Method of Obtaining Drug–Macromolecule Binding Parameters Directly from Dynamic Dialysis Data

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Abstract □ A new method of treating dynamic dialysis data to obtain binding parameters for drug–macromolecule interactions is presented. This method allows the determination of binding parameters directly from dialysis data according to a theoretical model. It is not necessary to determine the dialysis rate constant accurately in a separate experiment, and bias is not introduced due to differentiation. The proposed method should be applicable where the drug is substantially bound to the dialysis membrane.

Keyphrases □ Drug–macromolecule binding—parameters obtained directly from dynamic dialysis data □ Binding, drug–macromolecule—parameters obtained directly from dynamic dialysis data □ Dialysis data, dynamic—drug–macromolecule binding parameters obtained directly

The dynamic dialysis method for characterizing interactions of small molecules with macromolecules is well established (1–15) and has several advantages compared to equilibrium dialysis and ultrafiltration. A complete binding profile can be obtained rapidly in one experiment, and the method utilizes only a small macromolecule sample. Since the method is based on a dynamic process, an equilibrium state does not need to be defined, and, compared to ultrafiltration, there is no change in the macromolecule concentration.

Meyer and Guttman (3) designed a dynamic dialysis method to characterize drug–protein interactions, but their method has some limitations. The experimental data must be differentiated to evaluate binding parameters, which may introduce substantial errors, particularly if the number of data points is limited. An empirical equation was used to fit dialysis data to obtain instantaneous rates. A recent publication (13) showed that the various empirical equations used can yield substantially different binding parameters.

The technique of Meyer and Guttman (3) requires that the rate constant for dialysis be determined in a separate experiment in the absence of macromolecules. It is assumed that the same rate constant will apply in the presence of macromolecules. This assumption may be unreasonable because the rate constant depends on several factors, such as the physicochemical state of the dialysis membrane (16, 17), that may change between runs.

Several compounds are significantly bound to the membrane material (5, 11). When using previous methods, it has not been possible to determine the dialysis rate constant and to account for the membrane binding in the determination of macromolecule binding parameters of such compounds.

This paper presents a new approach. It rigorously describes the total kinetics of the system in a form that enables binding parameters to be estimated accurately, directly from dialysis data. It eliminates the need to determine an accurate dialysis rate constant separately. The method does not rely on differentiation of experimental data and should be applicable to membrane-bound compounds.

THEORY

Consider an interaction between small molecules and macromolecules that can be described by the general binding expression:

$$\bar{\nu} = \sum_{i=1}^{j} \frac{n_i K_i D_f}{1 + K_i D_f} \qquad \text{(Eq. 1)}$$

where $\bar{\nu}$ is the number of moles of small molecules bound per mole of macromolecule, n_i is the number of binding sites in the ith class of sites, K_i is the association constant for the interaction, and D_f is the molar concentration of unbound small molecules. If a model with two classes ($j = 2$) is assumed, then the total concentration of drug, D_t, in the protein compartment is given by (5):

$$D_t = D_f + P_t D_f \left(\frac{n_1 K_1}{1 + K_1 D_f} + \frac{n_2 K_2}{1 + K_2 D_f}\right) \qquad \text{(Eq. 2)}$$

If sink conditions prevail, the small molecules leave the protein compartment by a first-order process:

$$\frac{dD_t}{dt} = -K_e D_f \qquad \text{(Eq. 3)}$$

where K_e is the dialysis rate constant.

It is convenient to introduce a variable, s, defined as:

$$s = -\frac{dD_t}{dt} \qquad \text{(Eq. 4)}$$

so that $D_f = s/K_e$, and Eq. 2 can be written:

$$D_t = \frac{s}{K_e} + P_t s \left(\frac{n_1 K_1}{K_e + K_1 s} + \frac{n_2 K_2}{K_e + K_2 s}\right) \qquad \text{(Eq. 5)}$$

