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Research Article

Saturable Binding of Cyclosporin A to Erythrocytes: Estimation of Binding Parameters in Renal Transplant Patients and Implications for Bioavailability Assessment

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Cyclosporin (CyA) exhibits saturable binding to erythrocytes. A one-site binding model was fitted to data from renal transplant patients receiving CyA therapy. The average maximum binding capacity is 2560 μg CyA/liter of packed erythrocytes; the unbound CyA concentration associated with 50% saturation of the binding site is 67 μg/liter. Analysis indicates that whole-blood CyA measurement to monitor drug therapy should be viewed cautiously, particularly when the hematocrit varies considerably. The error in estimating absolute bioavailability at steady state from whole-blood measurements, resulting as a consequence of the saturable binding, has been explored. Although extrapolation to the therapeutic situation, which involves transient drug administration, is difficult, errors of up to 25% are anticipated. When an accurate estimate of bioavailability is required, analysis based on plasma data is proposed. For bioequivalence testing, both blood and plasma CyA data are equally acceptable.

KEY WORDS: cyclosporin; saturable binding; erythrocyte; bioavailability; therapeutic drug monitoring.

INTRODUCTION

Cyclosporin A (CyA) is a third-generation immunosuppressant dosed chronically to transplant patients (1). Owing to its narrow therapeutic index and relatively soft indices of efficacy and toxicity, both blood and plasma concentration monitoring of CyA has become commonplace. However, the quantitative distribution of CyA within blood is poorly defined.

Cyclosporin binds to both erythrocytes and plasma proteins, with the ratio of the concentration of CyA in blood to that in plasma (C_B/C_P) being concentration dependent (2). In man, the binding of CyA to plasma proteins is independent of concentration (3,4), which implies that any concentration dependence of C_B/C_P is due to saturable binding of the drug to erythrocytes. Saturable erythrocyte binding of CyA has also been observed in rabbit blood *in vitro* and a model, based on a single binding site, has been applied to these data (5).

In this paper we present data to confirm saturable erythrocyte binding in renal transplant patients undergoing CyA therapy and estimate the parameters for the single-binding site model. The implications of saturable erythrocyte binding in the assessment of bioavailability of CyA from plasma and whole-blood data are also examined by use of the model.

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MATERIALS AND METHODS

Patient Dosing and Sampling

The blood samples were taken during a study to investigate oral and intravenous pharmacokinetics of CyA in renal transplant patients; approximately 30 samples were taken from each of five patients over a 10-day period immediately posttransplant. The intravenous dose was given as a continuous infusion over 3 days at 7 mg/kg/day via a central venous line. This infusion was followed by an oral regimen of 8.5 mg, given as a freshly prepared emulsion with Caotina chocolate powder and cold milk, every 12 hr. A 12-hr oral profile was taken on day 8 for assessment of bioavailability.

Blood samples (10 ml) were taken by venipuncture into EDTA tubes and either processed immediately or stored at 4°C. Measurements of the blood-to-plasma concentration ratio (R), fraction unbound (fu), and hematocrit (H) were carried out within 24 hr of sampling.

Blood-to-Plasma Ratio (R) Measurements

The partitioning of CyA into erythrocytes was carried out at 37°C using a tritiated CyA tracer of specific activity 4000 dpm ng^{-1} (supplied by Sandoz Ltd., Basel). The radiochemical purity of this material was >99%.

A 5-μl aliquot of radiolabeled CyA dissolved in methanol was freshly spiked into a clean tube and dried in a stream of oxygen-free nitrogen. The CyA was then dissolved and equilibrated with 4.0 ml of blood by incubation for at least 30 min in a 37°C water bath, with intermittent gentle mixing. The sample was then quickly centrifuged in buckets

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preheated to 37°C and the concentration of radioactivity in 180-μl aliquots of the plasma (C_P*) was determined by liquid scintillation counting in an LKB Rackbeta 1218 counter using a radioimmunoassay (RIA) Luma (Fisons, Loughborough) scintillator. The remaining radioactive plasma was used for determination of the fraction unbound (fu).

