Prediction of *in Vivo* Tissue Distribution from *in Vitro* Data. 2. Influence of Albumin Diffusion from Tissue Pieces during an *in Vitro* Incubation on Estimated Tissue-to-Unbound Plasma Partition Coefficients (Kpu)

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Received February 13, 2003; accepted March 3, 2003

Purpose. To determine the extent of albumin diffusion from tissue pieces into medium during *in vitro* incubations, to develop and assess the utility of mathematical models describing this effect on the estimation of tissue-to-unbound plasma partition coefficients (Kpu) of drug substances and to derive factors to correct for associated errors.
Methods. Twelve separate tissues were obtained from rats sacrificed by cervical dislocation, 48 h after an intravenous dose of ^{125}I-human albumin, and tissue pieces incubated to determine the efflux of albumin into media over 2 to 4 h. A mathematical model was developed to predict and correct for the effect of albumin diffusion on the measured *Kpu* values of drugs.
Results. The model predicted that the effect of albumin diffusion from tissue pieces during *in vitro* incubation (ranging from 14 to 59% remaining in tissue) on *Kpu* values was generally minimal, except for compounds that are highly plasma bound and have a low measured *Kpu*. Under these circumstances, the measured *Kpu* substantially underestimates the true value. An equation was derived from readily available or measurable parameters to correct for this underestimation.
Conclusions. Albumin diffuses from tissue pieces into protein free media during *in vitro* incubations until equilibrium is reached, defined by the albumin *Kpu*. Model predictions indicated that for the majority of compounds albumin diffusion would have a minimal effect on the measured *Kpu* value and that a correction factor could be calculated to account for any deviation.

KEY WORDS: tissue distribution; albumin; protein binding; diffusion; *in vitro- in vivo*.

INTRODUCTION

Tissue-to-unbound plasma partition coefficients (Kpu) used in physiologically-based pharmacokinetic (PBPK) models have been estimated from both *in vivo* (1,2) and *in vitro* (3,4) studies. *In vitro Kpu* estimates have most often been made using tissue homogenates (5,6) although tissue slices have been used for some drugs (7,8). The advantage of using slices or pieces is that the cellular architecture of the tissue remains largely intact and consequently this preparation offers the possibility of more accurately predicting *in vivo Kpu* values than homogenates, as demonstrated in our earlier report (9), concerned with estimating the extracellular and total tissue water spaces of various tissues using specific marker compounds.

One observed complication in using tissue pieces to estimate *Kpu* is the imbibing of the medium during the incubation (9). The efflux of proteinaceous material, such as albumin, or other tissue components to which drugs bind during the same process is largely unknown or ignored (10) and may complicate the correct estimation of *Kpu*.

The objectives of this work are to determine the extent of albumin diffusion from tissue pieces during an *in vitro* incubation and to predict the effect of albumin diffusion on the measured *Kpu* value.

MATERIALS AND METHODS

Reagents

Hanks' Balanced Salt Solution containing D-glucose (HBSS) was supplied by Life Technologies Limited, Paisley, UK and ^{125}I-human serum albumin was purchased from Amersham Healthcare, Little Chalfont, Buckinghamshire, UK. All other chemicals were obtained from Sigma Chemical Company, Poole, Dorset, UK and used as received. Hanks/HEPES buffer was prepared by adding 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, sodium salt (HEPES) to HBSS at 10 mM. Fresh tissues (adipose, brain, heart, gastrointestinal tract, kidney, liver, lung, muscle, skin, stomach, spleen, testes, and thymus) were obtained from four male Wistar rats weighing between 214 and 355 g.

Albumin Diffusion from Tissue Slices during *in Vitro* Incubations

Male rats received an intravenous (i.v.) bolus dose of ^{125}I-human serum albumin (250 μCi/kg, 100 mg albumin/kg) and were sacrificed after 48 h. Terminal blood samples were centrifuged to provide plasma. Excised tissues were cut freehand with scissors into pieces (typically from 80 to 110 mg, except for specific experiments where the exact weight has been detailed) that were weighed before and after *in vitro* incubation with Hanks/HEPES buffer (2 ml) in a shaking waterbath at 37°C for 2 to 4 h. The radioactivity in each tissue slice was determined at set incubation times using a radioactivity counter (LKB Wallac 1277 Gammamaster).

The percentage of albumin remaining in the tissue slice at the end of the incubation was calculated as:

$$\textit{Albumin remaining}\ (\%) = \frac{cpm_T}{cpm_{T,\,pre}} \times 100 \qquad (1)$$

where $cpm_{T,pre}$ and cpm_T are the counts-per-minute of ^{125}I-albumin in the tissue piece before and after incubation.

