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Application of Spin Labeling to Drug Assays

II. Determination of the Binding of [^{14}C]-Phenytoin and Spin-Labeled Phenytoins to Albumin and Human Serum

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SUMMARY

The concentration-dependent binding of four nitroxide spin-labeled derivatives of phenytoin (Compound I) to bovine serum albumin and human sera has been investigated. The spin label moiety was attached to phenytoin either at position 3-*N* via a carbimidomethyl linkage (Compound II) or at a 4′-phenyl position via an amide linkage (Compounds III–V). Two of the phenyl labels (Compounds III and IV) differed only in the presence of a double bond in the pyrroline ring in Compound IV, and Compound V had the amide linkage reversed as compared with Compound III. The results of the binding studies were compared with those obtained by equilibrium dialysis of [^{14}C]phenytoin. The spin labels were found to bind less strongly than [^{14}C]phenytoin, with the order of binding of the spin-labeled phenytoins being IV > III > II > V. Studies with salicylic acid and phenylbutazone, known competitors of phenytoin binding, showed that the competition of spin label II paralleled that of [^{14}C]phenytoin more than did the 4′-phenyl labels (III–V). These results suggest that the phenyl rings play a significant role in the binding of phenytoin to albumin. The binding of the 3-*N* spin-labeled phenytoin (II) paralleled that of [^{14}C]phenytoin in human sera and should prove useful in the determination of free phenytoin levels in sera.

INTRODUCTION

The majority of drugs bind to serum proteins to some degree. Of the intravascular proteins, albumin serves as the primary carrier for many drugs, especially those with acidic or lipophilic characteristics. This fraction of bound drug ranges from as little as 17% for digoxin (1) to over 99% for warfarin (2). On the basis of both chemical principles and pharmacological evidence, it is clear that in general only the free drug fraction can interact with the target site. Assays for the free fraction are clinically relevant for drugs with highly bound fractions, since small changes in the intravascular environment, such as those caused by uremia (3), hepatic failure, or other drugs competing for the same protein binding sites, can cause dramatic alterations in the free drug levels. Recent interest in the determination of the free fractions for antiepileptic drugs (4–6) such as phenytoin (7) illustrates the value of measuring the free fraction in minimizing drug toxicity, especially for drugs with a narrow therapeutic ratio and high protein binding. Yet, most clinical assays for the determinations of free drug levels continue to be time-consuming, and are reliable only if performed under ideal conditions.

Both equilibrium dialysis and ultrafiltration through semipermeable membranes have proven to be accurate and quite reproducible techniques for determining free drug levels (8, 9). However, both methods are fraught with difficulties (9, 10). Equilibrium dialysis has been used as the reference method, but equilibrium between the inside and outside of the dialysis bag may require 24 hr or more at room temperature, thus making this method unsuitable for clinical use. Furthermore, drugs are known to bind to the bag (9). In addition, the method does not easily permit automation and is highly labor-intensive. Ultrafiltration has gained some popularity recently as a clinical laboratory assay of free phenytoin levels, especially since a kit has become available (Worthington Diagnostics, Millipore Corporation, Freehold,

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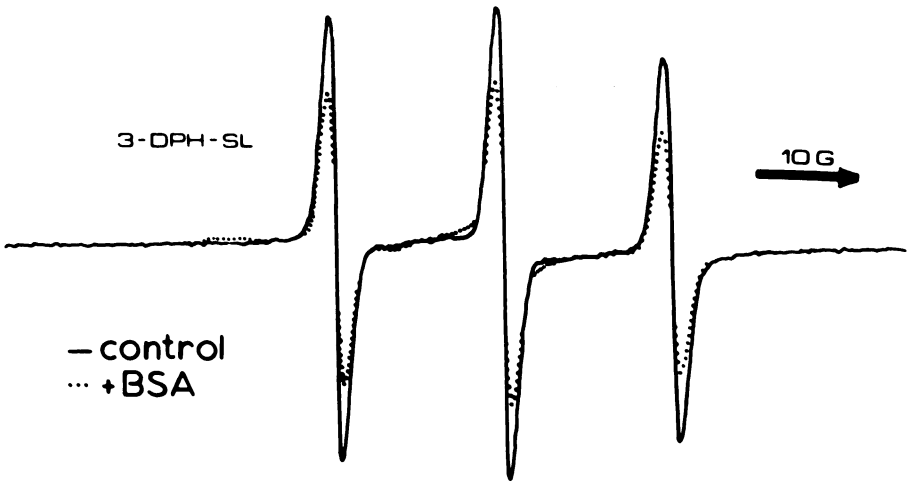


