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# Free fraction change with differing volume ratios in equilibrium dialysis: cases of non-linear drug-protein binding

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When equilibrium dialysis is used to measure drug-protein binding, an increase in the volume ratio (buffer solution/protein solution) causes a decrease in the total drug concentration at equilibrium. When binding is non-linear, this decrease in total drug concentration also causes a decrease in the free fraction. By use of the same volume ratio (3 : 1) binding curves were obtained for the binding of prednisolone to plasma proteins, and for the binding of salicylic acid and tolmetin to human serum albumin (HSA). When the volume ratio was changed, the observed free fractions were appreciably different from those expected by taking into account only the changes in the total equilibrium drug concentration. There is evidence that this phenomenon is the result of competition for binding sites between the drug and an impurity in the HSA. The effects are not the result of the presence of free fatty acids.

When equilibrium dialysis is used to measure drug-protein binding, an increase in the volume of the buffer solution relative to the protein solution causes a decrease in the total equilibrium drug concentration (Behm & Wagner 1979). When the binding is non-linear, this decrease in the total drug concentration causes a corresponding decrease in the fraction of free (unbound) drug. Binding curves were obtained using 3 ml of buffer solution and 1 ml of plasma or protein solution characterizing the non-linear binding of three drugs to plasma proteins. Experiments were then performed using the same three drugs but with larger ratios of volumes of buffer solution to plasma or protein solution. The observed free fractions were compared to the expected free fractions from the binding curve (3 ml buffer solution to 1 ml plasma or protein solution) at the same total equilibrium drug concentration. The observed free fractions were appreciably different from those expected from the theoretical binding curve. Thus, the change in fraction free with an increase in the volume ratio cannot be totally attributed to changes in total equilibrium drug concentrations.

## MATERIALS AND METHODS

Equilibrium dialysis was used to measure drug-protein binding (Ağabeyoğlu et al 1979). All experiments were performed at 37 °C. Plasma was obtained from a normal volunteer. Two types of human serum albumin (HSA) were used for these studies—a 4% solution diluted from a 25% solution (Michigan Department of Health, Lansing), termed albumin I, and a 4% solution of fatty acid-free albumin (Sigma, St Louis, MO), termed albumin II. Phosphate buffers (0·1 M, pH 7·4) were used throughout.

The h.p.l.c. assay used for the quantification of prednisolone in plasma and albumin solutions was published previously by Ağabeyoğlu et al (1980). An Amicon MMC diafiltration system with a PM10 membrane was used to purify the albumin solutions. A Hewlett-Packard Model 8450-OA UV/VIS Spectrophotometer was used to measure u.v. absorbance.

The unlabelled and [$^{14}$C] tolmetin were both kindly supplied by McNeil Laboratories (Fort Washington, PA). The unlabelled acid was obtained from Mallinkrodt (St Louis, MO) and the $^{14}$C-labelled salicylic acid was obtained from California Bionuclear Corporation (Sun Valley, CA). The unlabelled and [$^{3}$H]prednisolone were purchased from Sigma and New England Nuclear (Boston, MA), respectively.

Methods for determining total equilibrium drug concentration following equilibrium dialysis were published previously (Behm & Wagner 1979). Briefly, the total equilibrium drug concentration can be determined with equation 1 which allows for change in the ratio: volume of buffer/volume of plasma or protein solution.

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$$C_t = \frac{A_1}{f_b V_1 + (1\text{-}f_b)(V_o + V_1)} \quad (1)$$

where $C_t$ is the total concentration of free and bound drug in the plasma or protein compartment at equilibrium and $A_1$ is the total amount of drug initially in the equilibrium dialysis system; $f_b$ is the fraction of drug bound to protein at equilibrium, determined by subtracting from one the fraction free. The fraction free is equal to the ratio: concentration of free drug/concentration of total drug as measured by radiolabelled tracer drug. $V_1$ is the volume inside protein or plasma compartment and $V_o$ is the volume of buffer in buffer compartment.

