EFFECT OF HEMATOCRIT AND ALBUMIN CONCENTRATION ON HEPATIC CLEARANCE OF TACROLIMUS (FK506) DURING RABBIT LIVER PERFUSION

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ABSTRACT:
Tacrolimus is an immunosuppressive agent used for organ transplantation (1–3). The disposition of tacrolimus has been studied in transplant patients (4–7) and in animal models (8–11). Animal pharmacokinetic studies have shown that the disposition of this drug in rats is different than in humans. On other hand, rabbits exhibit pharmacokinetics of this drug similar to humans (10), with high plasma clearance and volume of distribution, and low whole blood clearance and volume of distribution. Rabbit liver perfusion studies have shown that the RBC binding of tacrolimus and slow efflux from RBC protect this drug from hepatic extraction. Computer simulations indicate that an increase in RBC binding of tacrolimus should increase the apparent plasma clearance (10). Clinical observations support this finding: patients with higher RBC binding of tacrolimus (greater whole blood:plasma ratio) exhibited higher plasma clearances (7). The purpose of the present study was to investigate the influence of different hematocrits and albumin concentrations on hepatic extraction of tacrolimus in a rabbit liver perfusion model with utilization of human erythrocytes. We had previously examined one experimental condition (hematocrit 0.1 with an albumin concentration of 1%).

Materials and Methods
Animals. Male New Zealand rabbits (Beckins Animal Farm, Sanborn, NY) weighing 3.5–4.0 kg were housed in a 12-hr light/dark, constant temperature (22°C) environment with free access to standard laboratory chow and tap water ad libitum. All rabbits were fasted overnight before experiments. The protocol was approved by the University Laboratory Animal Care Committee.

In Vitro RBC Binding Study. Erythrocytes were isolated from heparinized whole blood (Interstate Blood Bank, Memphis, TN) by centrifugation and were added to blank plasma or Krebs-Henseleit bicarbonate buffer (with 1 or 5% albumin) to achieve a hematocrit of 0.05 (buffer only), 0.1, 0.2, and 0.4, with a final concentration in reconstructed blood ranging from 5 to 150 ng/ml. After incubation for 30 min at 37°C, the plasma was separated by centrifugation at 37°C. Tacrolimus concentrations were determined in plasma, buffer, and whole blood.

In Vitro RBC Influx and Efflux Studies. For the influx study, plasma and RBCs were separated by centrifugation, and plasma or buffer were spiked with tacrolimus to achieve total (after reconstruction) concentrations near 5, 25, or 50 ng/ml. Spiked plasma (buffer: 1% albumin; temperature: 37°C) was mixed with RBCs to achieve hematocrits of 0.1, 0.2, or 0.4. The first sample was withdrawn immediately; the other samples were collected after incubation at...
37°C from 4 to 60 min. Plasma was separated by centrifugation (2 min). Tacrolimus concentrations were determined in plasma, buffer, and whole blood. RBC concentrations were calculated based on these data and hematocrits.

The efflux study was performed by mixing RBCs containing tacrolimus with blank plasma or buffer. In addition, buffer with 5% albumin and hematocrits of 0.05 and 0.2 were used.

**Perfusion Study.** The perfusion apparatus was a MX Amber Perfuser TWO/TEN (MX International, Inc., Aurora, CO). The 300–500 ml of perfusion medium was recirculated at a rate of 49–52 ml/min using a roller pump (Masterflex model; Cole-Palmer Instrument Co., Chicago, IL). Arterial and venous oxygen concentrations were measured with a D616 Thermostatted Cell with an E3036 pO2 electrode, a PHM71 MK2 acid/base analyzer, and a PHA 934 pO2 module (Radiometer, Copenhagen, Denmark).

**Surgical Technique.** Laparotomy was performed after the rabbits were anesthetized with 50 mg/kg ketamine HCl and 10 mg/kg xylazine HCl. The chest cavity was opened by cutting through the rib cage and pericardium. The heart was exposed and a catheter was placed in the left atrium for measurements of central venous pressure.

Tacrolimus concentrations were determined in plasma, buffer, and whole blood and plasma concentrations of tacrolimus (1 mg/ml) was added to the perfusate to achieve hematocrits of 0.05 or 0.2.

**Perfusion Experiment.** The ethanol solution of tacrolimus (1 mg/ml) was added to the perfusate to achieve a concentration of −50 ng/ml. A 400–600 ml aliquot of this perfusate was transferred to the beaker, incubated, and oxygenated with O2 : CO2 (95:5) at 37°C for 30 min. Before starting the experiment, the first sample was withdrawn from the reservoir, then 1-ml samples were taken from the reservoir and sampling port upon exit from the liver. Samples were split into two parts: after separating 0.1 ml of whole blood, one part was immediately centrifuged (4 min at 37°C), and the other part was incubated for 30 min at 37°C. After withdrawing 0.1 ml of whole blood, the sample was centrifuged as previously described. The plasma was separated immediately after centrifugation. We previously showed that tacrolimus does not bind to the perfusion device (10).