By taking the differential of this equation, noting $dD_t = -s dt$, it becomes:

$$dt = -\left\{\frac{1}{K_e s} + P_t K_e \left[\frac{n_1 K_1}{s(K_e + K_1 s)^2} + \frac{n_2 K_2}{s(K_e + K_2 s)^2}\right]\right\} ds \qquad \text{(Eq. 6)}$$

which, integrated from $t = 0$ to t, corresponding to $s = s_0$ to s, yields:

$$t = \left|\frac{1}{K_e} \ln s + P_t n_1 K_1 \left[\frac{1}{K_e + K_1 s} + \frac{1}{K_e} \ln\left(\frac{s}{K_e + K_1 s}\right)\right] + P_t n_2 K_2 \left[\frac{1}{K_e + K_2 s} + \frac{1}{K_e} \ln\left(\frac{s}{K_e + K_2 s}\right)\right]\right|_s^{s_0} \qquad \text{(Eq. 7)}$$

If the following function is defined:

$$f(x) = \frac{1}{K_e} \ln x + P_t n_1 K_1 \left[\frac{1}{K_e + K_1 x} + \frac{1}{K_e} \ln\left(\frac{x}{K_e + K_1 x}\right)\right] + P_t n_2 K_2 \left[\frac{1}{K_e + K_2 x} + \frac{1}{K_e} \ln\left(\frac{x}{K_e + K_2 x}\right)\right] \qquad \text{(Eq. 8)}$$

then Eq. 7 can be written more simply as:

$$t = f(s_0) - f(s) \qquad \text{(Eq. 9)}$$

The exact functional relationship describing the change of D_t with t is now described by Eqs. 5 and 9 in parametric form where the variable s is the parameter. With these equations, each value of s defines a unique pair of D_t and t values.

The quantity s_0 is the initial ($t = 0$) value of $-dD_t/dt$ (Eq. 4), which would normally be determined by extrapolation. To avoid the errors and problems of such an extrapolation, it is convenient to define $t = 0$ at the first sampling time. In this way, s_0 is $-dD_t/dt$ at the first sampling.

To determine the binding parameters by nonlinear regression, it is necessary to define the exact functional relationship between D_t and t for any values of n_1, K_1, n_2, K_2, s_0, and K_e, which are changing during the nonlinear fitting procedure. This step can be performed by determining the particular values of s that satisfy Eq. 9. These values are then used to determine the corresponding values of D_t by Eq. 5.

However, Eq. 9 cannot be expressed explicitly in terms of s, so some iterative procedure is needed to solve for s. The Newton–Raphson algorithm is particularly suitable because it is computationally compact and exhibits quadratic convergence. If:

$$\phi(s) = t - f(s_0) + f(s) \qquad \text{(Eq. 10)}$$

then Eq. 9 can be solved by the Newton–Raphson method using the following iteration[1]:

$$s_{i+1} = s_i - \frac{\phi(s_i)}{\phi'(s_i)} \qquad \text{(Eq. 11)}$$

where the functions ϕ and ϕ' are defined by:

$$\phi'(s) = f'(s) = \frac{1}{K_e s} + \frac{P_t K_e}{s}\left[\frac{n_1 K_1}{(K_e + K_1 s)^2} + \frac{n_2 K_2}{(K_e + K_2 s)^2}\right] \qquad \text{(Eq. 12)}$$

and:

$$\phi(s) = t + \frac{1}{K_e}\ln\frac{s}{s_0} + P_t n_1 K_1\left[\frac{K_1(s_0 - s)}{(K_e + K_1 s)(K_e + K_1 s_0)} + \frac{1}{K_e}\ln\frac{s}{s_0}\frac{(K_e + K_1 s_0)}{(K_e + K_1 s)}\right] + P_t n_2 K_2\left[\frac{K_2(s_0 - s)}{(K_e + K_2 s)(K_e + K_2 s_0)} + \frac{1}{K_e}\ln\frac{s}{s_0}\frac{(K_e + K_2 s_0)}{(K_e + K_2 s)}\right] \qquad \text{(Eq. 13)}$$

For the algorithm (Eq. 11) to converge within a given number of iterations, the initial estimates of s must not be too far from the s values for which Eq. 9 is satisfied. These initial estimates can, according to Eq. 4, be obtained as the (positive) values of $-dD_t/dt$ estimated from the observed values of D_t *versus* t. Any simple technique for slope estimation can be used since the actual accuracy of the estimates is of no importance for the final result.

