Effects of Temperature and Endogenous Factors in Blood on Concentrations of Cyclosporin in Plasma Measured by High-Performance Liquid Chromatography

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The effects of temperature, hematocrit (Hct), lipid level in plasma and cyclosporin A (CyA) level in whole blood on the concentration of CyA in plasma measured by high-performance liquid chromatography were studied *in vitro*. With rise in blood storage temperature before cells were removed, the concentration of CyA in plasma was increased in the temperature range between 10 °C and 37 °C, but was decreased between 4 °C and 10 °C. With rise in Hct, the concentration of CyA in plasma was decreased, and it was more markedly decreased at the blood storage temperature of 4 °C than at 37 °C. A lipid supplementation study showed that the concentration of CyA in plasma was increased with rise in plasma triglyceride level and in plasma cholesterol level at the storage temperature of 4 °C but not at 37 °C. Studies of the effect of CyA concentration in blood on the CyA distribution in blood demonstrated that the cellular/plasma concentration (C/P) ratio at low levels (<200 μg/ml) of plasma CyA ranged from 4 to 10 and was about 2 times higher than that at higher concentrations at 4 °C, but the ratio was relatively constant at 37 °C. The saturation capacity of the cellular fraction for CyA showed considerable individual variations, but there was no difference between the capacities at 4 °C and 37 °C. The separation of plasma after equilibration at 37 °C made it possible to avoid the variations in the distribution of CyA in whole blood associated with changes in Hct, lipid level in plasma and CyA level in whole blood, and to obtain a measurement reflecting the physiologically significant concentration of CyA in plasma.

Keywords cyclosporin A; blood-to-plasma distribution; monitoring therapy; cellular saturation; HPLC

Introduction

Cyclosporin A (CyA), a powerful immunosuppressive agent of fungal origin, is widely used in the prevention of graft rejection after solid organ transplantation [1] and in the prevention of graft-*versus*-host disease after bone marrow transplantation.[2] Monitoring of CyA levels after therapeutic drug administration is essential for several reasons: (a) there is a wide variability in CyA pharmacokinetics which is related to a patient's disease state; (b) a blood concentration of CyA suitable to prevent rejection of the transplant organ must be maintaind: (c) it is desirable to minimize hepatotoxicity and nephrotoxicity; (d) the patient's compliance can be monitored. Two analytical methods, radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC), are available, but they provide different results for CyA in serum, plasma and whole blood,[3] bacause the RIA measures CyA plus some of the CyA-related metabolites with which the assay cross-reacts. It was reported that several factors, such as temperature, lipid in plasma and hematocrit (Hct) of blood, affect the distribution of CyA in blood. However, there is disagreement in the literature on the distribution of CyA in blood due to differences in the analytical method, in the time and temperature of sample handling before cells are removed and in the plasma lipid or lipoprotein level.

The present investigation was undertaken *in vitro* using CyA-supplemented samples to estimate the effects of temperature, Hct, lipid level in plasma, and CyA level in whole blood on the distribution of CyA between cellular and plasma fractions, and to estimate the saturation capacity of the cellular fraction for CyA. Because the RIA for CyA lacks specificity, we performed the analysis by HPLC, which offers high specificity for the drug.

Experimental

Drugs and Reagents CyA and cyclosporin D (CyD, used as an internal standard for the HPLC determination) were kindly supplied by Sandoz Ltd., Basle, Switzerland. Tris(hydroxymethyl)aminomethane (Tris), polyoxyethylene sorbitan monolaurate (Tween 20) and cholesterol (CHO) were purchased from Nacalai Tesque Co., Ltd., Kyoto, Japan. Glycerol trioleate, used as triglyceride (TG), was purchased from Tokyo Kasei Co., Ltd., Tokyo, Japan. All other reagents were of analytical grade.

Preparation of Standard Solutions The CyA standard solution was prepared by dissolving CyA in buffer, pH 7.4, consisting of 0.05 mol/l of Tris and 10 ml/l of Tween 20 (Tris–Tween buffer) to yield a CyA concentration of 500 mg/l. Working CyA solutions for all the supplementation studies were prepared by diluting this CyA standard solution 1 : 5 to 1 : 50 with 0.9% saline. The TG and CHO standard solutions were prepared by dissolving TG or CHO in Tris–Tween buffer to yield concentrations of 0.5 and 1 mol/l, respectively, followed by sonication at 21 °C for 5 min with a Nissei ultrasonic generator (Tokyo, Japan), and used in the lipid supplementation study.