FIG. 1. *ESR spectra of spin label II (3-DPH-SL) in 50 mM phosphate-buffered 150 mM NaCl (——) and in the presence of bovine serum albumin (BSA) solution (4 g/100 ml) in buffer (····)*

The spin label concentration was 12 μM. The ESR spectrometer settings were as follows: calibrated modulation amplitude, 2.96 G; receiver gain, 1600; time constant, 1 sec; 100 G scan centered at 3240 G; scan time, 16 min; and microwave frequency, 9.12 GHz.

N. J.) (11). Although such kits considerably reduce the necessary labor and are relatively rapid, they are subject to the usual errors of ultrafiltration and dialysis which can lead to a failure to obtain the true free drug levels (1, 10). Others have reported that the permeability of free phenytoin through certain ultrafiltration filters is only 88% (7), which suggests another potential source of error. With care, clinical laboratories should find ultrafiltration methods usable, but such skills are frequently unavailable.

In light of these problems, we have been investigating ESR spectroscopy of spin-labeled drugs as an alternative method for the determination of free concentrations (12–16). This technique depends on the observation that spin-labeled drugs rotating freely in solution give characteristic ESR spectra distinct from the spectra of the spin-labeled drug bound to protein. In the case of nitroxide spin labels, the free drug typically appears as a sharp, three-line spectrum which broadens significantly as it becomes bound to albumin. The low-field peak height is a measure of the free drug concentration and can be used to determine the free fraction (Fig. 1).

We have synthesized two series of spin-labeled derivatives of phenytoin. In one, the nitroxide is covalently attached to position 3-*N* of the hydantoin ring (13–16); in the other, to one of the phenyl rings at position 4′ (16).[5] In this study we have compared the equilibrium association constants of phenytoin (Compound I), the phenytoin spin-labeled at position 3-*N* of the hydantoin ring (Compound II) (13, 14), and three phenytoins spin-labeled on one of the phenyl rings (Compounds III–V) (16)[5] (Fig. 2). From these comparisons, we have sought to determine (a) whether the spin labels bind to the same sites as the unlabeled phenytoin, (b) which portions of the molecule are attached to albumin, and (c) whether the free fraction of the drug is related to the free fraction as determined by the spin label assay for albumin solutions and human serum. Although numerous past studies (3–7, 11, 17–23) have examined the albumin binding of phenytoin, the widely differing results suggest that the observed binding constants are highly dependent on subtle experimental differences. For example, studies on the effect of fatty acids on the binding of phenytoin in serum have reached opposing conclusions (24, 25). As a result, we have repeated many previous experiments reported in the literature and have chosen to compare the spin-label studies with our own results.

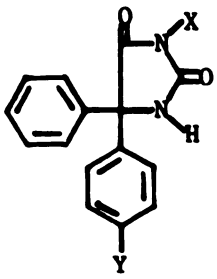
MATERIALS AND METHODS

Spin-labeled Compounds II–V were prepared as described elsewhere[5] (16). The purity was verified by thin-layer chromatographic, infrared, mass spectral, and melting point data. BSA[6] (Cohn Fraction V, 99% pure and recrystallized) and AAG (human) were obtained from Sigma Chemical Company (St. Louis, Mo.). Radioactive [4-^{14}C]phenytoin (specific activity 49.6 mCi/mM) was obtained from ICN Pharmaceuticals (Irvine, Calif.) and was repurified by thin-layer chromatography on silica gel GF plates. Liquid scintillation was performed with a Packard Model 3330 counter using a dioxane-2,5-diphenyloxdazole-naphthalene scintillator. Counting efficiency was determined by the addition of [^{14}C]toluene after initial counting.

