Cyclosporine Metabolism in Patients After Kidney, Bone Marrow, Heart-Lung, and Liver Transplantation in the Early and Late Posttransplant Periods

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Abstract

Cyclosporine is used in the prevention of allograft rejection. Owing to its narrow therapeutic index, regular monitoring of the whole blood levels of cyclosporine is required. We observed that immunoassays measured significantly higher cyclosporine levels than did high-performance liquid chromatography (HPLC) over time after transplantation. As cyclosporine metabolites cross-react even with immunoassays, this observation might be due to alterations of the cyclosporine metabolism. We analyzed cyclosporine metabolite concentrations in the early and in the late posttransplantation periods in 127 patients after kidney, bone marrow, heart-lung, and liver transplantation by HPLC and determined whole blood levels of cyclosporine by 4 immunoassays (enzyme-multiplied immunoassay [EMIT], cloned enzyme donor immunoassay [CEDIA], AxSYM [Abbott Laboratories, Chicago, IL], and TDx [Abbott Laboratories]). Despite reduced dose, we found significantly higher cyclosporine concentrations measured by the EMIT, AxSYM, and TDx assays in various patient groups. These results are due to the increased metabolite/cyclosporine ratio in the late posttransplantation period. In particular, the metabolites AM1 and AM19 increased significantly over time in bone marrow transplant recipients. Therefore, cyclosporine levels measured by immunoassays should be interpreted with caution.

Cyclosporine is a potent immunosuppressant drug that is used in the prevention of allograft rejection and treatment of certain autoimmune diseases. Its use is limited by severe toxic effects and a narrow therapeutic index. Therefore, to avoid adverse effects and to ensure effective immunosuppression despite intraindividual and interindividual variability in pharmacokinetics and metabolism, regular monitoring of the whole blood levels of cyclosporine is required.

Cyclosporine is metabolized extensively in the liver by the cytochrome P-450 oxidase. The primary metabolites are the monohydroxylated AM1 (M-17) and AM9 (M-1) and the N-demethylated AM4n (M-21). Further oxidation of AM1 and AM9 results in the dihydroxylated AM19 (M-8), AM49 (M-10), and AM69 (M-16).

The distribution of cyclosporine and its main metabolites in whole blood are reported to be as follows: cyclosporine (27%), AM1 (24%), AM9 (14%). Among different transplant groups (kidney, bone marrow, heart-lung, and liver), the highest quantities of cyclosporine metabolites (AM1 and AM9) were observed in patients after liver and heart transplantation, whereas only in liver recipients were the blood levels of AM1 found to be significantly (P < .02) higher than the parent drug levels.

Cyclosporine metabolites cross-react even with the newly introduced specific monoclonal immunoassays (fluorescence polarization immunoassay [FPIA]/AxSYM [Abbott Laboratories, Chicago, IL], modified enzyme-multiplied immunoassay [EMIT], and cloned enzyme donor immunoassay [CEDIA]).

By spiking a whole blood pool from healthy donors with the major cyclosporine metabolites AM1 and AM9, Hamwi et al found that the monoclonal FPIA/TDx (Abbott Laboratories) showed the highest cross-reactivity (7.1%) toward AM1, followed by FPIA/AxSYM (5.5%) and CEDIA (4.5%).
whereas the EMIT showed no detectable cross-reactivity with AM1. Furthermore, all assays displayed a cross-reactivity toward AM9; the highest cross-reactivity was seen with CEDIA (23%), followed by FPIA/TDX (18.2%) and FPIA/AxSYM (13.7%), and the lowest with EMIT (9.2%).

Hamwi et al9 also showed that these immunoassays measured significantly (P < .001) higher cyclosporine concentrations than those measured by high-performance liquid chromatography (HPLC). In the comparison studies between each of the 4 monoclonal immunoassays (CEDIA, EMIT, FPIA/AxSYM, and FPIA/TDX) for cyclosporine and the reference method, HPLC, the slopes of the regression lines were 1.25 for CEDIA, 1.12 for the EMIT, 1.15 for FPIA/AxSYM, and 1.4 for the FPIA/TDX.9 Furthermore, these differences depended not only on the assay used but also on the transplanted organ.9 In addition, the FPIA/TDX immunoassay measured significantly (P < .015) higher cyclosporine levels over time after transplantation than did HPLC.9

Cross-reactivity of immunoassays with metabolites when determining cyclosporine levels may result in inadequate immunosuppression. For example, the main metabolite AM1 shows only 10% to 20% of the parent compound’s activity.10 Therefore, exact knowledge of the metabolism of cyclosporine in different patient groups during the posttransplantation period is required for accurate interpretation of the results measured by monoclonal immunoassays.

