Improved Procedure for the Determination of Protein Binding by Conventional Equilibrium Dialysis

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Abstract ◻ The binding of drugs to plasma proteins has been studied extensively using a variety of methods, including equilibrium dialysis. Published information on controls used in these studies is frequently inadequate; in other cases, there are deficiencies in the experimental design for the controls. A method is described that eliminates many of the problems associated with artifactual errors in dialysis studies. Multiple replicated controls are performed at the same time as the test, under identical conditions. The controls are used to correct for concentration-dependent binding of drug to the membrane or other equipment. The method was used to determine the binding of sulfadimethoxine to CF-IV-1 α-globulin at therapeutic concentrations. The level of binding was low (9–13%), but the stringent control technique permitted statistical analysis which showed each mean test value to be significantly different from its corresponding control. Furthermore, there was a linear relationship between the control-corrected percentage binding values and total drug concentration, whereas there was no correlation between total drug concentration and the uncorrected percentage binding values.

Keyphrases ◻ Equilibrium dialysis—measurement of low-level protein binding, elimination of artifactual error, multiple replicated controls ◻ Protein binding—low levels measured by equilibrium dialysis, use of multiple replicated controls to eliminate artifactual error, correction for concentration-dependent binding ◻ Sulfadimethoxine—binding to CF-IV-1 α-globulin at therapeutic doses, equilibrium dialysis with multiple replicated controls

The binding of drugs and xenobiotic agents to blood proteins is an important parameter in pharmacokinetic studies, since the extent and affinity of this binding influences distribution of the compound in the body (1, 2). The concentration of unbound drug in the plasma can affect rates of metabolism and elimination (3). Numerous qualitative and quantitative techniques have been used to study the interaction between drugs and macromolecules. Equilibrium dialysis is the classical procedure and remains the most popular method (4). In the conventional method, the drug–protein mixture is contained within a sealed tube of semipermeable membrane, with protein-free buffer solution outside the membrane. At equilibrium the concentration of drug in the dialysate equals that of the unbound drug inside the dialysis tube. If a suitable range of drug concentrations is used, it is also possible to determine the extent of binding, the binding constants, and the number of binding sites on an isolated pure protein (5). A comprehensive study also should include an appropriate range of protein concentrations to detect any differences in binding due to changes in protein concentration.

Good controls are essential in any dialysis study since many drugs bind to the semipermeable membranes and apparatus (6), reducing the apparent concentration of free drug. Other factors influencing the extent of binding measured include temperature (7, 8), pH (9), and variation in the physicochemical properties of the protein (10). Additional influences are those parameters inherent in the design of the experiment, such as the nature and concentration of proteins present (11) and the drug used. Artifactual errors in the determination of protein binding can be minimized through adequate controls and consideration of experimental design. Literature reports of equilibrium dialysis studies for the determination of free drug concentrations are frequently deficient in details concerning the controls used.

All methods for determination of free drug concentrations in plasma, serum, or solutions of macromolecules at normal biological concentrations have some inherent problems that may influence the results (4). The most commonly used techniques involve separation of a fraction of the unbound drug from the bound component. One of the advantages of dialysis is that the free component retains access to the bound component when equilibrium is reached. Concentrations of free and bound drug are not affected to the same extent as in ultrafiltration, where a portion of the free drug is removed from the system. Ultrafiltration also results in some concentration of the protein solution, which may influence the binding (12).

Inadequacy of controls is a major contributor to the variations found in binding studies reported in the literature. Nonspecific adsorption to the semipermeable membrane is a problem in the dialysis of many compounds (13). The extent of adsorption to membranes is frequently <10% of the total drug present, but may exceed 50% (6) and vary according to the method of preparation and storage of the membrane (14). It can occur with all types of membrane and is a source of potential error in free drug determination. Nevertheless, a commonly used technique is to run limited preliminary controls which are used to generate a correction factor to be applied to all subsequent results obtained with solutions containing protein (15). In many cases, the binding to apparatus and membranes is considered negligible. But variations in these losses may become highly significant when working at low concentrations such as those encountered with many drugs at therapeutic doses. Furthermore, the variability in results obtained with conventional dialysis procedures often