ESR spectra were obtained in a Varian E-4 spectrometer with an E-231 cavity and a variable temperature accessory. Samples for quantitative analysis were run in 50-μl capillary pipettes (Corning Glass Works, Corning, N. Y.). The pipettes were placed in a 4-mm quartz tube which was placed in the ESR variable temperature dewar inside the cavity. The field scan was calibrated with Fremy's salt (26) and the field modulation was calibrated (27), as described previously. For the spin-label assays, the ESR spectra were run on the low-field line at a nominal power of 10 mW to avoid microwave heating effects (28) and at the modulation amplitude setting which maximized the peak-to-peak intensity.

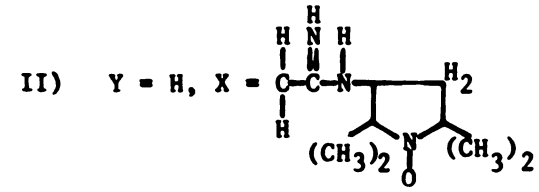
[5] C. F. Polnaszek, Y. Yost, E. A. Krauss, D. Chou, and J. L. Holtzman, to be published.

[6] The abbreviations used are: BSA, bovine serum albumin; AAG, α-acid glycoprotein; PBZ, phenylbutazone; SL, spin label; SA, salicylic acid.

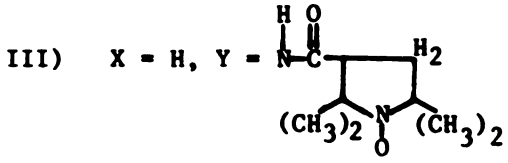


I) Diphenylhydantoin
(Phenytoin)

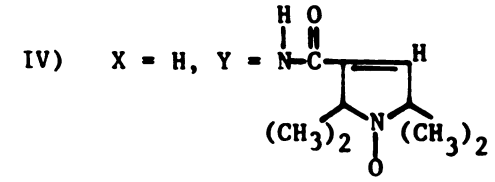
X = H
Y = H



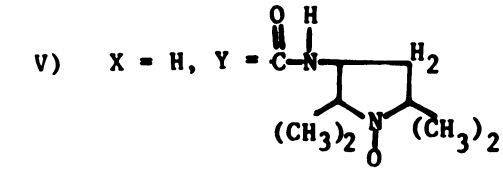
II) Y = H, X =



III) X = H, Y =



IV) X = H, Y =



V) X = H, Y =

FIG. 2. *Chemical structures of phenytoin (I), 3-N-hydantoin spin-labeled phenytoin (II), 4'-aminophenylpyrrolidinyl spin-labeled phenytoin (III), 4'-aminophenylpyrroline spin-labeled phenytoin (IV), and 4'-carboxyphenylpyrrolidinyl spin-labeled phenytoin (V)*

Equilibrium dialysis was performed in 50-ml Erlenmeyer flasks which had been silanized with dichlorodimethyl silane (Sigma Chemical Company)toluene (1–2: 100), followed by rinsing in toluene, methanol, and deionized water. Dialysis tubing (10-mm Spectrapor-4) was prepared by boiling for 5–10 min and rinsing in cold distilled water. Approximately 10 cm of tubing containing 1 ml of a solution of unlabeled phenytoin at various concentrations, and [^{14}C]phenytoin in an aqueous solution of BSA (4 g/100 ml) were immersed in 30 ml of dialysis solution containing unlabeled phenytoin in 50 mM phosphate buffer (pH 7.4) in 150 mM NaCl solution with sodium azide (0.01 g/100 ml). Silanized glass beads were included inside the dialysis bag to facilitate agitation and reduce buoyancy. The phenytoin concentrations in the system varied from 1 μM to a maximum around 45 μM. PBZ (1.98 mM) and sodium salicylate (2.94 mM) were also added to some solutions to examine drug competition. The entire flask was then incubated at 22–26° for 48–72 hr in a rotary shaker. Control samples containing no albumin were included to ensure that equilibrium had been reached. All experimental runs were performed on duplicate samples. Binding parameters were estimated by computer using the direct linear-plot method (29). Binding was analyzed using the following equation (30):