Assay of CyA in Plasma CyA in plasma was determined in duplicate samples by our HPLC method.[4] To 1 ml of plasma were added 20 μl of internal standard solution containing CyD at the concentration of 25 mg/l in methanol, 2 ml of 0.1 mmol/l dextran sulfate sodium salt (molecular weight of 500 kDa) solution containing 50 mmol/l magnesium chloride, and 10 ml of *n*-hexane. The mixture was agitated vigorously for 10 min and centrifuged at 3000 rpm for 10 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 the mobile phase (*n*-hexane: ethanol, 85 : 15), and 150 μl was injected into the column. The apparatus used for the HPLC 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 Lichrosorb 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. The within-run and between-run coefficients of variation for a CyA concentration of 25 μg/l in plasma were 2.5% and 3.0%, respectively.

Effect of Temperature on CyA Concentration in Plasma To 4 ml of fresh heparinized whole blood obtained from four healthy volunteers, 40 μl of CyA working solution was added to obtain a final concentration of 500 μg/l in whole blood. Aliquots of the samples were equilibrated at 4, 10, 15, 20, 25, 30 and 37 °C for 3 h, followed by centrifugation at the same temperature and prompt removal of the plasma.

Effects of Hct and Plasma Lipid on CyA Concentration in Plasma The erythrocytes were separated from fresh heparinized, pooled blood from healthy volunteers, discarding the buffy coat after centrifugation at 3000 rpm for 10 min, and washed three times with 0.9% saline. In the study on the effect of Hct, the washed packed erythrocytes were resuspended in preseparated plasma at seventeen different Hcts of 10 to 60%. To 4 ml of this specimen, 40 μl of CyA working solution was added to give a final concentration of 500 μg/l. In the study on the effect of plasma lipid, lipid-

supplemented plasma was prepared by diluting the lipid standard solutions 1:100 to 1:500 with preseparated plasma to give five different concentrations of 1 to 5 mmol/l for TG or five different concentrations of 2 to 10 mmol/l for CHO, followed by sonicating to ensure lipid dispersion. The washed packed erythrocytes were resuspended in this lipid-supplemented plasma at Hcts of 20% and 40%. To 4 ml of the resultant erythrocyte suspensions, 40 μl of CyA working solution was added to give a final concentration of 500 μg/l, and then the mixture was equilibrated at 4 °C and 37 °C for 3 h, followed by centrifugation at the same temperature and prompt removal of plasma.

Effect of the Concentration of CyA in Blood on the Distribution of Drug To 4 ml of fresh, heparinized, whole blood obtained from four healthy volunteers, 40 μl of CyA working solution was added to give seven different concentrations of 100 to 1000 μg/l. After being equilibrated at 4 °C and 37 °C for 3 h, plasma was separated from cells without delay. The concentration (C) of CyA in the cellular fraction of whole blood was calculated from the following relationship: $C=[B-P(1-\mathrm{Hct})]/\mathrm{Hct}$, where B and P are the concentrations of CyA (μg/l) in whole blood and plasma, respectively, and Hct represents hematocrit after the addition of CyA working solution. Then the cellular-to-plasma concentration (C/P) ratios of CyA were calculated.

Results and Discussion

Using whole blood samples from transplant patients receiving CyA, Yatscoff *et al.*[5] found that re-equilibration of whole blood stored at 4 °C to 37 °C occurred within 10 min and they recommended 30 min as the optimal equilibration interval. Niederberger *et al.*[6] reported that when the storage temperature is lowered from 37 °C to 21 °C, the new equilibrium of CyA is reached within 2 h. Moreover, after cooling to 6 °C, equilibrium is reached after 18 h. Our results in a preliminary study showed that when the blood samples were cooled from 37 °C to 4 °C and heated back from 4 °C to 37 °C, the new equilibrium was reached within 0.5—3 h and within 3—6 h, respectively. Considering the possible hemolysis of samples, we selected 3 h as the incubation time to ensure complete equilibration at any temperature.

In this study, we used Tween 20 to prepare the standard solutions. It is well known that Tween 20 makes lipophilic substances more soluble in the aqueous phase. Therefore, it is possible that Tween 20 affects the distribution of CyA between plasma and cells. In our preliminary study using two groups of CyA-spiked samples (500 μg/l in whole blood) containing Tween 20 at the concentration of 0.0002 or 0.02%, there was no significant difference between the two groups in plasma CyA concentration after equilibration for 3 h at 4 °C or 37 °C. Therefore, the effect of Tween 20 on the distribution of CyA in blood, if any, is negligible, because the concentration range of Tween 20 used in this study was below 0.02%.