The aims of the present study were to analyze by HPLC the metabolite patterns in the early (3 months or less after transplantation) and late (>3 months after transplantation) posttransplantation periods in different patient groups after kidney, bone marrow, heart-lung, and liver transplantation and to compare these results with results obtained by 4 immunoassays.

Materials and Methods

Patient Samples

During the posttransplantation period and during cyclosporine immunosuppression, 179 whole blood samples were obtained from 127 patients (89 men, mean ± SD age, 51.85 ± 12.22; 38 women, mean ± SD age, 52.05 ± 11.30) as follows: renal transplant recipients, 49 specimens (24 early and 25 late); bone marrow transplant recipients, 40 specimens (20 early, 20 late); heart-lung transplant recipients, 50 specimens (18 early, 32 late); and liver transplant recipients, 40 specimens (8 early, 32 late). The subdivision of the patients into early and late transplant groups on the basis of a 3-month posttransplantation period relied on the fact that administered cyclosporine doses are usually lowered 3 to 6 months after transplantation and after the patient’s condition is clinically and pharmacologically stable.11 Trough cyclosporine concentrations were determined, as sampling was performed in the morning before the next dose. The test period was 2 months.

Whole blood was collected into tubes coated with the anticoagulant EDTA. The first analysis was performed with the monoclonal FPIA/TDX method during the regular cyclosporine monitoring. Analyses of the cyclosporine level by FPIA/AxSYM, CEDIA, modified EMIT, and HPLC assays were performed on samples stored at 4°C for a maximum of 5 days or frozen at –20°C for a maximum of 4 weeks. According to the producer of the HPLC method, the determination of cyclosporine showed no difference between samples stored at 4°C for 1 week and those kept at –20°C for 4 weeks.

Cyclosporine Metabolites

Cyclosporine A metabolites were kindly provided by Dade Behring, Frankfurt, Germany, and were of highest purity available (>95% as stated by the supplier).

HPLC Method

Extraction and HPLC analysis of cyclosporine and its metabolites in whole blood were performed with minor modifications using the commercially available ClinRep kit (Recipe Chemicals and Instruments, Munich, Germany). The assay is based on the HPLC method described by Christians et al.12 Briefly, after hemolysis of 1 mL whole blood by the addition of a mixture of zinc sulfate/methanol (65:35, wt/vol; 2 mL), 50 µL (250 ng) of cyclosporin D was added as the internal standard. After centrifugation (5 minutes, 3,000 g), the supernatant was passed through a C-18 cartridge (Recipe Chemicals and Instruments) previously equilibrated with 2 mL of methanol and water (pH 3.0), respectively. The column was washed with methanol in water (50%, vol/vol; 2.3 mL) and heptane (0.5 mL), and cyclosporine A and its metabolites were eluted with ethanol (100%, 300 µL). The eluate was mixed with 100 µL of water (pH 3.0) and 1 mL of heptane and centrifuged for 5 minutes at 3,000g. An aliquot (80 µL) of the aqueous phase was injected onto the HPLC column.

HPLC was performed using a Merck La Chrom System (Merck, Darmstadt, Germany) equipped with an L-7250 injector, an L-7100 pump, an L7300 column oven (set at 75°C), a D-7000 interface, and an L-7400 UV-detector set at a wavelength of 205 nm. Separation of cyclosporine A and its metabolites was performed using a Hypersil (Astmoor, England) BDS-C18 column (5 µm, 150 x 4.6 mm inner diameter) at a flow rate of 0.6 mL/min. The mobile phase consisted of a continuous gradient mixed from solvent A (acetonitrile in water [35:75, vol/vol]) and solvent B (acetonitrile in water [85:15, vol/vol]). The column was equilibrated with 45% solvent B at time 0; after injection of the sample (80 µL), the content of solvent B was linearly increased to 93% at 19 minutes. Subsequently the percentage of solvent B was

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decreased to 45% within 2 minutes, to equilibrate the column for 8 minutes before application of the next samples.

The quantitation of cyclosporine A and its metabolites AM1, AM9, and AM19 is based on the comparison of cyclosporine (metabolites)/cyclosporin D peak height ratios with the ratio of cyclosporine (metabolites)/cyclosporin D in the whole blood calibrator. The HPLC was recalibrated daily.

