Parabolic Equation Relating Free and Total Drug Concentrations in Cases of Nonlinear Plasma Protein Binding

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Abstract □ In many cases of nonlinear drug–protein binding, investigators have fitted data to the classical Langmuir model with two classes of binding sites involving four parameters. For human serum albumin binding of tolmetin and salicylate, a simple two-parameter parabolic equation relating free to total concentrations fitted the binding data better. Thus, the free fraction corresponding to the serum or plasma drug concentration in the blood sample taken from a subject can be estimated directly from the parabolic model. This is not the case when the Langmuir model is used to describe nonlinear binding data since the positive root of a cubic equation must be obtained to estimate the free from the total concentration. The direct relationship between free and total drug concentrations would be useful in many clinical situations.

Keyphrases □ Drug–protein binding—Langmuir and parabolic models compared, parameter estimation, equilibrium dialysis of salicylic acid and tolmetin with human serum albumin □ Equilibrium dialysis—drug–protein binding, Langmuir and parabolic models compared for free drug concentration estimation □ Models, mathematical—Langmuir and parabolic models compared for free drug concentration estimation, equilibrium dialysis

When equilibrium dialysis is used to measure nonlinear drug–protein binding, the free fraction of drug obtained from the equilibrium concentrations in the buffer and plasma compartments of the dialysis apparatus is always less than that in the original plasma or serum sample. The free fraction corresponding to the initial total drug concentration must be estimated from a function describing the binding over a range of equilibrium drug concentrations. If only one serum or plasma sample containing drug is available, one cannot calculate the correct free and bound drug concentrations in the original plasma or serum sample (1).

BACKGROUND

Free fractions corresponding to the initial plasma or serum total drug concentrations can be estimated by solving a quadratic ($k = 1$) or cubic ($k = 2$) equation (1) when nonlinear drug–protein binding data, obtained using equilibrium dialysis, are fitted to the classical Langmuir model (2):

$$C_b = \sum_{i=1}^{k} \frac{n_i C_p C_f}{Kd_i + C_f} \qquad \text{(Eq. 1)}$$

where C_b is the protein-bound drug concentration at equilibrium, C_p is the protein concentration, C_f is the free (unbound) drug concentration at equilibrium, Kd_i is the dissociation constant for the ith class of binding sites, n_i is the number of identical independent binding sites in a class, and k is the number of classes. The disadvantage of this technique is that the free drug concentration corresponding to a total drug concentration cannot be estimated directly.

With tolmetin–human serum albumin ($k = 2$ or 3) (3) or salicylate–human serum albumin ($k = 2$) (4) binding data from equilibrium dialysis experiments, it was found empirically that a plot of the free fraction *versus* total equilibrium drug concentration was linear:

$$\frac{C_f}{C_t} = a + bC_t \qquad \text{(Eq. 2)}$$

where C_t is the total drug concentration and a and b are the intercept and slope of the straight line, respectively. The constant a is dimensionless, while b has dimensions of μM^{-1}. This equation can be rearranged to give a simple parabolic relationship that passes through the origin and predicts zero drug bound at zero total drug concentration:

$$C_f = aC_t + bC_t^2 \qquad \text{(Eq. 3)}$$

In many cases, the binding data were fitted much better to Eq. 3 than to the classical Langmuir model (Eq. 1 with $k = 2$). This parabolic relationship is useful in many clinical situations since C_f can be estimated directly for a given C_t value. The concentration range was 0–1200 μM for tolmetin and 0–2400 μM for salicylic acid, and the parameters of Eq. 3 (Tables I and II) are valid only in these ranges.

EXPERIMENTAL

All drug–protein binding assays were performed using equilibrium dialysis as described previously (5). Equilibration was attained when samples were placed in an oscillating water bath (at 37°) for 18 hr. Drug concentrations were estimated by methods published previously (2). The unlabeled[1] and [^{14}C]salicylic acid[2] were obtained commercially. Human serum albumin[3] solution (25%) was diluted with water to 4% and used in the binding studies with various buffers, while fatty acid-free human serum albumin[4] was used for the binding studies at various temperatures. The program NONLIN (6) was used for fitting the binding data to Eq. 1 ($k = 2$) or 3.