$$[\text{Albumin}]/[\text{bound}] = (1/nK_a)(1/[\text{free}]) + 1/n \quad (1)$$

where K_a is the equilibrium association constant and n is the number of binding sites. When there was competition by compound X, an apparent nK_a was measured which is related to the inihibition constant K_I by the equation:

$$K_I = [X]/(nK_a/(nK_a)_{app} - 1) \quad (2)$$

The above form was used since the type of competition, i.e., noncompetitive, uncompetitive, or competitive, could not be distinguished as the error in the calculated quantities n and K_a was considerable, whereas the values of nK_a were reproducible and varied significantly in the competitive binding studies.

Human serum was obtained from outdated blood bank plasma to which was added 40 mM $CaCl_2$. All data were obtained from a single unit of blood. The serum was centrifuged at 2000 rpm for 20 min and the clot was removed.

For the experiments with human serum, the methods were as described above with the following modifications. The solution in the dialysis tubing contained 0.9 ml of serum and 0.1 ml of [^{14}C]phenytoin in buffer. The spin-label experiments were performed with solutions containing the same ratio of serum to stock spin label in buffer solution as for the equilibrium dialysis experiments. For the competition experiments with the spin labels, the competitor compound was first dissolved in the serum. The concentrations of albumin and total protein in the serum were determined by the laboratory service at the Veterans Administration Medical Center. Serum was frozen between experiments with Compounds I, II, and IV. The experiment with Compound II was rerun 1 week later in order to investigate the effects of freezing and thawing on the binding. The free fraction of spin label

III in serum from patients taking phenytoin was determined and compared with that obtained by equilibrium dialysis and by ultrafiltration (11). For these samples the concentration of Compound I was determined by the EMIT assay (31).

The binding analysis for the human serum dialysis experiments was identical with that used for the experiments with albumin solutions. For the spin-label experiments with human serum it was not possible to vary the spin-label concentration over the range necessary to obtain binding curves, as was done for the albumin solutions, because of the low solubility of Compound II. The binding parameter nK_a was obtained in the following manner. Because the free fraction (F_{SL}) of spin label ([free SL]/[SL]) was determined at [SL] ≪ [albumin], Eq. 1 can be rewritten as

$$nK_a = (F_{SL}^{-1} - 1)/[\text{albumin}] \qquad (3)$$

As [SL] ≃ 2 μM and [albumin] = 437 μM, this equation is expected to give the correct result with very small error (<1%). The inhibition constants were then obtained by Eq. 2.

RESULTS

Although initial experiments with [^{14}C]phenytoin suggested that the inside and outside of the dialysis bag reached 95% of equilibrium at 16 hr, we found that we had to dialyze for 48 hr to obtain reproducible duplicates in the calculated values of nK_a. Our equilibrium dialysis results are presented as a double-reciprocal plot (30) (Fig. 3). Our values for nK_a of phenytoin of 8.3 ± 0.4 × 10^3 M^{-1} for BSA and 11.8 ± 1.4 × 10^3 M^{-1} for human serum agree well with the values of 10.5–11.4 × 10^3 M^{-1} for patient serum samples found by Odar-Cederlof and Borgå (3). However, the number of binding sites, n, in our studies approached 1 rather than 2.5–3.4 as suggested by their experiments. We attribute these differences to (a) the use of BSA rather than human proteins, and (b) their use of ethanol in their experiments (approximately 2:100 ethanol-serum), which we did not include in our experiments. The difficulty in solubilizing phenytoin (19) certainly contributes to the difficulty in estimating the number of binding sites accurately. Lunde *et al.* (17) observed that, at concentrations less than 79 μM, the number of binding sites did approach 1, a finding more consistent with our experiments.

