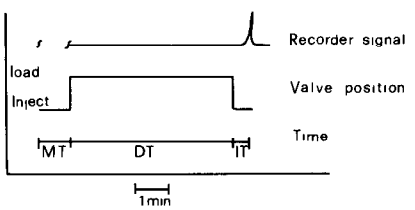
prompted to add the first portion of the standard stock solution of the drug under test, using a Hamilton microsyringe. The valve remains in this position, during which time the carrier solution (water) flows through the receiving chamber, for the preset mixing/washing time (1 min) in order to ensure adequate mixing and transference of the protein solution. Then the computer turns the valve to the load position during which time 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 dialyzate to the spectrophotometric manifold. The absorbance peak, corresponding to the concentration of the dialysed 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, because the valve is in 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

$$dD_i/dt = k_d(D_t - D_i) \quad (1)$$

where D_i is the instantaneous drug concentration in the receiving compart-



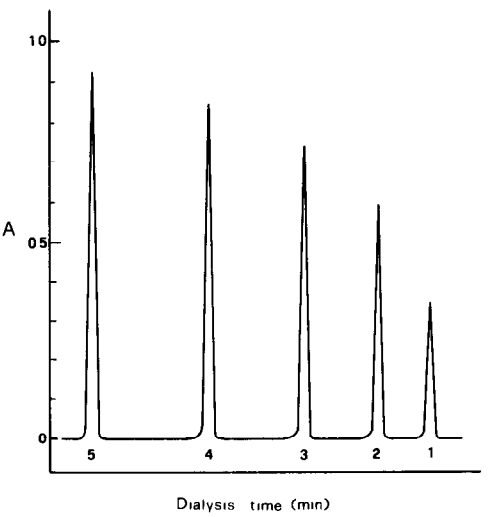


Fig. 3. Timing diagram of operation of the system: MT, mixing time; DT, dialysis time; IT, injection time.

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

ment, D_t is the concentration in the protein compartment and k_d is the intrinsic dialysis rate constant. When protein is included in the solution undergoing dialysis, small molecules bound to the protein do not diffuse. 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 $dD_i/dt = k_d(D_f - D_i)$, assuming that the dialysis rate constant is unaffected by the presence of protein. Integration of this equation gives

$$D_i = D_{fo}[1 - \exp(-k_d t)] \quad (2)$$

where D_{fo} is the initial free drug concentration in the protein compartment after equilibrium has been reached and before dialysis is initiated. Equation 2 actually describes the time profile of the dialysate concentration in the receiving compartment during a dialysis run. Because 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}. Clearly, if the experimental conditions and dialysis time remain constant, D_i is linearly related to D_{fo}.

With an appropriate flow-injection manifold, regardless of the type of detector (photometer, fluorimeter, electrochemical detector, etc), the required signal, P_i, is measured as a peak, which is related to D_i in a specified concentration range. Therefore, if an appropriate dialysis time is chosen, the signal measured, P_i, is directly related to D_{fo} over a specified range of concentrations. For the flow-injection analyzer used here with spectrophotometric detection, a calibration graph of absorbance peaks vs. free drug concentration is easily constructed by running a series of drug additions with subsequent dialysis in a buffer solution in the absence of protein. When the procedure is repeated with buffered protein solution and the absorbance peak of each successive drug addition/dialysis is measured, the free drug concentration D_{fo} can be estimated from the calibration graph. Because the total drug concentrations D_t are known from the successive drug additions, the binding parameters can be calculated by the well known Scatchard procedures [15]. In this study a computer program in BASIC based on nonlinear regression was used to obtain these parameters.