The *in vivo* albumin tissue-to-plasma partition coefficient (Kp) was calculated as:

$$Kp = \frac{cpm_T / wt}{cpm_{pl} / V_{pl}} \qquad (2)$$

where *wt* is the weight of tissue (g) and cpm_{pl}/V_{pl} is the counts per minute of ^{125}I-albumin per unit volume of plasma (V_{pl}, ml)

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Table I. Effect of Liver and Muscle Tissue Weight and Tissue-to-Medium Volume Ratio ($V_{T/M}$) on the Measured ^{125}I-Human Serum Albumin *Kp* and Percentage of Albumin Remaining in the Tissue Piece after 4 h Incubation in Hanks/HEPES Buffer (2 ml, pH 7.4)

Muscle				Liver			
Tissue wt (mg)	$V_{T/M}$[a]	Albumin *Kp*	% Albumin remaining	Tissue wt (mg)	$V_{T/M}$[a]	Albumin *Kp*	% Albumin remaining
20	0.01	0.004	6.3	23	0.01	0.022	21.9
45	0.02	0.003	7.1	40	0.02	0.024	25.6
103	0.05	0.011	24.3	92	0.05	0.035	33.8
135	0.07	0.009	16.5	123	0.06	0.036	34.8
140	0.07	0.012	22.6	220	0.11	0.043	44.5
536	0.27	0.017	34.1	496	0.25	0.043	45.7

[a] Specific gravity of muscle and liver assumed to be 1.0.

both at the time the tissue was excised. The specific gravity of tissue and incubation medium are assumed to be the same, hence *Kp* is a dimensionless term.

Effect of Albumin Diffusion on Tissue-to-Unbound Plasma Partition Coefficient

The loss of albumin from the tissue into the incubation medium affects the measured, or apparent, tissue-to-medium partition coefficient, designated Kpu_{app}. This value is related to the true value, *Kpu*, if no albumin loss from the tissue has occurred, by the following relationship expressed in experimentally known or readily determined parameters (see Appendix for derivation):

$$\frac{Kpu_{app}}{Kpu} = \frac{1 - \left\{ \dfrac{(1-f_R)\, f_P \left(\dfrac{1-fu}{fu}\right).}{1 + f_p\left(\dfrac{1-fu}{fu}\right) + \left(\dfrac{1}{\theta_E} - 1\right) \Big/ fu_I} \right\}}{1 + \theta_E \dfrac{V_T}{V_M} f_P \left(\dfrac{1-fu}{fu}\right)(1-f_R)} \qquad (3)$$

where $\theta_E = V_E/V_T$ (V_E and V_T are the extracellular and total tissue volumes respectively); V_M, is the volume of medium; f_P, the ratio of the binding protein concentrations originally in extracellular space to that in plasma (11); *fu*, the unbound fraction of compound in plasma; f_R, fraction of extracellular binding protein remaining in tissue at the end of the incubation; fu_I, is the unbound intracellular fraction of compound.

Calculation of a Factor to Correct Kpu_{app}, the Apparent or Measured Tissue-to-Plasma Medium Partition Coefficient, to Kpu

The loss of albumin from the tissue into the incubation medium affects the measured, or apparent, tissue-to-medium partition coefficient, designated Kpu_{app}. A correction factor (CF) can be applied to Kpu_{app} to give the true *Kpu*, where no albumin has diffused from the tissue, using experimentally determined or readily available variables, Eq. 4 (see Appendix for derivation).

$$CF = \frac{Kpu}{Kpu_{app}} = \left[1 + \theta_E\left(\frac{V_T}{V_M}\right) \cdot \left(\frac{1-fu}{fu}\right) f_p (1-f_R)\right] + \frac{\theta_E (1-f_R)\left(\dfrac{1-fu}{fu}\right) f_p}{Kpu_{app}} \qquad (4)$$

RESULTS

Diffusion of Albumin from Tissue Slices during *in Vitro* Incubations

The rate of diffusion and proportion of ^{125}I-human serum albumin retained in the tissue after *in vitro* incubation were dependent on the tissue-to-medium volume ratio ($V_{T/M}$); assuming the specific gravity of the tissue to be 1.0. For example, for $V_{T/M}$ ranging from 0.01 to 0.25 in liver, the ^{125}I-human serum albumin remaining in the tissue after 4 h incubation increased from 22–46%; for a similar $V_{T/M}$ range in muscle between 6.3 and 34% albumin remained, Table I.

