Cyclosporine Metabolites in Human Blood and Renal Tissue

T.G. Rosano, B.M. Freed, M.A. Pell, and N. Lempert

CYCLOSPORINE A (CsA) is a cyclic oligopeptide that undergoes extensive hepatic biotransformation with final elimination into bile, feces, and, to a limited extent, urine.[1,2] Primary and secondary metabolites have also been detected in the peripheral blood of healthy individuals who received tritiated CsA orally,[3] and we have detected three primary metabolites of CsA in trough blood samples from renal allograft recipients treated with CsA.[4] Metabolites in the circulation may equilibrate with the cellular fraction of blood and tissue and may result in an appreciable distribution of metabolites into cells. CsA metabolites have been detected in peripheral tissues in the rat,[3] but the pattern or level of metabolites in human tissue has not yet been defined.

Studies in our laboratory have demonstrated potent in vitro immunosuppressive activity of the primary CsA metabolites, M1 and M17.[4,5] If these metabolites are present in tissue, they might play a role in the efficacy of CsA therapy. We have now developed a high-performance liquid chromatographic (HPLC) method for measurement of CsA and its primary metabolites. The present study characterizes the level and distribution of primary metabolites in blood and renal tissue.

MATERIALS AND METHODS

Measurement of CsA and Metabolites

Specific quantitation of CsA, M1, M17, and M21 levels was performed by a modification of a previously reported HPLC procedure.[4] Briefly, 1 mL of hemolyzed blood, plasma, or homogenized tissues was extracted with diethyl ether under base and acid conditions. After ether evaporation, CsA and the metabolites were dissolved in 300 μL of mobile phase (43% acetonitrile-57% H_2O) and washed with 1 mL of heptane. A portion of the mobile-phase layer was then chromatographed on a cyanopropyl column (4.6 mm × 25 cm; DuPont Instruments, Wilmington, DE) maintained at 65°C with a flow rate of 0.5 mL/min. Peaks were detected at 210 nm, and the assay was standardized with purified CsA, M1, M17, and M21 (generous gift of Dr G. Maurer, Biopharmaceutical Department, Sandoz Ltd, Basel, Switzerland). CsA and the immuno–cross-reacting metabolites were also measured in blood and tissue by a radioimmunoassay (RIA) procedure from Sandoz (Basle, Switzerland).

Distribution of CsA and Metabolites in Blood

Partitioning of CsA and metabolites into the plasma and cellular fractions of blood was studied using heparinized blood from nonmedicated volunteers spiked with 500 ng/mL of CsA, M1, M17, and M21. Aliquots of blood were equilibrated for two hours at 22°C or 37°C and then centrifuged. The amounts of CsA and metabolites in the plasma and whole blood were then measured. The amount of CsA and metabolites in the cellular compartment was calculated from the levels in plasma and whole blood and the hematocrit. Distribution of CsA and the metabolites was expressed as the percentage of each compound in the cellular fraction of blood. The levels of CsA and metabolites were also determined in blood and plasma from renal allograft recipients. Blood was equilibrated and centrifuged at 22°C as described above.

Patients

The levels of CsA and metabolites were determined in 132 trough blood samples (12 hours after oral dosage) from 24 renal allograft recipients treated with CsA, azathioprine (Aza), and methylprednisolone (MP). Blood was obtained within 70 days of the transplant. Patients were initially treated with 2 to 5 mg/kg of CsA (at 12-hour intervals) as soon as they had achieved good renal function. Whole blood levels of CsA were then determined by RIA on a daily basis while the patients were in the hospital, then less frequently on an outpatient basis. The CsA dose was adjusted to maintain an RIA level of

From the Departments of Clinical Chemistry, Biochemistry, and Surgery, Albany Medical College and Albany Medical Center Hospital, Albany, NY.

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Address reprint requests to Dr. T.G. Rosano, Department of Clincial Chemistry, Albany Medical College and Albany Medical Center Hospital, Albany, NY 12208.

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CsA at 150 to 300 ng/mL. MP was given initially as a 300-mg bolus and then adjusted to 20 mg/day by 30 days posttransplant and 10 mg/day by 60 days. Aza (4 mg/kg) was initiated preoperatively and immediately tapered to 0.75 to 1.0 mg/kg and adjusted if the white cell count fell to <5,000 cells/cc. Analysis of CsA and metabolites was also performed in kidney tissue from nephrectomies performed at either Albany Medical Center Hospital or the Hospital of the University of Pennsylvania (tissue provided by Dr L.M. Shaw). Nephrectomies were performed as a result of allograft rejection. Patients were maintained on oral CsA therapy until the day of the nephrectomy. One of the patients received concomitant therapy with phenytoin (200 mg/day). Kidney tissue (500 mg) was homogenized in five volumes of a 0.9% NaCl solution using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The volume of the homogenates was adjusted with 0.9% NaCl to give a final tissue concentration of 100 mg wet weight/mL. Replicate 1-mL aliquots of tissue homogenates were then analyzed for CsA, M1, M17, and M21. In two of the patients, a trough blood sample obtained on the day of nephrectomy was also available for analysis.