RESULTS

The estimated parameters and their coefficients of variation for the two models (Eqs. 1 and 3) for salicylic acid–human serum albumin binding data are shown in Table I; coefficients of variation were obtained as indicated. For the Langmuir model, the estimated parameters, $P(i)$, in terms of previously defined constants were $P(1) = n_1C_p$, $P(2) = Kd_1$, $P(3) = n_2C_p$, and $P(4) = Kd_2$. Here, n_1C_p and n_2C_p are the binding capacities at 4% human serum albumin. With salicylic acid, fits to the parabolic relationship were much better than to the Langmuir model with two classes of binding sites ($k = 2$), as reflected by the much smaller coefficients of variation for the parabolic model than for the Langmuir model. However, fits to both models were excellent. Measures of fit[5], corr and r^2, for fitting of data to the parabolic model ranged from both being 0.997 to both being 1.00. The corr and r^2 values ranged from 0.998 and 0.999, respectively, in 0.05 M KH_2PO_4 buffer to 0.995 and 0.996, respectively, in triethanolamine buffer when data were fitted to the Langmuir model with $k = 2$.

The estimated parameters and their coefficients of variation for the two models (Eqs. 1 and 3) for the tolmetin–human serum albumin binding data are shown in Table II. The computer fitting of data obtained using imidazole buffer provided no standard deviations for two of the estimated parameters. Again, fits to the parabolic model (as reflected by the lower coefficients of variation) were better than to the Langmuir model but were not as dramatically different as the fits with the salicylic acid data. However, fits to both models were extremely good. Measures of fit, corr and r^2, for the parabolic model fitting ranged from 0.992 and

[1] Mallinckrodt.
[2] Prepared by California Bionuclear Corp.
[3] Michigan State Department of Health.
[4] Sigma Chemical Co.
[5] Corr is the correlation coefficient for the regression of expected *versus* observed concentrations, and r^2 is the coefficient of determination.

0022-3549/81/0700-0802$01.00/0

Table I—Estimated Parameters and Their Coefficients of Variation for Salicylic Acid

Buffer	Langmuir Model				Parabolic Model	
	n_1C_p, μM	Kd_1, μM	n_2C_p, μM	Kd_2, μM	a	b, μM^{-1}
Tromethanine	1247[a]	372	2,276	5,509	0.233	8.98×10^{-5}
	(200)[b]	(137)	(911)	(1578)	(2.2)	(3.7)
0.1 *M* KH_2PO_4	549	177	2,493	2,256	0.215	1.05×10^{-4}
	(67.0)	(50.2)	(42.8)	(110)	(3.2)	(4.2)
0.05 *M* KH_2PO_4	753	281	1,879	1,546	0.192	1.05×10^{-4}
	(194)	(106)	(27.3)	(195)	(2.2)	(2.4)
"House"[c]	1054	244	5,452	9,580	0.175	1.07×10^{-4}
	(30.6)	(28.5)	(187)	(253)	(3.3)	(3.3)
0.05 *M* KH_2PO_4	1086	308	16,610	40,925	0.218	1.03×10^{-4}
+ 0.05 *M* NaCl	(16.0)	(15.1)	(726)	(766)	(2.6)	(3.4)
Imidazole	890	297	—	—	0.244	1.04×10^{-4}
	(4.6)	(9.7)			(2.7)	(4.0)
Triethanolamine	684	308	2,744	3,615	0.271	9.43×10^{-5}
	(134)	(86.6)	(152)	(321)	(3.0)	(5.5)
0.1 *M* NaH_2PO_4	541	180	4,779	3,859	0.222	7.78×10^{-5}
	(61.7)	(48.9)	(83.3)	(130)	(3.3)	(5.5)

[a] Estimated parameter. [b] Coefficient of variation (%) = (standard deviation of estimate/estimate) × 100. [c] Consists of 0.093 *M* phosphate (0.696 g of dibasic potassium phosphate, 0.138 g of dibasic sodium phosphate monohydrate, and 2.25 g of sodium chloride and brought to volume of 500 ml with water).

