A Model to Account for the Blood-to-Plasma Distribution of Cyclosporin A in Human Blood

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A mathematical model to account for the blood-to-plasma distribution of cyclosporin A (CyA) in human blood was derived, and alterations in the blood-to-plasma distribution of CyA under several influencing conditions were examined using this model. The model was derived on the assumption that there are two CyA binding sites in human blood, which exist in plasma and cellular fractions. In the plasma fraction, the CyA binding sites are mainly lipoproteins (LPs), in which CyA exhibits non-saturable, low affinity binding with LPs independent of drug concentration. In the cellular fraction, the binding site of CyA is the CyA binding protein (CBP), in which CyA shows a specific binding in a temperature dependent, saturable process. The results obtained from simulative studies agreed with previous reports on the blood-to-plasma distribution of CyA, indicating the usefulness of this approach. And also, this model may be used to predetermine individual variations in the blood-to-plasma distribution of the drug in renal transplant patients.

Keywords — cyclosporin A; blood-to-plasma distribution; model; temperature-dependent binding; simulative study

Introduction

Cyclosporin A (CyA) is a third-generation immunosuppressive agent that has been widely used in organ transplantation.^{1–3)} Therapeutic monitoring of CyA has been advocated because of its optimal use, but no consensus has been reached on the usefulness of monitoring of CyA in blood or plasma because of the complex mechanism of CyA distribution in the blood.⁴⁾

As factors influencing the blood-to-plasma distribution of CyA, the temperature at which the whole blood sample is handled, hematocrit, plasma lipid levels are well known.^{5–11)} However, it is still not known how these factors may affect the blood-to-plasma distribution of CyA. It is well known that CyA has two different binding sites in the human blood system. In the plasma fraction, the main binding site of CyA is lipoproteins (LPs) and the binding of CyA to albumin is negligible.⁷⁾ The LPs constructed by very low density lipoprotein (VLDL), high density lipoprotein (HDL), low density lipoprotein (LDL) and chylomicrons in plasma exhibit a non-saturable, low affinity, high capacity uptake for CyA.⁶⁾ On the other hand, in the cellular fraction, the main binding site of CyA is CBP,¹²⁾ which exhibits a saturable, high affinity

binding for CyA in a temperature dependent manner.¹³⁾

In this study, on the basis of this assumption, we derived a mathematical model to account for the blood-to-plasma distribution of CyA, and simulated the effects of temperature, hematocrit and plasma lipid level on the blood-to-plasma distribution of the drug. We also applied this model to data obtained from a renal transplant patient.

Theory

The characterization of CyA binding to CBP is defined as follows:

$$C_b = \frac{N_c K_c C_f}{1 + K_c C_f} \quad (1)$$

where C_b and C_f are the concentration ($\mu\text{g/ml}$) of bound CyA to CBP in the cellular fraction and that of unbound CyA in the cellular fraction, respectively. N_c and K_c represent the capacity factor constant for the cellular fraction and the binding affinity constant for CyA to CBP, respectively. Then, a total concentration ($\mu\text{g/ml}$) of CyA inside cells (C) can be represented by the following relationship:

$$C = \frac{N_c K_c C_f}{1 + K_c C_f} + C_f \quad (2)$$

Since the main binding site of CyA in the plasma fraction is LPs, and the binding to LPs is nonspecific and independent of plasma CyA concentrations,⁶⁾ a concentration of bound CyA to LPs in the plasma fraction can be written,

$$P_b = N_p K_p P_f \quad (3)$$

where P_b and P_f are the concentration ($\mu\text{g/ml}$) of bound CyA to LPs and that of unbound CyA in the plasma fraction, respectively. $N_p K_p$ represents the apparent binding affinity for LPs in the plasma fraction. Likewise, a total concentration ($\mu\text{g/ml}$) of CyA in plasma (P) can be represented by the following relationship:

$$P = N_p K_p P_f + P_f \quad (4)$$

Based on the mass balance rule for the blood system, a concentration ($\mu\text{g/ml}$) of CyA in whole blood (B) can be represented by the following relationship:

$$B = (N_p K_p P_f + P_f) (1 - H) + H C_f + \frac{H N_c K_c C_f}{1 + K_c C_f} \quad (5)$$

where H represents hematocrit.