The effect of tissue mass on the rate of albumin diffusion (normalized to 100% at the start of the incubation) from rat liver pieces into incubation media is demonstrated in Fig. 1, although it should be noted that there may be variability here due to differences in the size and shape of the tissues. There were also substantial differences in the percentage of albumin

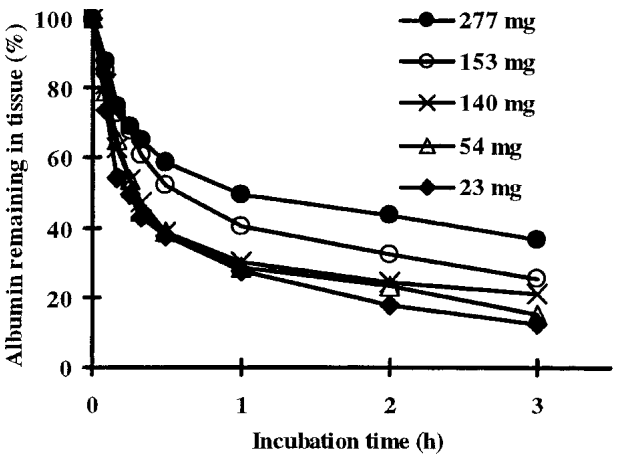

Fig. 1. The effect of tissue mass (23–277 mg) on albumin diffusion (normalized to 100% at the start of the incubation) from rat liver pieces into incubation media.

Table II. Mean (n = 5, ± Standard Error) ^{125}I-Human Serum Albumin Tissue-to-Plasma Ratios 48 h Post i.v. Injection, the Percentage Losses of Albumin during a 2 h *in Vitro* Incubation in Hanks/HEPES Buffer (Approx 80 mg Tissue, 2 ml Buffer, pH 7.4) and the Extravascular-to-Vascular Albumin Content, f_p, in Rat Tissues (11)

Tissue	f_p values	Albumin tissue-to-plasma ratio (± standard error)	% Albumin remaining after 2 h incubation (± standard error)
Adipose	0.042	0.031 (0.0005)	14.1 (1.53)
Brain	0	0.014 (0.0014)	42.6 (2.49)
Heart	0.055	0.155 (0.0097)	76.0 (2.70)
Intestine	0.065	0.125 (0.0045)	44.6 (1.87)
Kidney	0.042	0.124 (0.0018)	45.0 (1.96)
Liver	0.01	0.091 (0.0014)	50.8 (2.24)
Lung	0.13	0.256 (0.0029)	58.6 (1.75)
Muscle	0.003	0.057 (0.0040)	25.9 (0.50)
Skin	0.11	0.172 (0.0029)	42.5 (2.76)
Spleen	0.025	0.090 (0.0003)	49.7 (3.75)
Stomach	0.10	0.114 (0.0070)	32.3 (2.72)
Thymus	NR[a]	0.119 (0.0066)	28.2 (1.81)

[a] NR—no result.

remaining after a 2 h incubation between the tissues, ranging from 14% in adipose tissue to 59% in lung at a typical $V_{T/M}$ of 0.04 (80 mg tissue per 2 ml of media), Table II.

Effect of Albumin Diffusion on Measured Kpu

The model describing the relationship between the measured and actual tissue-to-medium partition coefficients during *in vitro* incubations (Eq. 3, Kpu_{app} and Kpu respectively) was used to simulate typical conditions found with *in vitro* tissue incubations, Fig. 2. Lung and liver tissue were chosen as they represented the extreme ratios of the extravascular-to-vascular albumin content, f_p = 0.13 and 0.01 respectively, Table II (11), although the profile for all tissues were similar. This demonstrated that Kpu_{app} for a compound could be substantially underestimated under certain conditions. For example, taking a typical value of 80% diffusion of albumin from the tissue piece at the end of the incubation period (f_R = 0.2), minimal difference between Kpu_{app} and Kpu values for compounds with plasma albumin binding of less than 99% (fu $\geq$ 0.01) is predicted for compounds having a high intracellular binding, including binding to cell wall ($fu_I \leq 0.1$). However, where plasma protein binding is very high (fu < 0.01), the ratio Kpu_{app}/Kpu decreases dramatically, so that measured Kpu_{app} could underestimate the actual value (Kpu) by 100-fold or more. This is most marked for tissues having a high extravascular binding protein concentration relative to plasma or for a drug that is restricted to the extracellular tissue space (Fig. 2). In this latter circumstance, even at low binding to albumin (fu $\geq$ 0.1), the measured Kpu_{app} is substantially lower when albumin diffuses into the medium.

