Factors affecting variability in distribution of tacrolimus in liver transplant recipients

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Keywords

blood distribution, liver transplantation, pharmacokinetics, plasma protein binding, tacrolimus, therapeutic drug monitoring

Received

21 March 2003

Accepted

12 August 2003

Aims

Therapeutic drug monitoring (TDM) of tacrolimus is complicated by conflicting data on the correlation between tacrolimus trough blood concentrations and the incidence of rejection. The aim of this cross-sectional study was to investigate the blood distribution and protein binding of tacrolimus in liver transplant recipients to explore better predictors of clinical outcome.

Methods

Blood and plasma distribution of ³H-dihydro-tacrolimus was investigated in 40 liver transplant recipients using Ficoll Paque and density gradient ultracentrifugation, respectively, and equilibrium dialysis to investigate plasma protein binding.

Results

In blood tacrolimus was mainly associated with the erythrocyte fraction (83.2%, range 74.6–94.9%), followed by diluted plasma (16.1%, range 4.5–24.9%), and lymphocyte fraction (0.61%, range: 0.11–1.53%). In plasma, lipoprotein deficient serum fraction (54.2%, range 38.5–68.2%) was the main reservoir of tacrolimus. The unbound fraction of tacrolimus was found to be $0.47 \pm 0.18\%$ (range 0.07–0.89%). The percentage of tacrolimus associated with the lymphocytes (0.8 ± 0.4 vs $0.3 \pm 0.1\%$, $P = 0.012$) and estimated unbound concentration (0.42 ± 0.21 ng l⁻¹ vs 0.24 ± 0.08 ng l⁻¹, $P < 0.001$) of tacrolimus were significantly different in stable transplant recipients and those experiencing rejection. Haematocrit and red blood cell count significantly influenced the percentage of tacrolimus associated with erythrocytes. The fraction unbound of tacrolimus was correlated with α_1 -acid glycoprotein and high density lipoprotein cholesterol concentrations.

Conclusions

Tacrolimus unbound concentration was observed to be lower in liver transplant recipients experiencing rejection and further study is required to evaluate its utility in the TDM of tacrolimus.

Introduction

The clinical use of tacrolimus is complicated by a relatively narrow therapeutic index which makes therapeutic drug monitoring (TDM) of tacrolimus essential [1]. Several studies have reported that the side-effects of tacrolimus are more closely correlated with tacrolimus plasma concentration rather than the dose [2, 3]. How-

ever, two research groups failed to find a relationship between tacrolimus concentration and the incidence of kidney allograft rejection [4, 5]. Furthermore, Kershner *et al.* [6] conducted a systematic review of data from three larger clinical trials in liver transplant recipients but failed to show a significant relationship between tacrolimus blood concentration and the incidence of

liver allograft rejection. In contrast, Backman *et al.* [7] have found that tacrolimus blood concentration measurements could be used to avoid drug related toxicity, but not to predict immunosuppressive efficacy in liver transplant recipients. Venkataramanan *et al.* [8] reported that the risk of acute rejection can be reduced by increasing tacrolimus concentration over a 7-day period. These researchers concluded that tacrolimus concentration alone was not able to predict the occurrence of rejection, however, with liver function tests (especially ALT concentrations) there was improved sensitivity of detecting clinically important outcomes. These researchers also reported a significant relationship between nephrotoxicity and trough tacrolimus blood concentration [8].

Refinement of TDM strategies for tacrolimus could improve dose optimization in transplant recipients. One hypothesis to be investigated is that improved outcomes could be provided by a comprehensive understanding of the distribution and plasma protein binding of tacrolimus in transplant recipients. A rigorous study of tacrolimus distribution in blood and plasma has the potential to provide a greater insight into the pharmacokinetic and pharmacodynamic variability and lack of correlation of blood concentration with clinical outcomes for tacrolimus.

Our research group has shown ^3H -dihydro-tacrolimus is mainly associated with erythrocytes ($85.3 \pm 1.5\%$ of total tacrolimus concentration) and to a lesser extent with plasma ($14.3 \pm 1.5\%$) and lymphocytes ($0.46 \pm 0.1\%$) in blood from healthy subjects [9]. In plasma, tacrolimus maximum association was with soluble proteins ($61.2 \pm 2.5\%$), followed by high density lipoproteins (HDL) $28.1 \pm 5.4\%$, low density lipoproteins (LDL) $7.8 \pm 1.6\%$ and very low density lipoproteins (VLDL) $1.4 \pm 0.3\%$ [9]. The *in vitro* fraction unbound of tacrolimus in plasma from healthy subjects was estimated to be $1.2 \pm 0.12\%$ [9]. This study also concluded that the distribution of ^3H -dihydro-tacrolimus in blood was not significantly different from unlabelled drug.