Prednisolone binding to plasma proteins can be described by the two term equation:

$$C_b = \frac{n_T k_{aT} P_T C_f}{1 + k_{aT} C_f} + n_A k_{aA} P_A C_f \quad (2)$$

where $C_b$ and $C_f$ are the bound and free concentrations of prednisolone, respectively, n is the number of binding sites per protein molecule, $k_a$ is the association constant, and P is the protein concentration. The subscripts 'T' and 'A' refer to transcortin and albumin, respectively (Rocci et al 1980; Ağabeyoğlu et al 1979).

Equation 2 can be rearranged to give an alternate form of this binding equation, letting $1/k_a = k_d$, the dissociation constant, resulting in

$$C_b = \frac{n_T P_T C_f}{k_{dT} + C_f} + n_A k_{aA} P_A C_f = \frac{P(1)C_f}{P(2) + C_f} + P(3)C_f \quad (3)$$

where $P(1) = n_T P_T$, $P(2) = k_{dT}$, and $P(3) = n_A k_{aA} P_A$. If parameters are known for the binding of prednisolone to plasma proteins, equation 3 may be rearranged to give a quadratic equation with positive root equal to the free drug concentration corresponding to a total drug concentration on the binding curve (Behm & Wagner 1979). This quadratic equation is:

$$[1 + P(3)]C_f^2 + [P(1) + P(2) + P(2)P(3) - C_t]C_f - P(2)C_t = 0 \quad (4)$$

The binding of tolmetin and salicylic acid to HSA was described by the two parameter equation (Behm 1980):

$$C_f = a\,C_t + b\,C_t^2 \quad (5)$$

where $C_f$ and $C_t$ are the free and total drug concentrations and a and b are constants. Using this model the expected free drug concentrations corresponding to any total drug concentration on the binding curve can be estimated directly.

The classical Langmuir model (Meyer & Guttman 1968) can also be used to describe tolmetin and salicylic acid-HSA binding. That model, assuming two classes of binding sites (1 and 2), leads to equation 6:

$$C_b = \frac{n_1 C_p C_f}{kd_1 + C_f} + \frac{n_2 C_p C_f}{kd_2 + C_f} = \frac{P(1)C_f}{P(2) + C_f} + \frac{P(3)C_f}{P(4) + C_f} \quad (6)$$

In equation 6, $C_p$ is the protein concentration, $P(1) = n_1 C_p$, $P(2) = kd_1$, $P(3) = n_2 C_p$, and $P(4) = kd_2$. If the parameters are known for the binding of tolmetin or salicylate to plasma proteins, equation 6 may be rearranged to give a cubic equation with one root equal to the free drug concentration corresponding to a total drug concentration on the binding curve (Behm & Wagner 1979). This cubic equation is:

$$C_f^3 + [P(1) + P(2) + P(3) + P(4) - C_t]C_f^2 + [P(1)P(4) + P(3)P(2) + P(2)P(4) - [P(2) + P(4)]C_t]C_f - [P(2)P(4)]C_t = 0 \quad (7)$$

## RESULTS AND DIFCUSSION

### *Dependence of free fraction on volume ratio: volume of buffer/volume of protein solution*

*Prednisolone.* Non-linear binding curves were prepared for the binding of prednisolone to plasma using 1 ml of plasma from a normal volunteer and 3 ml of buffer in the equilibrium dialysis apparatus. Equation 1 was used to determine the total prednisolone concentration at equilibrium. The prednisolone data were computer fitted using NONLIN (Metzler 1969) to equation 3 and the parameters (and standard deviations) obtained were estimate (standard deviation): $P(1) = 0{\cdot}476$ (0·103), $P(2) = 0{\cdot}0914$ (0·0143), and $P(3) = 0{\cdot}964$ (0·319). Measures of fit, 'corr' and '$r^2$'[1], were 0·995 and 0·997, respectively.