As CyA can pass through the membrane freely, and has no binding affinity for the cellular membrane^{7,14)} and the binding of CyA to plasma proteins is independent of plasma concentration within the range of 0.02 to 20 $\mu\text{g/ml}$,¹⁵⁾ we therefore defined the unbound fraction of CyA in the cellular fraction and the plasma fraction as follows:

$$f_p = P_f / P = C_f / P \quad (6)$$

where f_p is the unbound fraction of CyA in plasma. Substituting for C_f and P_f in Eq. (6), we get

$$B = (N_p K_p P f_p + P f_p) (1 - H) + H P f_p + \frac{H N_c K_c P f_p}{1 + K_c P f_p} \quad (7)$$

Rearranging Eq. (7) and solving the quadratic equation for P using only the plus sign, because the concentration in plasma is $> \text{zero}$, we get Eq. (8) which was used for data fitting and simulative studies.

Thus, the concentration of CyA in plasma (P) are expressed as a function of H and B with four distribution parameters of $N_p K_p$, N_c , K_c and f_p .

$$P = \frac{BK_c - N_p K_p - 1 - H(N_c K_c - N_p K_p) + \sqrt{(N_p K_p + 1 + H(N_c K_c - N_p K_p) - BK_c)^2 + 4K_c B(N_p K_p - HN_p K_p + 1)}}{2K_c f_p (N_p K_p - HN_p K_p + 1)} \quad (8)$$

Materials and Methods

Reagents — CyA and cyclosporin D (CyD, used as an internal standard for the high performance liquid chromatography (HPLC) determination) were kindly supplied by Sandoz Ltd., Basle, Switzerland. Ethanol and *n*-hexane were HPLC grade (Wako Pure Chemicals Co., Ltd., Osaka, Japan). All other chemicals and reagents were of reagent grade and were commercially available.

Preparation of Whole Blood Samples Containing Various Concentrations of CyA

— We put the individual mark A, B, C, D on fresh heparinized whole blood (400 ml) obtained from 4 healthy volunteers, and provide them separately as materials for the experiment. To avoid an alteration of the blood protein and a possible effect of medium dispersal, the whole blood samples containing various concentrations of CyA were prepared in the following manner. CyA was directly dissolved in the individual plasma, which was separated from a part of blood, to yield 12 different concentrations ranging from 10 to 500 $\mu\text{g/ml}$ -plasma (CyA standard plasma). To 4 ml of individual whole blood, 40 μl of this CyA standard plasma was added to produce 12 different concentrations of 0.1 to 5 $\mu\text{g/ml}$ in the whole blood. After equilibrating the CyA spiked whole blood sample at 4, 10, 21 or 37 °C for 3 h, the plasma was separated from cells at the same temperatures without delay.

The plasma was then frozen at -80°C until the day of assay. Hematocrits (Hct) of all blood specimens were measured after the addition of CyA standard solutions using a centrifugation method.

Estimation of the Distribution Parameters

— To estimate the distribution parameters ($N_p K_p$, N_c , K_c and f_p), the observed plasma (P) and known whole blood (B) concentrations in each blood sample were fitted to Eq. (8) by a non-linear least squares regression analysis program, MULTI¹⁶⁾ on a microcomputer, PC-9801 Vm2 with the damping Gauss-Newton method, where H was treated as an independent value. The goodness of fit of the model was assessed by calculating the coefficient of determination, R^2 , using the actual data, the sum of deviations squared and the number of data¹⁷⁾ and parameter estimates were compared to zero with the distribution F test. Initial estimation of the distribution parameters for the curve fitting was performed in the following manner. The initial value for f_p (f_{p0}) was calculated by the relationship described by Legg *et al.*⁹⁾ and is as defined by

$$f_{p0} = 1/(1.346[\text{TG}] + 2.815[\text{CH}] + 1) \quad (9)$$

where [TG] and [CH] are millimolar concentrations of plasma triglyceride and cholesterol, respectively. The initial values for $N_p K_p$, N_c and K_c ($N_p K_{p0}$, N_{c0} and K_{c0} , respectively) were estimated using the following linear equations:

$$1/C_b = \alpha(1/P_f) + \beta \quad (10)$$

$$P_b = \gamma P_f \quad (11)$$

where, α , β and γ are regression coefficients, then $N_p K_{p0}$, N_{c0} and K_{c0} were given by the following relationships.

$$N_p K_{p0} = \gamma$$

$$N_{c0} = 1/\beta$$

$$K_{c0} = \beta/\alpha$$

In the Eqs. (10) and (11), C_b , P_b and P_f were calculated by use of f_{p0} and P .