There are few studies on the distribution and protein binding of tacrolimus in transplant recipients. Piekoszewski *et al.* [10] have reported the plasma protein binding of tacrolimus to be $72.0 \pm 3.6\%$ in a group of eight liver transplant recipients. Another study by Warty *et al.* [11] examined the distribution of tacrolimus in plasma of 13 transplant patients. These researchers reported that in plasma $64.0 \pm 8.0\%$ of tacrolimus associates with lipoprotein deficient serum (LPDS), $21.0 \pm 8.0\%$ associates with HDL, $3.0 \pm 3.0\%$ associates with LDL and $11.0 \pm 3.0\%$ associates with VLDL [11]. However, no

data are available on blood distribution of tacrolimus in transplant recipients.

The aim of the present study was to investigate the distribution and plasma protein binding of tacrolimus in blood from a cohort of 40 liver transplant recipients. The physiological indices that affect the variability in blood distribution and protein binding of tacrolimus have also been studied and the relationship between clinical status and fraction unbound has been investigated.

Methods

Ethics approval was obtained from the University of Sydney and the Central Sydney Area Health Services prior to the commencement of the study.

Drugs and reagents

Tacrolimus was obtained as a gift from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). ^3H -dihydro-tacrolimus was prepared at Amersham International (Buckinghamshire, UK) by catalytic reduction of tacrolimus with $^3\text{H}_2$ gas such that 35 ng of ^3H -dihydro-tacrolimus corresponded to approximately 1 μCi . Prior to the protein binding experiments, radio-labelled drug was purified using HPLC [9]. HPLC grade methanol, heptane and acetonitrile were purchased from Selby Biolab (NSW, Australia). All other reagents and buffer components were AR grade and commercially available.

Patients

Forty adult liver transplant recipients were recruited from the AW Morrow Liver and Gastroenterology Centre of Royal Prince Alfred Hospital (Sydney, NSW, Australia). All participants provided informed consent before participation in the study. Blood samples (40 ml) were collected (trough, 12 ± 2.0 h postdose) into vacutainer tubes containing EDTA at the same time as routine biochemical, cytological and tacrolimus trough blood concentration measurements.

Immunosuppressant and other maintenance drug therapy

All patients were receiving tacrolimus as their primary immunosuppressant and some patients (12.5%) were receiving triple drug regimens which included tacrolimus, prednisolone, azathioprine or mycophenolate mofetil. The recipients had been receiving the same dose of tacrolimus for at least 20.6 ± 17.1 weeks (range, 4–64 weeks) ensuring each patient had achieved a steady state concentration of the immunosuppressant. Patients enrolled in this study were receiving a range of other medications including calcium supplements (50% of patients), omeprazole (40% of patients), azathioprine

(30% of patients), oral hypoglycaemic agent (20% of patients) and insulin (10% of patients).

In vitro distribution study in blood

Blood (5 ml) was incubated with 13.5 ng ml^{-1} of ^3H -dihydro-tacrolimus (equivalent to $0.39 \text{ } \mu\text{Ci ml}^{-1}$) for 2 h at 37°C . A portion of blood (2.5 ml) was diluted with an equal volume of isotonic phosphate buffer pH 7.3 and then layered with 2.5 ml Ficoll Paque reagent before centrifugation for 20 min at $850 g$ and 37°C . After centrifugation, diluted plasma, lymphocytes and erythrocytes fractions were separated and analyzed for tacrolimus associated radioactivity in each fraction [9].

Estimation of blood : plasma ratio

An aliquot (0.2 ml) of incubated whole undiluted blood sample was analysed for radioactivity of ^3H -dihydro-tacrolimus. A portion (1 ml) of the blood sample incubated at 37°C was centrifuged at 1500 r.p.m. for 10 min to separate plasma. The radioactivity of ^3H -dihydro-tacrolimus in the supernatant plasma layer was measured to calculate whole blood : plasma ratio of tacrolimus.

In vitro distribution study in plasma

A portion of plasma (10 ml) was incubated for 2 h with 3.5 ng ml^{-1} of ^3H -dihydro-tacrolimus (equivalent to $0.10 \text{ } \mu\text{Ci ml}^{-1}$) at 37°C . After incubation the density of plasma was adjusted using potassium bromide (1.21 g ml^{-1}) and the sample was subjected to density gradient ultracentrifugation using a Beckman XL-90 centrifuge [9, 12]. Approximately 20 fractions (0.5 ml) were collected after ultracentrifugation and each fraction was analyzed for the concentrations of tacrolimus associated radioactivity, cholesterol and albumin as described previously [9]. All distribution studies were conducted in duplicate.

