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Comparative *In Vivo* and *In Vitro* Studies of Phenytoin Protein Binding and *In Vitro* Lipolysis in Plasma of Pregnant and Nonpregnant Rats

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Abstract □ This investigation was designed to determine the cause of the changes in drug protein binding that occur in rat plasma, particularly in plasma from pregnant animals, during *in vitro* drug-protein binding measurements. *In vivo* estimates of phenytoin binding in plasma were obtained from steady-state CSF-plasma concentration ratios in pregnant and nonpregnant rats. Immediate ultrafiltration of heparin- or EDTA-anticoagulated plasma yielded phenytoin free fraction values that were in good agreement with *in vivo* estimates for nonpregnant rats but that were about one-third higher than *in vivo* estimates for pregnant animals. *In vitro* free fraction values tended to increase during incubation of plasma and/or during equilibrium dialysis. The concentrations of the four major endogenous free fatty acids were similar in plasma of pregnant and nonpregnant rats if determined immediately after blood collection. Six hours of incubation at 37°C caused fatty acid concentrations to increase about fivefold and twofold in heparin-anticoagulated plasma from pregnant and nonpregnant animals, respectively. The corresponding increases in EDTA-anticoagulated plasma were only about twofold and 1.14-fold, respectively. These changes were associated with decreased plasma protein binding of phenytoin. The *in vivo* differences between pregnant and nonpregnant rats with respect to phenytoin binding in plasma are not due to differences in fatty acid concentrations, but the *in vitro* differences are due primarily to corresponding differences in free fatty acid concentrations if extensive *in vitro* lipolysis occurs.

Keyphrases □ Phenytoin—plasma protein binding, lipolysis, pregnant and nonpregnant rats □ Lipolysis—*in vitro* and *in vivo* comparisons, plasma protein binding, pregnant and nonpregnant rats □ Anticoagulants—heparin, EDTA, plasma protein binding, *in vitro* and *in vivo* comparisons, pregnant and nonpregnant rats

Pregnancy is known to be associated with quantitative changes of drug biotransformation (1) and drug-protein binding in plasma or serum (2). Properly designed studies of the effect of pregnancy on the pharmacokinetics of drugs, therefore, require measurement of unbound drug in plasma, to differentiate between pharmacokinetic changes due solely to drug binding alterations and those due to altered intrinsic clearance or to a combination of both types of effects. Unfortunately, the drug-protein binding characteristics of plasma from pregnant rats and, to a lesser extent, from pregnant women are quite unstable (3, 4). A lack of appreciation of this problem can lead to serious misinterpretations in pharmacokinetic studies, particularly in the assessment of intrinsic drug clearance and drug concentration–pharmacological activity relationships.

This investigation was designed to determine the causes of *in vitro* alterations in protein binding of drugs in plasma from pregnant and nonpregnant rats and to assess the effects of the *in vitro* anticoagulant and of the methodology used for protein binding determinations. The study protocol also permitted a comparative determination of the effect of pregnancy on the total clearance and *in vivo* plasma protein binding of phenytoin in rats.

EXPERIMENTAL SECTION

Pregnant (day 20 of gestation) and nonpregnant Lewis rats[1] (the latter ≃ 200 g) received a loading dose of phenytoin, 14.7 mg/kg, by rapid injection

[1] Charles River Breeding Laboratories, Wilmington, Mass.

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Table I—Plasma Protein Binding of Phenytoin in Pregnant and Nonpregnant Rats: Effect of Methodology and *In Vitro* Anticoagulant on Free Fraction Determination [a]

	Pregnant Rats		Nonpregnant Rats	
	EDTA	Heparin	EDTA	Heparin
Total phenytoin in plasma, μg/mL	15.8 ± 1.7	16.9 ± 3.6	12.3 ± 4.7	12.4 ± 3.0
CSF-plasma conc. ratio × 100	19.1 ± 2.3	17.0 ± 0.9[b]	12.6 ± 2.3	13.8 ± 0.9[b]
Free fraction × 100				
Immediate ultrafiltration	25.5 ± 4.2	23.4 ± 7.3[e]	12.4 ± 1.5	13.7 ± 1.8
Ultrafiltration after 6 h	31.9 ± 4.9[d]	39.4 ± 12.9[d, e]	12.5 ± 1.8[c]	13.9 ± 1.9[c]
Equilibrium dialysis	26.2 ± 3.2	28.5 ± 7.3[d, e]	15.2 ± 1.4[d]	17.5 ± 1.9[c,d]

