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Ultrafiltration in Serum Protein Binding Determinations

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Abstract □ The ultrafiltration technique was evaluated theoretically and experimentally for use in clinical serum binding determinations. It is apparent from free energy considerations that the ultrafiltrate concentration approaches the true free concentration only as the pressure gradient causing flow reduces to zero. The theory presented accounts for the previously unexplained lower ultrafiltrate concentration observed at higher filtration pressures. Mathematical simulations of the molecular separation show that the ultrafiltrate concentration remains constant during filtration, and, thus, binding equilibria are not disturbed by this procedure, suggesting that an arbitrary restriction on the volume filtered is unnecessary. This finding greatly extends the value of the ultrafiltration technique in clinical binding determinations, especially for strongly bound, potent drugs where assays may be insufficiently sensitive to detect the extremely small free fractions reliably. These theoretical findings were verified experimentally by ultrafiltration of salicylate, ibuprofen, and carprofen in buffer, purified proteins, and whole serum.

Keyphrases □ Ultrafiltration—evaluation for use in clinical serum binding determinations □ Binding—evaluation of ultrafiltration technique for use in clinical serum binding determinations □ Salicylate—determination of serum binding by ultrafiltration □ Ibuprofen—determination of serum binding by ultrafiltration

Interest in the influence of plasma binding on drug disposition is increasing. The extent of binding partially controls drug distribution between the blood and extravascular fluids (1, 2) and may profoundly affect both hepatic and renal clearance (3, 4). Furthermore, there is ample evidence that the free fraction of drug may be substantially altered postoperatively (5), in the elderly (6), and following stress and disease (7) and, for certain drugs, may differ considerably with the plasma concentration and between individuals (8, 9).

BACKGROUND

Reliable routine methodology for estimating the fraction of free drug in plasma and whole blood is needed. Ultrafiltration appears to be more appropriate than dialysis techniques because it can be carried out rapidly without storage or addition of potentially competitive buffer components and electrolytes. The speed with which the free fraction can be estimated after sample collection is particularly important since the levels of fatty acids produced by lipolysis of triglycerides increase on storage (10) and during dialysis (11). Nonesterified fatty acids are known to decrease the binding of drugs *in vitro* (12) and *in vivo* (13).

Among the generally recognized limitations of the ultrafiltration technique are the polarization of protein on the membrane, the uptake of small molecules by the membrane, and the change in the protein concentration with the volume filtered. Polarization may be minimized by stirring, and membrane binding may be assessed independently. However, the influence of filtration pressure in selectively altering the transport of solvent and drug molecules is not widely appreciated. Furthermore, in estimating the extent of binding by molecular filtration, it has become accepted practice to ultrafilter only a small fraction of the total sample (often <10%) to avoid disturbance of the protein binding equilibria (14–17). The subsequent difficulty in estimating extremely small amounts of ultrafiltered drug (18) often presents insurmountable analytical problems, particularly with strongly bound, low serum concentration drugs.

This report discusses two important aspects of ultrafiltration. First, the influence of filtration pressure on the ultrafiltrate drug concentration is examined theoretically. The theory presented accounts for the previously unexplained dilution of the ultrafiltrate observed at higher filtration pressures by Spector *et al.* (19). Second, it is shown theoretically and

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experimentally that arbitrary restriction of the volume fraction ultrafiltered is unnecessary and that the equilibrium position and ultrafiltrate concentration are unaltered, even when the protein concentration increases almost twofold. This finding greatly extends the value of ultrafiltration, particularly for clinical monitoring of binding.

THEORY

Free Energy Considerations: Influence of Pressure—Modern ultrafiltration membranes do not contain pores of uniform dimensions that allow the passage of small molecules and retain the much larger binding species. The mechanism of transfer and retention is unclear. However, their characteristic of allowing free passage of the solvent and yet, at the same time, reflecting some small solute molecules (16, 20) is analogous to transfer of the drug and the solvent through traditional membranes containing heterogeneous pores and suggests the applicability of pore theory to the newer system. These membranes may even permit the passage of a small, but usually negligible, fraction of the macromolecule.