Correction Factor to Estimate Kpu from Kpu_{app}, the Measured Tissue-to-Medium Partition Coefficient

The correction factor (CF) has been estimated for a range of Kpu_{app} in lung and liver tissue with compounds having protein binding values of 99 and 99.7%, using typical values for an *in vitro* incubation (2 h incubation, 2 ml media, and fraction of albumin remaining detailed in Table 2 for approximately 100 mg of tissue), see Fig. 3. For liver, CF remains close to 1 for all values of Kpu_{app} where plasma albumin binding is less than 99.7% and only rises above 1 at a Kpu_{app} of 1 and >99.9% binding. The *CF* for tissues with high f_p values (e.g., lung and skin) are most sensitive to changes in Kpu_{app} primarily due to the higher relative extracellular albumin concentration in these tissues.

DISCUSSION

We envisage the efflux of proteins from tissues during *in vitro* incubation as occurring from the interstitial and vascular tissue spaces, due to diffusion into the initially protein-free incubation medium. Albumin was chosen for study as it represents a major binding protein for many drugs, especially acids, is the most abundant plasma protein, and information on its tissue distribution within the interstitial spaces is the best known of the plasma proteins. Nonetheless, the general principles explored with albumin may apply equally to other plasma proteins, which are also all located in extracellular tissue spaces. Taking tissue samples from rats 48 h post an i.v. bolus dose ensured distribution equilibrium between tissues and plasma and minimal degradation of albumin, such that

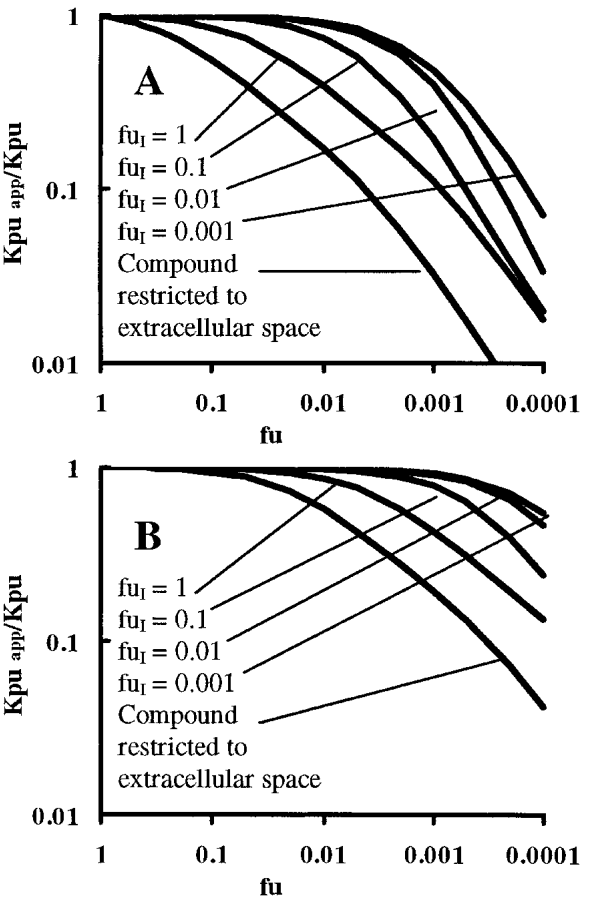

Fig. 2. The effect of plasma protein binding (fu) and intracellular binding (fu_I) on the ratio of measured (Kpu_{app}) to actual (Kpu) tissue-to-medium equilibrium partition coefficients for lung (A) and liver (B) pieces under typical *in vitro* conditions (4 h incubation, f_R = 0.2, f_P = 0.13 and 0.01 respectively, V_T = 0.1, V_M = 2, θ_E = 0.2, and for a compound restricted to extracellular tissue space).

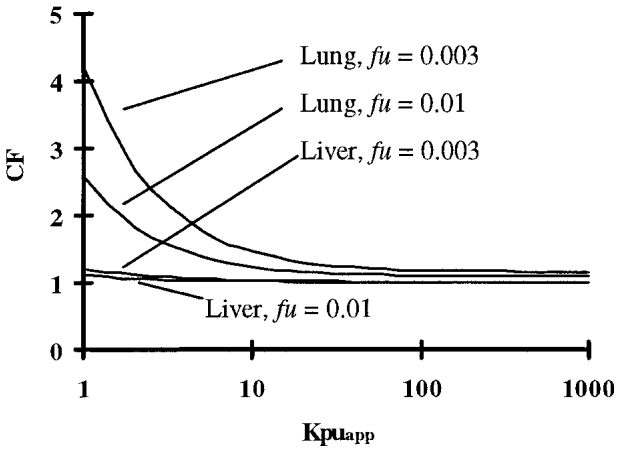

Fig. 3. Correction factor (CF) to account for underestimation of *Kpu* in the *in vitro* situation due to diffusion of albumin from lung and liver tissue to the incubation medium (f_R = 0.41 and 0.49 respectively, *fp* = 0.13 and 0.01 respectively, 2 h incubation, V_T = 0.1, V_M = 2, θ_E = 0.2, with *fu* values of 0.01 and 0.005).

total radioactivity in tissue is a good representation of intact albumin (12).