The total (whole-blood) concentration of radioactivity (C_B*) was obtained by counting 5-μl aliquots of the spiking solution, thus enabling the ratio C_B*/C_P* to be calculated. This ratio was taken to be the blood-to-plasma concentration ratio (R) of unlabeled drug.

Fraction Unbound

The fraction of CyA unbound in plasma was determined by ultracentrifugation (3) using radiolabeled CyA in unadulterated plasma, spiked as described above. The binding measurements were all carried out at 37°C.

CyA Concentration in Plasma (C_P)

This was measured by a high-performance liquid chromatographic (HPLC) method based on that of Carruthers *et al.* (6), using cyclosporin D as internal standard. Samples from each patient were analyzed simultaneously, and the intrabatch variability determined at a plasma concentration of 100 μg liter^{-1} was 4.1% (CV) and that at 1400 μg liter^{-1} was 5.0% (CV).

THEORY

Binding Isotherm

It is assumed that an equilibrium exists between unbound drug and drug bound to a single-site binding material associated with the erythrocyte, with the binding characterized simply by a dissociation constant K_d. From mass-action considerations,

$$K_d = \frac{C_u(nP_t - C_{be})}{C_{be}} \quad (1)$$

where C_u is the concentration of unbound drug, C_{be} is the concentration of drug bound to material in packed erythrocytes, P_t is the total concentration of binding material in packed erythrocytes, and n is the number of binding sites per unit of binding material. Rearrangement of Eq. (1) gives

$$C_{be} = \frac{nP_t \cdot C_u}{K_d + C_u} \quad (2)$$

Assuming that C_u is the same both inside and outside the erythrocyte, then the concentration of drug in packed erythrocytes, C_e, is given by

$$C_e = C_u + C_{be} \quad (3)$$

Combining Eqs. (2) and (3) gives

$$C_e = C_u + \frac{nP_tC_u}{K_d + C_u} \quad (4)$$

If C_B is the total-blood concentration of drug, then by mass balance and considering a unit volume,

$$C_B = C_P(1 - H) + C_e \cdot H \quad (5)$$

where C_P is the concentration of drug in plasma and H is the hematocrit. Substitution of Eq. (4) into Eq. (5), replacing nP_t (total binding capacity in packed erythrocytes) by K_1 and K_d by K_2, gives

$$C_B = C_P(1 - H) + H\left(C_u + \frac{K_1C_u}{K_2 + C_u}\right) \quad (6)$$

If the fraction unbound (fu) is independent of C_P (so that C_u = fu · C_P), then the blood/plasma concentration ratio (R) is derived from Eq. (6):

$$R = \frac{C_B}{C_P} = (1 - H) + H \cdot \text{fu} \cdot \left(1 + \frac{K_1}{K_2 + C_P\,\text{fu}}\right) \quad (7)$$

The parameters K_1 and K_2 can thus be determined from the values of R, H, fu, and C_P associated with each sample. Once K_1 and K_2 have been determined, then R can be calculated from the three variables H, C_P, and fu if they are either known or assumed to be constant.

The binding isotherm of Eq. (7) was fitted to the multivariate data (R, H, fu, and C_P) for each patient data set using nonlinear least-squares regression, to yield estimates of K_1 and K_2.

At high CyA concentrations the whole-blood concentration becomes lower than the plasma concentration. The minimum value of R indicated by Eq. (7) is always <1 and occurs as $C_P \rightarrow \infty$:

$$R_{min} = 1 - H(1 - \text{fu}) \quad (8)$$

Estimation of Bioavailability

The following derivation for estimation of bioavailability for a drug with saturable erythrocyte binding is from a consideration of steady-state conditions.