**CEDIA Immunoassay**

The reagents for this monoclonal antibody–based CEDIA cyclosporine assay were obtained from Boehringer Mannheim (Mannheim, Germany). The cyclosporine test was performed on a Hitachi 911 (Boehringer Mannheim) according to the manufacturer’s instructions. The test principle is based on the spontaneous association of a short recombinant NH2-terminal beta-galactosidase fragment (alpha-peptide) and a recombinant beta-galactosidase monomer with a deletion near the NH2 terminus to form active enzyme tetramer. Cyclosporine is attached chemically to the alpha-peptide, and an anticyclosporine antibody binds to both cyclosporine in the sample and cyclosporine coupled to the alpha-peptide. Because the latter process interferes with the formation of active enzyme, the amount of cyclosporine in the sample is directly proportional to the residual enzyme activity. Before the analysis, 200 µL of CEDIA cyclosporine lysing reagent was added to 100 µL of whole blood sample in the Hitachi sample cup. The sample cup was spun for 5 seconds and then directly placed into the analyzer without a centrifugation step. The assay is based on a 2-point calibration and is designed to measure cyclosporine A concentrations up to the highest standard level of 620 µg/L.

**EMIT Immunoassay**

The reagents for this monoclonal antibody–based EMIT 2000 Cyclosporine Specific Assay were obtained from Dade Behring. The cyclosporine test was performed on a Hitachi 911 analyzer according to the manufacturer’s instructions. Before the assay, 300 µL of pretreatment reagent was added to 100 µL of whole blood; the sample mixture was then spun for at least 10 seconds and centrifuged for at least 10 minutes in a microcentrifuge at 9,500 g. The supernatant then was decanted directly into the AxSYM sample cups, and the samples were assayed immediately. The assay was calibrated by a nonlinear calibration curve with 6 calibrators and is designed to measure cyclosporine concentrations up to the level of the highest standard of 1,500 µg/L.

**Monoclonal FPIA/AxSYM Method**

The reagents for this monoclonal antibody–based FPIA assay were obtained from Abbott Laboratories. The cyclosporine assay was performed on the Abbott AxSYM system according to the manufacturer’s instructions. Before assay, 50 µL of solubilization reagent and 300 µL of precipitation reagent were added to 150 µL of whole blood sample, and this mixture was spun for at least 10 seconds. The sample pretreatment is necessary to lyse erythrocytes, precipitate blood proteins, and extract cyclosporine. The pretreated sample tubes were centrifuged for at least 5 minutes in a microcentrifuge at 9,500g. The supernatant was then directly into the AxSYM sample cups, and the samples were assayed immediately. The assay was calibrated by a nonlinear calibration curve with 6 calibrators and is designed to measure cyclosporine concentrations up to 800 µg/L.

**Monoclonal FPIA/TDx Method**

The reagents for this monoclonal antibody–based FPIA assay were obtained from Abbott Laboratories. The cyclosporine assay was performed on the Abbott TDx system according to the manufacturer’s instructions. Before assay, 50 µL of solubilization reagent and 300 µL of precipitation reagent were added to 150 µL of whole blood sample, and this mixture was spun for at least 10 seconds. After centrifugation at 9,500g for 3 minutes, the supernatant was decanted and transferred immediately to a TDx analyzer for measurement of cyclosporine concentrations. The assay was calibrated by a nonlinear calibration curve composed of 6 calibrators and is designed to measure cyclosporine concentrations up to the level of the highest standard of 1,500 µg/L.

**Statistical Methods**

The unpaired t test was used for comparison of different result groups. They were calculated using PRISM from Graph Pad Inc (San Diego, CA).

**Results and Discussion**

**Comparison of Cyclosporine Concentrations Between Early and Late Posttransplantation Groups Measured by Monoclonal Immunoassays in Relation to HPLC**

To determine whether the elapsed time after transplantation has any effects on the measured cyclosporine concentrations by monoclonal immunoassays, we subdivided all patients into early and late posttransplantation groups. Mean cyclosporine concentrations were plotted as the percentage of HPLC results, and statistical differences between the early and late groups were calculated according to the method and organ. Results determined with 4 monoclonal immunoassays (CEDIA, EMIT, FPIA/AxSYM, and FPIA/TDx) for cyclosporine are summarized in [Figure 1](#).