[a] Mean ±*SD*, $n = 6$ except pregnant-heparin group where $n = 5$. [b] $n = 4$ since CSF could not be obtained from one or two animals. [c] $n = 5$ due to loss of sample or inadequate plasma volume. [d] The method of protein binding measurement had a significant effect on free fraction values, as determined by two-way ANOVA for pregnant and nonpregnant rats considered separately. The superscript indicates that the free fraction value differs significantly ($p < 0.05$ by Newman-Keuls test) from the CSF-plasma concentration ratio × 100. [e] One rat had only three fetuses and yielded unusually low free fraction values. Without that animal, the free fraction × 100 values for immediate ultrafiltration, ultrafiltration after 6 h, and equilibrium dialysis were 25.9 ± 4.3, 44.4 ± 4.5, and 31.3 ± 2.6, respectively.

Table II—Concentrations of Free Fatty Acids in Plasma of Pregnant and Nonpregnant Rats: Effect of Ultrafiltration and *In Vitro* Anticoagulant [a]

	Pregnant Rats		Nonpregnant Rats	
	EDTA	Heparin	EDTA	Heparin
Palmitic Acid[b]				
0 h	196 ± 48	179 ± 38	167 ± 26	152 ± 19
6 h	371 ± 119	792 ± 67	183 ± 29	260 ± 29
Ratio, 6 h/0 h	1.94 ± 0.58	4.54 ± 0.79	1.10 ± 0.14	1.75 ± 0.37
Stearic Acid				
0 h	58.8 ± 26.8	54.9 ± 23.6	51.0 ± 14.3	49.2 ± 9.9
6 h	89.4 ± 28.1	156 ± 27	64.6 ± 18.4	95.6 ± 24.5
Ratio, 6 h/0 h	1.65 ± 0.50	3.12 ± 0.93	1.42 ± 0.73	2.05 ± 0.73
Oleic Acid				
0 h	152 ± 78	130 ± 53	127 ± 44	118 ± 37
6 h	321 ± 137	743 ± 110	127 ± 28	209 ± 37
Ratio 6 h/0 h	2.27 ± 0.68	6.25 ± 1.76	1.09 ± 0.44	1.97 ± 0.81
Linoleic Acid				
0 hr	77.5 ± 45.4	65.7 ± 27.0	67.7 ± 26.0	66.1 ± 23.1
6 h	178 ± 88	379 ± 74	89.2 ± 38.5	145 ± 31
Ratio 6 h/0 h	2.49 ± 0.91	6.28 ± 1.84	1.37 ± 0.49	2.45 ± 0.99
Total fatty acids[c]				
0 h	484 ± 196	430 ± 125	422 ± 122	385 ± 87
6 h	960 ± 364	2071 ± 578	463 ± 105	709 ± 94
Ratio 6 h/0 h	2.08 ± 0.62	5.10 ± 1.20	1.14 ± 0.32	1.95 ± 0.62

[a] Mean ±*SD*, $n = 6$ except pregnant-heparin group where $n = 5$. All concentrations are expressed as μM/L. [b] Assayed immediately after blood collection (0 h) or after 6 h incubation of the plasma at 37°C, before ultrafiltration (6 h). [c] Based on the sum of the concentrations of the four individual free fatty acids.

and then a continuous infusion of phenytoin, 195 μg/min/kg, through a jugular vein cannula. Only saline solution without anticoagulant was used to maintain cannula patency before the experiment. CSF and blood (for plasma) from the abdominal aorta were obtained at 2 h after the beginning of the infusion and were assayed for phenytoin by HPLC. Details of these procedures have been described previously (5). The blood was collected in a plastic syringe[2] containing either heparin[3] or EDTA in amounts such that their concentrations in the blood were ~20 U/mL or 2 mg/mL, respectively. The syringes had been dried overnight at 37°C to remove the solvent from the anticoagulant solutions. Plasma was separated immediately after blood collection by centrifuging at 15,600×*g* for 1 min. Two microliters were ultrafiltered (3) immediately at 37°C to yield ≃ 90 μL of filtrate, and another portion of the plasma was similarly ultrafiltered after 6-h incubation (in a capped glass vial) in a waterbath at 37°C. A third portion of the plasma was used for equilibrium dialysis at 37°C for 6 h against an equal volume of isotonic sodium-potassium phosphate buffer, pH 7.4 (5). The cellophane membrane[4] used for these procedures had a pore permeability cut-off of between 12,000 and 14,000 Da. Phenytoin concentrations in the protein binding experiments were measured by an HPLC method, with a detection limit of ~1 μg/mL (5).