The experimental dialysis rate constant can easily be calculated from Eqn. 2. First, several standard solutions of the drug studied are introduced into the receiving compartment through ports 5 and 6 of the rotary valve (Fig. 1). Their absorbance peaks are measured and a calibration graph, 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 preset steps. Thus, the dialysis/time profile of the drug is obtained. The k_d value can be calculated from

$$\ln[(D_f - D_i)/D_f] = -k_d t \quad (3)$$

which is obtained from Eqn. 2 by rearrangement and logarithmic transformation. In addition, Eqn. 3 can provide the dialysis time required for a specified

percent of dialysis ($\%D_i$), say, 10% for sink conditions: $t = -\ln [1 - (\%D_i/100)/k_d]$. The percent dialysis for a specified dialysis time can then be obtained from

$$\%D_i = 100[1 - \exp(k_d t)] \quad (4)$$

RESULTS AND DISCUSSION

A typical dialysis profile of a sulfonamide obtained by using the proposed system is shown in Fig. 4. Dialysis time was increased stepwise by 1.0 min 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 statistical data. The excellent linearity of the plots and 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 graph of three standards and the dialysis runs (seven points) is only 20 min.

The precision of the system (dialysis and measurement) was studied by running five tests with 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 results from the precise timing control of the microcomputer and the stability of the flow rates of the pumps. After 30 min of dialysis, the difference in absorbance peaks was negligible, showing that the drug concentration remained practically constant.

TABLE 1

Calculation of dialysis rate constants of sulfonamides at pH 7.4 and 25°C

Drug	Concentration (M)	k_d (±SD) ($n = 5$)	r^a
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]Correlation coefficient of Eqn. 3.

TABLE 2

Precision of the proposed system for five runs with buffered 2×10^{-4} M sulfamethoxazole[a]

	No protein	With protein
Mean peak height	0.814	0.302
Standard deviation	0.011	0.003
RSD (%)	1.4	1.0

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

In order to study the effects of ionic strength and viscosity on the dialysis, experiments were done with standard sulfamethoxazole solutions containing various amounts of sodium chloride and sucrose. Sucrose was chosen for studying the effect of viscosity because it is not expected to bind sulfonamides and also, even if it is dialysable, it does not interfere with the analytical procedure. The results shown in Table 3 agree with the conclusions of Meyer and Guttman [5] that increased ionic strength and viscosity do not affect the rate of dialysis.

A typical dialysis experiment for the estimation of the binding parameters of sulfamethizole is shown in Fig. 5. The calibration graph (absorbance peaks vs. D_f) obtained for buffer solution in the absence of protein is shown along with the dialysis runs obtained in the presence of protein (5×10^{-4} M). The statistical data for the calibration graphs are shown in Table 4. The differences in the slopes of the calibration graphs are caused by the differences of k_d and also of the different molar absorptivities of the Bratton-Marshall reaction product for each sulfonamide. The dialysis time chosen for these experiments was 5 min, in order to increase the sensitivity of the measurement. The detection limit of the determinations was then about 5×10^{-6} M (concentration corresponding to an absorbance peak equal to twice the standard deviation of the most dilute standard). The percent dialysis ($\%D_i$) achieved with 5-min dialysis times, ranged from 37.8 to 50% for the three sulfonamides, but the loss of drug from the protein compartment was only 0.8–1.0%. Because the drug concentration is increased before each successive dialysis, the total loss is negligible. In addition the calibration graph serves to correct any loss of this kind. Although sink conditions were not maintained, linear calibration plots were obtained, showing the validity of Eqn. 2.

TABLE 3

Effect of ionic strength (I) and viscosity on dialysis[a]

	Variable	Absorbance peak	Relative change (%)
Effect of ionic strength			
	I (M)		
Buffer[b]	0.11	0.598	—
+1% NaCl	0.28	0.588	−1.7
+10% NaCl	1.82	0.591	−1.2
Effect of viscosity			
	Rel. viscosity		
Buffer[b]	1.000	0.491	—
+1% sucrose	1.026	0.498	+1.4
+10% sucrose	1.333	0.466	−5.0

[a]Dialysis time 3 min. [b]Phosphate 0.050 M, pH 7.4.