The addition of 2.9 mM salicylic acid reduced the nK_{app} to 4.7 ± 0.5 × 10^3 M^{-1} for the BSA solutions and 9.9 ± 0.9 × 10^3 M^{-1} for human serum. This value is in general agreement with Odar-Cedarlof and Borgå's value (3) of 5.9 × 10^3 M^{-1} for 2 mM salicylate and 4.6 × 10^3 M^{-1} for 6 mM salicylate (3). From the data of Odar-Cedarlof and Borgå (3), we calculate K_I = 3.7 ± 1.0 mM, which is in agreement with our results for BSA (Table 1) but less so for human sera (Table 2). Lunde *et al.* (17) observed that the concentrations of free phenytoin doubled in the presence of 3 mM salicylate, a finding also consistent with our results. Lunde *et al.* (17) also reported that 0.3 mM PBZ increases the free concentration of phenytoin from 6.7 to 11.1%. This figure compares well with a decrease in the nK_{app} for our experiments from 8,300 M^{-1} to 2,800 M^{-1} for albumin solutions and from 11,800 M^{-1} to 8,000 M^{-1} in human serum samples in the presence of 1.98 mM PBZ.

Spin-label experiments showed some interesting vari-

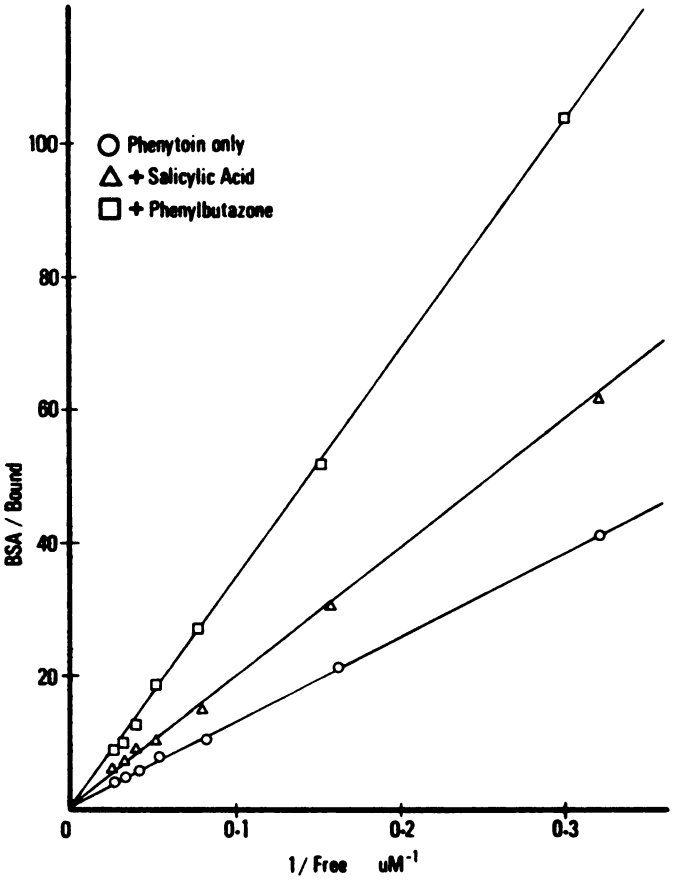


FIG. 3. *Binding plot of the [^{14}C]phenytoin equilibrium dialysis data in a double-reciprocal form*

The individual lines are for [^{14}C]phenytoin alone (○), [^{14}C]phenytoin plus 2.94 mM salicylic acid (△), and [^{14}C]phenytoin plus 1.99 mM PBZ (□). The BSA concentration was 588 μM in all samples.