In vitro protein binding study

Plasma samples (5 ml) from patients receiving tacrolimus were incubated for 2 h with 1.21 ng ml^{-1} of ^3H -dihydro-tacrolimus (equivalent to $0.03 \text{ } \mu\text{Ci ml}^{-1}$). Incubated samples were dialyzed against phosphate buffer (pH 7.3) using a Spectrum Equilibrium Dialysis apparatus for 13 h at 37°C . Samples were collected from both the buffer and plasma compartments and analyzed for tacrolimus associated radioactivity by liquid scintillation counting [9]. Plasma samples were analyzed for total protein content using the Biuret method [12] to allow for correction of fluid shifts that occurred during dialysis [13]. Protein binding studies were conducted in triplicate.

Biochemical and cytological measurements

Haemoglobin concentration, red blood cell count (RCC), lymphocyte cell count, bilirubin concentration, AST, ALT, GGT and triglyceride concentration were measured by the Pathology Department of Royal Prince Alfred Hospital, Sydney, NSW using standard methods. Total cholesterol, total protein and albumin concentration were measured by standard analytical methods [9]. HDL, LDL and VLDL were determined by density gradient ultracentrifugation [14]. Haematocrit was measured using a haemocytometer. The α_1 -acid glycoprotein concentration was measured using an immunodiffusion assay kit (CV range, 1.0–2.9%) (Dade Behring Marburg GmbH, Germany). Total concentration of tacrolimus was measured in each blood sample by the Department of Pathology, Royal Prince Alfred Hospital using IMX analyser (CV range, 8.4–14.5%) (Abbott Laboratories, Abbott Park, IL, USA).

Assessment of rejection status

Clinical end points were primarily determined on the basis of a patient's liver function tests and bilirubin concentrations. The specific criteria for presumed rejections were a serum bilirubin concentration of more than 18 mmol l^{-1} , cholestatic liver function test with high GGT values ($> 55 \text{ U l}^{-1}$), elevated ALT ($> 55 \text{ U l}^{-1}$) and AST ($> 55 \text{ U l}^{-1}$) concentrations.

Rejection cases were confirmed by liver biopsy. Biopsy reports were assessed using the Banff schema [15] to determine the rejection grade by Department of Surgical Pathology, Royal Prince Alfred Hospital, Sydney, NSW.

Data analysis

The observed blood to plasma ratio ($\text{BPR}_{\text{observed}}$) in patient blood sample (containing both the unlabelled and constant amount of spiked labelled tacrolimus) was calculated using Equation 1

$$\text{BPR}_{\text{observed}} = \frac{C_B}{C_P} \quad (1)$$

where C_B represents the concentration of radioactivity associated with ^3H -dihydro-tacrolimus in whole blood and C_P , the concentration of radioactivity in plasma. The distribution of tacrolimus between blood and plasma is concentration dependent [16] and the patients' blood samples which already contained unlabelled tacrolimus (as patients were receiving tacrolimus therapy) were spiked with an additional amount of ^3H -dihydro-tacrolimus (13.5 ng ml^{-1}). The corrected blood : plasma ratio ($\text{BPR}_{\text{corrected}}$) that accounts for concentration depen-

dent association of tacrolimus with erythrocytes was determined using Equation 2 [16].

$$\text{BPR}_{\text{corrected}} = 1 + \text{Hct} \frac{B_{\text{max}}}{K_d + C_p} \quad (2)$$

where B_{max} is the maximum concentration of tacrolimus bound to erythrocytes (ng ml^{-1}); Hct is the haematocrit; C_p is the concentration of tacrolimus in plasma (ng ml^{-1}); K_d is the erythrocyte binding affinity constant of tacrolimus (ng ml^{-1}). Empiric Bayes estimates of B_{max} and K_d for individual patients were determined using nonlinear mixed effects modelling (P-Pharm, Innaphase, Philadelphia, USA) using *ex vivo* data and starting estimates from Jusko *et al.* [16].

Tacrolimus unbound concentration (C_u) for individual recipients was calculated using Equation 3

$$C_u = \frac{f_u * C_p}{100} \quad (3)$$

where f_u is the fraction unbound (%) and C_p is the plasma concentration of tacrolimus calculated from the estimated plasma to blood ratio (Equation 2) and actual blood concentration of tacrolimus in each patient. Correlation between various biochemical (including total protein, α_1 -acid glycoprotein, haematocrit) and distribution parameters (such as f_u , percentage of tacrolimus

associated with different fractions) were examined using regression analysis.