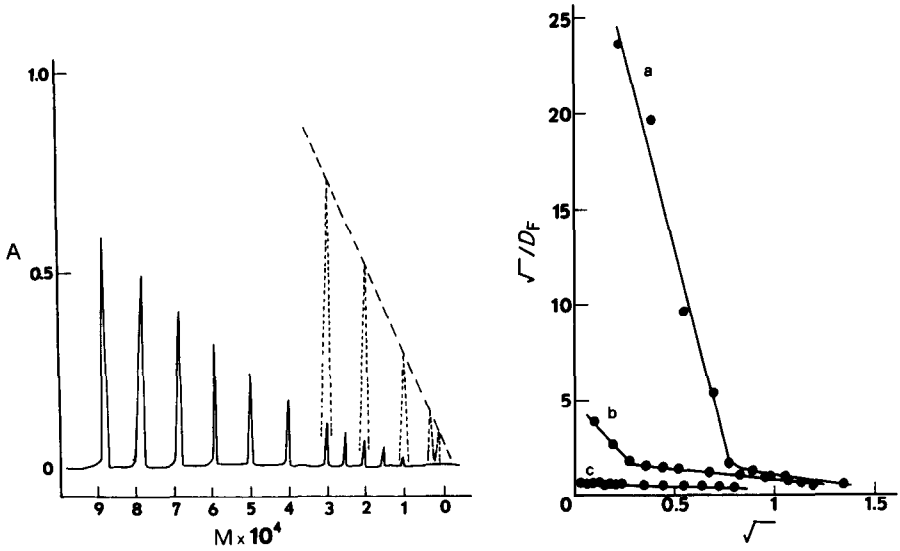


Fig. 5. Typical dialysis experiments for a binding study of sulfamethizole. Peaks for the calibration graph are shown as dashed lines. Protein concentration 5.0 × 10^{-4} M; dialysis time 5 min.

Fig. 6. Typical Scatchard plots for sulfonamide binding studies from data obtained in this study. (a) Sulfisoxazole; (b) sulfamethizole; (c) sulfamethoxazole. Dialysis experiments at pH 7.4, 25°C, with 5 × 10^{-4} M protein. $\sqrt{}$ = concentration of bound drug/total protein concentration.

The binding parameters estimated for each sulfonamide 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 exceeding 5 × 10^3 M^{-1}, while sulfamethoxazole is moderately bound. The parameters found were compared with literature values (Table 5); for sulfamethoxazole, the agreement is good. For sulfamethizole and sulfisoxazole, the present study revealed two groups of binding sites, which have been also reported for other sulfonamides [18]. The estimates for the secon-

TABLE 4

Calibration graphs for dialysis experiments used in sulfonamides binding studies

Drug	Calibration equation	Standard error	r^a	Detection limit (M)
Sulfamethizole	$A = 0.021(\pm 0.012) + 2415(\pm 71)C^b$	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	$A = 0.016(\pm 0.013) + 4347(120)C$	0.016	0.9992	5.1×10^{-6}

[a] Six standards. [b] Drug concentration in mol l^{-1}.

TABLE 5

Binding data for some 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
	$n = 2.9$	$K = 1.6 \times 10^3\ M^{-1}$	10
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
	$n = 2.0$	$K = 2.0 \times 10^4\ M^{-1}$	16
	$n = 2.9$	$K = 5.2 \times 10^3\ M^{-1}$	10
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
	$n = 2.0$	$K = 1 \times 10^5\ M^{-1}$	16
	$n = 2.5$	$K = 1.47 \times 10^4\ M^{-1}$	17
	$n = 2.9$	$K = 9.6 \times 10^3\ M^{-1}$	10

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

dary binding sites found here are close to the estimates of the single binding sites reported by Hsu et al. [10].

In conclusion, f.i.a. brings to the study of drug-protein binding its usual advantages of flexibility, speed, precise timing control, and automation of the sampling and measurement processes. By developing selective analytical methods (like the one proposed for sulfonamides) competitive binding studies can be done quickly and precisely.

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