Figure 1 shows CyA concentrations in plasma of four whole blood samples supplemented with a fixed amount of CyA and then equilibrated at seven different temperatures. The Hct values of the four whole blood samples ranged from 37 to 48%, and thus were in a normal range. Though the plasma CyA concentration showed considerable individual variations, it was minimum in all four subjects at 10 °C and increased with the change of temperature from 10 °C, especially with the rise in temperature from 10 to 37 °C. In order to check these findings, we performed the binding assay of CyA in hemolysate at 4, 10 and 37 °C using the charcoal separation method.[7] The result showed that CyA has a higher affinity for hemolysate at 10 °C than at the other storage temperatures (data not shown).

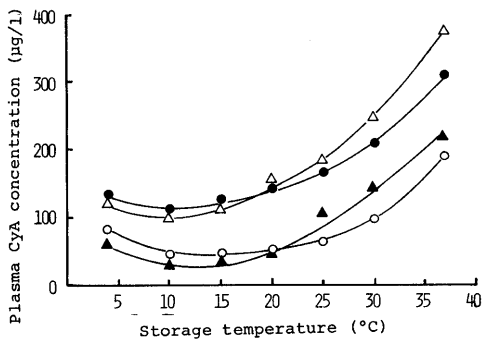

Fig. 1. Effect of Storage Temperature on Plasma CyA Concentration

Each symbol represents an individual subject. Whole blood samples spiked with CyA at a fixed concentration of 500 μg/l were stored for 3 h and centrifuged at the indicated temperatures.

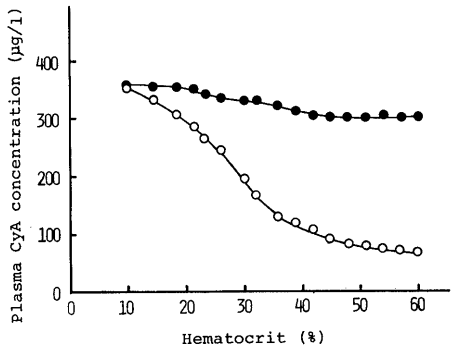

Fig. 2. Effect of Hematocrit on Plasma CyA Concentration

Washed, packed erythrocytes from normal individuals were resuspended at different Hcts as indicated in plasma from the same individuals. The suspensions were spiked with CyA at a fixed concentration of 500 μg/l, stored for 3 h and centrifuged at 4 °C (—○—) or 37 °C (—●—).

The considerable individual variations of plasma CyA (Fig. 1) suggested that there are some other factors than the temperature that affect the distribution of CyA in blood. The Hct is one possible factor, since Rosano[8] reported that the concentration of CyA in plasma exhibits a reciprocal relationship to Hct, though Agarwal *et al.*[9] reported that the distribution of CyA in blood varied independently of Hct. Our results shown in Fig. 2 agreed with the results of Rosano. At 4 °C, plasma CyA concentration was markedly decreased with the rise in Hct, and the concentration at Hct of 60% was only 1/5 of that at Hct of 10%. At 37 °C, plasma CyA concentration was decreased, but only slighly. As a result, at Hct of 10% the plasma CyA concentration at 4 °C was nearly equal to that at 37 °C, but at Hct of 60% the former was only 1/4 of the latter. Therefore, lower temperature of sample handling and higher Hct were found to result in lower plasma CyA concentration, though the CyA concentration in whole blood was unchanged.

Figure 3 shows CyA concentrations in plasma of erythrocyte-plasma suspensions with Hcts of 20% and 40% which were supplemented with different amounts of TG or CHO and a fixed amount of CyA and then equilibrated at 4 °C and 37 °C. At both Hcts of 20% and 40%, plasma CyA concentration was increased with the rise in plasma TG level and CHO level at 4 °C, but not at 37 °C. There is controversy in the literature concerning plasma lipid level as a factor affecting the distribution of CyA in blood. Lemire *et al.*[10] reported an effect of lipid level in plasma on