Given a constant-rate infusion to steady state by both intravenous (iv) and oral (o) routes, then for measurements of drug in plasma,

$$R_0(\text{iv}) = \text{CL}_P(\text{iv}) \cdot C_P(\text{iv}) \quad (9)$$
$$F_PR_0(\text{o}) = \text{CL}_P(\text{o}) \cdot C_P(\text{o}) \quad (10)$$

and for measurements of drug in blood,

$$R_0(\text{iv}) = \text{CL}_B(\text{iv}) \cdot C_B(\text{iv}) \quad (11)$$
$$F_BR_0(\text{o}) = \text{CL}_B(\text{o}) \cdot C_B(\text{o}) \quad (12)$$

where R_O is the drug infusion rate; CL_P and CL_B are the clearances from plasma and blood, respectively; C_P and C_B are the steady-state plasma and blood concentrations, respectively; and F_P and F_B are the bioavailability values derived from plasma and blood data, respectively. Assuming that neither CL_P nor CL_B changes either with concentration or between treatments [i.e., $CL_P(o) = CL_P(iv)$ and $CL_B(o) = CL_B(iv)$], then

$$F_P = \frac{C_P(\text{o})}{C_P(\text{iv})} \cdot \frac{R_0(\text{iv})}{R_0(\text{o})} \quad (13)$$

and

$$F_B = \frac{C_B(\text{o})}{C_B(\text{iv})} \cdot \frac{R_0(\text{iv})}{R_0(\text{o})} \quad (14)$$

From Eqs. (13) and (14) we can obtain the following ratio

$$\frac{F_B}{F_P} = \frac{C_B(o)}{C_P(o)} \cdot \frac{C_P(iv)}{C_B(iv)} \quad (15)$$

The respective C_B/C_P ratios can be substituted using Eq. (7) to give

$$\frac{F_B}{F_P} = \frac{(1 - H) + H \cdot fu \cdot (1 + \{K_1/[K_2 + fu\ C_P(o)]\})}{(1 - H) + H \cdot fu \cdot (1 + \{K_1/[K_2 + fu\ C_P(iv)]\})} \quad (16)$$

To simulate the variation of $C_P(o)$ with $C_P(iv)$ for various F_B/F_P values, Eq. (16) was rearranged to give $C_P(o)$ as a function of $C_P(iv)$.

RESULTS

Erythrocyte Binding

The plot in Fig. 1 confirms the anticipated nonlinearity of CyA binding to erythrocytes in blood from renal transplant patients receiving CyA therapy. The parameters obtained on fitting the model of Eq. (7) to the data from the five patients are given in Table I, together with their average values of the fraction of drug in plasma unbound (fu) and the average hematocrits observed.

The value determined for K_1 (the "capacity factor") ranges from 2089 to 3297 μg/liter, with an average of 2562 μg/liter (2.1×10^{-6} *M*), and is the concentration of CyA associated with the binding material in packed erythrocytes at saturation. It should be noted that this value is independent of the hematocrit. The value for K_2 (the dissociation constant) ranges from 52.8 to 75.3 μg/liter, with an average of 66.9 μg/liter (5.6×10^{-8} *M*), and represents the unbound concentration of CyA at which the erythrocytic binding material will be half-saturated.

Bioavailability Assessment

Estimates of the extent of absorption, or bioavailability (*F*), of CyA obtained from measurement of CyA in blood and plasma are likely to be different, especially if the intravenous concentration–time profile differs considerably from the oral profile.