Table II—Estimated Parameters and Their Coefficients of Variation for Tolmetin

Buffer	Langmuir Model				Parabolic Model	
	n_1C_p, μM	Kd_1, μM	n_2C_p, μM	Kd_2, μM	a	b, μM^{-1}
Tromethamine	301[a]	10.2	1077	59.8	1.27×10^{-2}	7.09×10^{-5}
	(291)[b]	(195)	(62.2)	(140)	(22.7)	(5.2)
0.1 *M* KH_2PO_4	140	3.94	1903	132	2.19×10^{-2}	6.96×10^{-5}
	(60.1)	(75.6)	(12.7)	(36.0)	(16.4)	(6.5)
0.05 *M* KH_2PO_4	132	4.12	2083	163	2.82×10^{-2}	6.97×10^{-5}
	(29.3)	(37.9)	(7.8)	(10.9)	(7.7)	(3.9)
"House"[c]	156	14.2	2109	216	4.75×10^{-2}	6.87×10^{-5}
	(162)	(142)	(24.1)	(77.6)	(9.3)	(7.8)
0.05 *M* KH_2PO_4	435	16.1	2082	300	2.85×10^{-2}	7.06×10^{-5}
+ 0.05 *M* NaCl	(62.8)	(53.3)	(60.0)	(134)	(7.1)	(3.6)
Imidazole	330	8.89	1816	232	1.50×10^{-2}	9.52×10^{-5}
	(13.9)	(14.6)	(10.3)	(25.0)	(8.5)	(1.9)
Triethanolamine	212	5.79	1650	142	1.64×10^{-2}	8.91×10^{-5}
	(22.5)	(25.5)	(5.8)	(19.5)	(10.8)	(2.7)
0.1 *M* NaH_2PO_4	332	4.74	1536	127	1.8×10^{-3}	8.19×10^{-5}
	(21.1)	(24.4)	(10.4)	(33.4)	(109)	(3.4)

[a] Estimated parameter. [b] Coefficient of variation (%) = (standard deviation of estimate/estimate) × 100. [c] See footnote to Table I.

0.990, respectively, in 0.1 *M* KH_2PO_4 to both being 0.999 in imidazole. The corr and r^2 values for fittings to the Langmuir model ranged from 0.993 and 0.992, respectively, in tromethamine to 0.999 and 1.00, respectively, in imidazole.

The binding of salicylic acid to two types of albumin was investigated at six temperatures, from 4 to 58 or 59°, with the higher temperature being different for the two types of albumin. These data were computer fitted to Eq. 3. With the salicylic acid–human serum albumin binding data, parameter b of Eq. 3 remained constant with a change in temperature when the highest temperature was excluded. The means and standard deviations of b were 1.06 (0.026) and 0.895 (0.047) for 4% albumin and 4% fatty acid-free albumin, respectively. The parameter a increased with an increase in temperature, and plots of a *versus* temperature exhibited convex curvature (not shown).

To compare estimated parameters obtained by the Langmuir and parabolic models, the models were manipulated to obtain limiting values for the ratios of bound to free drug concentrations as the total or free drug concentration approached zero (infinite dilution). The Langmuir model can be manipulated to obtain the limiting relationship (7):

$$\frac{C_b}{C_f} = \frac{P(1)}{P(2)} + \frac{P(3)}{P(4)} \qquad \text{(Eq. 4)}$$

For the parabolic model, the limiting relationship is:

$$\frac{C_b}{C_f} = \frac{1-a}{a} \qquad \text{(Eq. 5)}$$

The limiting values of C_b/C_f obtained from Eqs. 4 and 5 for the Langmuir and parabolic models, respectively, and for the binding of salicylic acid to human serum albumin using eight buffers are listed in Table III. The limiting values for the Langmuir and parabolic models were comparable in each case. Limiting values for the tolmetin–human serum albumin binding data (not shown) also were similar.

Table III—Limiting Values of Ratio of Bound to Free Drug Concentrations for Salicylic Acid

Buffer	Langmuir Prediction[a]	Parabolic Prediction[b]
Tromethamine	3.77	3.29
0.1 *M* KH_2PO_4	4.21	3.65
0.05 *M* KH_2PO_4	3.90	4.21
"House"[c]	4.89	4.71
0.05 *M* KH_2PO_4 + 0.05 *M* NaCl	3.93	3.59
Imidazole	3.46	3.10
Triethanolamine	2.98	2.69
0.1 *M* NaH_2PO_4	4.24	3.50

[a] See Eq. 4. [b] See Eq. 5. [c] See footnote to Table I.