Unless otherwise indicated ANOVA was used to test for significance (at the 5% level) between patients grouped according to their clinical status. When more than two groups are compared, Scheffe's *post hoc* analysis was done using SPSS (version 10). To test the distribution of data Kolmogorov-Smirnov test was used using SPSS (version 10).

Results

Patient characteristics

Forty adult liver transplant recipients were recruited into this study (Table 1). A quarter of the recruited patients had elevated bilirubin concentration, 40% of the patients had serum cholesterol concentration above 5 mmol l^{-1} and 63% had elevated α_1 -acid glycoprotein concentration (above 0.9 g l^{-1}). Haematocrit, albumin and total protein concentration were within expected normal ranges. The most common cause for liver transplantation in this patient cohort was liver cirrhosis due to hepatitis C infection. There was only one patient who had drug-induced (nitrofurantoin) liver damage prior to transplantation. Eight patients were experiencing mild rejection, six patients with moderate rejection and one

Table 1

Demographic and biochemical data for study population ($n = 40$)

Variables	Mean $\pm$ SD	Range (in patients)
Sex (male/female)	23/7	
Age (years)	48.3 ± 13.4	20–71
Time after transplant (days)	733 ± 445	71.0–1772.6
Haematocrit (%)	39.9 ± 4.3	31–49
Cholesterol (mmol l^{-1})	4.5 ± 1.2	2.2–6.9
Triglyceride (mmol l^{-1})	1.7 ± 1.0	0.6–5.8
Total protein (g l^{-1})	68.4 ± 5.0	57–75
Albumin (g l^{-1})	39.1 ± 3.8	29–46
α_1 -acid glycoprotein (g l^{-1})	0.95 ± 0.30	0.43–1.47
Bilirubin ($\mu\text{mol l}^{-1}$)	19.5 ± 36.1	4–233
Tacrolimus blood concentration (ng ml^{-1})	7.9 ± 2.9	3–15.2
Blood : plasma ratio	107.2 ± 13.4	75.3–134.8
Cause of transplant	Number of patients	
Hepatitis C cirrhosis	13	
Hepatitis B cirrhosis	8	
Alcoholic liver cirrhosis	5	
Cryptogenic liver cirrhosis	4	
Biliary atresia	2	
Hepatoma	2	
Primary cholangitis	2	
Autoimmune liver disease	2	
Others	2	

patient had severe rejection, assessed by liver function tests and biopsy results. It was found that for patients experiencing rejection, the time of blood sample collection was within 6.4 ± 4.9 days before or after the episode of rejection (range 2 days prerrejection to 15 days postrejection). There was no significant difference in demographic and various biochemical parameters in

patients experiencing rejection compared with those with stable liver function (Table 2). Significantly higher serum concentrations of ALT, GGT, AST and bilirubin were observed in patients experiencing rejection than those with stable liver function (Table 3). Pulse prednisolone therapy was initiated to treat episodes of rejection.

Table 2

Demographic and various biochemical parameters in stable liver transplant recipients and those experiencing rejection

Parameters	Stable* (n = 25)	Mild* (n = 8)	Moderate* (n = 6)	Severe* (n = 1)	P value
Age (years)	46.2	47.8	51.3	44.5	0.713
α_1 -acid glycoprotein (g l ⁻¹)	0.96 (0.79, 1.13)	1.06 (0.81, 1.32)	0.93 (0.69, 1.17)	0.54	0.523
Total protein (g l ⁻¹)	68.5 (65.7, 71.3)	69.4 (66.1, 72.6)	66.8 (61.2, 72.5)	65	0.783
Total cholesterol (mmol l ⁻¹)	4.38 (3.79, 4.99)	4.70 (3.95, 5.44)	5.03 (4.41, 5.66)	2.8	0.257
HDL-cholesterol (mmol l ⁻¹)	1.26 (1.09, 1.44)	1.51 (1.04, 1.99)	1.59 (1.27, 1.91)	1.78	0.207
Lymphocyte count (x 10 ⁹ l ⁻¹)	1.69 (1.29, 2.09)	1.09 (0.81, 1.37)	0.95 (0.38, 1.52)	0.81	0.062
Haematocrit (%)	39.7 (37.4, 42.0)	41.3 (37.7, 44.8)	39.8 (36.4, 43.3)	32	0.321

*Mean (95% confidence interval); *Mean (95% confidence interval not recorded due to low sample size).