An examination of Eq. (16) shows that $F_B = F_P$ only in the special case where $C_P(o) = C_P(iv)$, and consequently $C_B(o) = C_B(iv)$, that is, the situation when the rates of intravenous and oral administration are adjusted to produce the same steady-state concentration of CyA in plasma or blood. Under all other conditions the estimated F_B will not equal F_P. Figure 2 shows the predicted influence of differences in the steady-state plasma CyA concentration, following oral and intravenous infusion rates, on the bioavailability ratio F_B/F_P for a normal hematocrit ($H = 0.45$), using the average values of fu = 0.07, K_1 = 2562 μg/liter, and K_2 = 67 μg/liter (Table I). It is seen that the ratio F_B/F_P is less than unity when the plasma concentration after oral administration is greater than that after intravenous administration, and vice versa. It should also be noted that for a given F_B/F_P ratio, the deviation from unity is greater the higher the absolute concentrations. Further, a comparison between the results in Fig. 2 ($H = 0.45$) and those in Fig. 3 (using a low hematocrit, $H = 0.24$, with all other parameters held constant) clearly demonstrates that the lower the hematocrit the smaller is the difference between F_B and F_P.

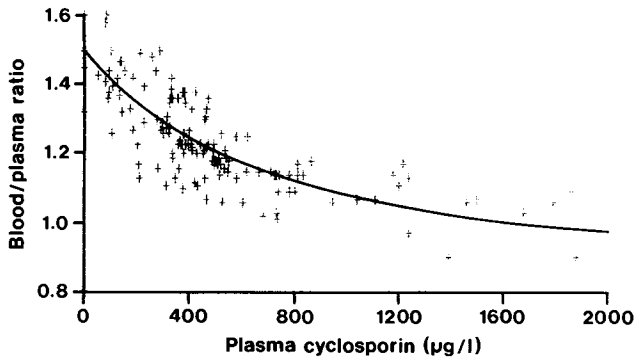

Fig. 1. Variation of blood/plasma concentration ratio (C_B/C_P) with plasma cyclosporin A concentration. The ratio is also a function of hematocrit and fraction unbound, which account for some of the scatter. The fitted line is according to Eq. (7) using $H = 0.24$, fu = 0.072, K_1 = 2209 μg/liter, and K_2 = 52.88 μg/liter.

DISCUSSION

Erythrocyte Binding Parameters

The values of K_1 (the capacity factor) and K_2 (the dissociation constant) found in samples from the renal transplant patients receiving CyA are comparable to the values found in spiked rabbit blood (with constant fu and *H*) of K_1 = 3590 μg/liter (3.0×10^{-6} *M*) and K_2 = 147 μg/liter (1.2×10^{-7} *M*) obtained from the data of Awni and Sawchuk (5). They estimated an fu of 0.405, a value sixfold higher than seen in the renal transplant patients, whose measured average fu was 0.073 (Table I).

The dissociation constant observed in our experiments ($K_2 = 5.6 \times 10^{-8}$ *M*) corresponds very closely to the value of 5×10^{-8} *M* obtained in *in vitro* hemolysates of human blood (7). The similarity may be fortuitous, as the parameter was measured under completely different conditions. In particular, the techniques used by these authors to determine the unbound drug (charcoal adsorption) and the temperature (4°C) were both different from those used by us. The capacity constant determined in the same paper (4 μ*M*, "within erythrocytes") compares favorably with the value obtained here (2.1 μ*M*, in packed erythrocytes), notwithstanding the above reservations.

The ratio K_1/K_2, which averaged 38.5, is a measure of the uptake of CyA by erythrocytes at a low CyA concentration. That is, at a low CyA concentration the concentration in the erythrocyte is approximately 40 times the unbound plasma concentration, falling to a factor of about 20 at an unbound concentration equal to K_2 (66.9 μg/liter). These results emphasize the high affinity between the erythrocyte binding component(s) and CyA.

Despite the acknowledged nonlinear binding to erythrocytes, various authors have suggested that the measurement of CyA should be carried out in whole blood (2,8–11). The main reason for this recommendation is to overcome the complexity of separating plasma at 37°C, which is necessary because of the temperature-dependent distribution of CyA into erythrocytes (2). Another reason is that higher concentrations of CyA generally occur in whole blood, enabling

Table I. Parameter Values Obtained by Fitting the Erythrocyte Binding Isotherm Model Represented by Eq. (7) to the Cyclosporin Data from Renal Transplant Patients