Calculation of the Cellular-to-Plasma Concentration (C/P) Ratio — The C/P ratio of

CyA was calculated from the following relationship:

$$C/P = (B/P - (1 - H))/H \quad (12)$$

where B and P are the concentrations of CyA ($\mu\text{g/ml}$) in whole blood and plasma, respectively.

Application of the Model to a Renal Transplant Patient — A renal transplant patient, whose immunosuppression after transplantation was achieved with CyA, azathioprine and prednisolone, participated in the study. He received CyA in two doses (4.0–10 mg/kg/d) orally for 2 to 120 d after transplantation. Whole blood was collected into heparinized tubes 12 h after dosing (trough level). Then, a plasma sample was obtained after equilibrating the whole blood sample at 37°C for 3 h and separating at the same temperature without delay. Whole blood and plasma samples from the patient were stored at -80°C until the day of assay.

Assay of CyA in Plasma and Whole Blood

— CyA in plasma or in whole blood was determined by our HPLC method.¹⁰⁾ Briefly, to 1 ml of plasma or hemolyzed whole blood sample was added 10 μl of internal standard solution containing CyD at the concentration of 25 $\mu\text{g/ml}$ in methanol, 2 ml of 0.1 mM dextrane sulfate sodium salt (molecular weight of 500 kDa) solution containing 50 mM magnesium chloride, and 10 ml of *n*-hexane. The mixture was vigorously agitated for 10 min and centrifuged at 3000 rpm for 5 min. The upper organic layer was transferred to a clean test tube and evaporated to dryness under reduced pressure at 40°C . The residue was dissolved in 200 μl of mobile phase (*n*-hexane:ethanol, 85:15 v/v), and 150 μl was injected into the column. The HPLC apparatus used consisted of a model 510 pump, a model U6K sample injector and a model 441 absorbance detector from Waters Assoc., Inc., Milford, MA, U.S.A. The column used was a Li-chrosorb Si-60 column (particle size 5 μm , 250×4.6 mm i.d.) from Chemco Co., Ltd., Osaka, Japan. The flow rate of the mobile phase was set at 1.0 ml/min, and the column effluent was monitored at 215 nm. Results were calculated using the peak area ratio method with a detec-

tion limit of 0.005 $\mu\text{g/ml}$.

Assay of TG and CH in Plasma — Tri-glyceride (TG) and cholesterol (CH) in plasma were measured colorimetrically using assay kits purchased from Iyatoron Co., Ltd., Tokyo, Japan.

Results and Discussion

The initial values of the distribution parameters from the linear equations (Eqs. (10), (11)) for the individual blood samples at four different temperatures are given in Table I. In preliminary study, the attempts to measure the free fraction of CyA in plasma using the ultrafiltration method or equilibrium dialysis method were not successful because adsorption, or binding of CyA to the membranes or the side walls of the apparatus was suspected. Therefore, we estimated the value of CyA free fraction in plasma using Eq. (9), and used it as an initial estimate of f_p for the computer curve fitting. In Fig. 1, plasma CyA concentrations obtained after equilibrating the blood samples at 4, 10, 21 and 37 °C for 3 h are plotted against whole blood CyA concentrations. Solid lines in each sample represent

the computer fits to Eq. (8) at four different temperatures. The lines seem to describe the data very well. The resultant data of the distribution parameters from the nonlinear regression for the individual concentration data sets are given in Table II. The values of R^2 were given closely to 1 in all samples (more than 0.999) at all storage conditions. This finding suggests that 99.9% of the variation of the plasma CyA concentration can be explained by the variance of the whole blood concentration and the distribution parameters, supporting the validity of the model proposed. In the temperature range between 10 °C and 37 °C, f_p was increased with rise in temperature, whereas $N_p K_p$ and K_c were decreased. At 4 °C, however, a counter result was observed in the relationship between these parameters and temperatures. These findings suggest that the binding of CyA to LPs in the plasma fraction and for CBP in the cellular fraction are temperature dependent. On the other hand, N_c was essentially constant among all of the sample storage temperatures, suggesting the independence of N_c for temperature. Agarwal *et al.*¹⁸⁾ also used a nonlinear saturable model to describe the relationship between cellular CyA and plasma