These derivations for two binding classes can easily be extended to any number (j) of classes for which then:

$$D_t = \frac{s}{K_e} + P_t s \sum_{i=1}^{j}\frac{n_i K_i}{K_e + K_i s} \qquad \text{(Eq. 14)}$$

and:

$$t = \left|\frac{1}{K_e}\ln s + P_t\sum_{i=1}^{j} n_i K_i\left[\frac{1}{K_e + K_i s} + \frac{1}{K_e}\ln\left(\frac{s}{K_e + K_i s}\right)\right]\right|_s^{s_0} \qquad \text{(Eq. 15)}$$

and the iteration, Eq. 11, can still be used with:

$$\phi'(s) = \frac{1}{K_e s} + \frac{P_t K_e}{s}\sum_{i=1}^{j}\frac{n_i K_i}{(K_e + K_i s)^2} \qquad \text{(Eq. 16)}$$

and:

$$\phi(s) = t + \frac{1}{K_e}\ln\frac{s}{s_0} + P_t\sum_{i=1}^{j} n_i K_i\left[\frac{K_i(s_0 - s)}{(K_e + K_i s)(K_e + K_i s_0)} + \frac{1}{K_e}\ln\frac{s(K_e + K_i s_0)}{s_0(K_e + K_i s)}\right] \qquad \text{(Eq. 17)}$$

Binding of Small Molecules by Dialysis Membrane—The outlined treatment is based on the assumption that binding occurs only to protein. However, some drugs, particularly those strongly protein bound, can become significantly bound to the dialysis membrane. This fact means that K_e cannot be estimated from plots of $\ln D_t$ *versus* t in the absence of protein using previous techniques because of curvature (Fig. 1).

A special technique is required to estimate the dialysis rate constant, and the membrane binding must be taken into account in the treatment of the dialysis behavior of the small molecule–macromolecule system.

Determination of K_e and Membrane Binding Parameters in Absence of Macromolecules—The binding of small molecules to the membrane can often be considered as a Langmuir-type adsorption phenomenon (5), which is mathematically analogous to binding to a single class of sites, and can be described by:

$$\bar{\nu}^* = \frac{n^* K^* D_f}{1 + K^* D_f} \qquad \text{(Eq. 18)}$$

[1] The function ϕ is not defined for $s \leq 0$. Therefore, it is necessary during the iteration procedure to prevent s from taking a nonpositive value by defining $s_{i+1} = s_i/2$ if $s_{i+1} \leq 0$ since ϕ is a monotone increasing function of s because $\phi' = f' > 0$ ($s > 0$).

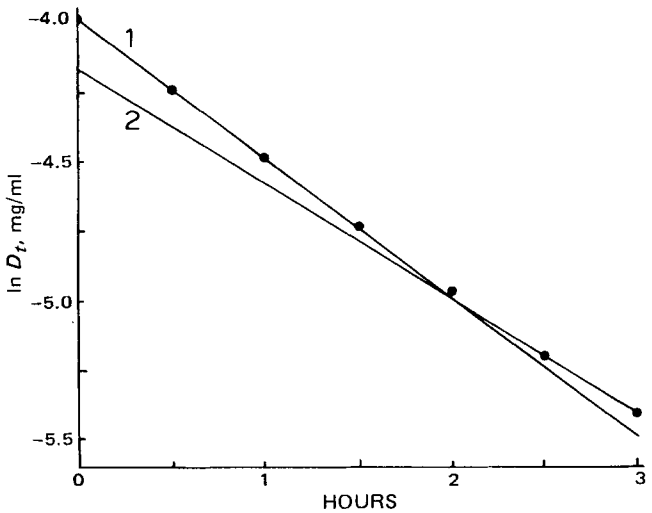

Figure 1—*Dynamic dialysis of glyburide in 0.067* M *phosphate buffer in the absence of macromolecules at pH 7.4 and 37°. The tangents illustrate a difference in the slope at* t = *0 and* t = *3 hr of 15%.*