Patient	No. of samples	Average fraction unbound (fu)	Average Haematocrit (H)	K_1 ($CV\%$) (μg/liter)	K_2 ($CV\%$) (μg/liter)	K_1/K_2
1	27	0.0546	0.271	2089 (15)	52.8 (20)	39.6
2	20	0.0879	0.234	2537 (14)	75.3 (19)	33.7
3	32	0.0685	0.230	2310 (14)	63.9 (20)	36.2
4	30	0.0714	0.240	3297 (12)	75.2 (14)	43.8
5	30	0.0811	0.227	2577 (21)	65.7 (29)	39.2
Average		0.0727	0.241	2562	66.9	38.5
SD				455	9.3	

concentrations to be measured following lower doses or for longer periods of time after administration than can be achieved with plasma measurements. If this recommendation is followed, and blood is used as the measurement medium for CyA, then the nonlinear distribution into erythrocytes raises questions in therapeutic monitoring and in bioavailability assessment.

Therapeutic Monitoring

For therapeutic monitoring the ideal would be to measure the unbound concentration, as it is generally accepted that in biological systems it is this concentration which correlates best with the pharmacological or toxicological effect. If the fraction unbound in plasma shows little variation, then the total plasma concentration is an equally good index of the potential effect. However, when, as commonly occurs, blood is used instead of plasma, the predictability of the effect will decrease because of the increased variability of the measured concentration for a given unbound concentration, due to variations in hematocrit and erythrocyte binding constants among patients. Normally, the hematocrit is about 0.45 but in renal patients it is required to be 0.25 or even lower immediately posttransplant, as observed in the current study (Table I). The effect of hematocrit on C_B/C_P is best seen by reference to Fig. 4, which shows, using Eq. (7), the predicted changes in the blood-to-plasma ratio with the plasma CyA concentration for different hematocrit values, assuming a constant typical fu value of 0.07 (Table I). During CyA therapy trough whole-blood concentrations are usually monitored with the recommendation that they lie below 250 μg/liter (12), which corresponds to an even lower plasma CyA concentration. It is particularly at these low concentrations that C_B/C_P is most sensitive to variations in hematocrit.

Assessment of Bioavailability

The estimated oral bioavailability of CyA is generally low and is highly variable (13,14). While much of this variability probably reflects the underlying variability in CyA absorption, some may be due to differences in methodology. Values based on RIA analyses must be questioned, given the acknowledged cross-reactivity of CyA metabolites in the commercial RIA kits using polyclonal antibodies (15). The present analysis also indicates that some differences exist between results based on measurement in blood and those based on plasma, even when a specific chemical assay is used. There are also recommendations in the literature that plasma should be separated from blood at temperatures lower than 37°C. CyA concentrations measured in such samples necessarily bear a complex relationship to concentrations in plasma at 37°C due to the variables involved in the redistribution process as the temperature is lowered. Bioavailability estimates using such samples should be viewed with suspicion.

The present theoretical analysis is based on the assumption that plasma clearance, rather than blood clear-

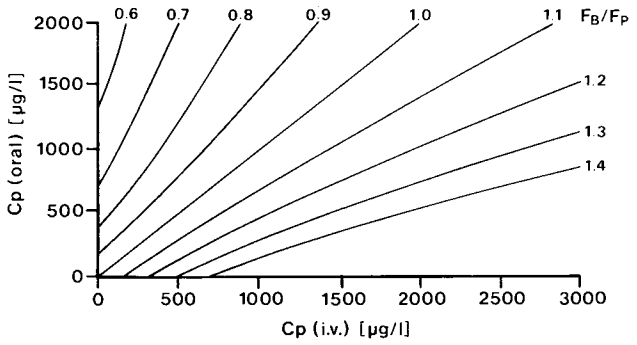

Fig. 2. Simulation of the error in F_B (expressed as the F_B/F_P ratio) as a function of the steady-state plasma concentrations of cyclosporin A during oral and iv infusion, using Eq. (16) with H = 0.45, fu = 0.07, K_1 = 2562 μg/liter, and K_2 = 66.9 μg/liter.