TABLE I. Initial Parameter Sets to Estimate Distribution Parameters for CyA in CyA Supplementation Study

Temp.		Hct ^{a)}	TG (mm)	CH (mm)	f_{p0} ^{b)}	$N_p K_{p0}$ ^{c)}	N_{c0} ^{c)} ($\mu\text{g/ml}$)	K_{c0} ^{c)} ($\text{ml}/\mu\text{g}$)
4 °C	A	0.35	0.58	5.56	0.0574	16.4	7.0	41.8
	B	0.38	6.18	4.24	0.0471	20.3	6.2	53.9
	C	0.41	0.82	4.45	0.0684	13.6	6.7	36.2
	D	0.45	4.17	3.75	0.0582	16.2	6.1	81.1
10 °C	A	0.35	0.58	5.56	0.0574	16.4	5.0	93.6
	B	0.38	6.18	4.24	0.0471	20.3	4.6	148.6
	C	0.41	0.82	4.45	0.0684	13.6	3.5	238.7
	D	0.45	4.17	3.75	0.0582	16.2	4.9	114.8
21 °C	A	0.35	0.58	5.56	0.0574	16.4	7.3	16.9
	B	0.38	6.18	4.24	0.0471	20.3	5.9	32.5
	C	0.41	0.82	4.45	0.0684	13.6	3.0	32.9
	D	0.45	4.17	3.75	0.0582	16.2	5.4	25.6
37 °C	A	0.35	0.58	5.56	0.0574	16.4	3.5	17.0
	B	0.38	6.18	4.24	0.0471	20.3	3.9	20.9
	C	0.41	0.82	4.45	0.0684	13.6	6.7	14.1
	D	0.45	4.17	3.75	0.0582	16.2	5.6	7.9

^{a)} Treated as a fixed value under the computing. ^{b)} Calculated by use of Eq. (9). ^{c)} Calculated by use of Eqs. (10) and (11).

Fig. 1. Plasma Concentration *versus* Blood Concentration of CyA in Human Blood at Four Different Temperatures
Solid lines represent computer fits to the individual concentration data sets using Eq. (8). Key: Δ , 4 °C; $\blacktriangle$, 10 °C; $\bullet$, 21 °C; $\circ$, 37 °C.

CyA concentrations in five renal transplant patients with saturation factor values between 4.75 and 10.4 $\mu\text{g/ml}$. The intensity of K_c at 37 °C was 3- to 10-fold less than at other temperatures, and K_c at 10 °C showed maximum intensity. Although it is still difficult to discuss the thermodynamic interaction between CyA and CBP, these findings suggest that structural changes in the CyA peptide due to changes in temperature may play an important role in the binding of CyA to CBP.

In the second place, to clarify the relationship between other influencing factors (hematocrit

and plasma lipid level) and the blood-to-plasma distribution of CyA, simulative studies were conducted in the clinically relevant blood concentration of CyA ranging from 0 to 1 $\mu\text{g/ml}$. According to Legg *et al.*,⁹⁾ changes in the amounts of plasma LPs are related to changes in f_p , and f_p is inversely proportional to plasma TG and CH levels which are constituents of the LPs. Therefore, we regarded changing f_p as changing LPs in plasma. Figure 2 represents computer-generated lines of plasma CyA to blood CyA concentration with changing H , by using the mean values of the distribution parameters

TABLE II. Distribution Parameters for CyA Computed Using the Model in CyA Supplementation Study