Consider a reversible interaction at equilibrium occurring in solution between a drug and protein at constant temperature and pressure. The reaction mixture is in contact with a selectively permeable membrane prior to ultrafiltration to determine the bound and free ligand concentrations (Scheme I).

$$[P] + [L] \rightleftharpoons [PL]$$

Scheme I

In Scheme I, $[P]$, $[L]$, and $[PL]$ are the protein, drug, and bound drug concentrations, respectively. Protein molecules with and without bound drug are unable to penetrate the membrane and, therefore, do not take part in the ultrafiltration process. The ultrafiltrate thus is produced from the interstitial solution in which the macromolecules are dispersed.

If the reaction mixture contains m_L and m_S moles of free drug and solvent, respectively, and the pressure on it is raised to $1 + \Delta P$ atmospheres (atm), a small volume of ultrafiltrate may be collected at 1 atm due to the pressure difference across the membrane of ΔP. The ultrafiltrate contains dm_L and dm_S moles of drug and solvent, respectively, and the solute concentration is not necessarily the same as in the reaction mixture:

$$\frac{dm_L}{dm_S} \neq \frac{m_L}{m_S} \qquad \text{(Eq. 1)}$$

When the pressure on the reaction mixture returns to 1 atm, then the free energy (ΔG_1) expended on the system to produce the ultrafiltrate is given by (21):

$$\Delta G_1 = (\bar{v}_L m_L + \bar{v}_S m_S)\Delta P \qquad \text{(Eq. 2)}$$

where $\bar{v}_L$ and $\bar{v}_S$ are the partial molal volumes of the free drug and solvent in the reaction mixture, respectively.

This free energy (ΔG_1) associated with the pressure applied to accelerate filtration is available to produce changes in the composition of the ultrafiltrate compared to the composition in the interstitial solution of the reaction mixture.

On the other hand, the free energy change required to convert an infinitesimal volume of the original reaction mixture to ultrafiltrate (ΔG_2) is given by (21):

$$\Delta G_2 = RT\left(dm_L \ln \frac{a_{L,uf}}{a_{L,rm}} + dm_S \ln \frac{a_{S,uf}}{a_{S,rm}}\right) \qquad \text{(Eq. 3)}$$

where T is the absolute temperature, R is the gas constant, and a_{uf} and a_{rm} are the atmospheric activities of the components of the ultrafiltrate and the reaction mixture, respectively. When the composition of the ultrafiltrate changes during filtration because the membrane is penetrated more readily by the solvent than the drug, ΔG_2 is positive. When the ultrafiltrate drug concentration is identical to the free drug concentration in the reaction mixture, it follows from Eq. 3 that ΔG_2 equals zero. Thus, to produce a true ultrafiltrate containing a drug concentration equal to the free concentration in the protein solution, pressure must be applied that is sufficient just to produce a finite flow rate but insufficient to supply free energy to change the ultrafiltrate composition.

Therefore, although ΔG_1 must be greater than ΔG_2, the ultrafiltrate drug concentration represents the free concentration most closely as the pressure gradient approaches zero.

Thus, if the membrane discriminates between solvent and drug molecules, whether on the basis of charge or dimension, then the drug concentration in the ultrafiltrate will not equal the free concentration in the original reaction mixture. The deviation will be an increasing function of the pressure gradient. This phenomenon may be explained by considering the events occurring at a particular channel through which only the solvent is able to pass. If the filtration pressure is greater than the osmotic pressure of the reaction mixture, then the solvent will pass through this pore and dilute the ultrafiltrate. In practice, this deviation may occur at pressures smaller than the osmotic pressure of the reaction mixture. The ultrafiltrate is in diffusional contact with the reaction mixture, and, thus, the pressure at which ultrafiltrate dilution occurs is simply the osmotic gradient between the reaction mixture and the ultrafiltrate.

Mass Balance Considerations: Influence of Volume Change—The effect of the protein and drug concentration changes that take place as a result of the reduction in volume and loss of drug in the ultrafiltrate is now considered.