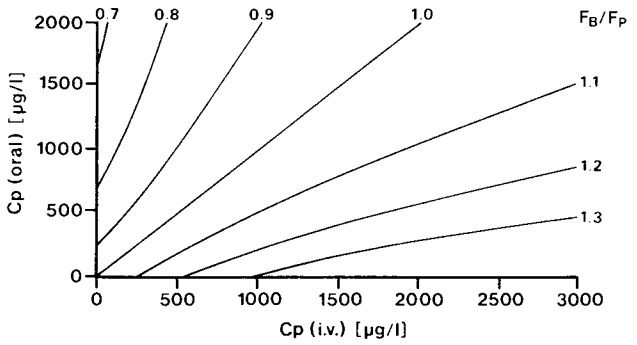

Fig. 3. Simulation of the error in F_B (expressed as the F_B/F_P ratio) as a function of the steady-state plasma concentrations of cyclosporin A during oral and iv infusion, using Eq. (16) with H = 0.24 and other variables as in the legend to Fig. 2.

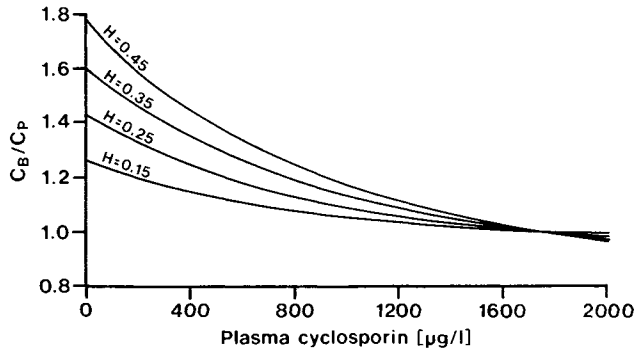

Fig. 4. Blood-to-plasma cyclosporin A concentration ratio as a function of plasma CyA concentration for various hematocrits, according to Eq. (7) with fu = 0.07, K_1 = 2562 μg/liter, and K_2 = 66.9 μg/liter.

ance, is constant and is independent of both drug concentration and hematocrit. That is, assessment of bioavailability based on measurements in plasma (separated at 37°C) will give a more accurate estimate than that based on measurement of CyA in blood. Support for this assumption centers around the extraction ratio of CyA.

Estimates of total-blood clearance of CyA in man, based on measurement of the whole-blood concentration using a specific HPLC assay, average about 6 ml/min/kg (14); elimination is predominantly by metabolism, as very little is excreted unchanged (16). Assuming a hepatic blood flow of 1500 ml/min/1.73 M^2 (17), and that all metabolism occurs in the liver, the hepatic extraction ratio of CyA is about 0.3, which indicates that CyA is a drug of low to medium extraction. Theory predicts that, for such a drug, the blood clearance (CL_B) varies reciprocally with the blood-to-plasma drug concentration ratio (R), whereas the plasma clearance (CL_P) is essentially independent of R (see Appendix). Accordingly, as the value of R varies with both CyA concentration and hematocrit, one would expect CL_B to vary much more than CL_P. Theory also predicts that for a drug of a low extraction ratio, both CL_B and CL_P vary proportionately with fu (see Appendix), but for CyA, fu is independent of the plasma concentration (3,4).

The current analysis indicates that the degree of discrepancy between the estimates of bioavailability based on blood (F_B) and those based on plasma (F_P) depends on both the absolute concentrations of CyA, after oral and intravenous administration, and the hematocrit. The ranges of concentrations and hematocrit values investigated are those commonly observed in practice, the hematocrit of patients for some weeks after organ transplant being particularly low. When the blood and plasma concentrations are comparable, the difference between F_B and F_P is small, but it can be appreciable if the concentrations differ by a large degree. For example, if the steady-state plasma concentrations were 500 and 2000 μg/liter after oral and intravenous infusions, then with a hematocrit of 0.45 (Fig. 2) F_B/F_P = 1.4, indicating that F_B would be 40% higher than the true value. In contrast, a reversal in the concentrations seen after oral and intravenous administrations indicates that F_B would be only 70% of the true value.