Binding studies were also conducted using prednisolone and plasma with changes in the volume of the buffer solution relative to the plasma volume. The volume ratio, buffer solution to plasma, varied from 2 to 20. After equilibrium was reached, the total equilibrium drug concentration was calculated using equation 1. The numerical values of the estimated parameters given above, along with equation 4, were used to estimate expected free drug concentrations corresponding to the total equilibrium drug concentrations obtained after dialysis with the volume ratios from 2 to 20. Expected free fractions were

[1] 'Corr' is the correlation coefficient for the regression of model-predicted *versus* observed concentrations, and '$r^2$' is the coefficient of determination.

obtained by dividing the expected free drug concentrations by the corresponding total equilibrium drug concentration.

The expected and observed free fractions (as percentages) for prednisolone binding to plasma proteins are plotted as a function of buffer volume in Fig. 1. Each point is the average of the three free fractions obtained at each different buffer volume.

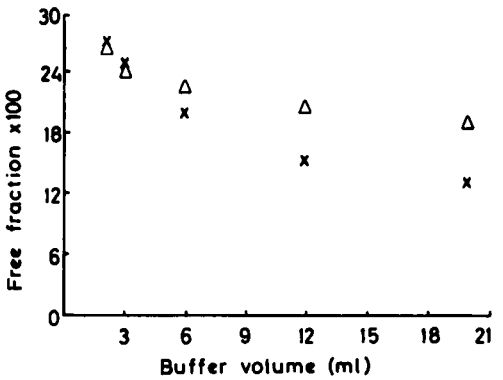


FIG. 1. Prednisolone free fraction vs volume of buffer. The x's (X) are the observed free fractions (average of three) and the triangles (Δ) are the expected free fractions from a binding curve obtained with a 3 to 1 ratio of volume of buffer solution to plasma volume.

Slight differences in total equilibrium prednisolone concentration caused by slightly different free fractions for the three values were ignored for the purposes of this plot, but would not cause any significant error. The free fractions observed were considerably, different from those expected by taking into account only the changes in the total equilibrium drug concentration. It can be seen that the free fraction is greater than expected with a buffer volume less than 3 ml and lower than expected with a buffer volume greater than 3 ml.

*Salicylic acid.* The binding of salicylic acid to 4% albumin I was investigated as described above. These data were fitted to equation 5 and the parameters (standard deviations) a and b. obtained were a = 0·175 (5·79 × $10^{-3}$) and b = 1·07 × $10^{-4}$ (3·6 × $10^{-6}$). 'Corr' and '$r^2$' were both 0·998. These data describe the salicylic acid—albumin I expected binding curve at a volume ratio (buffer solution to protein solution of 3).

After similar changes in the buffer to protein volume ratio, the total equilibrium drug concentrations were determined using equation 1. Using those total equilibrium drug concentrations, and equation 5, and the parameters given above, the expected free salicylic acid concentrations corresponding to the equilibrium total salicylic acid concentrations were estimated directly. The expected free fraction was obtained by dividing the expected free salicylic acid concentration by the total equilibrium salicylic acid concentration.

As in Fig. 1, Fig. 2 shows the expected and observed free fractions (average of three values) of salicylic acid. Again, the slight change in total equilibrium salicylic acid concentration in the triplicate samples was ignored. Again, the free fractions

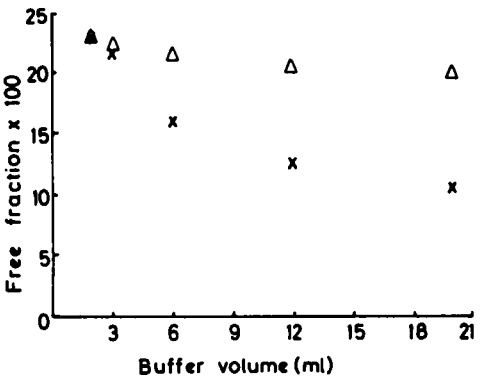


FIG. 2. Salicylic acid fraction free vs volume of buffer. The x's (X) are the observed free fractions (average of three) and the triangles (Δ) are the expected free fractions from a binding curve obtained with a 3 to 1 ratio of volume of buffer solution to HSA solution volume.

obtained were dramatically different from those expected by taking into account only the change in the total equilibrium salicylic acid concentration.