There are only sparse data available on the *in vitro* diffusion of proteins from tissue slices during incubation. One report indicates that 79% of lissamine rhodamine-labeled rat serum protein diffused out of rat brain slices during a 1 h incubation in Ringer bicarbonate solution containing glucose (13), which is similar to that for albumin in the current study (57% over 2 h).

In vitro drug distribution studies are often conducted using protein-free media to enable tissue-to-unbound plasma partition coefficients to be determined directly (9,14,15). However, the efflux of albumin from the tissue to initially protein-free media complicates this approach, necessitating the use of a correction factor, particularly for highly protein bound drugs.

One way to prevent a significant movement of proteins from the tissue and hence avoid a correction factor, would be to use plasma as the incubation medium. However, this creates its own problems for highly protein bound compounds where the free concentrations in media are low. Consequently the flux of compound into tissue is significantly reduced, resulting in a considerable increase in the time taken to achieve equilibrium. As the tissue pieces degraded significantly after 6 hours, it was not feasible to extend the incubation time further and therefore the option of using plasma as the incubation media was not pursued.

The observation that there is a decrease in the fractional loss of albumin with increasing tissue weight strongly suggests that albumin release from tissues is being diffusion controlled. The diffusion coefficients (all $\times 10^{-6}$ cm^2s^{-1}) of albumin effluxing from tissue pieces during the course of the *in vitro* incubation were estimated for all major rat tissues from equations based on a derivation of Ficks Law of diffusion (16), assuming the tissue to be a sphere in a bath of finite volume: adipose (1.8), intestine (1.1), thymus (0.95), muscle (0.95), stomach (0.75), skin (0.50), heart (0.50), kidney (0.40), brain (0.37), liver (0.34), spleen (0.32), and lung (0.20). It is perhaps surprising and encouraging that this model estimated diffusion coefficients in tissue that are similar to that of albumin in water (0.2×10^{-6} cm^2s^{-1}) (17). We observed a weak correlation (r^2 = 0.552) between increasing albumin diffusion coefficients and decreasing logarithm of fractional tissue vascular volume (17,18), but a stronger correlation (r^2 = 0.895, excluding skin) between diffusion coefficient and the ratio of extracellular-to-vascular tissue volumes (Fig. 4). That is, albumin tends to diffuse most rapidly from tissues where the extracellular-to-vascular volume is greatest.

An alternative way of investigating the effect of changing the tissue-to-incubation volume ratio on the rate of albumin efflux would have been to keep the tissue weight approximately constant and to vary the media volume. The advantage of this design is that a constant tissue surface area would have been maintained. Further work could be undertaken to see if results with this experiment design are comparable to those reported here.

The observed albumin *Kp* value for muscle and liver at the end of the incubation increased with increasing tissue mass, Table I. This finding, however, may simply reflect that equilibrium for this protein has not attained equilibrium within the typical 4 h incubation period, and that the final equilibrium value is expected to be lower and independent of the tissue-to-medium volume ratio.

The mathematical model (Eq. 3) describing the effect of albumin diffusion out of tissues on the equilibrium *Kpu* values of drugs has the boundary properties, with respect to plasma protein binding, that one would intuitively expect. Thus, when *fu* tends to 1, so does Kpu_{app}/Kpu; (i.e., for a compound that does not bind to albumin) the diffusion of albumin has no effect on the estimation of *Kpu*. When *fu* tends to 0, so does Kpu_{app}/Kpu; i.e., *Kpu* estimates will be most affected by albumin diffusion for compounds that bind highly to albumin.