**Drug Assay.** Tacrolimus concentrations in plasma, whole blood, and perfusate were determined by the two-step immunoassay described by Tamura et al. (13), as modified by Jusko and D’Ambrosio (14). Standards spanned the concentration ranges of 0–120 ng/ml in human blood and 0–20 ng/ml in plasma.

**Pharmacokinetic Calculations.** All computer fittings were done using Adapt II (Biomedical Simulations Resource 1992, University of Southern California) with general least squares estimation. Because no difference was observed between different buffer/albumin concentrations (see fig. 3), such data were treated as the same group.

**RBC Binding Model.** The whole blood and plasma concentrations of tacrolimus from the in vitro RBC binding study with different hematocrits were fitted simultaneously with eq. 1 to obtain the values of binding capacity (Bmax) and affinity constant (Kd):

\[
C_{wb} = C_p + Hct \cdot \left( \frac{B_{max} \cdot C_p^y}{K_d^y + C_p^y} + Nb \cdot C_r \right),
\]

where \(C_{wb}\) is whole blood concentration, \(C_p\) is plasma (buffer) concentration, \(C_r\) is free concentration (\(C_r = C_p\) for buffer and \(C_r = C_p \cdot f_a\) for plasma), \(y\) is the Hill coefficient, \(Nb\) is nonspecific binding constant, and \(f_a\) is free fraction in plasma.

**RBC Binding/Diffusion Model.** To characterize the diffusion of tacrolimus between RBCs and plasma (or buffer), the model shown in fig. 1 was used. The free concentrations [\(C_{wb}\) and \(C_{buff}\)] inside RBCs during partitioning with buffer or plasma were calculated by a bisection method (15) using eqs. 2 and 3, with the lower limit as 0 and the upper limit as total concentration in RBCs (\(C_0\)):

\[
C_{buff} + \frac{B_{max} \cdot C_{buff}}{K_h + C_{buff}} + Nb \cdot C_{buff} - C_{buff} = 0 \quad (2)
\]

\[
C_{buff} + \frac{B_{max} \cdot C_{buff}}{K_h + C_{buff}} + Nb \cdot C_{buff} - C_{buff} = 0 \quad (3)
\]

where \(C_{buff}\) and \(C_{buff}\) are the RBC concentrations in buffer and plasma. When the range of plasma concentrations is large enough to produce nonlinear binding in RBCs, the diffusion model fitting must use differential equations. Diffusion equilibrium was assumed to occur between plasma (or buffer) and free drug in RBCs [\(C_{buff}\) or \(C_{buff}\)] according to:

\[
\frac{dC_{buff}}{dt} = -CL_{buff} \cdot C_{buff} + CL_{buff} \cdot C_{buff} \quad (4)
\]

\[
\frac{dC_{buff}}{dt} = CL_{buff} \cdot C_{buff} - CL_{buff} \cdot C_{buff} \quad (5)
\]

\[
\frac{dC_{buff}}{dt} = -CL_{buff} \cdot f_a \cdot C_p + CL_{buff} \cdot C_{buff} \quad (6)
\]

\[
\frac{dC_{buff}}{dt} = CL_{buff} \cdot f_a \cdot C_p - CL_{buff} \cdot C_{buff} \quad (7)
\]

The values of \(B_{max}, f_a, K_d, y,\) and \(Nb\) estimated from the in vitro binding study were set as constants. \(V_p\) and \(V_b\) are the fractional volumes of buffer (or plasma) and RBCs based on hematocrit. Data for all hematocrits and initial concentrations were fitted simultaneously to solve for influx (\(CL_{buff}\)) and efflux (\(CL_{buff}\)) diffusion clearances.

**Perfusion Model.** The well-stirred model of hepatic clearance with first-order metabolism of tacrolimus from plasma was used to characterize the in situ rabbit liver perfusion data as shown in fig. 2.