TABLE 1

Binding of [^{14}C]phentyoin and the spin-labeled phenytoins in aqueous solutions of BSA (588 μM) at 25°

Phenytoin derivative	Inhibitor	Concentration	nK_a[a]	K_I
		mM	M^{-1}	mM
I	—	—	8300	—
	SA	2.94	4700	3.9 ± 1.0
	PBZ	1.98	2850	1.0 ± 0.2
II	—	—	2500	—
	SA	2.94	1700	6.0 ± 1.5
	PBZ	1.99	1200	1.8 ± 0.3
III	—	—	3400	—
	SA	2.94	1600	2.6 ± 0.4
	PBZ	1.99	2100	3.3 ± 0.5
IV	—	—	4500	—
	SA	2.37	2700	3.6 ± 1.2
	PBZ	1.17	3200	2.9 ± 0.4
V	—	—	1500	—
	SA	2.94	1000	5.9 ± 2.0
	PBZ	1.98	1200	7.6 ± 3.0

[a] In all of these studies, less than one binding site per albumin molecule was found. These low values are attributed to the use of Cohn Fraction V rather than electrophoretically pure BSA.

ations among the several phenytoin derivatives for the BSA solutions (Table 1). Spin label II in the ESR studies behaved in a fashion similar to [^{14}C]phenytoin (Compound I) in the dialysis experiments, both showing moderate displacement by 2.94 mM salicylate and more displacement by 1.98 mM PBZ.

Spin label III showed behavior quite different from [^{14}C]phenytoin being displaced more by salicylate than by PBZ. spin label IV showed little or no significant difference in its displacement by PBZ or salicylate relative to spin label III, but the apparent values of nK_a are larger for the compound with the double bond present. Spin label V had the weakest binding of all of the labels and qualitative similarity to III and IV in the competitive experiments. The results are summarized in Table 1.

The competitive binding of Compounds II and III in the presence of phenytoin showed that I displaced II to a greater extent (K_I = 2.8 mM) than III (K_I = 4.1 mM) from BSA solutions.

For the spin label experiments with human serum, qualitative results were obtained similar to those with the albumin solutions. Spin label II behaved in a manner similar to phenytoin in the dialysis experiments. In the competitive binding experiments the values of K_I for PBZ and SA were in good agreement for phenytoin and spin label II.

Only one phenyl spin label (IV) was examined in the competitive binding experiments with human serum. The results were again in qualitative agreement with those obtained for the BSA solutions in that K_I (PBZ) > K_I (SA) for IV, whereas the opposite K_I (PBZ) < K_I (SA) was observed for Compound I. IV also showed greater binding to serum (nK_a = 12,400 M^{-1}) than did II (nK_a = 4,200 M^{-1}) (Table 2), similar to that seen for the albumin solutions.

As the spin-label experiments with Compounds II and IV were not performed on the same days as the dialysis experiment with Compound I, the effect upon the binding of Compound II of freezing and thawing the serum over the period of 1 week was investigated. The results (Table 2) show that, although the binding affinity was reduced somewhat, the binding constants decreased in a similar manner when SA and PBZ were added, and the inhibition constants K_I obtained were in agreement with those obtained 1 week earlier. Furthermore, in a previous study Montgomery and Holtzman (12) found that spin-labeled morphine bound in a manner identical with that for fresh or refrozen human serum.

TABLE 2

Binding of [^{14}C]phenytoin and spin-labeled phenytoins to human serum at 25°[a]

Phenytoin derivative	Inhibitor	Concentration	nK_a[b]	K_I
		mM	M^{-1}	mM
I	—	—	11800 ± 1400	—
	SA	2.90	9850 ± 850	15.0 ± 1.5
	PBZ	1.97	8000 ± 1000	4.2 ± 1.5
II	—	—	4200 ± 500	—
	SA	3.23	3600 ± 200	18.8 ± 3.2
	PBZ	2.18	2800 ± 100	4.6 ± 0.7
II[c]	—	—	3000 ± 130	—
	SA	2.94	2500 ± 100	14.7 ± 1.2
	PBZ	1.98	2000 ± 70	3.9 ± 0.2
IV	—	—	12400 ± 1000	—
	SA	2.94	7100 ± 200	3.9 ± 0.4
	PBZ	1.98	9700 ± 800	7.1 ± 1.1

[a] Concentration of human serum albumin in serum was 3.3 g/100 ml; total protein, 5.3 g/100 ml. Stock [^{14}C]phenytoin or spin label (1 part) was added to 9 parts human serum.