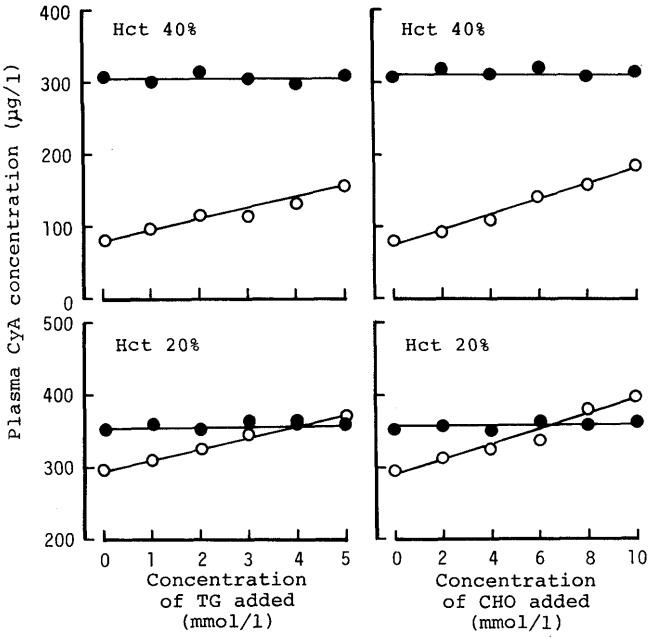

Fig. 3. Effect of Lipid on Plasma CyA Concentration

Washed, packed erythrocytes from normal individuals were resuspended at Hcts of 20 and 40% in plasma from the same individuals supplemented with TG or CHO. The suspensions were spiked with CyA at a fixed concentration of 500 μg/l, stored for 3 h and centrifuged at 4 °C (—○—) or 37 °C (—●—). Initial TG and CHO levels were 1.4 and 3.1 mmol/l, respectively.

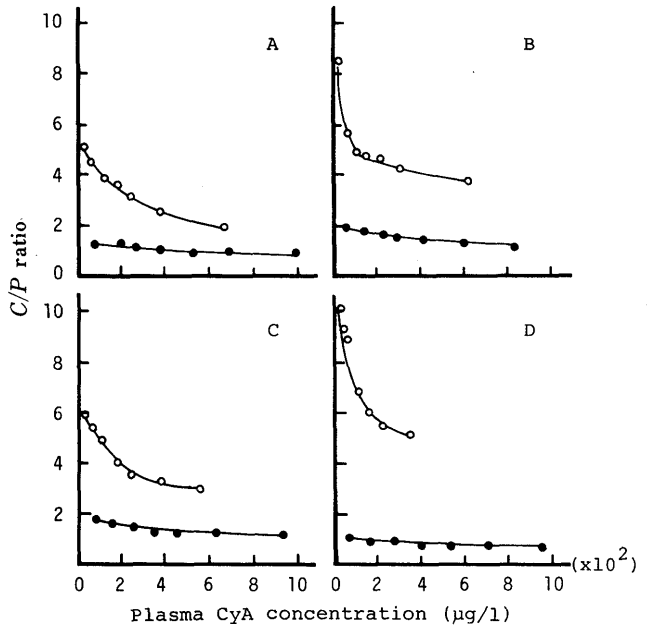

Fig. 4. Variation of Cellular/Plasma Concentration (C/P) Ratio of CyA as a Function of Plasma CyA Concentration

Whole blood from four healthy volunteers was spiked with CyA at seven different concentrations of 100 to 1000 μg/l, stored for 3 h and centrifuged at 4 °C (—○—) and 37 °C (—●—).

the distribution of CyA in blood, but more recently Rosano[8] found that the lipid level has no effect on it. This disagreement may be a consequence of differences in analytical methods and plasma separation conditions. Our results showed that plasma CyA concentration was increased with the rise in plasma lipid level at 4 °C, but not 37 °C. Although the reason is not yet clear, it may be that invasion of CyA into TG or CHO fractions is increased at