DISCUSSION

Many factors can cause variability in drug–protein binding from subject to subject or patient to patient. Variables include age (8, 9), drug therapy (10, 11), prolonged bedrest (12), cigarette smoking (13), and disease (14, 15). Circadian fluctuations in drug–protein binding also can occur (16). Because of the individual differences in drug–protein binding, the binding characteristics for each subject or patient undergoing pharmacokinetic evaluation should be determined rather than using pooled plasma or serum (17).

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ACKNOWLEDGMENTS

Supported in part by McNeil Laboratories and in part by a Lilly Endowment Fellowship in Pharmacy. McNeil Laboratories kindly supplied both unlabeled tolmetin and [^{14}C]tolmetin.

High-Pressure Liquid Chromatographic Assay of Theophylline, Ephedrine Hydrochloride, and Phenobarbital Tablets

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Received October 6, 1980, from the *Analytical Research Laboratories, Warner-Lambert Pharmaceutical Research, Morris Plains, NJ 07950.* Accepted for publication December 1, 1980.

Abstract □ An innovative high-pressure liquid chromatographic method is described in which theophylline, ephedrine hydrochloride (measured as benzaldehyde), and phenobarbital are determined simultaneously with butabarbital as the internal standard. Chromatographic conditions were selected to afford a pH sufficient for rapid oxidation of ephedrine and relatively high UV absorbance for the barbiturates and a detection wavelength near the maximum for benzaldehyde and the barbiturates and the minimum for theophylline. Chromatograms show peaks from iodate, theophylline, phenobarbital, butabarbital, and benzaldehyde, in order of increasing retention time, all within the dynamic range of a conventional recorder. Procedures are provided for the assay of conventional and sustained-action tablet formulations.

Keyphrases □ High-pressure liquid chromatography—simultaneous determination of theophylline, ephedrine hydrochloride, and phenobarbital, conventional and sustained-action tablet formulations □ Theophylline—simultaneous determination with ephedrine hydrochloride and phenobarbital by high-pressure liquid chromatography, conventional and sustained-action tablet formulations □ Ephedrine hydrochloride—simultaneous determination with theophylline and phenobarbital by high-pressure liquid chromatography, conventional and sustained-action tablet formulations □ Phenobarbital—simultaneous determination with theophylline and ephedrine hydrochloride by high-pressure liquid chromatography, conventional and sustained-action tablet formulations

The theophylline, ephedrine hydrochloride, and phenobarbital tablet is recognized in USP XX (1), which provides a laborious assay in which the drug components are separated by chromatography on two partition columns and solvent extraction and then determined by UV spectrometry. Elefant *et al.* (2) described a GLC assay for this formulation, and Schultz and Paveenbampen (3) reported one for a similar suspension dosage form. Both methods require derivative formation, and neither has proved satisfactory in speed and convenience.

High-pressure liquid chromatography (HPLC) affords easy separation of the three active components of the formulations; however, the enormous differences in their relative amounts and in their UV absorption maxima and absorptivities make their simultaneous determination by HPLC with UV detection a challenging analytical problem. This report describes an innovative solution to this problem, affording peaks within one dynamic range span of a recorder.

BACKGROUND

Conventional tablets, the USP formulation, declare 130 mg of hydrous theophylline, 24 mg of ephedrine hydrochloride, and 8 mg of phenobarbital; similar proportions are in sustained-action tablets. Hydrous theophylline shows a UV maximum at ~271 nm with an absorptivity (liters per gram centimeter) of ~48. Its UV spectrum is not affected greatly by pH. In acidic solution, ephedrine and phenobarbital have weak UV spectra due to their benzene ring structure, with maxima at ~256 nm and absorptivities of ~1.

Penner[1] developed a normal-phase HPLC method using detection at 254 nm and an attenuation change between elution of the phenobarbital and theophylline peaks to keep the latter on the recorder scale. Suraski and DiPede[2] developed this method further, using a separate injection of greater dilution to keep the theophylline peak within the recorder dynamic range. They suggested that use of a computing integrator could allow for one injection of all three drug components. In this method, ephedrine and phenobarbital are determined simultaneously and theophylline is determined separately, using the same chromatographic system. It is also possible to determine phenobarbital and theophylline together and ephedrine separately. The UV maximum of phenobarbital can be shifted to ~240 nm with an absorptivity of ~43 by raising the pH to 9–11, where the predominant UV chromophore is the monoanion of the ureide ring; however, a high pH is incompatible with HPLC column

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