Table 3

Fraction unbound, unbound concentration, dose and total concentration of tacrolimus and various biochemical parameters in stable liver transplant recipients and those experiencing rejection

Parameters	Stable* (n = 25)	Mild* (n = 8)	Moderate* (n = 6)	Severe* (n = 1)
Fraction unbound‡ (%)	0.53 (0.48, 0.60)	0.32 (0.15, 0.44)	0.43 (0.33, 0.45)	0.43
Estimated unbound concentration (ng l ⁻¹)‡	0.43 (0.35, 0.49)	0.21 (0.15, 0.27)	0.25 (0.19, 0.33)	0.22
Tacrolimus dose (mg day ⁻¹)	5.0 (4.4, 6.2)	4.8 (3.3, 6.3)	3.2 (3.2, 5.8)	6.0
Tacrolimus concentration (µg l ⁻¹)	6.6 (6.0, 8.5)	8.1 (5.9, 8.4)	10.6 (4.6, 9.0)	7.0
ALT (U l ⁻¹)\$‡	26.9	70.0	156.0	68
AST (U l ⁻¹)\$‡	22.6	45.2	104.4	76
GGT (U l ⁻¹)\$‡	55.2	325.1	302.8	391
Bilirubin (µmol l ⁻¹)\$‡	12.7	18.9	26.4	233.

*Mean (95% confidence interval); *Mean (95% confidence interval not recorded due to low sample size); ‡Significant difference between patients experiencing rejection and stable liver transplant recipients ($P < 0.001$); \$Data were not normally distributed, Mann–Whitney U-test was used to compare the groups.

In vitro distribution study in the blood of transplant recipients

In the blood ($n = 40$) ^3H -dihydro-tacrolimus was found to mainly be associated with erythrocytes (83.2%, range; 74.6–94.9%) followed by diluted plasma (16.1%, range; 4.5–24.9%) and lymphocyte fraction (0.61%, range; 0.11–1.53%). The percentage of drug associated with each component of blood varied widely among the patients but in all patients the erythrocyte fraction remained the main reservoir for tacrolimus in blood.

The observed blood : plasma ratio ($\text{BPR}_{\text{observed}}$) of tacrolimus was found to be 5.9 ± 3.6 (Table 1) as calculated based on radioactivity data over the observed tacrolimus concentration range of $16.5\text{--}28.7 \text{ ng ml}^{-1}$. However, on correcting the data for total tacrolimus concentration (labelled and unlabelled), the average estimated blood : plasma ratio over the clinically observed tacrolimus concentration range for these patients of $3\text{--}15.2 \text{ ng ml}^{-1}$ was found to be 107.2 ± 13.4 (range $75.3\text{--}134.8$). Plasma concentration of tacrolimus increased with blood concentration whereas the blood : plasma ratio decreased with increasing tacrolimus plasma concentration. The maximum concentration of tacrolimus bound (B_{max}) to erythrocytes and affinity constant (K_d) were estimated to be $417.6 \pm 1.2 \text{ ng ml}^{-1}$ and $3.8 \pm 0.5 \text{ ng ml}^{-1}$, respectively.

In the plasma ^3H -dihydro-tacrolimus was mainly associated with LPDS ($54.2 \pm 6.5\%$), followed by HDL ($29.1 \pm 7.4\%$), then LDL ($12.7 \pm 5.0\%$) and to a lesser extent with VLDL ($4.6 \pm 3.4\%$). There was wide inter-patient variability in the distribution of tacrolimus in the

plasma components across the study group. The percentage of tacrolimus associated with LPDS ranged from 38.5 to 68.2% (two-fold variation). There was a four-fold and six-fold variation in tacrolimus associated with HDL (range 10.9–47.2%) and LDL (range 5.8–30.3%) cholesterol fractions, respectively. Maximum variation was observed in the percentage of tacrolimus associated with VLDL (range 1.2–21.0%).

Significant differences were observed in the percentage of tacrolimus associated with HDL fraction ($26.0 \pm 6.4\%$ vs $33.2 \pm 6.6\%$; $P < 0.001$, Student's t -test) and HDL cholesterol content (1.2 ± 0.6 vs $1.6 \pm 0.5 \text{ mmol l}^{-1}$; $P = 0.03$, Student's t -test) between male and female recipients.

In vitro plasma protein binding study

In liver transplant recipients the unbound fraction of radiolabelled tacrolimus in plasma was found to be $0.47 \pm 0.18\%$ and there was a 15-fold variation (range 0.07–0.89%) in the fraction unbound (%) of tacrolimus in these patients (Figure 1). A significantly higher fraction unbound was observed in male recipients ($n = 23$) compared with female recipients ($n = 17$) ($0.5 \pm 0.1\%$ vs $0.4 \pm 0.1\%$; $P = 0.003$, Student's t -test).