[b] Calculated using human serum albumin concentration.

[c] Serum was frozen and thawed several times over the period of 1 week.

The increase in nK_a in human sera as compared with BSA solutions could be attributed to the binding of the compounds to other proteins found in the serum, such as AAG (32). Initial experiments showed that Compounds II and IV in 25 μM AAG solutions in the absence of BSA had bound fractions of 6.8% and 5.1%, respectively. Since in the calculation of nK_a for serum only the albumin concentration was used, the values of nK_a obtained will be higher owing to binding to other serum proteins.

Another reason for the higher binding constants in serum could be the reduction of the nitroxide spin labels by constituents of serum (33). This did not happen to any large extent, as (a) the observed free fraction of Compound II decreased by 15% within 4 hr and that of Compound IV by 2% within 2.5 hr, whereas the experiment is completed in about 10–15 min, and (b) similar increases in nK_a were seen for phenytoin itself.

DISCUSSION

In order for a spin-labeled compound to be useful for the assessment of the free drug levels of a drug, it must have binding characteristics similar to those of the parent compound. The affinity constants do not need to be identical with that of the parent compound, as determined from equilibrium dialysis, but we must be able to express the free fraction of drug (F_{drug}) as a function of the free fraction of spin label. In the simplest case, this expression would be a linear function of F_{SL}:

$$F_{drug} = [\text{drug free}]/[\text{drug total}] = A \times F_{SL} + B \quad (4)$$

where A and B are constants.

In order to use spin-labeled drugs for determining free drug levels, such a compound must be calibrated with a reference method. A calibration procedure consists of using a series of serum specimens in which the [free] to [bound] drug ratio has been determined by a reference method such as equilibrium dialysis. The spin-labeled drug would then be added in a tracer quantity to the serum references, and measurements of the low-field nitroxide peak height would be taken. These peak heights would then be compared with a blank consisting of the same tracer amount of the spin label in a phosphate-buffered solution. These peak height ratios would be plotted against the equilibrium dialysis data to form a calibration curve from which the percentage of free drug could be determined. This calibration curve would essentially be characteristic of the spin-labeled drug and would require infrequent recalibrations. To determine the free drug level on an unknown, a total drug level would be performed by any accepted technique followed by the addition of a tracer quantity of the spin label. The nitroxide peak-height ratio measured by ESR and the

calibration plot would then be used to determine the free drug fraction, from which the free drug level could be computed.

Clearly, the spin labels have different affinities for BSA and human serum than does [^{14}C]phenytoin (Tables 1 and 2). However, only spin label II fits the criteria for use in the assessment of the free drug levels. When we fit the data in Table 1 to Eq. 4, spin label II has a highly significant correlation coefficient (r = 0.996). On the other hand, for the phenyl-labeled probes, Compound IV (r = 0.783) and more so for Compound III (r = 0.582) and Compound V (r = 0.551), the correlations are considerably reduced. Similar results were found for serum samples (Table 3). The best fit values for spin label II are an A of 1.182 and a B of −0.317 in Eq. 4 for BSA. The strong correlation of the free fraction of Compound II with phenytoin suggests that the stereochemistry of the phenyl rings is important in the binding of this drug to albumin. Furthermore, it appears that Compound III may bind to different sites than I as shown by the results when I was used as a competitor for II and III.

