Improved Liquid-Chromatographic Determination of Cyclosporine and Its Metabolites in Blood

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We describe a liquid-chromatographic assay for cyclosporine (CyA) and four of its metabolites in blood. The method is sensitive and specific for this drug and its metabolites. The use of flow programming shortens total chromatographic run time to 30 min, resulting in acceptable separation of metabolites at a lower flow rate and yielding narrower peaks for CyA and the internal standard at the higher flow rate. CyA and metabolites Met 17 and Met 1 were measured in seven heart transplant patients who were undergoing chronic therapy with CyA in oral doses ranging from 3 to 13 mg/kg body wt per day. For the individual patients, the mean trough concentrations in blood of CyA, Met 17, and Met 1 ranged from 111 to 221, 159 to 334, and 36 to 90 μg/L, respectively. Met 8 and Met 21 were not observed in any of these patients' blood specimens.

Additional Keyphrases: immunosuppressants · chromatography, reversed-phase · flow programming · heart transplant

Cyclosporine (cyclosporin A, CyA) is a cyclic undecapeptide produced as a metabolite by a soil fungus, Tolypocladium inflatum Gams (1). It has been successfully used as an immunosuppressant in the field of organ transplantation (2). A major difficulty in the clinical use of CyA is its dose-dependent nephrotoxicity (3–5).

CyA is extensively biotransformed in humans and animals. Seventeen metabolites of CyA are known, 13 of which have been isolated and identified (6, 7). The biotransformation pathways are similar in mice, rats, rabbits, dogs, and humans (7, 8). All identified metabolites contain the intact cyclic oligopeptide structure of the parent drug. Structural modifications consist of mono- and dihydroxylation and N-demethylation at various sites on the CyA molecule (7–9).

In vitro immunosuppressive activity of four metabolites (Met 1, Met 8, Met 17, and Met 21) has been compared with that of CyA for inhibition of response of peripheral blood mononuclear cells, phytohemagglutinin, concanavalin A, pokeweed mitogen, and mixed leukocyte culture. Immunosuppressive activity has been shown for Met 17. Metabolites Met 1 and Met 21 are considerably less inhibitory than CyA or Met 17 (10, 11). The in vivo immunosuppressive activity and toxicity of these metabolites are yet to be established.

In general, CyA concentrations measured in blood by radioimmunoassay are higher than those determined by "high-performance" liquid chromatography (HPLC), because cross-reacting metabolites contribute to the value measured in the radioimmunoassay (12, 13). The metabolic activity of the liver may be changed in liver disease, recent liver transplantation, or the concomitant administration of other drugs. Thus, radioimmunoassay of CyA as a guide to therapy in these situations may be unreliable. The apparent immunosuppressive action of CyA metabolites seems to indicate the need for measuring these metabolites in blood of patients who are undergoing transplantation, particularly where quantitative alterations in CyA disposition are expected.

HPLC methods for assay of CyA and metabolites have been reported before (7, 10, 14, 15). These methods have the disadvantage of long chromatographic times. We have developed a rapid, sensitive, and specific HPLC method for CyA and four of its metabolites in blood.

Materials and Methods

Instrumentation

For liquid chromatography we used a "high-pressure" liquid chromatograph (Model 104B; Hewlett-Packard, Palo Alto, CA 94304) equipped with a variable-wavelength (190–
660 nm) detector and an automated sampling system. The separation was done on a 25 cm × 4.6 mm (i.d.) prepacked microparticulate (6-μm diameter) reversed-phase column (Zorbax CN; DuPont, Inc.; Wilmington, DE 19898) with the thermostated chromatograph oven maintained at 60 °C. The mobile phase was acetonitrile: methanol: water (26:14:60 by wt). Two flow rates of the mobile phase were used: 0.7 mL/min up to 20 min, and 1.4 mL/min from 21 to 28 min. At 29 min the flow rate was reduced to 0.7 mL/min. Total chromatographic run time was 30 min. The column effluent was monitored at 210 nm. Attenuation was 24, which is equal to 16 × 10^-4 absorbance units per cm, and the chart speed was 0.3 cm/min.

Reagents

Chemicals used were CyA; cyclosporin C (CyC); metabolites Met 1, Met 8, Met 17, and Met 21 (Sandoz Ltd., CH-4002, Basel, Switzerland); diethyl ether (American Burdick and Jackson, Muskegon, MI 49442); acetonitrile (UV-grade, glass-distilled; American Burdick and Jackson); n-hexane (HPLC grade; Fisher Scientific, Fair Lawn, NJ 07410); and methanol (glass-distilled; OmniSolv; EM Science, Cherry Hill, NJ 08034). Distilled water (Glenwood Inglewood, Minneapolis, MN 55405), methanol, and acetonitrile were filtered separately under reduced pressure through a prefiltor and a 0.4-μm (pore size) polycarbonate filter (both from Nucleapore Corp., Pleasanton, CA 94566) to remove any particulate matter. To prepare mobile phase, we used 235 g of acetonitrile, 127 g of methanol, and 539 g of distilled water. The premixed mobile phase was degassed on the liquid chromatograph under reduced pressure at 40 °C.