Factors affecting tacrolimus distribution and binding in blood

The percentage of tacrolimus associated with the erythrocyte fraction in blood tended to increase with an increase in haematocrit (Figure 2a, $r^2 = 0.47$) and red blood cell count (RCC) (Figure 2b, $r^2 = 0.49$). Further-

Figure 1

Variability in *in vitro* plasma protein binding of tacrolimus in individual liver transplant recipients. Dashed line indicates average fraction unbound in total cohort ($0.47 \pm 0.18\%$)

Figure 2

Percentage of tacrolimus associated with A) erythrocyte fraction vs haematocrit ($r^2 = 0.47$), B) erythrocyte fraction vs red blood cell count ($r^2 = 0.49$), C) lymphocyte fraction vs lymphocyte cell count ($r^2 = 0.58$), D) LPDS fraction vs α_1 -acid glycoprotein concentration (LPDS = lipoprotein deficient serum) ($r^2 = 0.53$) and E) HDL fraction vs cholesterol concentration (HDL = high density lipoproteins) ($r^2 = 0.63$)

Figure 2

Continued

more, there was also an increase in percentage of tacrolimus associated with lymphocytes with an increase in lymphocyte count (Figure 2c, $r^2 = 0.58$). A similar trend was also seen between the percentage content of tacrolimus associated with LPDS and total protein concentration ($r^2 = 0.38$) and α_1 -acid glycoprotein concentration (Figure 2d, $r^2 = 0.53$) but not albumin concentration. The percentage of tacrolimus associated with the high density lipoprotein fraction increased with increasing total cholesterol concentration (Figure 2e, $r^2 = 0.63$). The unbound fraction was found to be correlated to α_1 -acid glycoprotein ($r^2 = 0.50$, Figure 3a) and HDL-cholesterol concentration ($r^2 = 0.55$, Figure 3b).

Tacrolimus blood distribution and clinical status of the liver transplant recipients

A significant difference was observed in unbound fraction ($P < 0.001$) and unbound concentration ($P < 0.001$) of tacrolimus between stable recipients and those experiencing rejection (Table 3). But no significant difference was found in the unbound fraction ($P = 0.38$) and unbound concentration ($P = 0.68$) of tacrolimus among patients experiencing various extents of rejection. Interestingly, there was no significant difference in the total tacrolimus trough blood concentration ($P = 0.5$) or the

Figure 3

Fraction unbound (%) of tacrolimus vs A) α_1 - acid glycoprotein concentration ($r^2 = 0.50$) and B) HDL-cholesterol concentration (HDL = high density lipoproteins) ($r^2 = 0.55$)

tacrolimus dose ($P = 0.32$) among various groups of transplant recipients (Table 3).

A significant difference was observed in the percentage of tacrolimus associated with the lymphocytes of stable transplant recipients when compared with those experiencing rejection ($P = 0.012$) (Table 4). There was

Table 4

Blood distribution of tacrolimus in stable liver transplant recipients and those experiencing rejection

Parameters	Stable (<i>n</i> = 25)*†	Mild (<i>n</i> = 8)*†	Moderate (<i>n</i> = 6)*†	Severe (<i>n</i> = 1)*†
Lymphocyte fraction (%)‡	0.8 (0.6, 1.0)	0.3 (0.2, 0.4)	0.3 (0.3, 0.5)	0.5
Diluted plasma fraction (%)	16.7 (15.5, 17.9)	15.1 (11.4, 18.8)	13.6 (11.8, 15.4)	15.9
Erythrocyte fraction (%)	82.6 (81.3, 83.9)	84.4 (80.6, 88.2)	85.9 (84.2, 87.6)	83.6

*Mean (95% confidence interval); *Mean (95% confidence interval not recorded due to low sample size); †Data are presented as percentage of total blood concentration; ‡Significant difference between patients experiencing rejection and stable liver transplant recipients ($P = 0.012$).

