International Journal of Pharmaceutics, 11 (1982) 45–55
Elsevier Biomedical Press

Measurement of protein binding for drugs that are unstable in aqueous solution

Farahnaz Nouravarsani and John Cobby *

College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68105 (U.S.A.)

(Received May 28th, 1981)
(Revised version received December 15th, 1981)
(Accepted January 7th, 1982)

Summary

Differential model equations were proposed to describe the dynamics of drug–macromolecule binding for drugs which are inherently unstable in solution. Equations relating to stable drugs comprise a specific case of the general model.

To verify the model, binding parameters for the interaction of methyl diethyldithiocarbamate (MeDDC), a stable drug, and bovine serum albumin (BSA) were compared by using a rapid equilibrium dialysis system in its customary equilibrium mode and according to the dynamic model. No significant differences in the equilibrium association constant (K_a), the maximum molar bound concentration $(C_b)_{max}$, or their product were found. The dynamic model was then used to determine K_a and $(C_b)_{max}$ for diethyldithiocarbamate (DDC), an unstable drug.

The procedure does not require the prior measurement of a diffusion rate constant in the absence of BSA. Because experimentally measured data may be entered directly into the computer program (NONLIN) used to determine the binding parameters, statistically undesirable errors arising from the more common manipulation of such data prior to computation are minimized.

Introduction

The estimation of drug–protein binding parameters by dynamic dialysis is well established, yielding values which are equivalent to those obtained by equilibrium dialysis studies (Meyer and Guttman, 1968, 1970a, 1970b). The major advantages of

* Present address: ICI Pharma, 6299 Airport Road, Suite 605, Mississauga, Ont. L4V 1N3, Canada.

0378-5173/82/0000–0000/$02.75

dynamic dialysis include the need for only a small volume of protein solution and the rapidity with which the experimental measurements may be concluded.

In a traditional dynamic dialysis study, a fluid containing drug and protein in equilibrium is placed in a compartment from which only unbound drug can freely diffuse. The fluid may be a clinical plasma sample or a sample prepared by the addition of drug to a protein solution, such as albumin or blank plasma. The protein compartment is surrounded by a buffer into which the drug diffuses. Experimentally, it is necessary to know the initial drug concentration and the constant volume within the protein compartment, the constant volume of the buffer, and to measure the appearance of drug in the buffer at suitable time intervals (Meyer and Guttman, 1968, 1970a, 1970b; Crooks and Brown, 1973; El-Rashidy and Niazi, 1978). The manipulation of these data to determine binding parameters requires, except in one example (Pedersen et al., 1977), that an analogous experiment be conducted using buffer instead of the protein sample. This procedure determines the diffusion rate constant which is assumed, possibly incorrectly (Pedersen et al., 1977), to be identical both in the presence and absence of protein.

The equilibrium and dynamic dialysis procedures have been applied only to drugs which are inherently stable in the solution being examined. Where the equilibrium procedure is used, the experimentally required equilibration of unbound and total (bound and unbound) drug prevents the assessment of binding parameters for drugs which are unstable in solution. The dynamic procedure rests on the assumption that the appearance of drug outside the compartment, which constitutes the sole experimental observation, reflects the loss of drug within the compartment. This assumption is violated if the drug loss is due to instability as well as diffusion out of the compartment.

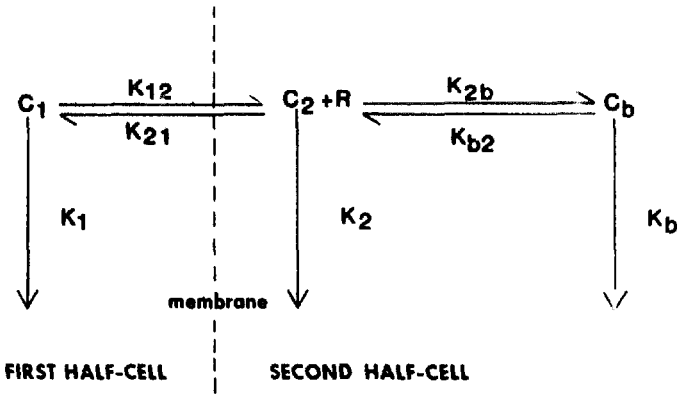
This report presents a dynamic procedure applicable to drugs which are unstable in solution while appearing to exhibit reversible binding properties. No manipulation of the experimentally measured data is necessary, nor are separate experiments required to determine the diffusion rate constant. The model drugs studied were two metabolites of disulfiram, diethyldithiocarbamate (DDC) and its methyl ester (MeDDC), reported to be unstable and stable, respectively, in blood and plasma (Cobby et al., 1977).