Precise comparisons between these predicitions of error using whole-blood data and those occurring experimentally are difficult. Consideration at steady state after constant-rate administration was chosen, as it afforded an analytical solution to the question of differences between F_B and F_P. Although experimentally constant-rate intravenous infusion to steady state is possible, it will not be achieved after oral administration. CyA is usually given every 12 hr, resulting in very wide fluctuations in the plasma (and blood) concentrations within a dosing interval, even at steady state (18). In practice, even the intravenous dose is commonly given as a relatively short-term infusion, over 2–6 hr, which also leads to a large variation in concentration. Where data are provided that allow comparisons, frequently the maximum concentrations after intravenous administration are greater than that seen after oral administration (19). However, the problem is complicated by the difference between F_B and F_P being dependent not only on the maximum concentration found but on the entire shape of the concentration–time curve, which is different after oral and intravenous administration. Theoretically, the discrepancy between F_B and F_P would disappear if the intravenous input profile was programmed to produce a concentration–time profile that matched the oral profile. In practice, this is very difficult to achieve, especially when, as occurs with CyA, oral absorption cannot be defined by a simple kinetic process.

Although exact statements cannot be made, it appears that in most situations the ratio F_B/F_P will lie between 0.7 and 1.4. Such a deviation from unity may be acceptable when a decision is being made whether the oral absorption of CyA in an individual or group of patients is, say, 20% as compared to 50%. However, when a precise estimate of absolute bioavailability is required, assessment should be based on measurement of drug in plasma separated from whole blood at 37°C.

The one situation where blood measurements should serve equally well as those in plasma is in the assessment of bioequivalence, for example, between two oral formulations of CyA, where the profiles would be expected to be quite similar.

APPENDIX: DEPENDENCY OF BLOOD CLEARANCE ON R FOR DRUGS OF LOW EXTRACTION

The relationship between plasma and whole-blood clearances is given by

$$CL_P = R \cdot CL_B \tag{A1}$$

where R is the blood-to-plasma concentration ratio.

Assume that the hepatic clearance of a drug is described by the well-stirred model of hepatic elimination (20).

$$CL_B = Q \cdot E = Q \cdot \left(\frac{fu_B \cdot CL_{int}}{Q + fu_B \cdot CL_{int}} \right) \tag{A2}$$

where E is the extraction ratio of the drug, Q is the blood flow, CL_{int} is its intrinsic clearance, and fu_B is the ratio of the unbound concentration in plasma (C_u) to the concentration in whole blood (C_B). The parameter fu_B can be related to the fraction in plasma unbound (fu) and R thus:

$$fu_B = \frac{C_u}{C_B} = \frac{fu \cdot C_P}{C_B} = \frac{fu}{R} \tag{A3}$$

Substituting Eq. (A3) into Eq. (A2) yields

$$CL_B = Q\left[\frac{(fu/R) \cdot CL_{int}}{Q + (fu/R) \cdot CL_{int}}\right] \quad (A4)$$

For a drug of low extraction ratio, $Q > [(fu/R) \cdot CL_{int}]$, Eq. (A4) reduces to the approximation

$$CL_B \simeq fu \cdot CL_{int}/R \quad (A5)$$

from which it is seen that CL_B is sensitive to variations in R. In contrast, substitution of Eq. (A5) into Eq. (A1) gives

$$CL_P = fu \cdot CL_{int} \quad (A6)$$

and indicates that CL_P is independent of R.

However, it should be noted that for a drug with a high extraction ratio, Eq. (A4) gives the approximation $CL_B \simeq Q$ and consequently $CL_P \simeq Q \cdot R$.

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