*Tolmetin.* The binding of tolmetin to 4% albumin I was also investigated as described above and the data were fitted to equation 5 resulting in the parameters (standard deviations) as follows: a = 2·82 × $10^{-2}$(2·18 × $10^{-3}$) and b = 6·97 × $10^{-5}$ (2·74 × $10^{-6}$). 'Corr' and '$r^2$' were both 0·997.

Again experiments were performed with tolmetin at different buffer to protein volume ratios. Because of the high degree of binding of tolmetin to HSA, theoretically the free fraction should change only slightly due to the small changes in tolmetin equilibrium total concentration following equilibrium dialysis. As in Figs 1 and 2, Fig. 3 shows the expected and observed free fractions (average of three values) of tolmetin. The slight change in total equilibrium tolmetin concentration in the triplicate samples are ignored in preparing this plot. Again, the free fractions obtained were dramatically different from those expected by taking into account only the

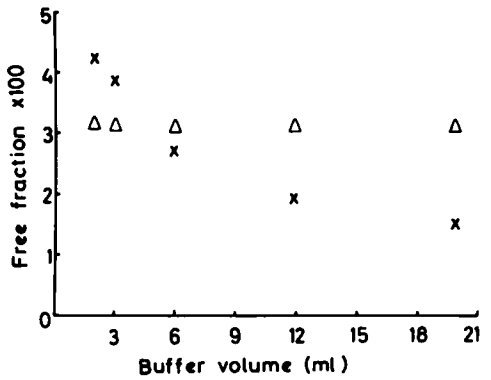


FIG. 3. Tolmetin fraction free vs volume of buffer. The x's (X) are the observed free fractions (average of three) and the triangles (Δ) are the expected free fractions from a binding curve obtained with a 3 to 1 ratio of volume of buffer solution to HSA solution volume.

change in the total equilibrium tolmetin concentration.

*Detection of an impurity in protein solutions and plasma*

During the h.p.l.c. assays of prednisolone, samples from buffer and plasma compartments following equilibrium dialysis, an impurity peak was observed. The buffer and protein solutions were from equilibrium dialysis experiments where the buffer/plasma volume ratio had ranged from 1 to 25. In order to compare relative 'impurity' concentrations in these buffer and plasma samples, peak height ratios (PHR) were obtained by dividing the height of the impurity peak by the peak height of the internal standard used for the prednisolone assay.

The data for the h.p.l.c. analysis of the 'impurity' peak are given in Table 1. One ml of plasma was dialysed against various volumes of buffer. As would be expected if the 'impurity' were being diluted from the plasma compartment, the PHR of the impurity in the plasma compartment decreased consistently with an increase in the buffer/plasma volume ratio. However, the buffer/plasma ratios of the PHR's of the 'impurity' peak are constant. This constant buffer/plasma ratio is consistent with linear binding where the free fraction of 'impurity' is constant, even though the concentration of 'impurity' in the plasma compartment decreased with increase in the buffer/plasma volume ratio.

Table 1. H.p.l.c. analysis – impurity peak.

| Vol buffer* | Plasma PHR (Impurity/IS) | Buffer PHR (Impurity/IS) | Buffer/ Plasma |
|---|---|---|---|
| 5 ml | 3·04 | 2·42 | 0·796 |
| 10 ml | 1·51 | 1·12 | 0·742 |
| 15 ml | 1·01 | 0·813 | 0·805 |
| 20 ml | 0·84 | 0·65 | 0·774 |
| 25 ml | 0·72 | 0·56 | 0·782 |

Buffer vol. 1, 2, 3 ml were off scale in cols 2 and 3.