Theoretical

The general model (Scheme 1) represents the dynamic situation after a drug is placed in a multiple, rapid equilibrium dialysis system [1]. The drug is added in a simple aqueous solution to the first half-cell of the apparatus and a protein solution, initially containing no drug, is placed in the second half-cell. Thereafter, only unbound drug can passively and reversibly diffuse across the membrane separating the two half-cells. Drug present in the second half-cell may either remain unbound or reversibly bind with the protein to form a complex. As with simple equilibrium

[1] Dianorm, Innovative-Medizin AG, Esslingen, Switzerland.

binding theories (Goldstein, 1949), only one type of binding site per protein molecule is assumed; the interaction of drug with the membrane is assumed to be negligible. An unstable drug may degrade either when unbound or bound.



Scheme 1. General model representing the dynamics of drug–macromolecule binding in a multiple, rapid equilibrium dialysis system. For details and symbolism, see text.

Symbolically, the general model (Scheme 1) may be described as follows. C_1 and C_2 are molar unbound drug concentrations in the first and second half-cells, respectively, at time t; C_b is the molar bound drug concentration in the second half-cell at time t; and R is the 'molar' concentration of unbound binding sites on the protein molecule in the second half-cell at time t.

The diffusional rate constants for unbound drug movement across the membrane separating the two half-cells, K_{12} and K_{21}, are expressions of the drug clearance across the membrane (Cl) divided by the respective half-cell volumes (V_2 and V_1); where these volumes are experimentally equalized, the rate constants will also be equal. K_{2b} and K_{b2} are the directional rate constants for the binding and dissociation of the drug and protein. For an unstable drug, K_1, K_2 and K_b are the possible first-order degradation rate constants; for a stable drug, all 3 have a value of zero.

Simultaneous differential equations may be written for the unbound and bound drug concentrations:

$$\frac{dC_1}{dt} = K_{21}(C_2 - C_1) - K_1C_1 \tag{1}$$

$$\frac{dC_2}{dt} = K_{12}(C_1 - C_2) + K_{b2}C_b - K_{2b}C_2[(C_b)_{max} - C_b] - K_2C_2 \tag{2}$$

$$\text{and } \frac{dC_b}{dt} = K_{2b}C_2[(C_b)_{max} - C_b] - K_{b2}C_b - K_bC_b \tag{3}$$

where $(C_b)_{max}$ is the maximum molar bound drug concentration, hypothetically representing a situation where all the binding sites on the protein molecule are

occupied by drug[2]. The total molar drug concentration in the second half-cell (C_T) is the sum of the unbound (C_2) and bound (C_b) drug concentrations. Thus,

$$\frac{dC_T}{dt} = K_{12}(C_1 - C_2) - K_2C_2 - K_bC_b \quad (4)$$

In accordance with the law of mass action (Klotz, 1946), the equilibrium association constant of the drug–protein complex is defined as:

$$K_A = K_{2b}/K_{b2} \quad (5)$$

The equilibrium association constant and the maximum molar bound drug concentration represent parameters which are descriptive of the affinity and capacity of binding, respectively.

Experimental

Solutions

Bovine serum albumin[3] (BSA) solution (4.47%) was freshly prepared before use in Sørensen isotonic phosphate buffer (0.067 M, pH 7.38). Diethyldithiocarbamate[4] (DDC) and methyl diethyldithiocarbamate (MeDDC), prepared as previously reported (Cobby et al., 1977), were dissolved in buffer immediately before use.

Dialysis apparatus

A multiple, rapid equilibrium dialysis system[1] was used for both the dynamic and equilibrium dialysis studies. This system contains 20 polytef cells, with the two halves of the cell separated by a cellulose tubing membrane[5]. The construction of the system permits the entire contents of each half-cell to be removed by pipette.