One hundred-microliter portions of plasma were taken before and after incubation for analysis of free fatty acid concentrations. The "before incubation" sample was extracted into hexane within 5 min after blood collection to minimize *in vitro* lipolysis. Fatty acid concentrations were determined by GC after extraction and derivatization as described by others (6) except that *n*-heptadecanoic acid was used as the internal standard and sample volumes were reduced.

To determine the effect of *in vitro* addition of free fatty acids to serum on phenytoin protein binding, 200 μL of an ethanolic solution of fatty acids (palmitic, ≃40 mM; stearic, ≃8 mM; oleic, ≃60 mM; linoleic, ≃25 mM) was added to 20 mL of pooled rat serum (from 10 nonpregnant female animals) containing added phenytoin, 23 μg/mL. Another portion of that serum with only ethanol added was used as a control. Protein binding of phenytoin was determined by ultrafiltration at 37°C of seven aliquots of each pooled serum portion.

RESULTS

The results of the phenytoin infusion studies on pregnant and nonpregnant rats are summarized in Table I. Based on the plasma concentrations, which should be at steady state under the experimental conditions (5), the total clearance of phenytoin was significantly lower in the pregnant rats (12.2 ± 2.0 mL/min/kg; mean ±*SD*) than in the nonpregnant rats (17.1 ± 4.9 mL/min/kg; $p < 0.01$). This was also the case for the total clearance of free drug (70.0 ± 14.9 *versus* 136 ± 35 mL/min/kg; $p < 0.001$) which was calculated on the basis of the *in vivo* free fraction of phenytoin (*i.e.*, CSF concentration–plasma concentration ratio). These results represent the combined data obtained from the EDTA and heparin experiments because of their excellent replication and the identical *in vivo* experimental conditions, and were analyzed statistically by the unpaired *t* test.

The CSF-plasma concentration ratio of phenytoin was significantly higher in pregnant than in nonpregnant rats ($p < 0.001$ by *t* test for the combined EDTA and heparin data). There was excellent agreement between this ratio and the *in vitro* phenytoin free fraction in plasma from nonpregnant rats that was ultrafiltered within 5 min after blood collection (Table I). On the other hand, the *in vitro* free fraction of phenytoin in plasma from pregnant rats, determined under the same conditions, was about one-third higher than the CSF-plasma concentration ratio in these animals. Free fraction values of phenytoin determined by ultrafiltration after incubating plasma from pregnant rats for 6 h at 37°C were even higher. Equilibrium dialysis for 6 h at 37°C also yielded appreciably higher free fraction values than the *in vivo* estimates (CSF-plasma concentration ratio), for both pregnant and nonpregnant rats. The statistical significance of these differences are indicated in Table I.

Table II contains a summary of the results of the assays of the plasma samples for palmitic, stearic, oleic, and linoleic acids. The concentrations of these endogenous free fatty acids in plasma assayed within 5 min after blood

[2] Plastipak; Becton, Dickinson and Co., Rutherford, N.J.
[3] Heparin from beef lungs, 1000 U/mL solution; The Upjohn Co., Kalamazoo, Mich.
[4] Visking; Union Carbide Corp., Chicago, Ill.

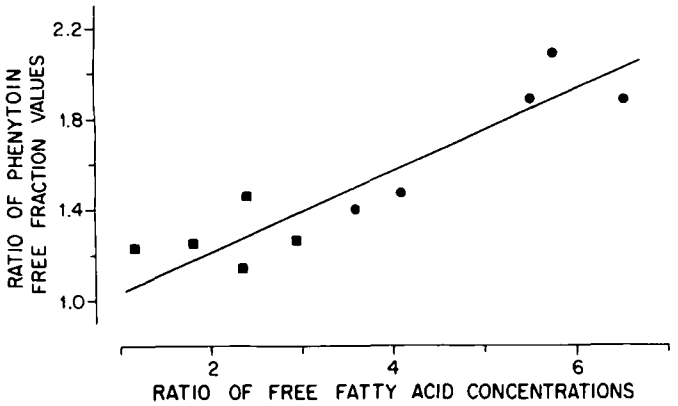

Figure 1—*Relationship between the ratios of the phenytoin free fraction values obtained by ultrafiltration (after 6 h incubation at 37°C-immediately, without incubation) and the ratios of the total free fatty acid concentrations (measured concomitantly in plasma of rats at day 20 of gestation) when either EDTA (■) or heparin (●) was used as the* in vitro *anticoagulant (r = 0.90, p < 0.001).*