where $\bar{\nu}^*$ is the amount of small molecules bound per amount of available membrane material, and K^* is the association constant for membrane binding. The quantity n^* does not have the same meaning as n previously defined but is introduced to establish a mathematical analogy leading to the following relationship between D_t and t, similar to Eqs. 14 and 15 ($j = 1$):

$$D_t = \frac{s}{K_e} + \frac{K's}{K_e + K^* s} \qquad \text{(Eq. 19)}$$

and:

$$t = \left|\frac{1}{K_e}\ln s + \frac{K'}{K_e + K^* s} + \frac{K'}{K_e}\ln\frac{s}{K_e + K^* s}\right|_s^{s_0} \qquad \text{(Eq. 20)}$$

where:

$$K' = \frac{Mn^* K^*}{WV} \qquad \text{(Eq. 21)}$$

and M is the amount of membrane material available for binding, W is the molecular weight of the small molecule, and V is the volume of the protein compartment. Equations 19 and 20 can then be used to determine K_e and the membrane binding parameters K^* and K' in the absence of protein using the described technique.

Determination of Drug–Macromolecule Binding Parameters in Presence of Membrane Binding—Once K_e, K', and K^* have been determined using this approach, it is possible to account for membrane binding and to determine parameters for binding to the macromolecule.

Simultaneous binding to the membrane and the macromolecule leads to the following expressions:

$$D_t = \frac{K's}{K_e + K^* s} + \frac{s}{K_e} + P_t s\sum_{i=1}^{j}\frac{n_i K_i}{K_e + K_i s} \qquad \text{(Eq. 22)}$$

$$t = \left|\frac{K'}{K_e + K^* s} + \frac{K'}{K_e}\ln\frac{s}{K_e + K^* s} + \frac{1}{K_e}\ln s + P_t\sum_{i=1}^{j} n_i K_i\left[\frac{1}{K_e + K_i s} + \frac{1}{K_e}\ln\left(\frac{s}{K_e + K_i s}\right)\right]\right|_s^{s_0} \qquad \text{(Eq. 23)}$$

which enables the D_t, t functional relationship to be evaluated using the iterative procedure discussed.

By comparing Eqs. 22 and 23 with Eqs. 14 and 15, it is seen that, in the presence of membrane binding, the dialysis behavior is mathematically analogous to a system where the small molecule is binding to two macromolecular species.

EXPERIMENTAL

The dynamic dialysis technique was described previously (3). To evaluate the new method of data treatment, chlorpropamide was dialyzed from 1% bovine serum albumin in 0.067 *M* phosphate buffer, pH 7.4, at 37° in the presence of a fixed free concentration of warfarin (1.6×10^{-5}

Table I—Chlorpropamide–Bovine Serum Albumin Binding Parameters Obtained in the Presence of 1.6×10^{-5} *M* Free Warfarin by the New Method and Two Derivative Methods

Parameter	New Method	Derivative Method	
		I[a]	II[b]
n_1	2	1.94	5.28
K_1, M^{-1}	2.64×10^4	1.09×10^4	2.92×10^3
n_2	9	9.14	7.77
K_2, M^{-1}	1.94×10^2	1.86×10^2	3.61×10^{-5}
K_e, hr^{-1}	0.714	0.711	0.711
SS[c], mM^2	2.60×10^{-3}	15.0×10^{-3}	5.50×10^{-3}

[a] A fourth-order least-squares polynomial was used to estimate $-dD_t/dt$. The binding parameters were calculated by the method of Hart (19) as used by Crooks and Brown (9). [b] The binding parameters were estimated by nonlinear regression analysis of $\bar{\nu}$ on D_f, obtained by the method of Meyer and Guttman (3) from a three-exponential fitting of D_t *versus* t data. [c] Sum of squared differences (Figs. 3–5) between observed D_t values and exact theoretical values calculated according to Eqs. 5 and 9 for the estimated values of n_1, K_1, n_2, K_2, and K_e.