The dialyzing membranes were prepared as 25 cm^2 squares. After soaking in distilled water for 15 min, the membranes were boiled in water for 5 min, rinsed 5 times with water, and stored in 30% v/v ethanol. When required for use, the membranes were thoroughly rinsed with water and soaked in buffer, twice for 15 min periods and finally overnight. The membranes were then placed between the two half-cells of the multiple, rapid equilibrium dialysis system[1].

Dynamic dialysis

Buffered drug solution (1 ml) was dialyzed against BSA solution (1 ml) at 37.0 ± 0.5°C. Samples (0.5 ml) of each solution were removed from successive cells at appropriate intervals over 4 h. The unbound (C_1) and total (C_T) drug concentrations in the samples were determined by published assays for DDC and MeDDC (Cobby et al., 1977).

---

[2] The value of R, which is a necessary constituent of Eqns. 2 and 3, is given by $(C_b)_{max} - C_b$ at time t.

[3] Sigma Chemicals, St. Louis, MO.

[4] Eastman Kodak, Rochester, NY; purchased as the sodium salt (trihydrate).

[5] Tubing 8-667C, Fisher Scientific, Pittsburg, PA.

Equilibrium dialysis of MeDDC

Preliminary investigations using dynamic dialysis indicated that the two assayed concentrations of MeDDC exhibited an equilibrium after 2 h dialysis. Therefore, buffered drug solutions were dialyzed against BSA solution and samples of each solution removed for assay at 240 min.

Data analysis

For dynamic dialysis, the experimentally measured concentrations (C_1 and C_T) were subjected to weighted least-squares regression of Eqns. 1–4 using the computer program NONLIN (Metzler et al., 1974) in its differential equation mode. In the subroutine DFUNC, the values of C_2 and K_{2b} were replaced by $(C_T - C_b)$ and $K_A K_{b2}$, respectively. Based on the variance of the replicate assay calibration curves, the measured concentrations (C_1 and C_T) were each weighted by the squared reciprocal of their values (Boxenbaum et al., 1974; Cobby et al., 1977). The unmeasured concentrations (C_2 and C_b) were given dummy input values, each with a zero weighting.

For equilibrium dialysis of MeDDC, the experimentally measured equilibrium concentrations $(C_1)_\infty$ and $(C_T)_\infty$ were subjected to weighted least-squares regression of the positive root of the following quadratic equation (Endrenyi and Kwong, 1972) using NONLIN in its integrated mode:

$$(C_1)_\infty = \frac{-b \pm \sqrt{b^2 - 4K_A(C_T)_\infty}}{2K_A} \tag{6}$$

$$\text{where } b = 1 + K_A[(C_b)_{max} - (C_T)_\infty] \tag{7}$$

Initial estimates of K_A and $(C_b)_{max}$ were obtained by manipulating the measured concentrations to yield data appropriate to Rosenthal (1967) axes.

The product $K_A(C_b)_{max}$ was calculated from the derived parameters (Sellers and Koch-Weser, 1970) for both dynamic and equilibrium dialysis.

Results

Dynamic dialysis of MeDDC

The dialysis of MeDDC in buffer against BSA solution resulted in an observed equilibrium between unbound and total drug concentrations after approximately 120 min. Therefore, the values of K_1, K_2, and K_b in the general model (Scheme 1) were designated as zero and Eqns. 1–4 modified appropriately to reflect a stable drug. Solution of these equations gave an excellent fit (Fig. 1) to the experimentally measured concentrations (C_1 and C_T) and a prediction of the unmeasured concentrations (C_2 and C_b); the parameters are reported in Table 1. The values of K_A and $(C_b)_{max}$ were 1.81 ± 0.07 mM^{-1} (mean ± S.D.) and 3.00 ± 0.20 mM, respectively, with their product having a value of 5.41 ± 0.19. It is apparent from the