With the FPIA/TDx in all patient groups except after liver transplantation, the late posttransplantation groups showed significantly higher cyclosporine levels than those in the early
groups. After liver transplantation, cyclosporine concentrations for the late group were significantly lower (mean ± SD HPLC, 180% ± 24% vs 161% ± 17%) than those for the early group when cyclosporine levels were determined with the FPIA/TDx method. With the CEDIA method, late kidney (119% ± 17% vs 109% ± 15%) and heart-lung groups (121% ± 10% vs 109% ± 14%) showed significantly lower cyclosporine concentrations than the corresponding early groups.

The EMIT showed a significant increase of the mean cyclosporine concentrations in late heart-lung (109% ± 14% vs 119% ± 10%) and liver (112% ± 14% vs 128% ± 17%) groups compared with the corresponding early groups. With the FPIA/AxSYM, cyclosporine whole blood concentrations in the late bone marrow group were significantly higher (123% ± 19% of HPLC) than those for the early group (114% ± 10%).

These differences between transplant recipients in the early and late groups could be due to different cyclosporine metabolite patterns over time after transplantation and the consequence of different cross-reactivity of cyclosporine metabolites with antibodies of the immunoassays. Hamwi et al. found that the FPIA/TDx and the FPIA/AxSYM assay had high cross-reactivity with the cyclosporine metabolites AM1 and AM9.
Cyclosporine Metabolite Patterns (Concentrations) in Early and Late Posttransplantation Recipients in 4 Groups

To explain the differences in the measured cyclosporine concentrations by the different immunoassays between the early and late groups, we studied the changes of the metabolite patterns over time after transplantation in 4 organ recipient groups (kidney, bone marrow, heart-lung, and liver). For this purpose, we measured the concentrations of the parent drug by 4 immunoassays and by HPLC and determined the AM1, AM9, and AM19 cyclosporine metabolite concentrations by HPLC. Statistical differences were calculated between the early and late groups. Mean ± SE concentrations of the parent drug and its metabolites of the groups are plotted in Figure 2.

After kidney transplantation, cyclosporine concentrations were significantly lower in the late than in the early group (mean ± SD: 48 ± 17 µg/L vs 79 ± 47 µg/L), whereas no statistically significant differences for metabolites were found between the early and late groups. As shown in Figure 1, these results did not correspond with concentrations measured by immunoassays.

After bone marrow transplantation, cyclosporine concentrations were also significantly lower in the late than in the early group (108 ± 57 µg/L vs 162 ± 47 µg/L). However, AM1 and AM19 concentrations were significantly higher in the late
than in the early groups (late, 408 ± 260 µg/L and 174 ± 82 µg/L vs early, 274 ± 101 µg/L and 105 ± 55 µg/L). Owing to these high metabolite concentrations and the cross-reactivity of the AxSYM and TDx assays, these assays measured higher cyclosporine concentrations in the late group.

After heart-lung transplantation, HPLC-cyclosporine parent drug and AM1 concentrations were significantly lower in the late than in the early group (late, 127 ± 48 µg/L and 191 ± 107 µg/L vs early, 165 ± 68 µg/L and 366 ± 257 µg/L). Nevertheless, parent drug concentrations, which were measured by the EMIT or TDx immunoassays, were indeed significantly higher parent drug concentrations in the late groups than in the early groups (Figure 1).

After liver transplantation, cyclosporine concentrations showed no significant differences between the late and early groups. However, AM19 concentrations were significantly lower in the late than in the early group (29 ± 19 µg/L vs 51 ± 36 µg/L). The parent cyclosporine concentrations, which were measured by the TDx immunoassay, were indeed significantly lower in the late group (Figure 1). These results do not correspond with concentrations determined by HPLC.

Cyclosporine Metabolite/Parent Drug Ratios in Early and Late Posttransplantation Recipients in 4 Groups

To eliminate the influence of the applied cyclosporine dose on the measured metabolite concentration, we calculated metabolite/parent cyclosporine ratios for AM1, AM9, and AM19. Results are plotted as mean ± SE metabolite/parent drug ratios in Figure 3.

After kidney transplantation, the ratios of all 3 measured metabolites (AM1, AM9, and AM19) were slightly but not significantly higher in the late compared with the early group (mean ± SD ratios: [early group] AM1, 3.04 ± 2.03; AM9, 0.60 ± 0.41; and AM19, 0.92 ± 0.63; [late group] 3.54 ± 1.58, 0.89 ± 0.62, and 1.17 ± 0.57, respectively).