M) maintained to within ±2% as described previously (12). Although warfarin displaces some chlorpropamide, the latter still appears to bind to two classes of sites (12). This system was selected so that such a model of two classes of sites could be appraised by the new data treatment method.

The starting concentration of chlorpropamide was 3.62×10^{-3} *M*, and the external compartment was sampled at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 7 hr. Chlorpropamide and warfarin were estimated by differential UV spectrophotometry at 231 and 310 nm using simultaneous equations. Absorbances of each component were additive at both wavelengths.

Glyburide was dialyzed from 0.067 *M* phosphate buffer, pH 7.4, at 37°. ^{14}C-Labeled material was used and assayed by liquid scintillation counting. All calculations were made to an accuracy of at least eight significant digits using a digital computer.

RESULTS AND DISCUSSION

Data for the dialysis of chlorpropamide from bovine serum albumin in the presence of 1.6×10^{-5} *M* free warfarin were treated according to the proposed method. Binding parameters n_1, K_1, n_2, and K_2 and the dialysis rate constant K_e were estimated using the function relating D_t and t given in Eqs. 5 and 9. This function was evaluated using Eqs. 11–13 and 5, programmed in a subroutine that was executed with the highly interactive time-sharing Fortran program, FUNFIT, written for general nonlinear least-squares regression (18).

The least-squares fit of the model with two classes of binding sites (Fig. 2) agreed well with the experimental data (r = 0.99986). The binding parameters estimated by the method are summarized in Table I. The

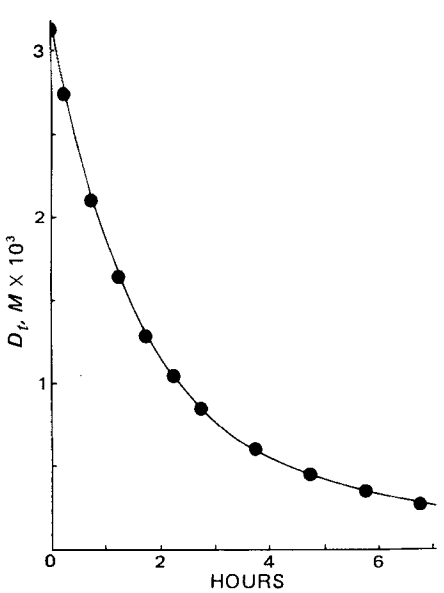

Figure 2—*Dynamic dialysis data for the binding of chlorpropamide to 1% bovine serum albumin at pH 7.4 and 37° in the presence of 1.6 × 10^{-5} M free warfarin. The curve fitted by least squares is the function relating* D_t *and* t *(Eqs. 5 and 9), which provides estimates of* n_1, K_1, n_2, K_2, *and* K_e.

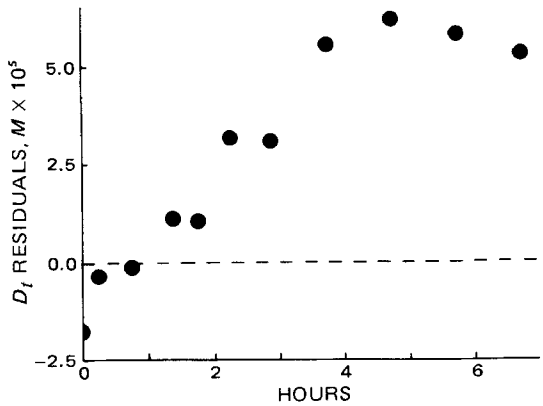

Figure 3—*Differences between observed* D_t *values and exact theoretical values calculated according to Eqs. 5 and 9 for the values of* n_1, K_1, n_2, K_2, *and* K_e *(Table I) estimated by the derivative method of Crooks and Brown (9).*

values for the number of binding sites, n_1 and n_2, were so close to 2 and 9 in a preliminary computation that their values were fixed as integers. Such a model has greater conceptual value, and the slight change in n_1 and n_2 did not substantially alter the values of the other parameters.