Evaluation of the results for human sera suggest that spin label II should be useful in measuring free drug levels in the clinical laboratory. The parameters in Eq. 4 are now an A of 0.987 and a B of −0.193. This discrepancy may be due to different binding constants of human serum and BSA. More likely, the presence of other binding proteins in the sera, such as AAG (see results above) and thyroxine-binding globulin (34), caused the differences between BSA and human serum.

Compounds III and IV do provide some insight into the nature of phenytoin binding to bovine albumin. The sensitivity of the binding of the phenyl rings of phenytoin to the nitroxide substituents strongly suggests their participation in hydrophobic bonding to albumin. The qualitative similarity of the competitive binding of spin label II and [^{14}C]phenytoin suggests that the hydantoin ring remains outside the attachment area and that its main function is to increase the solubility of Compound I (34).

The differences in binding between the single III and double-bonded IV spin labels was unexpected but suggests that the double bond in the pyrroline ring of IV may simply provide an area of electrostatic interaction through the π bonds. Alternatively, the planar nature of the double-bonded nitroxide molecule may provide less steric hindrance to binding. Either or both factors may help to explain the greater affinity of IV for BSA and human serum. The difference seen in the binding of spin label III (nK_a = 3400 M^{-1}) and the same compound in which the amide linkage is reversed, V (nK_a = 1500M^{-1}), suggests that the steric factor is important.

A major disadvantage to the use of the spin label II in this drug assay is that this compound is even less soluble than phenytoin itself (20 μM versus 80 μM). The spin labels II–V are several times more soluble in buffer than is II, presumably because of the presence of the 3-N hydrogen atom, which is missing in spin label II. However, they are only somewhat more soluble than phenytoin itself, presumably owing to the polar amide and nitroxide groups. To eliminate this problem, alcohol was used in some previous studies to aid in the dissolution of phenytoin and its derivatives (16, 17). In our earlier study (16) we found that spin label III behaved in a manner similar to [^{14}C]phenytoin (r = 0.90), whereas II correlated poorly in the competitive binding experiments (r = 0.30). However, ethanol (buffer-EtOH, 23:1) was added to aid in dissolving the phenytoin spin labels. This was almost a 1000-fold excess on a molar basis over the BSA concentrations used. Alcohols have been shown to bind to BSA (35) and would be expected to compete more strongly with the more polar salicylate than with the phenytoin spin labels or PBZ. This may be the cause of the previous observations (16) that salicylate did not affect the binding of II rather than displace it. Further studies on the binding of phenytoin and its spin labels in the presence of ethanol are currently in progress.[5]

The ESR method using spin-labeled compounds provides a rapid and effective alternative for the clinical assessment of free drug levels. The analyses can be performed using spin-label concentrations which are much less than serum levels found in patients. Further studies of patients taking phenytoin will be needed to evaluate whether spin label II can be used to measure free phenytoin levels in clinical situations. Other modifications of the hydantoin moiety are being considered both for evaluation as spin labels and for further characterization of the nature of the interaction between phenytoin and albumin. These modifications may reduce the size of the labeling group and increase the binding constants and solubility of the spin label without perturbing the hydrophobic binding of phenytoin. Finally, ^{15}N-enriched nitroxide spin labels are being synthesized to increase the spin-label peak heights and the sensitivity of the technique. This would allow the use of tracer quantities of the spin label at quantities less than the 2 μM currently used and reduce the likelihood of unlabeled drug-labeled drug interaction at low serum drug levels.

TABLE 3

Correlation of the free fraction of phenytoin as determined by equilibrium dialysis to the free fraction of spin-labeled phenytoins

System	Spin label	r
BSA (4 g/100 ml)	II	0.996
	III	0.582
	IV	0.783
	V	0.551
Human serum	II	0.967
	II[a]	0.950
	IV	0.401
	III[b]	0.604
	III[b, c]	0.864
	III[b]	0.571

[a] Serum frozen and thawed several times.

[b] Albumin concentration not determined.

[c] Free fraction of phenytoin determined by ultrafiltration (11).

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