The correction factor (CF) is calculated from readily determined or available parameters and is used to convert the experiment determined Kpu_{app} to the true *Kpu*. From Fig. 3, *CF's* are seen to increase with increasing protein binding (decreasing fu) and decreasing Kpu_{app}. This latter effect can be explained by the unbound intracellular fraction of compound. For example, a drug that binds avidly to plasma proteins but is predominantly unbound intracellularly will consequently

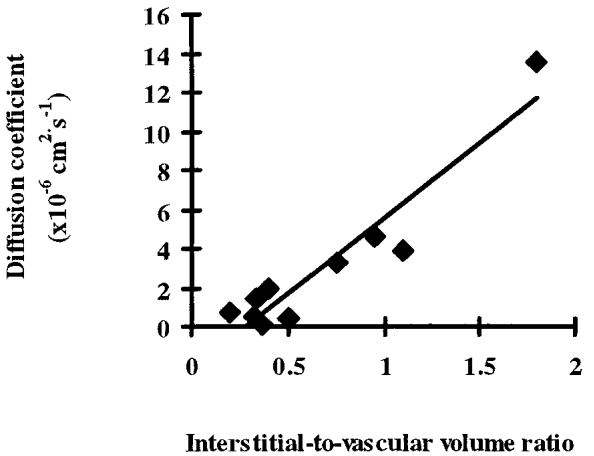

Fig. 4. Relationship between the diffusion coefficient of albumin from rat tissue pieces *in vitro* and the extracellular-to-vascular volume ratio. The line represents a linear regression through the data (r^2 = 0.895)

have low intracellular drug concentrations relative to plasma, effectively ensuring that the bulk of the drug is present in the extracellular tissue compartment, if no diffusion of proteins from the tissue were observed. Since extracellular tissue proteins are observed to diffuse from the tissue into the medium, however, a substantial amount of the drug placed in the incubation medium *in vitro* either remains there bound to effluxed protein or, on entering the tissue, subsequently returns to the medium bound to effluxing proteins.

In conclusion, although albumin diffusion has been demonstrated to occur from tissue pieces during *in vitro* incubations, it is unlikely to result in an underestimate of *Kpu* for the majority of compounds that bind to this protein. The compounds for which *Kpu* is most likely to be underestimated experimentally are those that bind avidly to albumin and do not enter cells to any great extent. In these circumstances a factor can be readily calculated to correct for the underestimation. The authors have applied this correction to the study of the *Kpu* values of a series of barbiturates discussed in a companion paper (19).

ACKNOWLEDGMENTS

Peter Ballard thanks AstraZeneca for sponsoring this research.

APPENDIX

Symbols

Cu, the unbound drug concentration
Cb, the bound drug concentration
fu, the unbound fraction of drug in plasma
fu_I, is the unbound intracellular fraction of compound.
f_P, the ratio of the binding protein concentrations originally in extracellular space to that in plasma
f_R, fraction of extracellular binding protein remaining in tissue at the end of the incubation
K_A, is the protein binding association constant
Kpu, the true tissue-to-medium partition coefficient.
Kpu_{app}, measured or apparent *in vitro* tissue-to-medium partition coefficient
P_E, extracellular protein concentration
P_L, is the difference in protein concentration between the start and end of the incubation
V_E, extracellular tissue volume
V_M, the volume of medium
V_{pl}, the volume of plasma
V_T, total tissue volume
θ_E, the extracellular to total tissue volume ratio

Derivation of an Expression Detailing the Effect of Diffusion of Albumin from Tissue Pieces to Media during *in Vitro* Incubation on the Distribution of Compounds

The schematic model displayed in Fig. 5 was used to derive an expression detailing the effect that the diffusion of albumin from tissue pieces to medium during *in vitro* incubations has on the equilibrium distribution of compounds. The derived expression,

$$Kpu \,/\, Kpu_{app},$$

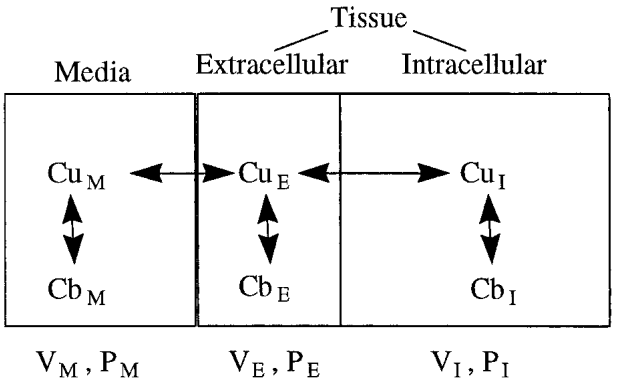

Fig. 5. *In vitro* model of drug distribution indicating that only unbound drug moves between media, extracellular and intracellular tissue spaces and unbound drug is in dynamic equilibrium with bound drug in each compartment.

expresses the relationship between compound distribution in an *in vitro* model in the absence of any movement of albumin (Kpu), the theoretical value, to that where albumin has diffused to the medium (Kpu_{app}), the measured value.

Definition of terms: Cu and Cb are the unbound and bound drug concentrations; V is the compartmental volume; P is the protein concentration in the compartment; M, E and I are the subscripts referring to the medium, extracellular and intracellular spaces respectively, as applied to C, V and P.