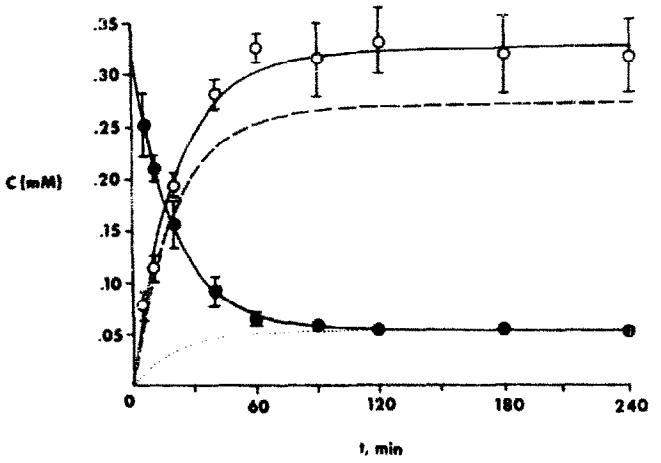


Fig. 1. Dynamic plot of MeDDC concentrations, C, during dialysis against BSA solution. Key: ●———●, measured concentrations (C_1) and computer-fit of unbound drug in first half-cell; ○———○, measured concentrations (C_T) and computer-fit of total drug in second half-cell; — — —, predicted concentrations (C_b) of bound drug in second half-cell; · · · · · ·, predicted concentration (C_2) of unbound drug in second half-cell. The S.D. of the measured concentrations, where visually significant, are shown.

inequality of K_{12} and K_{21} (Table I) that movement of fluid across the dialysis membrane to the protein side resulted in mean half-cell volumes (V_1 and V_2) that differed from their initial experimental equality, an observation common in equilibrium dialysis studies also.

Equilibrium dialysis of MeDDC

The dialysis of MeDDC in buffer against BSA solution gave observed equilibrium data well described (Fig. 2) by the quadratic equation (Eqn. 6). Over 5 separate series of experiments and an 80-fold range of initial unbound MeDDC concentrations, the

TABLE I

MODEL PARAMETERS[a] DETERMINED FROM EXPERIMENTALLY MEASURED DATA FOR THE DYNAMIC DIALYSIS OF MeDDC AGAINST BSA SOLUTION

	Experiment			Mean	(%CV)[b]
	1	2	3		
K_{12} (min^{-1})	0.0533	0.0467	0.0507	0.0503	(6.5)
K_{21} (min^{-1})	0.0415	0.0378	0.0380	0.0391	(5.3)
K_{b2} (min^{-1})	2.10	2.07	2.04	2.07	(1.5)
K_a (mM^{-1})	1.89	1.75	1.79	1.81	(3.9)
$(C_b)_{max}$ (mM)	2.76	3.12	3.12	3.00	(6.8)
$K_a(C_b)_{max}$	5.21	5.44	5.59	5.41	(3.5)

[a] Assuming $K_b = K_2 = K_1 = 0$ (see Scheme 1).
[b] Percentage coefficient of variation.

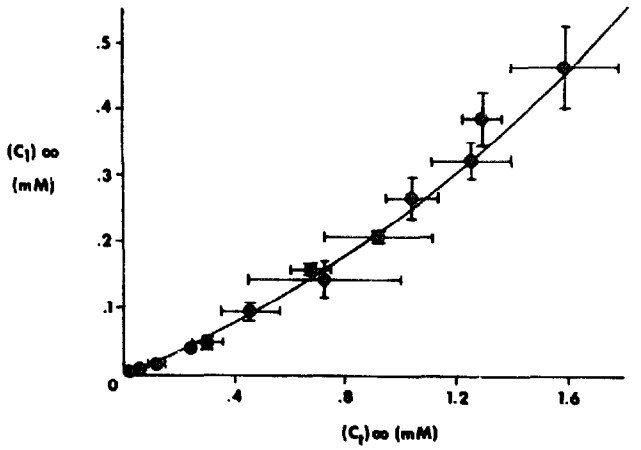


Fig. 2. Equilibrium dialysis binding plot of measured unbound, $(C_I)_\infty$, and total, $(C_T)_\infty$, drug concentrations of MeDDC against BSA solution. The S.D. for both measured concentrations, where visually significant, are shown.

values of K_a and $(C_b)_{max}$ were 1.95 ± 0.24 mM^{-1} and 2.58 ± 0.34 mM, respectively, with their product having a value of 5.02 ± 0.80 (Table 2). These parameters values and their product were not significantly different (unpaired t-test, $2P < 0.05$) from those obtained by the dynamic dialysis procedure (Table 1).