The RBCs and buffer data were fitted simultaneously with the following differential equations:

\[
\frac{dC_{buff}}{dt} = -Q_p \cdot C_{buff} + Q_p \cdot C_{buff} - CL_{buff} \cdot C_{buff} - CL_{buff} \cdot C_{buff} + CL_{buff} \cdot C_{buff} \quad (8)
\]

\[
\frac{dC_{buff}}{dt} = -Q_f \cdot C_{buff} + Q_f \cdot C_{buff} - CL_{buff} \cdot C_{buff} - CL_{buff} \cdot C_{buff} + CL_{buff} \cdot C_{buff} \quad (9)
\]

\[
\frac{dC_{buff}}{dt} = -Q_i \cdot C_{buff} + Q_i \cdot C_{buff} - CL_{buff} \cdot C_{buff} - CL_{buff} \cdot C_{buff} + CL_{buff} \cdot C_{buff} \quad (10)
\]

\[
\frac{dC_{buffer}}{dt} = Q_a \cdot C_{buff} + Q_a \cdot C_{buffer} - CL_{buffer} \cdot C_{buffer} - CL_{buffer} \cdot C_{buffer} + CL_{buffer} \cdot C_{buffer} \quad (11)
\]

The subscripts R and H define the reservoir and hepatic compartment volumes (\(V\)), flows (\(Q\)), and concentrations (\(C\)) for RBC (\(B\)) and plasma (\(P\)). \(CL_{buff}\) is the intrinsic metabolic clearance, whereas \(CL_{buff}\) and \(CL_{buff}\) are sampling clearances. Influx (\(CL_{buff}\)) and efflux (\(CL_{buff}\)) clearances were fixed as estimated from in vitro diffusion fittings. \(C_{buff}\) is the concentration entering the liver, and \(C_{buff}\) is the concentration exiting the liver. For the well-stirred model, the aforementioned equations were used as shown. For the parallel-tube model, the \(CL_{buffer}\) product was replaced by \(C_{buff} = \frac{C_{buff} h (C_{buff} C_{buff})}{C_{buff} h + C_{buff} h}\), and the \(CL_{buff}\) product was replaced by \(C_{buff} = C_{buff} h (C_{buff} C_{buff}) - C_{buff} h + C_{buff} h\). The same subroutines using eqs. 2 and 3 were applied to generate free drug concentrations inside RBCs in the reservoir (\(C_{buff}\)) or hepatic compartments (\(C_{buff}\)).
and $V_{RB}$ were recorded before each experiment. Hepatic RBC volume at hematocrit 0.05 [$V_{HP,0.05} = V_{HP,0.05} \cdot (0.05/0.95)$] and hematocrit at 0.2 [$V_{HP,0.2} = V_{HP,0.05} \cdot (0.8/0.95)$] were expressed as functions of $V_{HP,0.05}$ and hematocrit. Data from all six rabbits were then fitted simultaneously to estimate the $CL_{M}$ and hepatic plasma volume at hematocrit 0.05 ($V_{HP,0.05}$).

**Results**

**Effect of Albumin on Whole Blood:Plasma Ratio.** To determine if the concentration of albumin influences the binding of tacrolimus with RBC, human erythrocytes were reconstructed with Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 1 and 5% albumin. The hematocrits were 0.05, 0.1, 0.2, and 0.4, and concentrations of tacrolimus ranged from 5 to 150 ng/ml. The albumin concentration had no influence on the blood distribution of tacrolimus (fig. 3). Over the range of tacrolimus concentrations studied, the whole blood:plasma ratios were similar for both concentrations of albumin. On the other hand, hematocrit was an important factor governing blood distribution of tacrolimus (figs. 3 and 4). Another phenomenon observed in this study was the lower whole blood:plasma ratio in plasma reconstructed blood spiked with tacrolimus when compared with buffer reconstructed blood (fig. 4).

Initial fittings of *in vitro* binding data also showed no difference between 1 and 5% buffer albumin concentrations. Binding parameters (table 1) were obtained from fitting both plasma and buffer reconstructed whole blood binding data together using eq. 1. The fitted result is shown in fig. 5. The binding capacity ($B_{max}$) was 440 ng/ml RBCs, binding affinity ($K_M$) was 0.870 ng/ml, $\gamma$ was 1.33, and $N_{sb}$

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**Fig. 1. Binding/diffusion model for tacrolimus equilibration between plasma or buffer ($C_p, V_p$) and RBCs ($C_B, V_B$).

Unbound drug in plasma is $fu \cdot C_p$, whereas that in RBCs is $C_r$.

**Fig. 2. Perfusion/diffusion/binding model for hepatic disposition of tacrolimus.

Symbols are defined in Abbreviations.
was 4.22 for plasma-reconstructed RBCs and buffer suspension. The free fraction \((f_u)\) was 0.537 for plasma in whole blood.