At equilibrium, the following is assumed for all compartments:

$$Cu_M = Cu_E = Cu_I \tag{5}$$

The total concentration in each compartment can be expressed in terms of the bound and unbound concentrations:

$$C_X = Cb_X + Cu_X \tag{6}$$

where $x = M$, E or I.

The unbound fraction is then:

$$fu_x = Cu_x \,/\, C_x \tag{7}$$

Condition *A*

Where no extracellular binding protein has diffused into the protein-free medium.

The mass balance can be defined as:

$$Dose = V_M \cdot Cu_M + V_E[Cu_E + Cb_E] + V_I[Cu_I + Cb_I] \tag{8}$$

where *dose* is the total amount of drug added to the incubation medium.

At a concentration below saturation of the binding protein

$$Cb_E = K_A \cdot P_E \cdot Cu_E \tag{9}$$

where K_A is the protein binding association constant.

Substituting Eq. 6 into Eq. 9:

$$C_E = Cu_E \cdot (1 + K_A \cdot P_E) \tag{10}$$

and Eq. 7 and 10 into Eq. 8 gives:

$$Dose = V_M \cdot Cu_M + V_E \cdot Cu_E \cdot [1 + K_A \cdot P_E] + V_I \cdot Cu_I \,/\, fu_I \tag{11}$$

where fu_I is the intracellular unbound drug fraction.

From the definition for the tissue-to-unbound plasma partition coefficient (Kpu)

$$Kpu = \frac{C_T}{Cu} = \frac{[Dose - V_M \cdot Cu] / V_T}{Cu} \quad (12)$$

where C_T is the total tissue concentration and V_T is the volume of tissue.

But from Eq. 5 and substituting for Eq. 11:

$$Kpu = [V_E(1 + K_A \cdot P_E) + V_I / fu_I] / V_T \quad (13)$$

Finally, defining θ_E as the extracellular volume fraction of the tissue $= V_E/V_T$, it follows that:

$$Kpu = \theta_E[1 + K_A \cdot P_E] + (1 - \theta_E) / fu_I \quad (14)$$

Condition *B*

Where extracellular binding protein has diffused from tissue into the initially protein-free medium and the final concentration remaining in the extracellular fluid $= P_E'$

Hence, the loss of protein from the tissue ($V_T.P_L$, where P_L is the difference in protein concentration between the start and end of the incubation), matched by the gain in the medium, is given by:

$$V_T \cdot P_L = V_E \cdot (P_E - P_E') \quad (15)$$

The amount of drug in the medium, $V_M \cdot C_M'$ where the symbol $'$ refers to Condition B, is therefore equal to the sum of the amount unbound in the media and that bound to diffused protein:

$$V_M \cdot C_M' = V_M \cdot Cu_M' + V_E \cdot Cu_E' \cdot K_A \cdot (P_E - P_E') \quad (16)$$

Dividing by V_M and substituting for θ_E gives:

$$C_M' = Cu_M' + \frac{V_T}{V_M} \cdot \theta_E \cdot Cu_E' \cdot K_A \cdot (P_E - P_E') \quad (17)$$

Defining the tissue-to-medium partition coefficient when albumin has diffused into the medium (Kpu_{app}) as:

$$Kpu_{app} = \frac{C_T'}{C_M'} = \frac{[Dose - V_M \cdot C_M'] / V_T}{C_M'} \quad (18)$$

Substituting Eq. 11 (where $Cu_X = Cu_X'$, $P_E = P_E'$ *and* $Cu_M = C_M'$ for the mass balance) into Eq. 18 yields:

$$Kpu_{app} = \frac{[V_E Cu_E'[1 + K_A \cdot P_E'] + V_I \cdot Cu_I' / fu_I] / V_T}{C_M'} \quad (19)$$

Substituting θ_E and Equation 19 gives:

$$Kpu_{app} = \frac{\theta_E \cdot Cu_E'[1 + K_A \cdot P_E'] + (1 - \theta_E) Cu_I' / fu_I}{Cu_M' + \theta_E \frac{V_T}{V_M} Cu_E' K_A \cdot (P_E - P_E')} \quad (20)$$

But at equilibrium all unbound concentrations are equal and as equilibrium is not disturbed by movement of protein, it follows that $Cu' = Cu$, so that Kpu_{app} simplifies to:

$$Kpu_{app} = \frac{\theta_E \cdot [1 + K_A \cdot P_E'] + (1 - \theta_E) / fu_I}{1 + \theta_E \frac{V_T}{V_M} K_A \cdot (P_E - P_E')} \quad (21)$$