Also included in Table I are values for binding parameters estimated by fitting dialysis data to a fourth-order polynomial and then evaluated using a modification of the method of Hart (19) (Method I) as described elsewhere (9). Method II was that of Meyer and Guttman (3), where a three-exponential expression:

$$D_t = \sum_{i=1}^{3} A_i e^{-\alpha_i t} \qquad \text{(Eq. 24)}$$

is used to fit dialysis data. By differentiation at various t values, a $\bar{\nu}$ *versus* D_f profile was constructed; the binding parameters were estimated by a nonlinear regression technique using Eq. 1.

The dialysis rate constant, K_e, used for the derivative methods of data treatment was determined in a separate experiment.

There was good agreement between binding parameters determined using the new method and Method I, particularly for n_1, n_2, K_2, and K_e, although K_1 determined by the new method was somewhat greater. However, entirely different binding parameters were determined by Method II, probably because of the choice of the empirical equation, Eq. 24. The selection of this equation probably was based on a requirement for "smoothness" of the fitted curve and its first derivative. Although this requirement is satisfied, Eq. 24 does not have the same flexibility as a polynomial, which could be just as important. The flexibility of polynomials to approximate arbitrary functions is explained by the well-known Taylor series theorem.

The difference in flexibility is clearly demonstrated by fitting a fourth-order polynomial and Eq. 24 to exact dialysis data generated using the least-squares binding parameter estimates obtained using the new method (Table I)[2]. For the polynomial fitting, the D_t residuals (expressed as percent of calculated values) were 0.286, −0.217, −0.519, −0.0609, 0.466, 0.779, 0.381, −1.49, −1.42, 3.14, and −1.12. For the triexponential fit, the values were 1.73, 1.91, 1.46, 3.38, −1.19, −2.65, −3.91, −4.55, −2.19, 3.10, and 10.7.

These results show that the polynomial is considerably more flexible. The triexponential fitting resulted in significant systematic deviation in residuals, leading to a bias in the slope values and D_t values and, therefore, a bias in the final results. The residual sum of squares (mM^2) were 5.85×10^{-4} and 1.04×10^{-2} for the polynomial and triexponential fittings, respectively.

A further disadvantage of using Eq. 24 is that multiple solutions are possible; this equation is nonlinear (in α) and may result in several sum-of-squares minima. This is not the case with a polynomial, which has a unique least-squares solution.

The fitting of Eq. 24 to the generated exact D_t, t data was repeated several times with different initial estimates for A_i and α_i, but the same solution was obtained each time, suggesting that this fit is the best pos-

[2] The exact theoretical data used were simply the values calculated from the curve using the new method. Any other arbitrary values of the binding parameters could have been used. Theoretical D_t, t data can readily be calculated for given values of $(D_t)_{t=0}$, n_1, K_1, n_2, K_2, P_t, and K_e by first employing Eq. 5 to get s_0 and then using Eqs. 11–13 and 5.

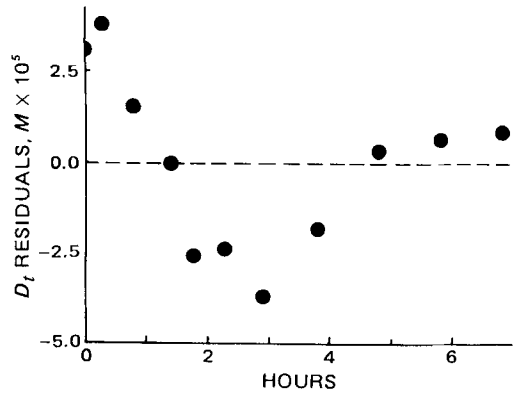

Figure 4—*Differences between observed* D_t *values and exact theoretical values calculated according to Eqs. 5 and 9 for the values of* n_1, K_1, n_2, K_2, *and* K_e *(Table I) estimated by the derivative method of Meyer and Guttman (3).*

sible using the triexponential equation. However, the use of a least-squares polynomial to represent dialysis data is expected to be less suitable than the triexponential when experimental errors are large or significant "gaps" exist between observation points because the ordinary least-squares polynomial fitting completely disregards derivative values. The derivative values are commonly in large error at the first and last observation points and just before or after gaps in the data.