Standard Solutions

*Standard solution of CyA. CyA, 6.36 mg, was dissolved in methanol and the volume was made up to 100 mL. A working standard of 2.544 μg/mL was prepared by further dilution of this stock solution.

*Internal standard solution. CyC, 4.51 mg, was dissolved in methanol and the volume was made up to 100 mL. A working standard of 4.51 μg/mL was prepared by further dilution.  

*Standard solution of metabolites. Metabolites Met 1, Met 8, Met 17, and Met 21 were dissolved, as received, in 10 mL of methanol. The concentrations ranged from 25 to 1000 ng/mL. Exact concentrations of these metabolites were determined by assuming the same molar absorptivity as that of CyA. Peak areas, determined by integration for the metabolites, were compared with those for known amounts of CyA, to calibrate the metabolite standard solutions.

Procedures

*Sample preparation. CyA and metabolites were extracted from the blood samples by the method of Sawchuk and Cartier (16), with modifications as described below. Table 1 lists the concentration ranges of CyA and metabolites in the standard curves. One milliliter of drug-free human whole blood was added to the standard-curve tubes, and 1-mL samples of patients' blood were added to the appropriate sample tubes. Each pipette was rinsed with 1 mL of distilled water and the rinse was added to the corresponding standard or sample tube. From this point on, all tubes were treated identically. To the standards and blood samples 11 mL of diethyl ether was added with an automated pipette (Repipet; Lab Industries, Berkeley, CA 94710). The centrifuge tubes were stoppered with ground-glass stoppers, placed horizontally on a mechanical shaker (Eberbach Corp., Ann Arbor, MI 48106), and shaken at 180 cycles per minute for 15 min. All tubes were centrifuged at 750 × g for 5 min.

A 10-mL aliquot of the ether phase was transferred by pipette to a clean 35-mL centrifuge tube, evaporated (Evapomix), and 1.0 mL of HCl (25 mmol/L), 2.0 mL of methanol, and 7.0 mL of n-hexane were added. The standards and samples were shaken for 5 min and centrifuged for 5 min as before. The n-hexane layer was aspirated and discarded, and 7.0 mL of fresh n-hexane was added. The shaking, centrifuging, and aspirating of the hexane layer was repeated as described. Finally, 1.0 mL of a 25 mmol/L solution of NaOH and 7.0 mL of ether were added to the remaining aqueous phase. The samples were shaken for 10 min as before. The ether layer (6 mL) was transferred to a clean 35-mL centrifuge tube, evaporated (Evapomix), and reconstituted with 105 μL of mobile phase. The solutions were transferred into 0.2-mL microvials (cat. no. 3-3208; Supelco, Bellefonte, PA 16823-0048) for automated injection into the chromatograph.

*Chromatography. Approximately 90 μL of the samples reconstituted with the mobile phase was injected into the liquid chromatograph. Peak heights were measured electronically with an integrator (Model 3390A, Hewlett Packard) and the ratios of peak heights of CyA and metabolites to that of the internal standard, CyC, were calculated.

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**Table 1. Day-to-Day Variability In the Analytical Method for CyA, Met 17, and Met 1**

<table>
<thead>
<tr>
<th>CyA concn, ng/mL</th>
<th>Met 17 concn, ng/mL</th>
<th>Met 1 concn, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>63.6</td>
<td>25.0</td>
<td>24.0</td>
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<tr>
<td>127.2</td>
<td>50.0</td>
<td>48.0</td>
</tr>
<tr>
<td>178.1</td>
<td>100.0</td>
<td>120.0</td>
</tr>
<tr>
<td>254.4</td>
<td>250.0</td>
<td>240.0</td>
</tr>
<tr>
<td>381.6</td>
<td>500.0</td>
<td>480.0</td>
</tr>
<tr>
<td>508.8</td>
<td>750.0</td>
<td>3.20</td>
</tr>
<tr>
<td>636.0</td>
<td>1000.0</td>
<td>4.48 ± 0.03</td>
</tr>
</tbody>
</table>

*PHR = peak-height ratio, mean ± SD, n = 5. Values in parentheses are CV, %.

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**Calculations.** Concentrations of CyA and metabolites were determined from the regression equations relating peak-height ratios of the standards to their concentrations.

**Results**

Figure 1 shows representative chromatograms for CyA and its four metabolites. The metabolites were eluted at the slower flow rate, 0.7 mL/min. The change in flow rate to 1.4 mL/min after the elution of Met 21 resulted in elution of CyC and CyA within 30 min. In the absence of a change in flow, isocratic elution at the rate of 0.7 mL/min would extend the total elution time to 45 min. Thus, the change in flow rate from 0.7 to 1.4 mL/min, which occurs linearly between 20 and 21 min, shortens the total run time substantially and provides narrower peaks for CyC and CyA. Total chromatographic run times of 45 to 80 min for the analysis for CyA and metabolites have been reported by other investigators (8, 10, 14, 15). The use of CyC rather than CyD (14) also provides shorter chromatographic run times.