Drug–protein interactions (Scheme I) often can be described by equations derived from the law of mass action. Thus, for a single class of binding sites:

$$\bar{\nu} = \frac{nKm_L}{V + Km_L} \qquad \text{(Eq. 4)}$$

where $\bar{\nu}$ is the average drug–protein molar binding ratio and n is the number of sites on each protein molecule of association constant K. The volume of the reaction mixture is V, and m_L is as defined previously.

It is assumed here that the ultrafiltration membrane acts as a perfect molecular sieve, completely retaining the macromolecule and neither binding nor reflecting the smaller drug molecule, such that when pressure is applied, its passage across the membrane, like that of the solvent, is unimpeded.

In the situation where a small volume (dV) of solution containing dm_L moles of free drug is ultrafiltered through the membrane, if the new average molar binding ratio is denoted by $\bar{\nu}'$, then:

$$\bar{\nu}' = \frac{nK(m_L - dm_L)}{(V - dV) + K(m_L - dm_L)} \qquad \text{(Eq. 5)}$$

This expression can be rearranged:

$$\frac{\bar{\nu}'(V - dV)}{K} = n(m_L - dm_L) - \bar{\nu}'(m_L - dm_L) \qquad \text{(Eq. 6)}$$

Hence:

$$\frac{\bar{\nu}'}{K} = \frac{n(m_L - dm_L)}{V - dV} - \frac{\bar{\nu}'(m_L - dm_L)}{V - dV} \qquad \text{(Eq. 7)}$$

If the membrane does not differentiate between solvent and ligand molecules, then:

$$\frac{m_L - dm_L}{V - dV} = \frac{m_L}{V} \qquad \text{(Eq. 8)}$$

Substitution in Eq. 7 and rearrangement give:

$$\bar{\nu}' = \frac{nKm_L}{V(1 + Km_L/V)} = \bar{\nu} \qquad \text{(Eq. 9)}$$

It is apparent that the molar binding ratio remains constant throughout ultrafiltration, even though the protein and total drug concentrations increase markedly. Because of this constancy in $\bar{\nu}$, the drug concentration in the ultrafiltrate also will be constant and will be the same as the equilibrium free concentration in the original reaction mixture. In the treatment of the ultrafiltration process, no limitation is placed on the fraction of original reaction mixture that may be filtered. Thus, $\bar{\nu}$ and $[L]$ remain constant and independent of the volume of sample filtered. This treatment assumes constancy of both n and K as filtration proceeds, which may not be true at very high protein concentrations due to protein conformational change or formation of dimers or high-order macromolecular aggregates.

Similar reasoning leading to the same conclusions can be applied to more complex reaction mixtures consisting of many binding species, including proteins with more than one class of binding sites.

EXPERIMENTAL

[^{14}C]Ibuprofen[1] (specific activity 13.11 ± 0.19 μCi/mg) and [^{14}C]carprofen[2] (specific activity 13.47 ± 0.34 μCi/mg) were used as received or diluted with unlabeled material. [^{14}C]Salicylic acid[3] was diluted with

[1] Boots Pure Drug Co. Ltd., Nottingham, England.
[2] Roche Pharmaceuticals, Sydney, Australia.
[3] New England Nuclear, Boston, Mass.

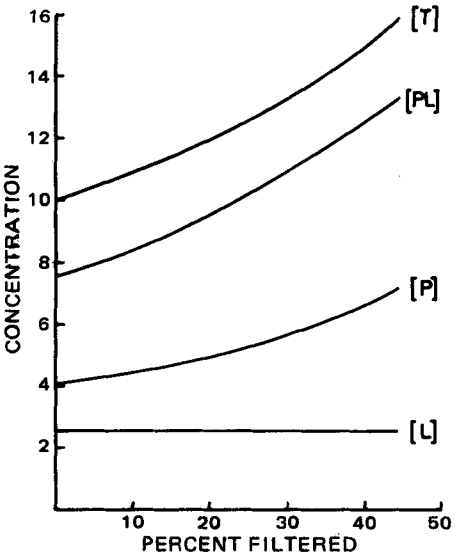

Figure 1—*Simulation of ultrafiltration process for hypothetical drug of binding constants* $K_1 = 10^4\,M^{-1}$ ($n_1 = 1$) *and* $K_2 = 10^2\,M^{-1}$ ($n_2 = 7$), *showing changes in the total ([T], micrograms per milliliter* $\times 10^{-1}$), *bound ([PL], micrograms per milliliter* $\times 10^{-1}$), *free ([L], micrograms per milliliter* $\times 10^{-1}$), *and protein ([P], gram percent) concentrations with the percent volume of the reaction mixture filtered.*