Temp.		$N_p K_p$	N_c ($\mu\text{g/ml}$)	K_c ($\text{ml}/\mu\text{g}$)	f_p	R^2	p^b
4 °C	A	8.4	5.1	54.7	0.1205	0.9991	< 0.001
	B	20.9	5.3	55.5	0.0593	0.9998	< 0.001
	C	3.9	5.5	33.7	0.2005	0.9995	< 0.001
	D	14.7	4.6	81.5	0.0831	0.9998	< 0.001
	Mean ^{a)}	12.0	5.1	56.4	0.1159		
	S.D.	7.4	0.4	19.6	0.0618		
10 °C	A	22.7	5.3	90.0	0.0526	0.9998	< 0.001
	B	55.1	5.9	114.5	0.0238	0.9994	< 0.001
	C	37.9	4.9	222.9	0.0339	0.9998	< 0.001
	D	23.5	5.0	112.6	0.0508	0.9997	< 0.001
	Mean ^{a)}	34.8	5.3	135.0	0.0403		
	S.D.	15.2	0.5	59.6	0.0138		
21 °C	A	20.1	5.3	42.5	0.0532	0.9992	< 0.001
	B	36.7	5.3	32.3	0.0343	0.9995	< 0.001
	C	20.8	5.5	45.4	0.0520	0.9997	< 0.001
	D	18.1	4.9	27.9	0.0595	0.9994	< 0.001
	Mean ^{a)}	23.9	5.3	38.5	0.0498		
	S.D.	8.6	0.3	6.5	0.0108		
37 °C	A	25.0	5.7	11.7	0.0420	0.9995	< 0.001
	B	25.6	4.6	10.3	0.0467	0.9997	< 0.001
	C	16.1	3.6	24.8	0.0732	0.9998	< 0.001
	D	17.7	5.1	9.2	0.0606	0.9996	< 0.001
	Mean ^{a)}	21.1	4.8	14.0	0.0556		
	S.D.	4.9	0.9	7.3	0.0141		

^{a)} Values are shown as means $\pm$ S.D. for 4 determinations at each temperature. ^{b)} Parameter estimates were compared to zero with the distribution F test.

listed in Table II.

Keeping the distribution parameters ($N_p K_p$, N_c , K_c and f_p) constant, the value of H was changed from 0.1 to 0.6. With a rise in H , the plasma CyA was markedly decreased at temperatures below 37 °C (especially at 10 °C). On the other hand, at 37 °C, the relationship between plasma CyA and blood CyA was almost linear even at the value of H . In our previous report of an *in vitro* study⁸⁾ using CyA spiked samples (0.5 $\mu\text{g/ml}$ in whole blood), plasma CyA at 4 °C was markedly decreased with a rise in H , and the concentration at an H of 0.6 was only one-fifth of that at an H of 0.1. At 37 °C, plasma CyA decreased, but only a little. Moreover, several other reports of *in vivo* studies support these findings from the simulation with changes in hematocrits, namely, plasma CyA exhibits a reciprocal relationship to hematocrits at room temperature,⁷⁾ but at 37 °C the relationship was

not observed.¹⁹⁾ Figure 3 represents computer-generated lines of the C/P ratio to plasma CyA concentration with changing f_p , using the distribution parameters listed in Table II. Keeping the distribution parameters ($N_p K_p$, N_c , K_c) and H (0.398, a mean value of blood samples) constant, the value of f_p changed from 0.01 to 0.2. The range of f_p used here was nearly equal to that of f_p demonstrated by Legg *et al.*, using renal transplant patients (0.036 to 0.1).⁹⁾ At temperatures below 37 °C, changing f_p has a profound effect on the shape of the curves (especially, at 10 °C). On the other hand, at 37 °C, the relationship between plasma CyA and blood CyA concentration was relatively linear compared to the other temperatures. In addition, as the value of f_p is reduced, the shift of CyA to the plasma fraction is increased. CyA is exclusively associated with LPs and enters the hydrophobic core regions of the LPs where it is distributed ac-

Fig. 2. Simulation of Plasma Concentration *versus* Blood Concentration of CyA with Changing H at Four Different Temperatures

The mean values of the distribution parameters ($N_p K_p$, N_c , K_c and f_p) listed in Table II were used for the simulation.

cording to its high partition coefficient.⁵⁾ Theoretically, a large variation of plasma CyA is related to the change in free plasma CyA, and these findings from the simulation with changes in plasma lipid levels agreed with several other reports.^{9,20,21)} In clinical practice, since LPs are altered in renal impairment as well as in liver disease, the binding of CyA to LPs can vary considerably with the degree of lipoproteinemia. Therefore, variability in CyA binding to the LPs resulted in misleading pharmacokinetic properties with clinical implications, because the free CyA concentration in plasma represents the pharmacologically active species. The transition of the C/P ratio below 37 °C is in agreement with the results of Agarwal *et al.*,¹⁸⁾ namely, the cellular associated drug estimated from the concentration data of renal transplant patients was 10- to 28-fold greater than that found in plasma at low plasma levels of the drug ($< 0.1 \mu\text{g/ml}$), when the plasma separation was performed at

4 °C. Although the variation of the C/P ratio for CyA essentially arose by the differences in the binding affinities of CyA between the cellular fraction and the plasma fraction, the free fraction of CyA in plasma has an important role for the blood-to-plasma distribution of the drug.