**In Vitro Influx and Efflux Studies.** The diffusion of tacrolimus from human RBCs into buffer was fast, and equilibration was observed within 2 min (the first sampling point) (fig. 6). The diffusion from buffer into RBC was a much slower process. The diffusion clearance (table 1) was greater from RBCs to buffer (0.940 ml/min) than from buffer to RBCs (0.576 ml/min). The time to equilibration of tacrolimus concentrations in plasma with RBC (fig. 7) was slower compared with the buffer-RBC mixture. The diffusion clearance was also greater from RBC to plasma (1.70 ml/min) than from plasma to RBC (0.276 ml/min).

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Buffer</th>
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<th>Plasma</th>
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</thead>
<tbody>
<tr>
<td>Binding parameters</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hct fitted</td>
<td>0.05, 0.1, 0.2, 0.4</td>
<td>0.1, 0.2, 0.4</td>
<td></td>
</tr>
<tr>
<td>(K_D) (ng/ml)</td>
<td>0.870 (6.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B_{\text{max}}) (ng/ml)</td>
<td>440 (3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\gamma)</td>
<td>1.33 (3.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N_{sb})</td>
<td>4.22 (10.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(f_u)</td>
<td>0.537 (4.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Diffusion parameters | | | |
| Hct fitted | 0.05, 0.1, 0.2, 0.4 | 0.1, 0.4 |
| \(CL_{12}\) (ml/min) | 0.576 (1.1) |
| \(CL_{21}\) (ml/min) | 0.940 (1.6) |

All data are presented as the parameter estimate (coefficient of variation, %). See **Abbreviations** for more information.
Liver perfusion experiments were performed with two hematocrits: 0.05 and 0.2. In both conditions, there were no differences observed in tacrolimus concentrations in plasma isolated immediately after sampling or after 30 min of post-sampling incubation at 37°C. This is in good agreement with data for diffusion of tacrolimus from RBCs into buffer (fig 6). Typical disposition profiles of tacrolimus concentrations in plasma with whole blood collected from reservoir and exiting the liver for the two hematocrits and an albumin concentration of 1% are shown in fig. 8. The decline of concentrations in plasma and RBCs was more rapid for experiments with the hematocrit of 0.05. The initial plasma concentrations of tacrolimus were 5-fold higher for hematocrit 0.05 than for 0.2 in the same range of RBC concentrations. For the well-stirred model, the estimated metabolic clearance ($CL_M$) was 8.43 ml/min, and the hepatic plasma volume at hematocrit 0.05 ($V_{HP,0.05}$) was 115.0 ml (table 2). The calculated metabolic rate ($k$) was 0.073 min$^{-1}$, and the ERs were 0.151 and 0.174 for hematocrits 0.05 and 0.2. For the parallel-tube model, the estimated $CL_M$ was 17.44 ml/min and $V_{HP,0.05}$ was 115.4 ml. The calculated $k$ was 0.181 min$^{-1}$ and ERs were 0.147 and 0.172 for hematocrits 0.05 and 0.2. Hepatic volumes were expressed as a function of $V_{HP,0.05}$ and hematocrit, and the estimated $V_{HP,0.05}$ did not differ statistically between the two models.

**Discussion**

There are differences in blood distribution of tacrolimus among species. However, the pharmacokinetic parameters of clearance and $V_{SS}$ of tacrolimus in human and rabbit adjusted for body size are similar (4, 7, 10, 11). We had previously used the rabbit liver perfusion model to assess hepatic extraction of tacrolimus (10). In both species, a major part of the drug is bound with RBCs. Human erythrocytes are able to accumulate more drug than rabbit RBCs, and the observed whole blood:plasma ratios in humans approach 50, whereas ratios in rabbits are <20.

The present RBC binding, RBC influx and efflux, and liver perfusion studies were undertaken to answer questions of whether RBCs or plasma protein binding controls hepatic extraction of tacrolimus. These experiments were performed with human erythrocytes, and rabbit livers were used for evaluation of the intrinsic clearance of tacrolimus.

The blood distribution of tacrolimus was not affected by albumin concentration. In the range of hematocrits used (0.05–0.4), differences in whole blood:plasma ratios were not observed, despite an increase of albumin concentration from 1 to 5%. On the other hand, hematocrit was a factor strongly influencing the distribution of tacrolimus between RBCs and buffer. At low hematocrit, independent of albumin content, and for the same concentrations of tacrolimus in whole blood, drug concentrations in buffer were higher than results.
When comparing plasma and buffer, the concentration of this drug was higher in plasma (whole blood: plasma ratio was lower) than in the buffer medium (fig. 3). This allowed us to conclude that a plasma protein other than albumin protects tacrolimus from diffusion into RBCs. Tacrolimus exhibits moderate plasma protein binding in humans with a fraction unbound of ~28%. More than 50% of the drug in buffer containing 3.5% albumin is bound; however, another protein such as a α1-acid glycoprotein participates in binding of the drug (~39%) (17). Lipoproteins were not found to be important in binding of tacrolimus in plasma in one study (4), whereas another study (18) showed considerable attachment of the drug to this fraction of plasma. It is probable that differences in whole blood:plasma ratios between plasma and buffer are caused by binding of tacrolimus with α1-acid glycoprotein.