Now, let the fraction of extracellular binding protein remaining in tissue $(f_R) = P_E'/P_E$ and adding and subtracting $\theta_E(1 - f_R)K_A \cdot P_E$ to the numerator of Eq. 21:

$$Kpu_{app} = \frac{\theta_E \cdot [1 + K_A \cdot P_E] + (1 - \theta_E) / fu_I - \theta_E (1 - f_R) K_A \cdot P_E}{1 + \theta_E \frac{V_T}{V_M} K_A \cdot P_E (1 - f_R)} \quad (22)$$

So that, dividing Eq. 14 by Eq. 22 yields:

$$\frac{Kpu}{Kpu_{app}} = \frac{\theta_E \cdot [1 + K_A \cdot P_E] + (1 - \theta_E) / fu_I}{\left\{ \frac{\theta_E \cdot [1 + K_A \cdot P_E] + (1 - \theta_E) / fu_I - \theta_E \cdot (1 - f_R) K_A \cdot P_E}{1 + \theta_E \frac{V_T}{V_M} K_A \cdot P_E (1 - f_R)} \right\}} \quad (23)$$

which on dividing the numerator and denominator by $\theta_E[1 + K_A \cdot P_E] + (1 - \theta_E)/fu_I$, reduces to:

$$\frac{Kpu}{Kpu_{app}} = \frac{1 + \theta_E \frac{V_T}{V_M} K_A \cdot P_E (1 - f_R)}{1 - \left\{ \frac{\theta_E \cdot (1 - f_R) K_A \cdot P_E}{\theta_E \cdot [1 + K_A \cdot P_E] + (1 - \theta_E) / fu_I} \right\}} \quad (24)$$

It remains to parameterise Eq. 24 using experimentally determined variables. Let fu = fraction of drug unbound in plasma and the total concentration of binding protein in plasma $= P_P$, then under non-saturating conditions:

$$fu = \frac{1}{1 + K_A \cdot P_P} \quad (25)$$

So that:

$$K_A \cdot P_E = K_A \frac{P_E}{P_P} P_P = f_P \left(\frac{1 - fu}{fu} \right) \quad (26)$$

where $f_P = P_E/P_P$

hence, substituting into Equation 26 yields:

$$\frac{Kpu}{Kpu_{app}} = \frac{1 + \theta_E \frac{V_T}{V_M} f_P \left(\frac{1 - fu}{fu} \right) (1 - f_R)}{1 - \left\{ \frac{(1 - f_R) f_P \left(\frac{1 - fu}{fu} \right) \cdot}{1 + f_P \left(\frac{1 - fu}{fu} \right) + \left(\frac{1}{\theta_E} - 1 \right) \Big/ fu_I} \right\}} \quad (27)$$

Given that Kpu_{app} underestimates Kpu, it is easier to view the reciprocal of this expression, i.e., by comparing the measured to the theoretical tissue-to-unbound plasma partition coefficient (Eq. 3 in the body of the text).

Derivation of a Correction Factor to account for the effect of the diffusion of albumin from tissue pieces during *in vitro* incubations on Kpu.

It follows that the measured Kpu_{app} is related to the true Kpu through a correction factor (CF):

$$Kpu = CF \cdot Kpu_{app} \quad (28)$$

hence

$$CF = Kpu / Kpu_{app} \quad (29)$$

Although Eq. 27 parameterizes this expression, it contains the term fu_I, which cannot be readily determined experimentally.

However, substituting for *Kpu* in Eq. 14 into Eq. 22 yields:

$$Kpu_{app} = \frac{Kpu - \theta_E(1-f_R)K_A \cdot P_E}{1 + \theta_E \frac{V_T}{V_M} K_A \cdot P_E(1-f_R)} \tag{30}$$

or, on rearrangement:

$$\frac{Kpu}{Kpu_{app}} = \left[1 + \theta_E\left(\frac{V_T}{V_M}\right) \cdot K_A \cdot P_E(1-f_R)\right] + \frac{\theta_E(1-f_R)K_A \cdot P_E}{Kpu_{app}} \tag{31}$$

Substituting Eq. 26 into Eq. 31 gives an expression for a correction factor (CF) accounting for the effect of albumin diffusion on *Kpu* during *in vitro* incubations from experimentally determined variables:

$$CF = \frac{Kpu}{Kpu_{app}} = \left[1 + \theta_E\left(\frac{V_T}{V_M}\right) \cdot \left(\frac{1-fu}{fu}\right) f_p(1-f_R)\right] + \frac{\theta_E(1-f_R)\left(\frac{1-fu}{fu}\right)f_p}{Kpu_{app}} \tag{32}$$

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