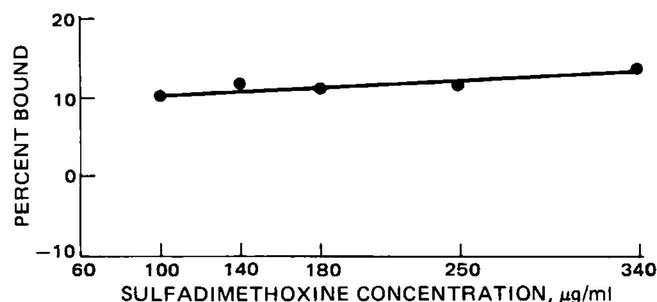

Figure 1—*Plot of percentage binding* versus *concentration of sulfadimethoxine. Percentage binding values are calculated directly from the optical density mean values shown in Table I.*

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Table I—Percentage Protein Binding of Sulfadimethoxine Calculated Directly from the Mean Optical Density Values (First Method) [a]

Initial Concentration, μg/ml	Mean Optical Density: Control (Y_c)	Test (Y_t)	Protein Binding,[b] %
100	0.3783 ± 0.0025	0.3394 ± 0.0041	10.28 ± 1.09
140	0.5338 ± 0.0066	0.4710 ± 0.0082	11.76 ± 1.54
180	0.7170 ± 0.0129	0.6378 ± 0.0096	11.05 ± 1.33
250	1.049 ± 0.0051	0.9276 ± 0.0084	11.57 ± 0.80
340	1.452 ± 0.0080	1.253 ± 0.0094	13.71 ± 0.65
	$r = 0.9997$	$r = 0.9994$	$r = 0.8811$

[a] Expressed as mean ± SE; $n = 5$. [b] $[(Y_c - Y_t)/Y_c] \times 100$.

precludes accurate determination of binding for drugs that show a low percentage bound.

In the present study, an equilibrium dialysis method is described which employs calibration and control procedures that minimize artifactual errors and permit statistical evaluation of control, as well as test data. The method permits compensation to be made for concentration-dependent binding to apparatus and membranes and allows for variations in conditions in individual experiments. Sulfadimethoxine and Cohn fraction (CF) IV-1 α-globulin were selected as a model drug–protein system that gives a low percentage binding. The concentration of the protein was constant at the normal blood level of 0.81% w/v, with the drug present in amounts equivalent to those found in therapeutic application.

EXPERIMENTAL

Equilibrium dialysis was performed using 20-cm strips of dialysis tubing[1] (1-cm diameter, 4.8-nm pore diameter) with a molecular weight cut-off not greater than 12,000. These membranes were immersed in boiling water and stirred for 2 hr as the water cooled. The tubing was then stirred with 70% methanol for 30 min, stored in 50% methanol overnight, rinsed with distilled water, and soaked in phosphate buffer (pH 7.4) for 2–3 hr prior to use. The membranes were used immediately after preparation. The tubing was tied with a double knot at one end and filled with 2 ml of protein solution (CF-IV-1 α-globulin[2] in phosphate buffer, 0.81% w/v; pH 7.4) containing sulfadimethoxine. The drug concentration range was 100–340 μg/ml. The tubing was sealed and placed in a glass culture tube (16 × 125 mm) with a polytetrafluoroethylene cap. Four milliliters of phosphate buffer (pH 7.4) was placed in each culture tube, and the solutions were dialyzed for 24 hr at 37°[3]. The tubes were rotated 12 times per minute using a rotary mixer[4]. The protein solution and dialysate were assayed for sulfadimethoxine using the Bratton–Marshall method (16). Absorbance was measured at 420 nm; five replicates of each test concentration were dialyzed. A set of five controls was prepared for each concentration studied and placed in the rotary mixer with the test samples. Controls were identical to the test samples, except the solution inside the dialysis membrane contained no protein. Glassware and buffer were sterilized by autoclave[5] prior to use. Preparation and transfer of solutions were carried out under aseptic conditions in a laminar-airflow hood[6]. The dialysate was tested for the absence of protein both visually (absence of frothing) and by means of a semiquantitative colorimetric indicator[7].