Albumin I solutions, diluted to 4%, were also analysed by the h.p.l.c. assay used to measure prednisolone. The 'impurity' was also present in the HSA solution giving large absorption peaks upon h.p.l.c. analysis at 254 nm.

The 'impurity' was isolated by fractional collection from the h.p.l.c. analysis of the HSA solutions (4% diluted from 25%) and dissolved in n-hexane. The u.v. analysis (Fig. 4) shows absorption peaks at 220, 267, and 274 nm. 'Impurity' samples, obtained as described above, were subjected to analysis by a

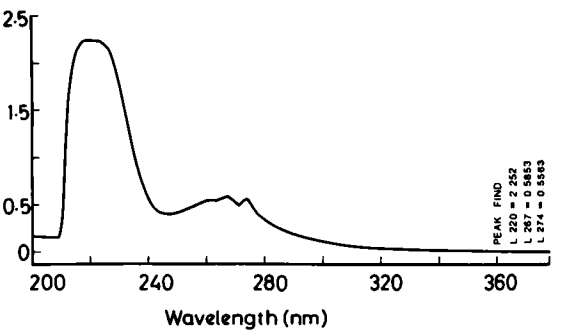


FIG. 4. The u.v. absorbance of the 'Impurity'.

mass spectrometer. The largest peaks in the spectrum appeared at temperatures of around 120 °C, but the sample was heated to 360 °C. The analysis of the spectra suggest the possible presence of a long-chain hydrocarbon from 30 to 40 carbon atoms in length. This compound may contain some smaller alcoholic or acidic groups but a mixture of compounds may also be present.

The presence of one or more impurities in the plasma or protein solutions could cause differences in observed and expected free fractions of drugs when the ratio of buffer to protein solution or plasma is changed. By increasing the volume of the buffer solution relative to the protein solution, the concentration of this competing substance in the plasma or protein solution would be decreased as would be the drug concentration. Thus, an increase in binding of the drug as compared with what is expected with a smaller buffer/plasma volume ratio would be observed.

*Change in free fraction following diafiltration of HSA solutions*

Diafiltration was used to purify the 4% albumin I. Buffer was passed through the stirred albumin solution at a constant rate. The purpose of these experiments was to determine not only if binding was increased following the removal of any dialysable substances, but also if the effect was dependent upon the length of time of the diafiltration. In this case, since the buffer was passed through the albumin solution at a constant rate, the longer the albumin was dialysed, the greater the opportunity for purification.

In separate procedures, 4% albumin I solutions were dialysed using the diafiltration system for 0, 1·5, 4, 8 and 16 h. The binding of tolmetin in these dialysed HSA solutions was then determined using equilibrium dialysis with buffer/HSA solution volume ratios from one to thirty. The initial amount of tolmetin in the equilibrium dialysis apparatus was 350 $\mu$g (1367 $\mu$M) in all cases. When compared with the undialysed 'control' HSA solution (0 h), the longer the solution was dialysed, the greater was the reduction in free fraction at a particular buffer/protein solution volume ratio. These results are shown graphically in Fig. 5. This plot ignores differences in equilibrium drug concentrations.

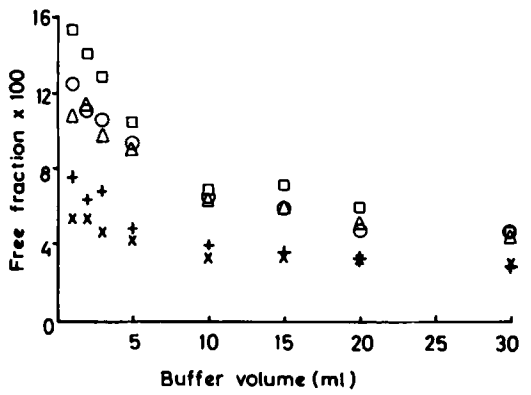


FIG. 5. Free fraction following diafiltration of HSA obtained at buffer/HSA solution volume ratios of 1 to 30. Points are results from HSA dialysed 0 h (□), 1·5 h (○), 4 h (Δ), 8 h (+), and 16 h (X).