unlabeled compound to a specific activity of 0.144 ± 0.004 μCi/mg. Solutions were analyzed by counting ^{14}C-labeled drug in Bray's solution to a standard deviation of <3.5% in a liquid scintillation spectrometer[4]. Correction for quenching was made using an automatic external standard technique. Quinidine sulfate[5] was assayed by fluorescence spectroscopy[6] using excitation and emission wavelengths of 245 and 375 nm, respectively.

Bovine serum albumin[7] and human serum albumin[8] were reconstituted with 0.033 *M* phosphate buffer (pH 7.4). Whole human serum was collected by venipuncture from young healthy volunteers and used immediately.

Real ultrafiltration data were obtained at ambient temperature using an 80-ml polycarbonate cell fitted with a molecular filtration membrane[9] with a surface area of 17.4 cm^2 stated to retain molecular weights of $>10^4$.

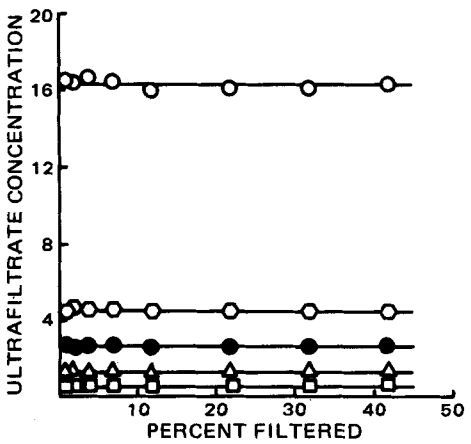

Figure 2—*Variation of the ultrafiltrate concentration (micrograms per milliliter* $\times 10^{-1}$) *with the percent volume filtered for salicylate in 4% bovine serum albumin (solid symbol) or whole human serum (open symbols). The initial total concentrations were 387 (○), 187 (○), 95 (△), 48 (□), and 194 (●) μg/ml.*

[4] Hewlett-Packard model 3375.
[5] BDH Laboratory Chemicals Ltd., Poole, England.
[6] Perkin-Elmer model 204.
[7] Fraction V, Sigma Chemical Co., St. Louis, Mo.
[8] Fraction V equivalent, Behringwerke AG, Marsburg, West Germany.
[9] PTGC 04710, Millipore Corp., Bedford Mass.

Solutions (25 ml) were stirred at 40 rpm during filtration under nitrogen at 10 psi. Filtrates were checked periodically for protein leakage using trichloroacetic acid. Since these membranes are quite robust, new membranes were used for each drug or macromolecular solution. Between ultrafiltrations of different concentrations of the same drug, the membrane was rinsed in several changes of phosphate buffer (pH 7.4) and 100 ml of the drug-free buffer was filtered.

The extent of drug binding to the membrane was evaluated by ultrafiltration of drugs dissolved in phosphate buffer (pH 7.4) and assay of successive aliquots of the ultrafiltrate. Concentrations were those that could reasonably be expected to be found free in serum clinically.

Ultrafiltration simulations were carried out on a programmable calculator[10] for hypothetical drugs of known binding constants. The filtration membrane was assumed to be impervious to the macromolecule and neither reflected nor bound the drug. In addition, the pressure gradient producing hydrodynamic flow was assumed to be infinitesimally small, such that any pressure artifact could be neglected. The Scatchard equation:

$$\bar{\nu} = \frac{[T] - [L]}{[P]} = \sum_{i=1}^{i=i} \frac{n_i K_i [L]}{1 + K_i [L]} \qquad \text{(Eq. 10)}$$

where $[T]$ is the total drug concentration, $[P]$, $\bar{\nu}$, n_i, K_i, and $[L]$ are as defined previously, and $i = 2$, was used to calculate the free drug concentration and, hence, the ultrafiltrate concentration for any particular total drug concentration. The algorithm for this iterative calculation was reported previously (22). The ultrafiltration process thus was simulated by allowing the unfiltered volume to decrease by small increments. Mass balance adjustments were made sequentially to account for loss of the drug to the ultrafiltrate, the reduced volume, and the increased protein concentration in the reaction mixture. Hypothetical free drug concentrations were calculated after each increment of filtered volume. Simulations were carried out for several hypothetical drugs with wide ranging binding constants.