RESULTS AND DISCUSSION

The mean optical density values ($n = 5$) for control and test solutions are shown in Table I. Each set of controls is an exact replicate of the corresponding set of test solutions, except that the dialysis bags in the control tubes contained no protein. The controls therefore take into account any spurious loss of drug due to adsorption to the tubes, cap liners, membrane, transfer pipets, or any other equipment. Furthermore, the controls obviate any day-to-day variation in the assay procedure or experimental conditions because they are run in parallel with the tests, and they may be subjected to the same statistical analyses as the tests.

Calculation of Protein Binding—*First Method*—The difference in mean optical density values between the test and its corresponding control ($Y_c - Y_t$) is attributable to binding of the drug to protein since artifactual discrepancies are represented in the control value. The control value is the optical density reading that would be obtained in the corresponding test if there were no binding of drug to the protein. Thus, the value obtained by subtracting the test optical density from that of the control, expressed as a percentage of the control $[100\ (Y_c - Y_t)/Y_c]$, corresponds to the percentage binding of the drug to protein (Table I). The percentage binding in the present experiment was ~10% throughout the concentration range studied (Fig. 1). Nevertheless, the stringent control technique permits statistical analysis, which showed each test optical density mean to be significantly different from its corresponding control (Student's two-tailed t test; $p < 0.001$ except for the 180-μg/ml concentration where the limit was $p < 0.01$). It is interesting to note that there is a linear relationship between the percentage drug bound and concentration ($y = 0.01183x + 9.279$; $r = 0.8811$, $p' = 0.05$). We would point out however that the y-intercept value of 9.279% is meaningless because we have no data for concentrations <100 μg/ml, and there is no reason to presume that linearity in the relationship would continue to hold at very low drug concentrations.

Second Method—As an alternate approach to the calculation, the controls (Table I) may be used to generate a calibration curve ($y = 0.004525x - 0.08808$; $r = 0.9970$) from which the test optical density values (Y_t) may be converted to percentage binding values. In this procedure, the experimental test optical density mean value (Y_t) is inserted in the regression equation to calculate a value ($\hat{X}_t$) for the concentration of free drug in the test solution at equilibrium. The percentage drug bound to the protein is then calculated by the expression $100\ (X_0 - \hat{X}_t)/X_0$, where X_0 is the initial concentration of the drug (Table II). There is no correlation (Fig. 2) between these uncorrected percentage binding values and initial drug concentration ($r = 0.6842$, not significant).

However, the present experimental design also permits the control optical density mean value (Y_c) to be converted similarly to a value ($\hat{X}_c$) for the concentration of free drug in the control solution at equilibrium. The percentage "spurious" binding in the control is then given by the expression $100\ (X_0 - \hat{X}_c)/X_0$. The "spurious" binding values calculated by this method are shown in Table II. Theoretically, the controls should show no binding because they contain no protein. In practice, positive

Table II—Percentage Protein Binding of Sulfadimethoxine Calculated by a Regression Equation (Second Method) [a]

Initial Concentration, μg/ml	Protein Binding, %: Test[b]	Control[c]	Corrected Protein Binding, %[d]
100	5.53 ± 0.81	−3.06 ± 0.47	8.59 ± 0.91
140	11.75 ± 1.16	1.84 ± 0.94	9.91 ± 1.30
180	10.87 ± 1.05	1.16 ± 1.42	9.71 ± 1.18
250	10.22 ± 0.66	−0.51 ± 0.40	10.73 ± 0.74
340	12.84 ± 0.54	−0.097 ± 0.46	12.93 ± 0.61
	r = 0.6842	r = 0.2243	r = 0.9670

[a] Expressed as mean ± SE; $n = 5$. [b] $[(X_0 - \hat{X}_t)/X_0] \times 100$. [c] $[(X_0 - \hat{X}_c)/X_0] \times 100$. [d] $[(\hat{X}_c - \hat{X}_t)/X_0] \times 100$.