The HSA solutions before and after diafiltration and the buffer passed through the diafiltration cell were analysed using the h.p.l.c. prednisolone assay. The PHR of the 'impurity' peak in the HSA decreased with increase in time of filtration. No 'impurity' peak was present in a control buffer solution, but the 'impurity' peak was present upon h.p.l.c. analysis of the eluent buffer following diafiltration.

*The binding of tolmetin to fatty acid-free albumin*

Tolmetin binding studies were performed using 4% fatty acid-free HSA (albumin II) to determine if tolmetin binding was different from that in the 4% albumin I solution. Tolmetin-albumin I binding data were obtained using 1 ml of HSA solution and 1 ml of buffer. The data were computer fitted to equation 5 resulting in the parameters (standard deviations): $a = 2{\cdot}89 \times 10^{-2}$ ($2{\cdot}3 \times 10^{-2}$) and $b = 1{\cdot}98 \times 10^{-5}$ ($2{\cdot}09 \times 10^{-6}$). Measures of fit, 'corr' and '$r^2$', were 0·993 and 0·992, respectively.

It was shown earlier that with albumin I, observed free fractions of tolmetin differed from the expected free fraction (from a binding curve obtained at a constant volume ratio) and that difference became greater as the buffer/HSA solution volume ratio increased. No such effect was observed for the binding of tolmetin to the fatty acid-free HSA (albumin II). In Fig. 6, the observed and expected free fractions

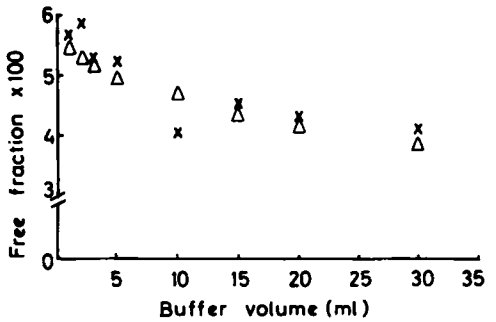


FIG. 6. The binding of tolmetin in fatty acid-free HSA solutions at different buffer solution/HSA solution volume ratios. The x's (X) are the observed free fractions and the triangles (Δ) are the expected free fractions.

are plotted against volume of buffer. It can be seen from this plot that a change in the buffer/HSA solution volume ratio does not result in unexpected free fractions of tolmetin in fatty acid-free albumin solutions when compared with a binding curve obtained at a constant volume ratio on one.

It was also determined that diafiltration of the fatty acid-free albumin does not increase the binding of tolmetin to fatty acid-free albumin.

All of the above data suggested that fatty acids are the impurities which cause the discrepancy between the observed and expected free fractions with increase in the ratio of the buffer solution/HSA solution or plasma volumes. A closer look was necessary. Many

investigators have reported that the presence of fatty acids alter drug-plasma protein binding (Gugler et al 1974; Brand & Toribara 1975; Fredholm et al 1975; Birkett 1978). However, it is also known that fatty acids of carbon chain length 12 or greater (greater than 99% of the fatty acids in the body) do not pass through ordinary dialysis membranes (Spector 1975). If dilution of competing substances in the albumin solutions or plasma during equilibrium dialysis increases drug binding, then fatty acids should be excluded since these competitors do not pass through the dialysis membranes. Also, u.v. absorption at 220, 267 and 274 nm is not characteristic of fatty acids, if, in fact, this 'impurity' is the competitor in question. It is feasible that 'competitive' substances other than fatty acids could be removed from albumin during the diafiltration process. The fatty acid-free HSA was analysed using the h.p.l.c. assay for prednisolone. The fatty acid-free HSA did not show an 'impurity' peak upon analysis and fractions collected characteristic of where the impurity peak should have been, showed no u.v. absorbance. Upon mass spectrometer analysis, the sample from the h.p.l.c. fraction collection where the 'impurity' peak should have been gave a flat plateau spectrum at 120 °C. At 120 °C, the sample from the h.p.l.c. fractional collection of an albumin I solution gave the highest peaks of the spectrum and those peaks were used for the subsequent mass spectrometer analysis.