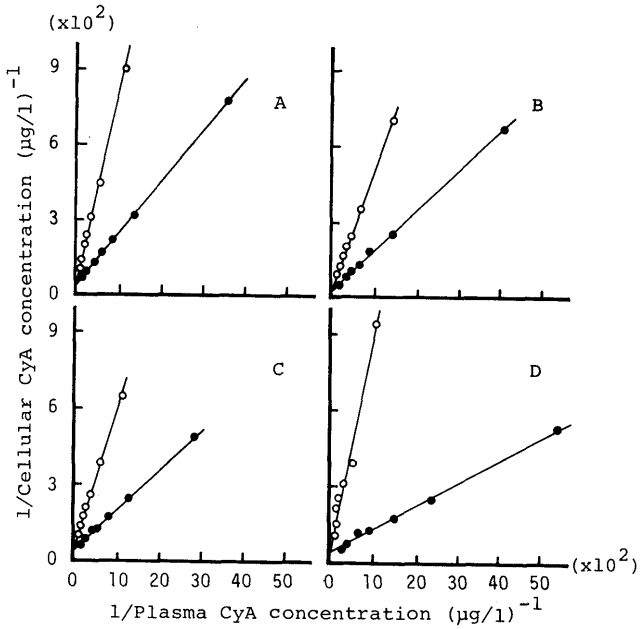

Fig. 5. Double-Reciprocal Plots of Cellular CyA and Plasma CyA Concentrations

Whole blood from four healthy volunteers was spiked with CyA at seven different concentrations of 100 to 1000 μg/l, stored for 3 h and centrifuged at 4 °C (—○—) and 37 °C (—●—).

lower temperature, but not higher temperature. Thus, a blood with a higher plasma lipid level, *e.g.* in lipemia, stored at a lower temperature shows a higher plasma CyA concentration.

Figure 4 illustrates the variation of the C/P ratio as a function of the plasma CyA concentrations found in the four subjects studied. At 4 °C, the ratio ranged from 4 to 10 at low levels (<200 μg/l) of plasma CyA and markedly decreased at higher concentrations of the drug, and the ratio at higher concentrations was about one-half of that at low levels of the drug. On the other hand, at 37 °C, the ratio ranged from 1 to 2 and hardly changed with rise in plasma CyA concentration. The relationship between C/P ratio and plasma CyA concentration at 4 °C shown in Fig. 4 is essentially in agreement with the results of Agarwal *et al.*[9] and Hamberger *et al.*,[11] but we found considerable individual variations. At 37 °C, the ratio ranged from 1 to 2 and was practically unchanged throughout the plasma CyA concentration range studied. The value of the ratio at 37 °C must be the clinically significant one because it was obtained at normal body temperature (37 °C), and because the C/P ratios of many other hydrophobic drugs, such as imipramine, chlorpromazine are around 3 at Hct of 45%.

A nonlinear relationship between CyA concentrations in plasma and cellular fractions was established.[11] The relationship can be expressed by using a Michaelis-Menten model. The maximum uptake of CyA into the cellular fraction, namely the saturation capacity of the cellular fraction for CyA was reported by several investigators,[9,11] but its implications for the distribution of CyA in blood are not clear. Then, to elucidate the implications of the saturation capacity of the cellular fraction and its involvement in the distribution of CyA in blood, we performed double-reciprocal plot analysis using the same blood samples as in Fig. 4. In Fig. 5, the intercept of the regression line gives the

TABLE I. Saturation Capacity of the Cellular Fraction for CyA

Subject	Hct (%)	Saturation capacity (μg/l)	
		4 °C	37 °C
A	49	1961	1846
B	40	4363	3905
C	38	2072	2178
D	45	2616	2718

reciprocal of the saturation capacity of the cellular fraction for CyA, and the slope gives the affinity of the cellular fraction for CyA. It was found that CyA has 3- to 9-fold higher affinity for the cellular fraction at 4 °C than at 37 °C, and that the saturation capacity of the cellular fraction at 4 °C is not different from that at 37 °C. Thus, the affinity and the saturation capacity of the cellular fraction for CyA are independent of each other. Table I lists the values of Hcts and the saturation capacities calculated from the plots. Since the saturation capacity of the cellular fraction for CyA had no relationship with Hct, and showed considerable individual variations, it is suggested that there are large individual variations in the amount of the CyA binding protein in erythrocytes; the protein was reported to be present in sufficient quantity to account for the saturation of CyA uptake into erythrocytes and to be distinct from hemoglobin, carbonic anhydrase, calmodulin and cytochrome b_5.[12] Therefore, change in the amount of CyA binding protein in erythrocytes in a transplant recipient may result in variations in the pharmacokinetics of CyA.

In conclusion, when the blood sample is handled at below 37 °C before cells are removed, the distribution of CyA between cellular and plasma fractions is significantly influenced by Hct, lipid level in plasma and CyA level in whole blood. Transplant recipients often have low Hct levels due to chronic disease or intraoperative blood loss and have high lipid levels due to persistent administration of CyA,[13] resulting in altered CyA distribution in these patients. Therefore, CyA measurements in plasma of blood from these patients may be misleading in terms of pharmacokinetics and clinical implications, when the blood sample are handled at below 37 °C. The concentration of CyA in plasma when the separation is done at 37 °C may be more significant to predict physiological tissue toxicity induced by the drug, because the plasma fraction is adjacent to the tissues.[14]

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