The method used for the present in vitro binding study was based on the erythrocyte partitioning method described by Trung et al. (19). It was shown that estimates of free fraction in plasma by this method agreed well with the classical ultrafiltration and equilibrium dialysis methods. From the in vitro binding study with the presence of RBCs, we were able to obtain the free fraction of tacrolimus. This demonstrated the influence of a plasma protein other than albumin on RBC binding.

The FKBP that binds tacrolimus with a $K_d$ value of 0.4 nM (20) was detected in both the cytosolic fraction (21) and membrane of human erythrocytes. (22) These findings partly explain the marked accumulation of tacrolimus in RBCs and the nonlinearity of the whole blood:plasma ratios over the therapeutic concentration range. However, the quantitative role of FKBPs in human erythrocytes on elimination was not previously investigated. Data from our RBC binding study (fig. 5) yield specific binding with $B_{\text{max}}$ of 440 ng/ml of RBC, $K_m$ of 0.87 ng/ml (1.1 nM), and nonspecific binding with a constant of 4.22. Our $K_m$ value is similar to the $K_d$ value for purified FKBP.

The efflux of tacrolimus from rabbit erythrocytes to buffer (10) was much slower than observed in the present study with human cells. Using centrifugation for separation of plasma requires a processing time (~2 min) that is too long for examining the time course of drug movement from RBCs to buffer. The diffusion of tacrolimus in the opposite direction, from buffer into RBCs, is slower (fig. 6). A similar phenomena was observed during studies of protein binding using an equilibrium dialysis method. The equilibration was slower from spiked buffer than from spiked plasma (23). Another factor influencing the time of equilibration was the initial condition (concentration in compartment containing drug). The equilibration was slower when the concentration of tacrolimus was lower (fig. 7). The same behavior was observed with MK-927 (a carbonic anhydrase inhibitor) by Lin et al. (24).

When plasma was replaced by buffer in diffusion experiments, influx into RBCs was slower and efflux was faster (table 1). There was a greater difference between influx and efflux in the plasma-RBC mixture than in buffer-RBC mixture. This difference can be explained by the presence of other binding proteins in plasma.

The results obtained from liver perfusion experiments show that the major factor regulating hepatic disposition of tacrolimus is hematocrit. The albumin concentration seems to have a negligible influence on this process. We applied the well-stirred and parallel-tube perfusion models to describe our data. Both models generated similar estimated values of hepatic volumes and ER for both hematocrits. As expected from model assumptions, we obtained different intrinsic clearances and hepatic elimination rates for these two models. However, fittings from both models provided the same degree of goodness-of-fit.
models reflect tacrolimus as a low extraction/low clearance drug. The ER of 0.15 for hematocrit = 0.05 is statistically different than ER of 0.17 for hematocrit = 0.2 for both hepatic models, but are very similar. The difference in RBC content is thus the sole factor affecting tacrolimus elimination by the liver. The increase of hematocrit from 0.05 to 0.2 produced a decrease of perfusate flow by 15.8%. In the well-stirred model, tacrolimus clearance was 17.7% of the perfusate flow in low hematocrit and 21.0% in high hematocrit. In the parallel-tube model, tacrolimus clearance was 36.7% of perfusate flow in low hematocrit and 43.6% in high hematocrit. The decreased perfusate flow to liver by increasing hematocrit does not significantly alter the clearance/perfusate flow ratio.

In summary, this study shows that the influence of protein (albumin) concentration on RBC binding, uptake, and release, and liver extraction of tacrolimus is negligible. The major factor controlling these processes is binding of this drug with RBCs and is directly related to hematocrit. This behavior of tacrolimus is expected to occur for both rabbits (10) and humans.

In effect, the intrinsic clearance and ER with respect to hepatic clearance of tacrolimus were not markedly affected by hematocrit. However, the higher hematocrit sequesters the drug, thus protecting it from extraction by the liver, while generating lower plasma concentrations. A traditional pharmacokinetic interpretation of the data, wherein clearance is represented by Dose/AUC, would make it seem that clearance is greater at higher hematocrit. This is obviously not true. The true hepatic disposition of this type of drug may not be discernible without experiments such as these.

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References