*The effect of fatty acid addition on the binding of tolmetin to fatty acid-free albumin*

The binding of tolmetin to albumin II was investigated in the presence of from 0 to 20 moles of lauric acid (a 12-carbon chain fatty acid) per mole of albumin. The data showed that the presence of lauric acid does alter tolmetin-HSA binding. The binding of tolmetin to albumin II in the presence of 5 moles of lauric acid per mole of albumin was investigated using 3 ml of buffer and 1 ml of HSA solution. Data were fitted to equation 6. Estimated parameters (and standard deviations) were: $P(1) = 1278\ (302)$, $P(2) = 457\ (174)$, $P(3) = 292\ (15{\cdot}3)$, and $P(4) = 1{\cdot}95\ (0{\cdot}19)$. Measures of fit 'corr' and '$r^2$' were both 0·999.

Binding studies were conducted using tolmetin and albumin II in the presence of 5 moles of lauric acid per mole of HSA with changes in the volume of the buffer solution relative to the volume of the HSA solution. The volume ratio, buffer solution to HSA solution, varied from 1 to 30. Data were fitted to equation 6, then the estimated parameters and equation 7 were used, along with the equilibrium drug concentrations (estimated using eqn 1), to estimate the free concentrations and free fractions expected at the various total tolmetin equilibrium concentrations. The expected and observed free fractions are plotted vs buffer volume in Fig. 7. The observed free fractions are comparable to the expected free fractions. Even though the addition of the lauric acid to the fatty acid-free HSA does change the tolmetin-HSA binding characteristics, the presence of the fatty acid does not cause a discrepancy in the observed vs expected free fraction when the buffer solution/HSA solution volume ratio is changed. These results are similar to observations made when tolmetin-albumin II binding was investigated.

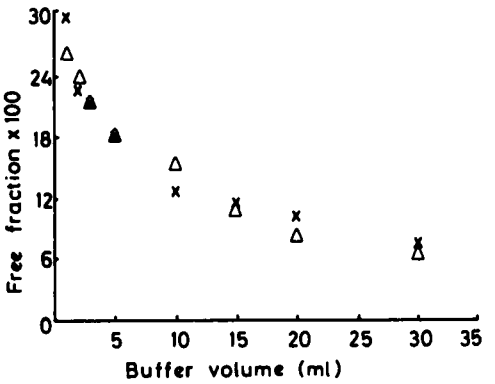


FIG. 7. The binding of tolmetin in fatty acid-free HSA solutions at different buffer solution/HSA solution volume ratios in the presence of 5 moles of lauric acid per mole of HSA. The x's (X) are the observed free fractions and the triangles (Δ) are the expected free fractions.

Experiments were also performed using 2 moles of lauric acid per mole of HSA (rather than 5 moles) in a manner identical to that described above. The expected and observed free fractions (not shown) were comparable in this case also.

We conclude that the dilution of an 'impurity' from the plasma and albumin solutions by the buffer solution during dialysis causes the discrepancy in the expected vs observed free fractions with change in buffer solution/HSA solution volume ratio. We are uncertain as to the nature of the impurity, but can rule out fatty acids. Since the dilution of this 'impurity' in the buffer causes changes in the binding characteristics of a drug to plasma, we recommend that equilibrium dialysis be performed with a buffer solution/plasma volume ratio as small as possible to minimize this effect.

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