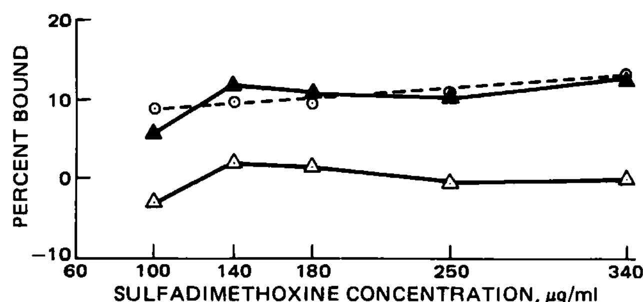

Figure 2—*Plots of percentage binding versus concentration of sulfadimethoxine. Key:* (▲) *mean test values;* (△) *mean control values;* (⊙) *adjusted test values (test less control).*

[1] Fisher Scientific Co., Toronto, Ontario, Canada.
[2] United States Biochemical Corp., Cleveland, Ohio.
[3] Thelco Model 4 Incubator, Fisher Scientific Co., Toronto, Ontario, Canada.
[4] Hematology/Chemistry Mixer Model 346, Fisher Scientific Co., Toronto, Ontario, Canada.
[5] Castle Autoclave, Fisher Scientific Co., Toronto, Ontario, Canada.
[6] Enviralab Sterility Module, Bio-Dynamics, Burlington, Ontario, Canada.
[7] Albustix, Ames Co. Division, Miles Laboratories, Rexdale, Ontario, Canada.

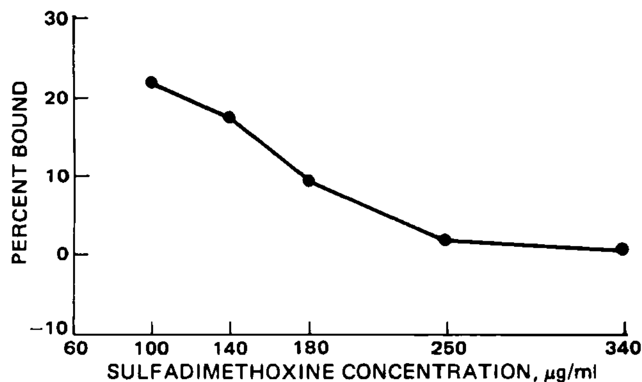

Figure 3—*Erroneous percentage binding values calculated from the regression equation of an unpaired calibration curve.*

or negative deviations from 0% binding reflect nonideal behavior in the system, brought about by the binding of drug to the equipment or other experimental error. Thus, the control ("spurious") binding values were subtracted from the corresponding test (uncorrected) binding values to give the percent binding. The simplified expression for the calculation of percent binding values by this method is $100 (\hat{X}_c - \hat{X}_t)/X_0$. There is a good correlation between the corrected percentage binding and initial concentration of drug ($y = 0.01650x + 7.042$; $r = 0.9670$, $p' = 0.01$).

Comparison of data in Tables I and II reveals that the percentage binding values calculated by the two methods are similar, but not identical. The disparity arises because of the different types of arithmetic manipulation of data employed. In the first method (Table I), the experimental optical density mean values of the test and control are used directly to calculate percentage binding values. By contrast, the second method (Table II) depends on the ability of the regression equation to predict the drug concentration ($\hat{X}$) which corresponds to an experimentally determined optical density value (Y). Thus the numerical difference between the percentage binding values generated by the two methods may be predicted by the expression $100 (1/Y_c - 1/mX_0)(Y_c - Y_t)$, where m is the slope of the regression equation. Full details of the derivation of this expression will be published elsewhere.

The percentage binding values calculated by the second method are consistently smaller than those calculated by the first method. This results in high t values (>7.0 with 4 degrees of freedom) when individual results contributing to a pair of mean values are compared, term by term, in a paired t test. However, the scatter of results about each mean is sufficiently wide to produce low t values (<1.0 with 8 degrees of freedom) in an unpaired t test. This means that, for practical purposes, there is no significant difference between mean percentage binding calculated by the two methods. We therefore prefer the first method because it is simple and direct.

Variability in the System—To assess day-to-day variability in the system, control-calibration experiments were run on nine different occasions by the same technician. Slope values generated from this data varied considerably (mean, 0.003970; *SD*, 0.0004974; coefficient of variation, 12.53%). This reflects not only variation in the analytical method, but also any day-to-day differences in the adsorption of drug to the membrane. The latter is suspect because the membranes have to be prepared for each run (see *Experimental*) and it is possible that there is interbatch variation in the extent of drug adsorption.