collection were similar in pregnant and nonpregnant animals, irrespective of the anticoagulant used. However, heparin-anticoagulated plasma underwent pronounced lipolysis during 6 h of incubation, particularly plasma from the pregnant rats. Total free fatty acid concentrations increased by an average of 410% in plasma from pregnant rats and 95% in plasma from nonpregnant rats during incubation. These changes were much smaller in EDTA-anticoagulated plasma: 108% and 14%, respectively. The relative increase in fatty acid concentrations was associated with a corresponding relative increase in the free fraction of phenytoin in plasma from pregnant rats (Fig. 1). Addition of a mixture of the four free fatty acids, similar in proportion to that found *in vivo*, to serum from nonpregnant rats caused an increase of phenytoin free fraction values similar in relative magnitude to that found on lipolysis (Fig. 2).

DISCUSSION

The therapeutic management of pregnant patients with seizure disorders is difficult (7). The most frequently used drug for this purpose is phenytoin. Pregnancy may be associated with alterations in the pharmacokinetics of phenytoin and in the phenytoin concentration–effect relationship. Investigations of these possibilities in patients and in intact animals must be designed to distinguish between apparent effects referable to pregnancy-associated

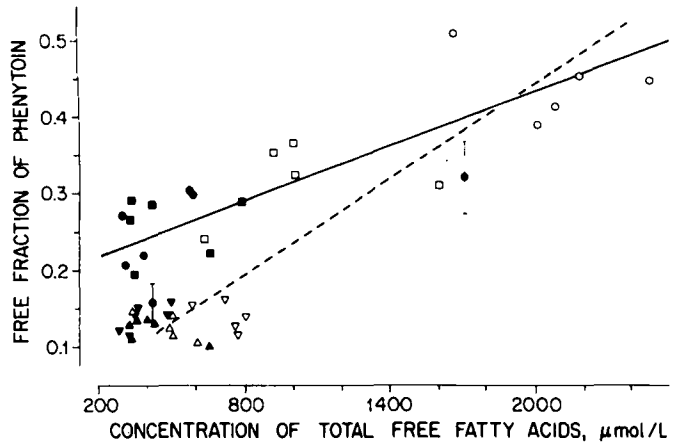

Figure 2—*Relationship between free fraction values of phenytoin and concentration of total free fatty acids in plasma. The lines were determined by bivariate linear regression. The two closed circles with the vertical bars (±SD) are the free fraction values obtained with serum from nonpregnant rats with and without addition of a physiological mixture of free fatty acids. Key: (——) relationship for pregnant rats at day 20 of gestation (r = 0.85, p < 0.001); (- - -) relationship for both nonpregnant and pregnant rats after incubation of plasma for 6 h at 37°C (r = 0.86, p < 0.001); (■,□) plasma from pregnant rats with EDTA as anticoagulant; (●,○) plasma from pregnant rats with heparin as anticoagulant; (▲,△) plasma from nonpregnant rats with EDTA as anticoagulant; (▼,▽) plasma from nonpregnant rats with heparin as anticoagulant. Closed symbols: before incubation; open symbols: after 6-h incubation at 37°C.*

decreased plasma protein binding of phenytoin and actual changes in intrinsic clearance of the drug and in the relationship between biophasic drug concentration and pharmacological response. It became evident in the course of such an investigation (8) that the protein binding of phenytoin in plasma from pregnant rats undergoes rapid and pronounced alterations *in vitro* during the time between blood collection and plasma protein binding determination (3). The present study was designed to determine the cause of these alterations and to explore possibilities for minimizing them.

An important, although incidental, result of this investigation is the demonstration of a decreased steady-state plasma clearance of phenytoin by pregnant as compared with nonpregnant rats. The clearance of free (unbound) phenytoin in nonpregnant (control) animals was about twice as high as that in pregnant rats when both groups of animals received infusions of the drug at the same rate per unit of body weight. Since phenytoin pharmacokinetics are nonlinear and subject to product inhibitory effects in rats (9, 10), quantitatively different results may have been obtained if the clearance determinations had been made at comparable plasma concentrations rather than at comparable infusion rates. The results of more detailed and extensive studies of the effect of pregnancy on phenytoin elimination kinetics in rats will be presented in another report and a discussion of the subject will, therefore, be deferred.