Dynamic dialysis of NaDDC

The dialysis of NaDDC in buffer against BSA solution did not result in an observed equilibrium between unbound and total drug concentrations. Therefore, these data were evaluated according to various potential dynamic models (Scheme 1) reflecting an unstable drug. When Eqns. 1–4 were modified assuming no degradation of bound drug ($K_b = 0$) and the intuitively reasonable assumption that the

TABLE 2

PARAMETERS DETERMINED FROM EXPERIMENTALLY MEASURED DATA FOR THE EQUILIBRIUM DIALYSIS OF MeDDC AGAINST BSA SOLUTION

Experimental series	$(C_I)_0$ [a] (mM)	K_a (mM^{-1})	$(C_b)_{max}$ (mM)	$K_A(C_b)_{max}$
1	0.032–1.594	1.89	2.83	5.35
2	0.398–1.594	1.95	2.83	5.52
3	0.025–1.594	2.16	2.71	5.85
4	0.398–1.992	1.58	2.48	3.92
5	0.199–1.992	2.19	2.04	4.45
Mean	–	1.95	2.58	5.02
(%CV) [b]	–	(12.5)	(13.0)	(16.0)

[a] Range of initial unbound MeDDC concentrations.

[b] Percentage coefficient of variation.

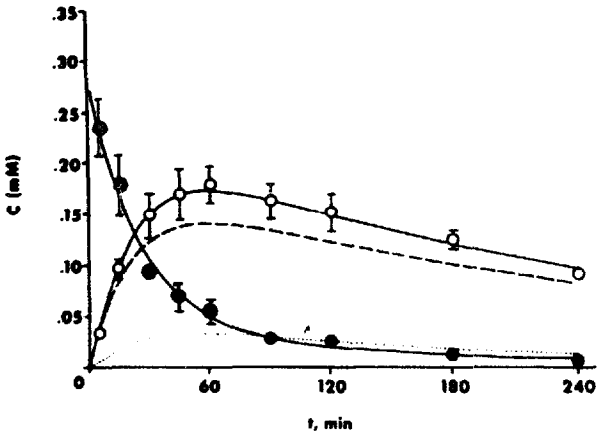


Fig. 3. Dynamic plot of NaDDC concentrations, C, during dialysis against BSA solution. Key: see Fig. 1.

degradation rate constants of unbound drug were equal in both half-cells ($K_1 = K_2$), an excellent fit to the experimentally measured concentrations (C_1 and C_T) was obtained (Fig. 3) together with a prediction of the unmeasured concentrations (C_2 and C_b). The parameters reported (Table 3) were not significantly different (unpaired t-test, $2P < 0.05$) from those obtained from an alternate model in which K_1 and K_2 had no constraint of equality. The values of K_A and $(C_b)_{max}$ were 37.0 ± 3.6 mM^{-1} and 0.253 ± 0.040 mM, respectively, with their product having a value of 9.85 ± 0.97 (Table 3).

Two other possible models were evaluated, both assuming the degradation of bound drug could occur. These models, one assuming K_1 and K_2 to be equal and the

TABLE 3

MODEL PARAMETERS [a] DETERMINED FROM EXPERIMENTALLY MEASURED DATA FOR THE DYNAMIC DIALYSIS OF NaDDC AGAINST BSA SOLUTION

	Experiment			Mean	(%CV) [b]
	1	2	3		
K_{12} (min^{-1})	0.0276	0.0277	0.0333	0.0295	(11.0)
K_{21} (min^{-1})	0.0172	0.0249	0.0202	0.0208	(18.8)
K_1 (min^{-1})	0.0179	0.0130	0.0149	0.0153 [c]	(16.4)
K_{b2} (min^{-1})	1.42	1.47	1.32	1.40	(5.5)
K_a (mM^{-1})	33.0	37.9	40.1	37.0	(9.7)
$(C_b)_{max}$ (mM)	0.299	0.234	0.225	0.253	(15.9)
$K_A(C_b)_{max}$	9.87	8.87	10.81	9.85	(9.9)

[a] Assuming $K_b = 0$ and $K_2 = K_1$ (see Scheme 1).