RESULTS

Figure 1 shows chromatograms from our simultaneous HPLC analysis of M1, M17, M21, and CsA. The chromatogram of blood supplemented with pure CsA, M1, M17, and M21 shows the retention and resolution obtained in this assay (Fig 1A). Multiple ultraviolet (UV)–absorbing compounds eluting prior to the metabolites were observed in all blood samples, but, as seen in Fig 1B for blood obtained prior to the initiation of CsA therapy, no significance interference with the measurement of CsA or the primary metabolites was observed. A patient treated with CsA shows appreciable levels of M17, M1, and CsA present in the trough sample (Fig 1C). The measurement of CsA and primary metabolites was linear up to 1,000 ng/mL with a detection limit in blood of 20 ng/mL. The extraction efficiencies and recoveries for all analytes was 82% and 99%, respectively.

CsA, M17, and M1 were found to distribute preferentially into the cellular components of blood (Fig 2). Although metabolites M17 and M1 are more polar than CsA, they exhibited even higher cellular affinities. In contrast, approximately 65% of M21 was found in the plasma at 37°C. The effect of temperature was observed for each of the cyclosporines. A decrease from 37°C to 22°C resulted in a greater partitioning of both CsA and the metabolites into the cellular compartment.

The levels of CsA and metabolites were also determined in blood and plasma of 10 renal allograft recipients. The average CsA level in blood was 161 ng/mL, compared with 32 ng/mL in plasma. M17, M1, and M21 levels in blood averaged 313 ng/mL, 64 ng/mL, and <20 ng/mL, respectively. The mean plasma level was <20 mg/mL for all of the metabolites.

Table 1 shows the levels of CsA and metab-

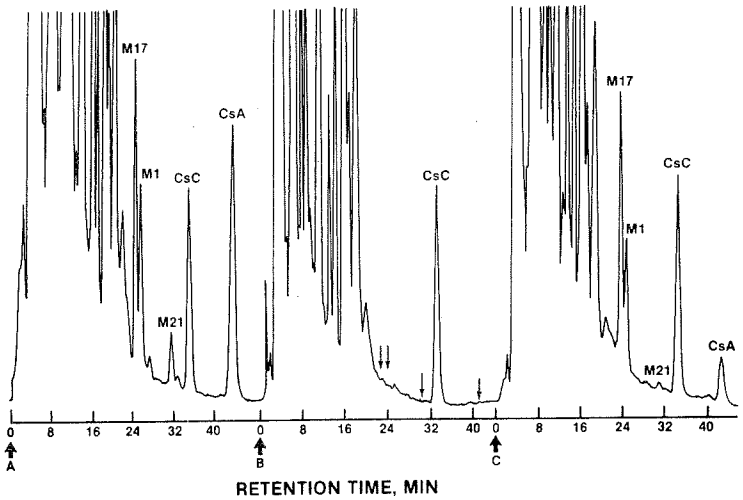

Fig 1. HPLC analysis of M1, M17, M21, and CsA in blood. Chromatogram A is from blood spiked with M17 (745 ng/mL), M1 (365 ng/mL), M21 (140 ng/mL), and CsA (870 ng/mL). Chromatogram B is from blood obtained from a patient prior to CsA therapy and spiked with CsC. Chromatogram C is from blood (trough) obtained from a patient treated with CsA in which levels of M17 (590 ng/mL), M1 (235 ng/mL), M21 (29 ng/mL), and CsA (136 ng/mL) were determined.

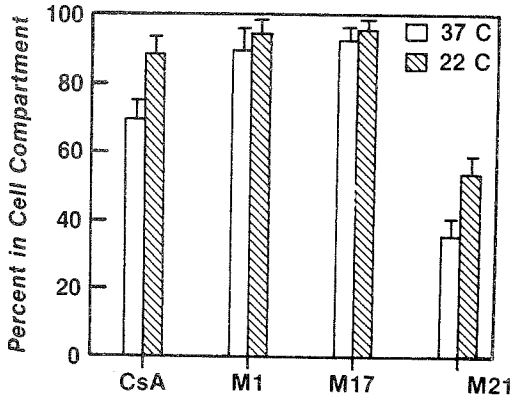

Fig 2. Distribution of CsA, M1, M17, and M21 in blood. Data are expressed as mean ± SD. Data are calculated from the hematocrit and the levels in whole blood and plasma collected at 22°C and 37°C.

olites in kidney tissue from three patients maintained on CsA therapy up until the time of nephrectomy. Results from patient 1 show high levels of M17, M1, and CsA in cortical and medullary kidney tissue. The level of M17 was 3.5 to 4 times the level of CsA in both regions of the kidney. High levels of M17 were also observed in tissue from patient 2. In this case, peripheral blood drawn just prior to nephrectomy was also analyzed. In both whole blood and tissue, the level of M17 exceeded that of CsA by two- to threefold. Results from patient 3 show a different pattern for this patient, who was given concomitant therapy with phenytoin. The levels of CsA and metabolites in this patient were low in both renal tissue and peripheral blood, and the CsA level exceeded that of the metabolites.