In several reports^{8,22,23)} related to the blood-to-plasma distribution of CyA using transplant patients, nonlinear and linear relationships between plasma CyA and blood CyA concentrations have been observed, and this contradiction has not yet been settled. However, linear regression analysis of measurements of plasma CyA and blood CyA is inappropriate because the cellular fraction has a higher affinity with the temperature dependent saturable process than the plasma fraction (Table II). From our simulative studies shown in Figs. 2 and 3, it appears that there may be some situations using this model in which linear results may be shown with transplant patients who exhibit low hematocrit values

Fig. 3. Simulation of Cellular/Plasma Concentration (C/P) versus Plasma Concentration of CyA with Changing f_p at Four Different Temperatures

The mean values of the distribution parameters ($N_p K_p$, N_c and K_c) and hematocrit listed in Table II were used for the simulation. The value of f_p was changed as follows: a, 0.01; b, 0.05; c, 0.1; d, 0.2.

Fig. 4. Application of the Model to a Renal Transplant Patient

The data displayed here were obtained from a renal transplant patient. a) Solid line represents computer-fit to Eq. (8) using the concentration data obtained within 30 d of transplantation, where the initial values of the distribution parameters were given by use of the mean distribution parameters (37°C) obtained in Table II. b) Predicted plasma concentrations were estimated from the measurements in whole blood, obtained on and 30 d after the transplantation ($Y = 0.014 + 0.937X$, $n = 40$, $r = 0.981$, $p < 0.001$).

or low levels of plasma lipids, and whose plasma samples are separated from cells at 37 °C.

To evaluate the adaptation of this model to clinical practice, we applied the model to the concentration data obtained from a renal transplant patient (Fig. 4). Using the CyA concentration data set of blood and plasma (37 °C equilibrated for 3 h) from the patient, obtained within 30 d of transplantation, we computed the distribution parameters for the patient in the same way as in Fig. 1. The initial values of $N_p K_p$, N_c , K_c and f_p for the patient were given by use of the mean distribution parameters (37 °C) obtained in Table II. The resultant data of the distribution parameters ($N_p K_p$, N_c , K_c and f_p) from the nonlinear regression for the patient were 17.2, 2.6 $\mu\text{g}/\text{ml}$, 8.6 $\text{ml}/\mu\text{g}$ and 0.1819, respectively. Using these parameters, we predicted plasma CyA levels at body temperature (37 °C) from the measurements of blood CyA, on and 30 d after the transplantation, and compared the predicted plasma CyA levels with those observed (37 °C equilibrated for 3 h). As shown in Fig. 4, there was a good correlation between the predicted plasma CyA levels and the observed plasma CyA levels (37 °C equilibrated for 3 h), suggesting that the model proposed here adequately describes the relationship between plasma CyA and blood CyA *in vivo*.

Since the CyA measurements in plasma separated from cells at temperatures below 37 °C do not reflect the true plasma concentrations *in vivo*, the values of pharmacokinetic parameters derived from such plasma concentration measurements are inadequate.²⁰⁾ To overcome this problem of temperature dependent CyA distribution in blood, the monitoring of CyA was assessed in whole blood in previous investigations. However, plasma measurements which reflect the true plasma concentration *in vivo* allow for the predetermination of therapeutic CyA effects and tissue toxicity induced by the drug, because the plasma fraction *in vivo* is associated with tissues.^{8,24)} Also, from this perspective, the model we have proposed here is useful for the therapeutic drug monitoring of CyA.

In conclusion, the model we described here clearly demonstrated the distribution of CyA in human blood *in vitro* and *in vivo*. Since trans-

plant patients often exhibit alterations in hematocrit values due to chronic disease or intraoperative blood loss, and show alterations in plasma lipid levels due to the persistent administration of CyA,²⁵⁾ this approach is useful for the therapeutic drug monitoring of CyA in transplant patients.

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