[b] Percentage coefficient of variation.

[c] When K_1 and K_2 had no constraint of equality, the mean parameter values were $K_1 = 0.0151$ min^{-1}, $K_2 = 0.0151$ min^{-1}, $K_{12} = 0.0312$ min^{-1}, $K_{21} = 0.0178$ min^{-1}, $K_{b2} = 1.53$ min^{-1}.

other removing this constraint, did not provide an adequate description of the observed data.

Discussion

To these authors' knowledge, no studies on the binding of unstable drugs with plasma proteins have been reported previously. Neither the equilibrium nor the traditional dynamic dialysis procedures can be used when degradation rate constants are included in the binding model.

By definition, equilibrium drug concentrations are required as a prelude to the determination of binding parameters from equilibrium dialysis experiments. The continuous degradation of an unstable drug perturbs the desired equilibrium, rendering the data unmanageable.

Traditional dynamic dialysis procedures require the assumption of the conservation of drug mass and the use of a diffusion rate constant determined from a separate experiment in the absence of protein (Meyer and Guttman, 1968, 1970a, 1970b; Crooks and Brown, 1973; El-Rashidy and Niazi, 1978). The assumption, necessary to calculate total and bound drug concentrations within a dialysis compartment, is violated with an unstable drug. Mathematically, the diffusion rate constant of a drug exiting from a compartment cannot be determined from drug concentrations outside the compartment if the drug within is being reduced by degradation in addition to diffusion (Notari et al., 1972). Hence this rate constant, necessary to calculate unbound drug concentrations within a dialysis compartment, cannot be determined with an unstable drug.

The procedures described herein offer two advantages over traditional dynamic dialysis procedures which permit the determination of binding constants for unstable drugs. The use of the rapid equilibrium dialysis system permits the experimental measurement of total drug concentrations (C_T) within the dialysis compartment in addition to those of unbound drug (C_1) outside the compartment. Secondly, the numerical integration of differential equations describing the binding model permits the determination of degradation and diffusion rate constants without the need for prior experimentation.

Statistically, it is desirable to use experimentally measured data as direct inputs into computer programs to determine whether a given model might describe drug–macromolecule binding (Whitlam and Brown, 1980). This consideration has been made possible by the use of NONLIN in its differential equation mode for dynamic dialysis and by a quadratic equation (Eqn. 6) for equilibrium dialysis. Therefore, C_1 and C_T are used directly, in contrast to other procedures for both dynamic and equilibrium dialysis wherein the input data are not those experimentally measured but arise from prior manipulations of the measured data.

The values of K_A and $(C_b)_{max}$ were not significantly different for a stable drug whether determined from dynamic or equilibrium dialysis data (Tables 1 and 2), indicating that the simple binding model affords an adequate description of the drug–macromolecule interaction and that the two procedures are equivalent. Sellers

and Koch-Weser (1970) have commented that K_a and $(C_b)_{max}$ may have little significance as independent binding parameters of clinical value; instead, the product of the two parameters may have greater descriptive merit. The appearance of $(C_b)_{max}$ in the model equations (Eqn. 2 and 3) is only in conjunction with K_{2b}, a rate constant inherent in the definition of K_a (Eqn. 5). The interdependence of K_a and $(C_b)_{max}$ may be visualized by substituting for K_{2b} in equations 2 and 3, yielding the product $K_a(C_b)_{max}$, which is effectively a single parameter. As anticipated, the values of this product parameter were not significantly different (Tables 1 and 2) regardless of the dialysis procedure.

Because K_a is by definition (Eqn. 5), a ratio of two rate constants and because K_a and $(C_b)_{max}$ cannot be obtained entirely independently, the computer-fitting procedures could lead to unconstrained individual values for these two parameters. To preclude this possibility, the mean experimentally measured data following dynamic dialysis were fitted ($r^2 > 0.99$) initially to empirical polyexponential equations (Kanfer and Cooper, 1976). The ideal data generated from this fit were then fitted to the model equations (Eqns. 1–4) using initial parameter estimates with wide (±100%) limits. The final values of K_a and $(C_b)_{max}$ obtained from the ideal fit were then the initial parameter estimates when the measured data for individual experiments were subsequently fitted; these two parameters were constrained within narrower (±20%) limits.