CsA and metabolite levels were measured in a series of trough blood samples (N = 132) from 24 renal allograft recipients. As can be seen in Table 2, the RIA level exceeded that of CsA and the metabolites measured in the HPLC. The mean level of CsA and metabolites followed the order of M17 > CsA > M1 > M21. A poor correlation was observed between CsA and M17 levels (data included in Table 2).

Figure 3 shows the results of serial monitoring of CsA and metabolite levels in a patient experiencing good renal function. The trough level of M17 (mean, 238 ng/mL; range, 65 to 374 ng/mL) exceeded that of CsA (mean, 124; range, 39 to 220 ng/mL) throughout the treatment period displayed. Levels of M1 were lower than CsA, and M21 (data not shown) was undetectable throughout most of the period. In serial monitoring of two patients with early posttransplant episodes of liver dysfunction, elevations in CsA, M17, and M1 paralleled the rise in serum bilirubin level, alkaline phosphatase, and gamma glutamytransferase. These patients also showed high RIA levels.

DISCUSSION

The distribution of CsA and metabolites in the peripheral circulation may have important implications for monitoring the efficacy of CsA therapy. Previous studies have shown that M17 and M1 have appreciable in vitro immunosuppressive activity.[4,5] We now report that these metabolites avidly bind to the cellular components of blood and tissue. Our data for CsA distribution in blood are consistent with prior isotopic studies that reported that approximately 70% of the circulating CsA was bound to erythrocytes and leukocytes.[6] M17 and M1 distributed into cells to a greater

Table 1. Levels of CsA and Metabolites in Human Blood and Kidney Tissue

	Patient 1		Patient 2		Patient 3	
Analyte	Renal Cortex	Renal Medulla	Renal Cortex	Peripheral Blood	Renal Cortex	Peripheral Blood
CsA	609	659	710	160	340	67
M17	2120	2800	1720	493	80	22
M1	555	882	ND	53	ND	ND
M21	ND	ND	ND	ND	ND	ND

Tissue and blood concentrations are reported in nanogram per gram of wet weight and nanogram per milliliter, respectively. Values represent the mean of replicate determinations; ND, none detected.

Table 2. Levels of CsA and Metabolites in Blood of Renal Allograft Recipients

Compound	Whole Blood Trough Level (ng/mL) Range	Mean ± SD
CsA	<20–310	125 ± 58
M17	20–612	217 ± 120
M1	<20–307	95 ± 60
M21	<20–73	7 ± 14

Patients (N = 24) were treated with CsA by the protocol described in the Materials and Methods section. Trough levels were measured in 132 whole blood samples collected immediately prior to the next dose. Statistical correlation of CsA and M17 levels resulted in the regression line $y = 1.34x + 50$ and a correlation coefficient of only 0.64.

extent than CsA, with greater than 90% of these metabolites in the cellular fraction of blood.

Cellular binding of CsA, M17, and M1 in blood appears to correlate with peripheral tissue storage of these immunosuppressive metabolites. Other investigators have demonstrated high levels of CsA or metabolites in tissue, but the nonspecific RIA method used in those studies did not allow an assessment of the actual CsA and metabolite content.[7,8] Parallel analyses of CsA by RIA and HPLC suggested significant levels of metabolites in tissue.[9] Our direct measurement of metabolites in kidney tissue showed high levels of CsA and M17, except for the patient treated with phenytoin. In the cases studied, the levels of CsA and primary metabolites in tissue reflected those found in whole blood. M17 was the major metabolite in both renal tissue and blood, and the ratio of M17:CsA in both compartments was similar. The patient treated with phenytoin had the lowest levels of CsA and metabolites in blood and tissue, even though the dose of CsA was maintained at