Unbound phenytoin in plasma equilibrates readily with phenytoin in CSF; the CSF–plasma concentration ratio of the drug represents, therefore, its free fraction value in plasma under *in vivo* conditions (5). It was found that the free fraction of phenytoin in rat plasma is substantially (≃50%) increased in late pregnancy. This result is not a consequence of the higher phenytoin concentrations in the pregnant animals, since the protein binding of phenytoin in rat serum is independent of concentration over a wide range (11). Nor are these *in vivo* results subject to uncertainties due to the technical problems and possible artifacts associated with *in vitro* protein binding determinations.

The ability of nonesterified (free) fatty acids to affect (usually decrease) the plasma protein binding of drugs is well established (12). Recent research emphasis has been on the elevation of fatty acid concentrations in plasma after systemic administration of heparin; this elevation is now recognized to be largely an *in vitro* artifact due to continuing lipolysis in plasma during the time between blood collection and the determination of fatty acid concentrations (13, 14). While injected heparin mobilizes and activates lipases which then cause formation of free fatty acids from triglycerides in plasma, even plasma from individuals (humans and rats) who have not received heparin contains appreciable lipolytic activity (15–17). Consequently, the concentrations of free fatty acids increase *in vitro* even in plasma of individuals who have not been treated with heparin (4, 18–22), albeit at a much lower rate than in post-heparin plasma.

The free fatty acids that accumulate in plasma *in vitro* derive from triglycerides as well as from lecithin (19, 23, 24). The plasma concentrations of these sources of fatty acids increase substantially during pregnancy (25), thereby increasing both the absolute rate and the extent of *in vitro* free fatty acid formation in pregnancy plasma. Such increase apparently does not occur to any significant extent *in vivo*, probably due to distribution of the formed free fatty acids from blood to extravascular sites and metabolic clearance. Neither distribution nor clearance occur *in vitro* so that accumulation of free fatty acids in plasma can be substantial under *in vitro* conditions.

The four fatty acid concentrations quantitatively determined in this investigation (palmitic, stearic, oleic, and linoleic acids) account for most (≃90%) of the fatty acids in human plasma (20, 26), and the sum of their concentrations is probably a reasonable estimate of total fatty acid concentration in rat plasma. It is desirable, however, to determine the concentrations of these fatty acids individually, because they may have quantitatively different displacing effects on drugs bound to plasma proteins. By the same token, confirmatory protein binding experiments involving *in vitro* addition of free fatty acids to plasma should be carried out with fatty acid mixtures with a composition and ratio of concentrations similar to that found *in vivo*. The effect of free fatty acids on the plasma protein binding of different drugs is a function of the capacity of the binding sites, the relative concentrations and affinity of the ligands, and other variables. Consequently, the rate and extent of protein binding alterations of phenytoin reported here apply to that drug only.

The results of this investigation show that the concentrations of free fatty acids in plasma from nonpregnant rats increase about twofold within 6 h at 37°C, *i.e.*, under conditions typical of equilibrium dialysis, if heparin is used as the *in vitro* anticoagulant. This lipolysis is almost prevented by use of EDTA instead of heparin. Total free fatty acid concentrations in heparinized plasma from pregnant rats increase about fivefold during 6 h at 37°C; use of EDTA instead of heparin reduces this increase to twofold. When EDTA and heparin are used together, the extent of lipolysis is similar to that in heparinized plasma[5]. This is consistent with reports that EDTA is not a good lipoprotein

[5] Unpublished results.

lipase inhibitor (14, 27) and indicates that *in vitro* heparin increased lipolytic activity, particularly in pregnancy plasma, under our experimental conditions. According to the literature, heparin may activate or inhibit lipases, depending on its concentration (28, 29). To significantly reduce the *in vitro* accumulation of free fatty acids in plasma, it will be necessary to inhibit not only the lipolysis of triglycerides but also the formation of fatty acids from phospholipids (23, 24).