RESULTS AND DISCUSSION

Influence of Pressure—Spector *et al.* (19) observed variations of 40–75% in unbound digoxin and of 80–97% in unbound ouabain when ultrafiltration was carried out at pressures ranging from 45 to 2 psi. They were unable to explain these findings; however, it was suggested that partial blocking of the filter pores or polarization of the proteins on the filter at higher pressures might have contributed to the artifact.

A more likely explanation is that molecules of the solvent and free drug cross the membrane at different rates, and this effect is exaggerated at higher pressures, as already described. This may be supported by the observations of Kurz *et al.* (16) that membrane reflection of drug molecules increases with molecular weight; thus, the dilution artifact would be greater for the ultrafiltration of larger molecules. This concept is consistent with the data of Spector *et al.* (19) showing that the pressure effect was much greater for digoxin (mol. wt. 781) than for ouabain (mol. wt. 585).

Influence of Volume Change—The data presented in Fig. 1 are typical of all of the simulation data generated and are for the filtration of a drug with binding constants $K_1 = 10^4\,M^{-1}$ ($n_1 = 1$) and $K_2 = 10^2\,M^{-1}$ ($n_2 = 7$). As filtration proceeds, the total, bound drug, and protein concentrations increase markedly, but the free concentration appearing in the filtrate and, thus, the concentration ratio of bound drug to protein ($\bar{\nu}$) remain constant. This finding is of substantial relevance in clinical binding estimations since estimates of the free concentration are independent of the filtered volume.

Ultrafiltration data for various salicylate concentrations from 4% bovine serum albumin and whole human serum (Fig. 2) provide an experimental verification of the simulations. Experimentally obtained ultrafiltrate concentrations remained constant and independent of the volume fraction of the protein solution filtered up to 42% of the total volume. In these data, the percent filtered was limited to 42%; however, this limit is somewhat arbitrary. Beyond ~40% filtered, the serum became quite viscous and filtration slowed. However, if necessary, a greater fraction can be filtered. These findings are consistent with the theory. Not only does ultrafiltration not disturb binding equilibria, but a large fraction of serum may be ultrafiltered, when necessary, to compensate for a lack of assay sensitivity. Although the protein concentration was doubled throughout these experiments, this fact had no effect on the ultrafiltrate concentration, indicating the absence of any Donnan membrane error.

The ultrafiltrate concentration might be expected to deviate from the

[10] TI58, Texas Instruments, Dallas, Tex.

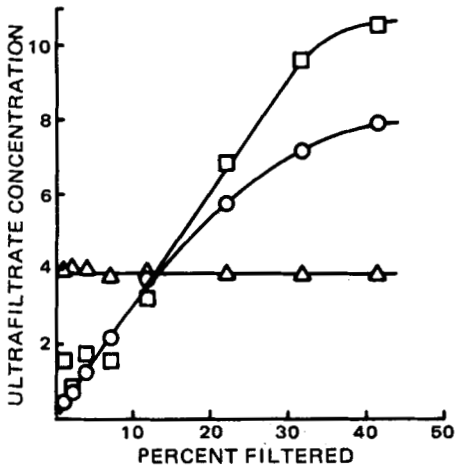

Figure 3—*Evaluation of membrane binding characteristics by filtration of drug in phosphate buffer (pH 7.4). Key for ultrafiltrate concentrations: △ salicylate (micrograms per milliliter × 10^{-1}); ○, ibuprofen (micrograms per milliliter × 10^2); and □, quinidine sulfate (micrograms per milliliter × 10). The initial concentrations were 38, 0.1, and 1.3 μg/ml, respectively.*