To illustrate the distortion that may be brought about by the use of an unpaired calibration curve, the test optical density mean values in Table I were converted to percent protein binding values by means of a calibration curve ($y = 0.003522x + 0.06394$; $r = 0.9981$) selected at random from the nine calibration-control experiments described above. The resulting percentage binding–drug concentration plot (Fig. 3) has a negative slope ($y = -0.09193x + 28.81$; $r = -0.9406$) and bears little resemblance to the authentic uncorrected plot (Fig. 2).

In the execution of the experiments, care was taken to standardize the manipulative procedures to minimize experimental error. The use of tubes with polytetrafluoroethylene-lined caps avoided the problem of leaching from rubber- or polyvinylchloride-lined caps, which can cause displacement of drugs from protein binding sites (17). All solutions and glassware were sterilized before use, and solution transfers were made under aseptic conditions in a laminar-airflow hood. This eliminates the possibility of microbial growth during dialysis and permits the experiments to be conducted for 24 hr without the use of preservatives that could influence binding characteristics. All dialysates were checked for the absence of protein, both visually (absence of frothing) and by the use of a semiquantitative colorimetric indicator.

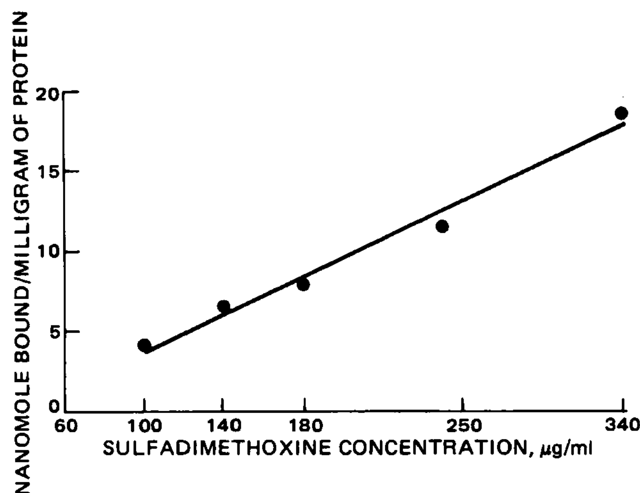

Figure 4—*Relationship between nanomoles of sulfadimethoxine bound per milligram of protein and total drug concentration.*

The majority of protein-binding experiments described in the literature are concerned with drugs which have a high percentage binding to plasma proteins and with protein fractions that transport a major fraction of a drug in the blood. The significance of minor proteins may increase in disease or other conditions, and it can be important to know their contribution to the overall binding of drugs in the blood.

The variability in results obtained with conventional dialysis procedures often precludes accurate determination of the binding of drugs which show a low percentage bound or a low affinity for proteins. The results (Fig. 1) obtained by the present method however, reveal a low, but statistically significant percentage binding throughout the drug concentration range studied. Conventional percentage binding–drug concentration plots can be difficult to interpret when the slope is shallow as in Fig. 1 ($y = 0.01183x + 9.297$). However, if the percentage binding is expressed in terms of nanomoles of drug bound per milligram of protein, it is clear that the amount of drug bound increases with total drug concentration in a linear manner ($y = 0.05866x - 2.116$, $r = 0.9913$; Fig. 4).

CONCLUSION

The method developed in this study provides an accurate technique for the minimization of errors that arise in equilibrium dialysis and is particularly suitable for use when the percentage binding is low. The stringent control technique permits correction to be made for concentration-dependent binding of drug to the membrane or other equipment. Most significant in the present study is the linear relationship between total drug concentration and control-corrected percentage binding, whereas there was no correlation between total drug concentration and conventional (uncorrected) percentage binding. The method may be adapted to other experimental designs (*e.g.*, fixed concentration of drug *versus* varied concentration of protein) and can be employed with any suitable method of drug analysis.