The rapid and pronounced *in vitro* lipolysis in the plasma of pregnant rats can easily lead to artifactual overestimation of *in vivo* free fatty acid concentrations. One group of investigators reported total plasma free fatty acid concentrations of $\simeq$360, 565, and 848 μM in heparinized plasma from nonpregnant rats, rats at day 19 of gestation, and rats at day 21 of gestation, respectively (30). The control value is similar to that in this study ($\simeq$400 μM), but the values for the pregnant rats are substantially higher than that for rats on day 20 of gestation determined in this study ($\simeq$430 μM) under conditions that minimize *in vitro* lipolysis. When proper precautions are taken to avoid artifacts, there appears to be no appreciable difference in plasma free fatty acid concentrations of pregnant and nonpregnant rats. This is also true for humans (26).

While plasma free fatty acid concentrations are of direct biochemical and physiological interest, their significance in pharmacology and pharmacokinetics relates principally to their effect on the plasma protein binding of drugs (12, 31). This effect is clearly evident in the strong correlation between the relative increase in the phenytoin free fraction and the relative increase of total free fatty acid concentrations in plasma found in the present investigation (Fig. 1). Moreover, direct addition of a physiological mixture of fatty acids to serum from nonpregnant rats, thereby duplicating the elevation of fatty acid concentrations found in the plasma of pregnant rats after 6 h of incubation at 37°C, results in an increase of the phenytoin free fraction to a value similar to that of the incubated pregnancy plasma (Fig. 2).

It is easy to come to the erroneous conclusion that pregnancy-associated changes of *in vivo* plasma protein binding of phenytoin (or other drugs) are due to the displacing effect of increased concentrations of free fatty acids. The dashed regression line in Fig. 2 represents the relationship between phenytoin free fraction value and total free fatty acids concentration in plasma from both pregnant and nonpregnant rats when that plasma had been incubated for 6 h at 37°C (*i.e.*, under conditions typical of equilibrium dialysis). The correlation is strong ($r = 0.86$) and highly significant ($p < 0.001$), but the conclusion is incorrect because it is based on inappropriate experimental conditions.

The solid symbols in Fig. 2 demonstrate that, when *in vitro* lipolysis is minimized, the protein binding of phenytoin is much less pronounced in plasma of pregnant rats than in plasma of nonpregnant controls at comparable free fatty acid concentrations. Viewed from another perspective, *in vivo* plasma protein binding of phenytoin is much less in pregnant than in nonpregnant rats (Table I), even though fatty acid concentrations are similar in the two groups when measured under conditions that minimize *in vitro* lipolysis (Table II). The notion that the diminished *in vivo* plasma protein binding of drugs during pregnancy is due to elevated fatty acid concentrations is, therefore, untenable. Lower plasma albumin concentrations during pregnancy (2, 32), possible accumulation of endogenous displacers other than fatty acids, and possible conformational changes of albumin are more likely causes of decreased plasma protein binding of albumin-bound drugs during pregnancy (11).

The *in vitro* lipolysis and corresponding changes in drug–protein binding demonstrated here with respect to plasma from rats in late pregnancy have also been investigated in humans (4, 33). The degree of *in vitro* lipolysis in plasma obtained from pregnant women during 6 h of equilibrium dialysis was, generally, somewhat less pronounced than in plasma from pregnant rats while the protein binding changes associated with the increased fatty acid concentrations ranged from negligible (phenytoin), to modest (salicylate, ibuprofen), to pronounced (diazepam). Rapid *in vitro* lipolysis may also occur in plasma from other species and in other states of altered physiology. It will be necessary to identify or rule out these factors in investigations of drug kinetics and dynamics in disease states.

Unfortunately, an *in vivo* estimation of plasma protein binding based on the steady-state CSF-plasma concentration ratio is not always feasible, because not all drugs equilibrate to equal concentrations in CSF and plasma water (5). Rapid separation of plasma after blood collection (which is facilitated by use of a high speed centrifuge), use of EDTA (or other suitable compounds) rather than heparin as the *in vitro* anticoagulant (provided that EDTA does not interfere with the drug–protein binding), and rapid ultrafiltration will minimize *in vitro* lipolysis and thereby reduce the likelihood of artifactual protein binding estimates. Particularly, if ultrafiltration is not feasible due to membrane binding or other technical problems and equilibrium dialysis must be used, determinations of fatty acid concentrations in plasma immediately after blood collection and again after dialysis (33) will provide an indication of the magnitude of *in vitro* lipolysis under the experimental conditions.

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