true free concentration if the drug binds extensively to the filtration membrane. Variable binding has been reported for various drugs and types of membranes. For example, binding was reported for phenytoin (18), salicylate (23, 24), and calcium ion (20). Some investigators reduced drug losses by minimizing the area of the membrane in contact with the reaction mixture (17). In other studies, urate (25), salicylate, acetazolamide (18), and calcium ion (26) were shown not to bind. Results from this laboratory indicate the strong binding of diazepam, quinidine sulfate, and ibuprofen to Millipore PTGC series membranes. Greater than 40% of the buffer solutions of ibuprofen and quinidine sulfate was filtered before the membrane became loaded and the ultrafiltrate concentration approached the initial drug concentration (Fig. 3). Uptake by the 17.4-cm^2 membrane was 0.02 μg of ibuprofen and 0.5 μg of quinidine sulfate/cm^2. However, this uptake would be expected to vary with the concentration of drug to be filtered if membrane binding obeys the law of mass action. Binding of salicylate to these membranes apparently is negligible (Fig. 3). Membrane binding sites probably are rapidly saturated by the much greater salicylate concentration used here to represent therapeutic free levels.

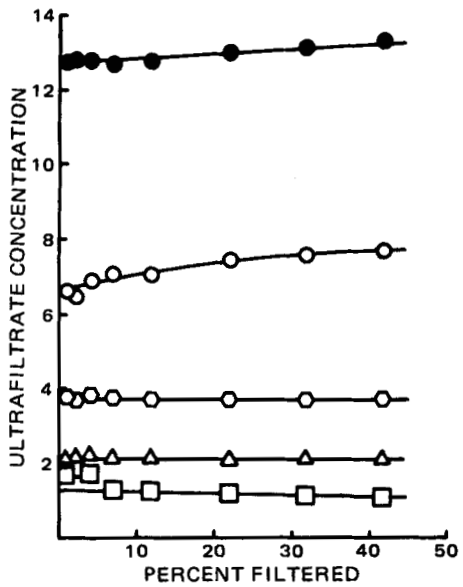

Figure 4—*Variation of ultrafiltrate concentration (micrograms per milliliter × 10^2) with the percent volume filtered for ibuprofen and carprofen in 4% bovine serum albumin (solid symbol) or whole human serum (open symbols). The initial total concentrations were 4.5 (○), 2.2 (○), 1.2 (△), and 15.0 (●) μg/ml for ibuprofen and 12.7 μg/ml for carprofen (□).*

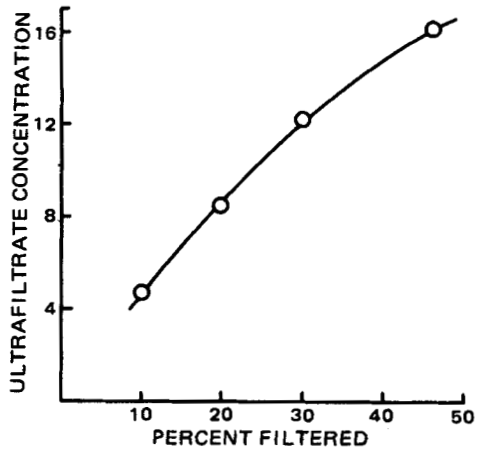

Figure 5—*Variation of the ultrafiltrate concentration (nanograms per milliliter) for diazepam in whole human serum at an initial total concentration of 400 ng/ml (Marianne J. Ridd, unpublished data).*

Data from the filtration of protein solutions also were obtained for the much more highly bound drug ibuprofen (Fig. 4). However, in this case, concentrations of 15.0 μg/ml in 4% bovine serum albumin and of 4.5 μg/ml in whole serum showed a slight increase in the ultrafiltrate concentration as filtration proceeded because of binding to new membranes. However, this artifact was not as great as would be predicted from the membrane binding data (Fig. 3) and would be negligible for most clinical purposes.