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Stereospecific High-Performance Liquid Chromatographic Analysis of Warfarin in Plasma

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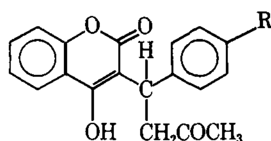
Abstract □ A stereospecific high-performance liquid chromatographic assay has been developed to determine $R(+)$- and $S(-)$-warfarin simultaneously in plasma. The method involved the formation of diastereoisomeric esters, using carbobenzyloxy-L-proline, with subsequent separation using silica as the stationary phase. The method permits characterization of the pharmacokinetics of warfarin enantiomers following administration of racemic drug.

Keyphrases □ Warfarin—stereospecific quantitation in plasma, high-performance liquid chromatography □ Stereoisomers—of warfarin, quantitation in plasma using the racemate, high-performance liquid chromatography □ High-performance liquid chromatography—of warfarin in plasma, quantitation of the stereoisomers using the racemate

Warfarin (I) is administered clinically as a racemic mixture. In humans, $S(-)$-warfarin is five times more potent and is more rapidly eliminated than the R-isomer (1, 2). Consequently, the concentration of each isomer in plasma varies with time within an individual and also between individuals following a dose of racemic warfarin. The response to warfarin is also variable (3). Drugs interact with the isomers differently (4–6). Thus, a more complete understanding of the sources of variability in response to warfarin, and the nature of interactions of drugs with warfarin, requires either giving each isomer separately (a rare clinical procedure) or determining the concentration of each isomer in plasma following administration of the prescribed racemic drug.

Stereospecific analysis of a mixture of enantiomers is difficult. Several specialized analytical techniques, including the synthesis of stable isotopes (pseudo-racemates) coupled with mass spectrometry (7, 8) and a stereospecific radioimmunoassay (9) have been developed to overcome this difficulty.



Chromatographic separation of enantiomers is possible if a diastereoisomeric relationship is established between them either through the use of chiral solvents (10–12) or derivatization with a suitable chiral reagent (13–15). This paper describes a simple method for the quantitative estimation of the isomers of warfarin in plasma using carbobenzyloxy-L-proline to form diastereoisomeric esters, which can be separated by high-performance liquid chromatography (HPLC) using silica as the stationary phase.

EXPERIMENTAL

Reagents and Materials—Racemic warfarin was obtained from its sodium salt[1] by precipitation with 0.1 *M* HCl. The dried material was recrystallized from absolute ethanol. $R(+)$- and $S(-)$-warfarin[2], the internal standard[3] (3-[α-(4′-fluorophenyl-)-β-acetylethyl]-4-hydroxycoumarin; 4′-fluorowarfarin) (II), imidazole[4], dicyclohexylcarbodiimide[5], carbobenzyloxy-L-proline[5], hexane (HPLC grade)[6], methanol[7], and ethyl acetate (HPLC grade)[6] were used as supplied. Peroxide-free ether[8] was prepared by passage through a column of activated alumina[9] (45 g, Brockman type 1 alkaline).

Extraction of *RS*-Warfarin—Plasma (0.2 ml) and internal standard (0.1 ml; 0.846 μg/0.1 ml of water) were added to a clean culture tube[10] (16 × 125 mm). The solution was made alkaline with 0.1 *M* K_2CO_3 (1 ml), shaken manually for 3 min with ether (4 ml), and separated by centrifugation[11] at 3000 rpm for 5 min. After aspiration of the organic layer, the aqueous layer was acidified with 1 *M* HCl (1.5 ml) and shaken manually for 3 min with ether (6 ml), followed by centrifugation at 3000 rpm for 3 min. The aqueous layer was quickly frozen by immersion in liquid nitrogen (40–60 sec) to allow the organic layer to be decanted into a culture tube whose tip was drawn out to a capacity of 0.2 ml. An antibumping

[1] Sorex, U.K.
[2] Gifts from Endo Laboratories, Inc., U.S.A.
[3] Gifts from Ciba Geigy Ltd., Switzerland.
[4] Sigma Chemical Co., U.S.A.
[5] Aldrich Chemical Co., U.S.A.
[6] Rathburn, Scotland.
[7] Analar; Fisons, U.K.
[8] May and Baker, Dagenham, U.K.
[9] B.D.H., U.K.
[10] Corning, U.S.A.
[11] MSE, Super Minor Centrifuge, No. 533A: MSE, U.K.

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