This disadvantage of polynomials can be reduced considerably by imposing constraints on the derivative values by using least-squares spline polynomials. Such polynomials compete favorably with Eq. 24 on data with large errors and gaps, particularly considering the fact that the problem of multiple minima, using Eq. 24, is much larger for large residual problems. However, no matter which empirical equation is used, the results obtained will theoretically never be as exact as those obtained using the true equation as in the proposed method.

To investigate the bias introduced by Methods I and II, the exact D_t, t profile was calculated for the parameter values (Table I) obtained with the two methods. The differences between the observed and calculated D_t values (Figs. 3 and 4) show that the residuals are significantly larger for those methods than for the new method (Fig. 5). The residuals from Method I are particularly biased in a positive direction (Fig. 3), although the pattern resembles that from the new method (Fig. 5). The residuals from Method II (Fig. 4) show an entirely different pattern, consistent with the fact that the binding parameters obtained using Method II represent an entirely different solution.

A final check on the bias introduced by Methods I and II was made by applying them to exact D_t, t data generated from the parameter values (Table I) obtained using the new method. Method I found binding parameters relatively close to the true values, although K_1 seems to be somewhat different (Table II). Method II found an entirely different solution, similar to the one obtained using the real experimental data.

The value for the dialysis rate constant, K_e, estimated by the new method agrees very well with the value determined experimentally (Table I). As stated previously, this result may not always be true since the permeability of the membrane may change between experiments.

Chlorpropamide does not appear to be significantly membrane bound. Figure 1 shows the kinetics of dialysis of glyburide in the absence of bovine serum albumin. The curvature indicates significant membrane

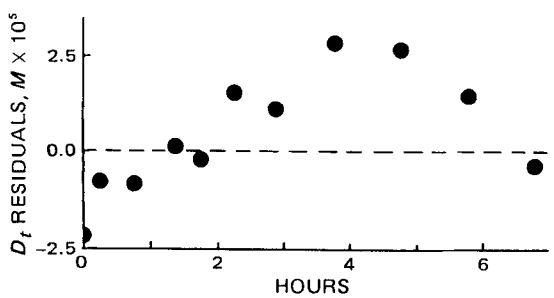

Figure 5—*Differences between observed* D_t *values and exact theoretical values calculated according to Eqs. 5 and 9 for the values of* n_1, K_1, n_2, K_2, *and* K_e *(Table I) estimated by the proposed method.*

Table II—Binding Parameters Obtained from Exact Generated Dialysis Data Using Two Derivative Methods [a]

Parameter	Exact Values	Estimated Values	
		Method I	Method II
n_1	2	1.96	5.02
K_1, M^{-1}	2.64×10^4	1.21×10^4	3.04×10^3
n_2	9	8.78	12.5
K_2, M^{-1}	1.94×10^2	1.74×10^2	3.16×10^{-5}

[a] $K_e = 0.714\ hr^{-1}$, and $P_t = 1.45 \times 10^{-4}\ M$.

binding. For this drug, it would not be possible to determine K_e and the binding parameters using previous approaches. However, they can be determined by the new method in two ways:

1. The membrane binding parameters K' and K^*, the dialysis rate constant K_e, and the drug–macromolecule binding parameters n_i and K_i can all be determined simultaneously and directly from experimental D_t, t data by applying Eqs. 22 and 23.
2. Since many parameters are involved in the first approach, it would be more reliable to determine K_e, K', and K^* in a separate experiment in the absence of the macromolecule and then to use the K_e value as an initial estimate with K' and K^* fixed as constants in the second experiment where n_i and K_i are determined.

As a result of these findings, the question naturally arises as to whether earlier reported binding parameters determined using previous techniques are valid. For certain combinations of binding parameters yielding a D_t *versus* t profile, some previous methods may give sufficiently accurate results. However, an excessively large number of simulation studies would be required to establish for which parameter combinations and sampling times previous methods are deficient. In general, the observer should not be too confident about the accuracy of the results obtained by previous approaches.

Studies on the use of dynamic dialysis for membrane-bound drugs are in progress.

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