Strömme (1965) studied the interaction of [^{35}S]labelled DDC with serum proteins by a gel filtration technique using a dilution of human serum yielding 3.6% proteins. In a range of DDC concentrations (0.01–0.4 mM) which encompass those used in this study, he found that DDC was loosely adsorbed onto serum proteins. The present study confirms an interaction between DDC and BSA, while also indicating that DDC is unstable in the presence of BSA, an observation not possible using an assay based on radiolabelled material (Strömme, 1965).

Acknowledgement

The assistance of Barbara Hildebrand is gratefully appreciated.

References

Boxenbaum, H.G., Riegelman, S. and Elashoff, R.M., Statistical estimations in pharmacokinetics. J. Pharmacokin. Biopharm., 2 (1974) 123–148.

Chorbajian, T. and Coull, J., Digital analog simulation of pharmacokinetic models II. CSMP. Drug Intell. Clin. Pharm., 5 (1971) 290–297.

Cobby, J., Mayersohn, M. and Selliah, S., The rapid reduction of disulfiram in blood and plasma. J. Pharmacol. Exp. Ther., 202 (1977) 724–731.

Crooks, M.J. and Brown, K.F., Binding of sulfonylureas to serum albumin. J. Pharm. Sci., 62 (1973) 1904–1907.

El-Rashidy, R. and Niazi, S., Binding of butylated hydroxyanisole to human albumin using a novel dynamic method. J. Pharm. Sci., 67 (1978) 967–970.

Endrenyi, L. and Kwong, F.H.F., Design and analysis of hyperbolic kinetic and binding experiments. In Analysis and Simulation, North-Holland, Amsterdam, 1972, p. 219.

Goldstein, A., The interaction of drugs and plasma proteins. Pharmacol. Rev., 1 (1949) 102–165.

Kanfer, I. and Cooper, D.R., The use of empirical equations to describe dynamic dialysis 'escape curves' in drug–macromolecule binding measurements. J. Pharm. Pharmacol., 28 (1976) 58–60.

Metzler, C.M., Elfring, G.L. and McEwan, A.J., A package of computer programs for pharmacokinetic modeling. Biometrics, 30 (1974) 562.

Meyer, M.C. and Guttman, D.E., Novel method for studying protein binding. J. Pharm. Sci., 57 (1968) 1627–1629.

Meyer, M.C. and Guttman, D.E., Dynamic dialysis as a method for studying protein binding I: Factors affecting the kinetics of dialysis through a cellophane membrane. J. Pharm. Sci., 59 (1970a) 33–38.

Meyer, M.C. and Guttman, D.E., Dynamic dialysis as a method for studying protein binding II: Evaluation of the method with a number of binding systems. J. Pharm. Sci., 59 (1970b) 39–48.

Notari, R.E., DeYoung, J.L. and Reuning, R.H., Effect of parallel first-order drug loss from site of administration on calculated values for absorption rate constants. J. Pharm. Sci., 61 (1972) 135–138.

Pedersen, P.V., Crooks, M.J. and Brown, K.F., Method of obtaining drug-macromolecule binding parameters directly from dynamic dialysis data. J. Pharm. Sci., 66 (1977) 1458–1461.

Rosenthal, H.E., A graphic method for the determination and presentation of binding parameters in a complex system. Anal. biochem., 20 (1967) 525–532.

Sellers, E.M. and Koch-Weser, J., Displacement of warfarin from human albumin by diazoxide and ethacrynic, mefenamic and nalidixic acids. Clin. Pharm. Ther., 11 (1970) 524–529.

Strömme, J.H., Interactions of disulfiram and diethyldithiocarbamate with serum proteins studied by means of a gel-filtration technique. Biochem. Pharmacol., 14 (1965) 381–391.

Whitlam, J.B. and Brown, K.F., Computation of multiclass drug–protein binding parameters. Int. J. Pharm., 5 (1980) 49–58.