In bone marrow recipients, the mean ± SD AM1/cyclosporine and AM19/cyclosporine ratios were significantly higher in the late group than in the early group: late group, 4.15 ± 1.61 and 2.06 ± 1.36; and early group, 1.78 ± 0.68 and 0.70 ± 0.38, respectively. The AM9/cyclosporine ratio also was higher in the late group than in the early group, 1.94 ± 0.85 vs 1.33 ± 0.48; however, this increase was not significant.

After heart-lung transplantation, the AM1/cyclosporine ratio was lower in the late group than in the early group (1.61 ± 0.74 vs 2.16 ± 0.97; not significant). During the same period, the ratios for AM9/cyclosporine and AM19/cyclosporine were slightly but not significantly higher in the late group than in the early group: AM9, 1.21 ± 0.54 vs 1.02 ± 0.60; AM19, 1.20 ± 0.91 vs 0.91 ± 0.43.

In liver recipients, all 3 ratios were lower in the late group than in the early group; however, only the AM19/cyclosporine ratio was significantly lower (0.39 ± 0.28 vs 0.80 ± 0.89).

In summary, in all patient groups except after liver transplantation, the parent drug concentration was significantly lower in the late than in the early group when determined by HPLC. After liver transplantation, the cyclosporine whole blood concentration was slightly but not significantly lower in the late group than in the early group. This is due to the decrease of the applied cyclosporine dose after the patient’s condition was clinically and pharmacologically stable.

However, immunoassays yielded partially significantly higher concentrations in the late group, which is due to the metabolic changes over time and the cross-reactivity of these assays. The concentrations of certain metabolites were higher in the late group than in the early group, although the dose and the concentration of the parent drug was lower. The most significant finding was in patients after bone marrow transplantation. The metabolite/parent drug ratio was significantly higher in the late group than in the early group for the metabolites AM1 and AM19, respectively. The cross-reactivity of both immunoassays, TDx and AxSYM, toward AM1 can explain the significantly higher cyclosporine levels measured by these assays in the late vs the early groups.

After kidney or heart-lung transplantation the AM1/cyclosporine and/or AM9/cyclosporine ratios were slightly but not significantly higher in the late vs the early groups. This increase and the cross-reactivity of the antibody might explain the significantly higher cyclosporine levels measured by the FPIA/TDx assay in the late posttransplantation period.

After liver transplantation, we observed a significantly lower AM19/cyclosporine ratio; however, the ratios for AM1 and AM9 did not change over time, and the parent cyclosporine concentration did not show a significant difference between the early and the late patient groups. Wang and coworkers observed an increase of the AM1/cyclosporine ratio in the late posttransplantation period after liver transplantation. High cyclosporine metabolite concentrations after liver transplantation also were described by Yatscoff and coworkers. It might be that our observation is due to the fact that liver transplantation is accompanied initially by poor oral cyclosporine absorption. As liver function improves, cyclosporine bioavailability increases. It is also important to note that the selection of liver transplant recipients was done irrespective of hepatic function, as even severe alterations of the hepatic function were reported not to be associated with changes of the cyclosporine metabolism.
Conclusion

Cyclosporine metabolites cross-react even with newly introduced monoclonal immunoassays, such as the FPIA/AxSYM, the modified EMIT, and the CEDIA.\(^8,9\) Hamwi et al found that immunoassays measured higher cyclosporine concentrations than HPLC.\(^9\) The measured concentrations increased over time and differed according to the type of transplantation.\(^9\)

In the present study, we proved by HPLC analysis of cyclosporine metabolites that changes in the cyclosporine metabolite pattern over time and the cross-reactivity of immunoassays are responsible for the overestimation of cyclosporine concentrations measured by immunoassays. In bone marrow, kidney, and heart-lung transplant recipients, the metabolite/parent drug ratio for AM1 and/or AM9 was higher in the late groups than in the early groups, which led to higher cyclosporine concentrations measured by immunoassays. Despite decreased cyclosporine doses, metabolites significantly accumulated in bone marrow transplant recipients over time after transplantation.

We conclude that the positive bias of cyclosporine measured by immunoassays from HPLC depends not only on the type of transplantation but also on the elapsed time after transplantation. For accurate interpretation of cyclosporine concentrations measured by immunoassays, the accumulation...
of metabolites should be considered. This is important to avoid overestimation of cyclosporine results and inadequate immunosuppression of the patient.

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