Table 5

Plasma distribution of tacrolimus in stable liver transplant recipients and those experiencing rejection

Parameters	Stable (<i>n</i> = 25)*†	Mild (<i>n</i> = 8)*†	Moderate (<i>n</i> = 6)*†	Severe (<i>n</i> = 1)*†
Lipoprotein deficient serum fraction (%)	54.6 (52.1, 57.1)	53.3 (49.9, 56.7)	54.7 (47.4, 62.0)	50.2
High density lipoprotein fraction (%)	29.2 (50.4, 56.2)	30.1 (26.0, 34.2)	30.0 (24.9, 35.1)	30.3
Low density lipoprotein fraction (%)	12.0 (10.1, 13.9)	13.1 (11.6, 14.6)	12.1 (9.4, 14.8)	10.9
Very low density lipoprotein fraction (%)	5.0 (3.2, 6.8)	3.5 (2.5, 4.5)	3.2 (2.3, 4.1)	8.8

*Mean (95% confidence interval); *Mean (95% confidence interval not recorded due to low sample size); †Data are presented as percentage of total plasma concentration.

no difference in the percentage of tacrolimus associated with the plasma ($P = 0.28$) and erythrocytes ($P = 0.23$) in stable transplant recipients and those patients experiencing rejection (Table 4) nor with plasma protein fractions (Table 5).

Discussion

Tacrolimus whole blood concentrations are routinely monitored to attain optimum immunosuppressant concentrations. However, the correlation between tacrolimus concentration and clinical outcome is yet to be established [2–8]. This study has examined the blood distribution and the fraction unbound of tacrolimus in liver transplant recipients to explore the hypothesis that the pharmacological response correlates more closely with the concentration of unbound drug [17]. Based on the same hypothesis it is expected that variability in

blood distribution of tacrolimus may result in variability in unbound fraction which may result in varied clinical outcomes. The consequences of such variability are likely to be more pronounced for a highly protein bound drug like tacrolimus that has a fraction unbound of less than 1% [9].

In a previous study we have shown that it is possible to investigate the distribution and protein binding of tacrolimus at clinically relevant concentrations using ^3H -dihydro-tacrolimus [9]. This experimental approach to investigate the association of tacrolimus with blood components has been employed in other studies including investigations of tacrolimus and cyclosporin distribution [12, 18, 19]. The results from the present study show that like healthy subjects [9], the erythrocyte is the main reservoir for tacrolimus in the blood of transplant recipients, but the percentage of total drug associated

with these cells varied widely. Furthermore, the blood : plasma ratio of tacrolimus was also found to vary from 2.9 to 134.8 over a blood concentration range of 3–28.7 ng ml⁻¹ supporting the concentration dependent nonlinear binding of tacrolimus to erythrocytes [16, 20]. Other researchers have reported a similar range of tacrolimus blood : plasma ratio (4–39 and 4–114) in transplant recipients [16, 21–23]. This variability in tacrolimus blood : plasma ratio is likely to be due to inter-patient differences in haematocrit (range, 31–49%), concentration dependent distribution of the drug between blood and plasma and the drug binding capacity of erythrocytes. Furthermore, in the present study it was found that with an increase in red blood cell count (RCC) and haematocrit there is an increase in the percentage of tacrolimus associated with erythrocytes (Figure 2a,b). The maximum concentration of tacrolimus bound ($B_{\max}$) and the association constant (K_d) determined in this study were similar to those reported by Jusko *et al.* [16]. Minor differences in these parameters could be due to the difference in the study populations especially with respect to post-transplant study period and patients' status. Jusko *et al.* [16] made their observations in patients in the early post transplant phase during which both the biochemical status of the patients and tacrolimus concentration are likely to be variable leading to the interpatient variability in $B_{\max}$ and K_d . In the present study, patients were studied at least 2 months after the transplantation by which time in most cases tacrolimus concentration has reached steady state.

The variability in the percentage of tacrolimus content in lymphocytes was more remarkable than other cellular components of blood ranging from 0.11% to 1.5%. This observation may have significant clinical implications because lymphocytes have been postulated to be the site of action for tacrolimus [23, 24]. Furthermore, the percentage of tacrolimus associated with the lymphocyte fraction was significantly different in stable transplant recipients and those experiencing rejection suggesting this could be an indicator of tacrolimus associated immunosuppression. However, the majority of patients in this study were receiving corticosteroids and all patients experiencing rejection were managed with pulse doses of prednisolone. Christians *et al.* [25] indicated that corticosteroids are time-dependent inducers and inhibitors of CYP3A enzymes and inducers of P-glycoprotein. These authors speculate that changes in tacrolimus clearance and bioavailability may occur during concomitant therapy with corticosteroids due to changes in the expression of CYP3A and P-glycoprotein in hepatic and intestinal tissues [25]. There is no data suggesting prednisolone influences tacrolimus protein

binding, plasma distribution or erythrocyte distribution. However, the possibility that concomitant prednisolone therapy may have contributed to variability in lymphocyte distribution of tacrolimus, via effects on lymphocyte P-glycoprotein and CYP3A4 expression, cannot be excluded.