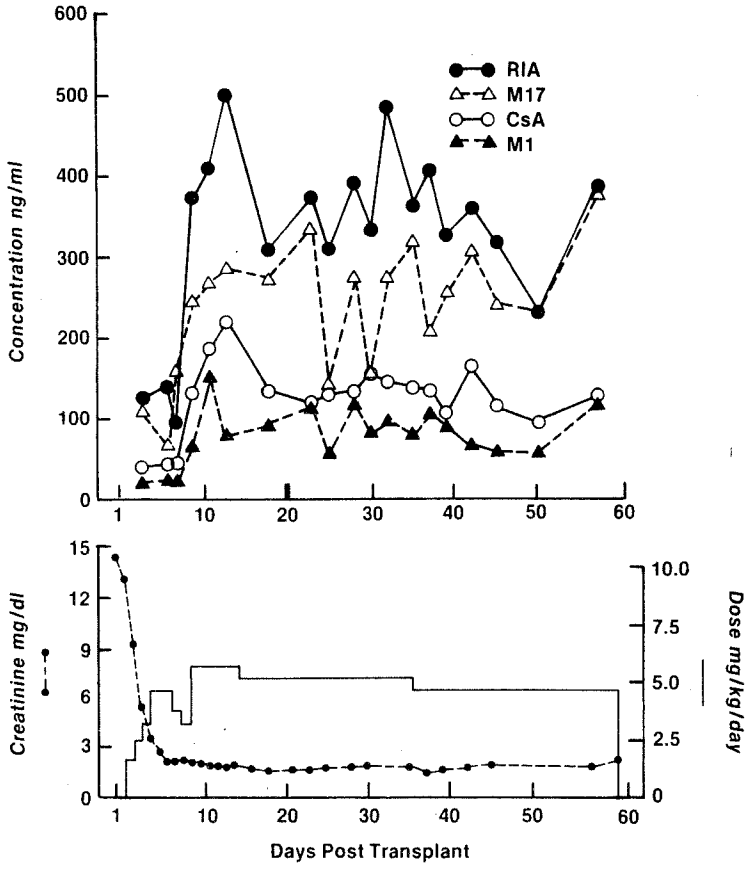

Fig 3. Profile of CsA and metabolite levels in a renal allograft recipient. Trough blood samples were monitored by the RIA and HPLC methodology. (A) Results. (B) CsA dose and serum creatinine.

11.7 mg/kg/day up until the time of nephrectomy. This effect of an inducer of the cytochrome P-450 system is consistent with the pharmacokinetic studies of Freeman et al,[10] in which the levels of both CsA and M17 in blood were reduced during concomitant treatment with CsA and phenytoin. Since whole blood levels of CsA and metabolites appear to reflect the levels in tissue, where immunosuppressive and nephrotoxic events are occurring, whole blood monitoring of CsA and the biologically active metabolites should provide a more accurate assessment of CsA's efficacy and toxicity. We caution, however, that our tissue studies were performed on kidneys with impaired function and that further studies with functional graft tissue as well as other peripheral tissue will be necessary in order to validate this hypothesis.

The affect of metabolite structure on binding to cellular components of blood suggests the presence of specific binding sites on or within cells. M17 and M1, for instance, bound more avidly to cells than did CsA. This finding would not be predicted from the more polar nature of the metabolites, if cellular binding was based on nonspecific interaction with lipophilic membrane structures. M21, which by chromatographic elution appeared closest in lipophilic nature to CsA, had the least cellular affinity, lending further support for the presence of a specific binding site. A binding protein has been reported for CsA,[11] but binding to metabolites was not investigated. Cellular binding properties of CsA and the metabolites, however, showed a similar temperature dependence, as previously reported for CsA,[12,13] suggesting a common binding site for CsA and the metabolites. Characterization of this binding site and its physiological significance will require further investigation.

Based on our tissue and distribution data, we have begun to monitor the levels of CsA and metabolites in the blood of renal allograft recipients. Our preliminary data showed significant levels of CsA and metabolites in trough bloods. M17 was the major metabolite, and its trough levels exceeded those of CsA. The relationship between CsA and M17 levels was variable and may reflect differing rates of metabolism and clearance. Monitoring the CsA level alone will not, therefore, predict the level of M17 in the circulation. Appreciable levels of M1 were also present in blood, as opposed to M21, which was not detected in a majority of the assays.

Metabolite levels may also be important in cases of drug toxicity. In serial samples from two patients with early posttransplant episodes of acute liver dysfunction, a rise in the levels of CsA, M17, and M1 paralleled the elevation in bilirubin, alkaline phosphatase, and gamma glutamyltransferase. Reduced hepatic clearance of metabolites may therefore be a component of hepatotoxicity. Further studies of the role of metabolites in hepatotoxicity and nephrotoxicity may provide a better understanding of these adverse reactions.

The data on the blood and tissue levels of metabolites and their in vitro immunosuppressive activity obviate the need for further investigation of the role of metabolites in CsA therapy. Although technically demanding, HPLC is an accurate method for determining the level of CsA and the primary metabolites. Further development of specific and sensitive monoclonal antibodies for CsA and the biologically active metabolites may widen the availability of metabolite measurements. Application of these techniques to studies of the cellular mechanism of immunosuppression and toxicity should lead to a more fundamental understanding of immunotherapy with CsA.

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