It is suggested that as free drug binds to the membrane, drug dissociates from the protein to reestablish the equilibrium. The net effect on the ultrafiltrate concentration thus is negligibly small when the amount of drug in the reaction mixture is much greater than that binding to the membrane. This reservoir effect may eliminate the need to correct for membrane uptake. Ultrafiltration of salicylate from 4% bovine serum albumin and whole human serum (Fig. 2), as expected, showed no membrane binding effect. It thus is probable that membrane binding is of practical importance only for extensively membrane-bound drugs where therapeutic plasma concentrations are sufficiently low that the reservoir effect is insufficient to saturate the membrane binding sites. Figure 5 shows ultrafiltration data for diazepam in whole serum at a concentration of 400 ng/ml. The marked membrane binding effect is evident from the fact that the ultrafiltrate concentration increased continuously with the volume filtered up to 45% of the 5-ml sample. At this low diazepam concentration, membrane sites are never saturated.

Table I—Comparison of Ultrafiltration with Other Methods for Measuring Protein Binding

Drug	Protein	Total Concentration, μg/ml	Free Concentration, μg/ml	
			Ultrafiltration	Other[a]
Salicylate	4% Bovine serum albumin	194.15	26.59	20.96[b]*
	Serum	187.62	44.26	42.90[c]
	Serum	94.93	12.31	14.70[c]
Ibuprofen	4% Bovine serum albumin	14.97	0.13	0.02[d]*
	1% Human serum albumin	24.35	0.61	0.29[e]*
Carprofen	1% Human serum albumin	12.71	0.013	0.019[f]*

[a] The asterisk indicates that the value was calculated using iterative algorithm (*cf.*, Ref. 22). [b] Dynamic dialysis: $n_1 = 1.18$, $K_1 = 2.10 \times 10^5\ M^{-1}$; and $n_2 = 5.03$, $K_2 = 1.67 \times 10^3\ M^{-1}$ (27). [c] Interpolation on serum binding profile obtained by equilibrium dialysis against plasma water (J. B. Whitlam and K. F. Brown, unpublished data). [d] Equilibrium dialysis: $n_1 = 0.92$, $K_1 = 1.37 \times 10^6\ M^{-1}$; and $n_2 = 6.66$, $K_2 = 1.94 \times 10^4\ M^{-1}$ (33). [e] Equilibrium dialysis: $n_1 = 0.80$, $K_1 = 2.73 \times 10^6\ M^{-1}$; and $n_2 = 6.27$, $K_2 = 1.95 \times 10^4\ M^{-1}$ (33). [f] Equilibrium dialysis: $n_1 = 3.53$, $K_1 = 1.39 \times 10^6\ M^{-1}$; and $n_2 = 6.31$, $K_2 = 1.28 \times 10^4\ M^{-1}$ (J. B. Whitlam and K. F. Brown, unpublished data).

The agreement between these ultrafiltration data and data obtained using dialysis procedures generally is good (Table I). Free concentrations obtained by both equilibrium dialysis and ultrafiltration of carprofen in 1% human serum albumin from a single batch are essentially identical. Free salicylate concentrations obtained by ultrafiltration of 4% bovine serum albumin solutions agree well with free concentrations obtained by calculation using binding parameters obtained previously by dynamic dialysis (27). Good agreement also was found in comparing serum free concentrations obtained by ultrafiltration with those obtained by equilibrium dialysis against plasma water.

Exact equivalence between methods would not be expected due to slight differences in experimental conditions. Different albumin batches may differ in their content of potentially displacing contaminants (28, 29). Thus, binding constants obtained for one system will not be universally applicable. In addition, binding parameter estimates may be substantially biased by the data reduction technique used (30–32). Binding constants for ibuprofen were obtained previously (33) using 1% albumin and then used in calculations for 4% albumin, assuming binding to be protein concentration independent. Protein concentration-dependent binding of drugs has been reported frequently (34, 35) and may provide a partial explanation for the observed discrepancies.

The observation that serum free concentrations determined by ultrafiltration are independent of the fraction of sample filtered should extend greatly the value of the technique. In clinical samples where free drug levels may approach the limit of detection, the ability to collect a large fraction of ultrafiltrate greatly improves the reliability of the analytical technique.

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