In transplant recipients, the plasma distribution pattern of tacrolimus was similar to that observed in healthy subjects [9] and was also in close agreement with that reported by Warty *et al.* [11]. This confirms that tacrolimus mainly associates with soluble proteins (such as albumin and α_1 -acid glycoprotein) which are the main constituents of the LPDS fraction obtained after ultracentrifugation. This observation is further supported by a strong correlation between the percentage of tacrolimus associated with LPDS and α_1 -acid glycoprotein concentrations (Figure 2d). Variation in the concentrations of these proteins during the early post transplant period may lead to variation in free drug concentration [21] and potentially undermine the utility of TDM of tacrolimus. To a lesser extent tacrolimus is also associated with HDL, LDL and VLDL. This distribution pattern in plasma is different from that of the immunosuppressant cyclosporin [11]. Moreover, the results suggest that with an increase in cholesterol and HDL-cholesterol concentration there is an increase in the percentage of tacrolimus associated with the HDL fraction (Figure 2e). As most of the liver transplant recipients receive corticosteroids as a part of their triple immunosuppressant therapy, there is a possibility of an increase in cholesterol concentration over the post-transplant period. Ichimaru *et al.* [26] have reported that over the post transplant period, there is an increase in cholesterol content in HDL2 and VLDL fractions in renal transplant recipients receiving tacrolimus. Variability in lipoprotein concentrations have the potential to affect the percentage of tacrolimus content in lipoprotein fractions leading to possible variation in unbound fraction of tacrolimus and thus clinical outcomes.

Only $0.47 \pm 0.18\%$ of tacrolimus was found to be unbound in plasma from transplant recipients. Furthermore, in these patients the fraction unbound was considerably lower than that reported for healthy subjects ($1.20 \pm 0.12\%$ [9]). This could be due to several factors like elevated bilirubin, α_1 -acid glycoprotein and cholesterol concentrations in most of the recipients (Table 1). The reported fraction unbound of tacrolimus is remarkably lower than that reported by Piekoszewski *et al.* [10] which could be due to the differences in the methodology used. Piekoszewski *et al.* [10] used ultracentrifugation at 22–24 °C. In the present study it was also found that the unbound fraction is significantly lower in female

recipients than males. Such a difference was also observed in the case of percentage of tacrolimus associated with HDL fraction and HDL-cholesterol concentration in male and female patients in the study group. Claesson *et al.* [27] has reported significantly higher concentrations of cholesterol in female transplant recipients than males during post-transplant phase but did not describe changes in various lipoprotein fractions over the post-transplant period. The low fraction unbound of tacrolimus observed in females in this study could be in part attributed to the observed high concentrations of HDL-cholesterol in female recipients. Furthermore, a significant correlation was observed between fraction unbound (%) of tacrolimus and α_1 -acid glycoprotein and HDL-cholesterol concentration (Figure 3a,b). Immediately after the transplant the high α_1 -acid glycoprotein concentration may cause lower unbound concentration and thus lesser immunosuppressive action of tacrolimus resulting in higher dose requirement. Over the post-transplant period, α_1 -acid glycoprotein concentration decreases whereas, lipoproteins concentrations may vary depending on the time elapsed after transplantation [26, 27]. This may affect the unbound concentration and clinical outcome of tacrolimus at any given dose over the post-transplant phase.

The present study indicates that there is a significant difference in the unbound fraction and unbound concentration of tacrolimus between stable transplant recipients and those experiencing rejection but no difference was observed for total tacrolimus trough blood concentration and daily dose among various patient groups. This finding supports the lack of a correlation between tacrolimus whole blood concentration and incidence of rejection as reported by several research groups [4–6] and suggests unbound concentration of tacrolimus could be a better correlate. Further study in a larger cohort of patient is needed to fully evaluate this observation.

The results from the present study indicate that there is wide variability in the blood distribution and plasma protein binding of tacrolimus in liver transplant recipients. A wide variability in demographic and biochemical indices was also observed in this patient cohort and to some extent these indices correlated with the distribution and protein binding pattern of tacrolimus. This variability in biochemical changes and the associated distribution and protein binding could reflect the clinical status of patients. Moreover this variability may account for the inability of the previous clinical studies to detect a significant relationship between tacrolimus blood concentration and clinical outcome. Further detailed study in this regard will be beneficial for a better understanding of the TDM of tacrolimus.

We acknowledge the help of Graham Kyedd, Dr Simon Strasser, Dr Watson Ng and Dr Samuel Douglas of Royal Prince Alfred Hospital in the clinical studies. We also thank Professor Kenneth Brown for his valuable advice on the design of this study.

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