The limit of detection for CyA and the four metabolites was 5 to 15 ng/mL, based on the acceptance of a minimum signal-to-noise ratio of 2/1.

Relative recovery of the four metabolites was 0.9–1.3 times the recovery of CyA. This was calculated by assuming the equal molar absorptivities of the metabolites and CyA, as described earlier. Absolute recovery of CyA from the whole blood is 50% (16) with this extraction procedure.

No interfering peaks were detected in blood samples. Retention times for metabolites Met 8, Met 17, Met 21, CyC, and CyA were 10.7, 15.5, 16.6, 20.8, 23.4, and 27.8 min, respectively. The quantification limit for the metabolites and CyA was 25 and 50 ng/mL, respectively. Standard curves for the metabolites were linear over the concentration range of 25 to 1000 ng/mL. Standard curves for CyA were linear over the range of 50 to 3000 ng/mL. The precision of the analytical procedure was established by day-to-day reproducibility of standard curves for CyA and metabolites, as shown in Table 1. Standard curves for Met 8 and Met 21 were not prepared daily because these metabolites were not detected in the patients' blood samples. The retention times of CyA and metabolites were found to be extremely sensitive to the composition of mobile phase, and varied for different batches of mobile phase. To minimize this variability, we weighed each component of mobile phase. The average useful life-time of the CN column at 60 °C was 500 injections, after which the resolution of metabolites was poor, but CyA could still be quantified.

We have measured (Table 2) concentrations of CyA and metabolites in the blood of heart-transplant recipients undergoing chronic therapy with oral doses of CyA ranging from 3 to 13 mg/kg of body weight per day. These patients also received various other medications including prednisone, azathioprine, pipercillin, co-trimoxazole, trimethoprim, tobramycin, and clotrimazole. For the analysis to determine the concentrations of CyA and metabolites, the blood samples were drawn under steady-state conditions just before dosing.

Profiles of blood concentration vs time for CyA and two metabolites for two patients (patients 1 and 2 in Table 2) are shown in Figure 2. Met 17 and Met 1 were detected in blood samples, but Met 8 and Met 21 were not observed in any of the seven patients monitored. Met 17 was present in all samples; however, Met 1 was detected in all samples from only five of the seven patients. In the other two patients, Met 1 was detected only in a few samples. These two patients had liver dysfunction, and one of them also had renal failure. In patient 1, the concentrations of Met 17 in blood were higher than the CyA concentrations in most of the samples (Figure 2A); in patient 2 they were considerably higher than those of the parent drug (Figure 2B). Both were being treated chronically with oral CyA. That the latter patient had liver dysfunction was indicated by increased concentrations of bilirubin, alkaline phosphatase, aspartate aminotransferase, and lactate dehydrogenase in serum.

**Discussion**

CyA is monitored in transplant patients by using radioimmunoassay or HPLC because the drug's dose-related nephrotoxicity can be prevented by lowering the dose. Radioimmunoassay overestimates CyA by also quantifying the cross-reacting metabolites. In spite of its deficiencies, however, radioimmunoassay is commonly used to monitor concentrations of CyA in patients (17, 18).

CyA is slowly but extensively metabolized by the liver. Metabolism of CyA is catalyzed by the hepatic cytochrome P-450 monooxygenase system, resulting in hydroxylated and N-demethylated derivatives (7–9). Cytochrome P-450 inducers such as rifampicin, phenytoin, phenobarbital, and Aroclor 1254 accelerate biotransformation of CyA (19). On the other hand, the cytochrome P-450 inhibitor ketoconazole produces an increase in CyA concentrations, which may lead to nephrotoxicity (7, 20–22).

In animal studies, CyA administration has resulted in impaired hepatic drug metabolism (23–25). During the administration of CyA (25 mg/kg per day) for 49 days to Sprague–Dawley rats, the activity concentrations of cytochrome P-450 did not change, whereas aminopyrine N-demethylase and NADPH-cytochrome c reductase activity

<table>
<thead>
<tr>
<th>Table 2. Summary of Trough (Steady-State) Blood Concentrations (Mean ± SD) of CyA, Met 17, and Met 1 Observed in Seven Heart-Transplant Patients</th>
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<tbody>
<tr>
<td>CyA</td>
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<tr>
<td>ng/mL</td>
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<tr>
<td>183 ± 64</td>
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<td>151 ± 91</td>
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<td>188 ± 74</td>
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<td>121 ± 34</td>
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<td>169 ± 43</td>
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was decreased (23). This decrease in monoxygenase enzyme activity resulted in a decrease in hepatic metabolism and increased CyA concentrations, which in turn led to nephrotoxicity (23). Augustine and Zemaitis (25) reported impairment of cytochrome c reductase and, to a lesser extent, cytochrome P-450 during the 50 mg/kg per day administration of CyA to Wistar rats. As a result of the decrease in N-demethylation and aromatic hydroxylation drug-metabolizing reactions, these workers implied that CyA can impair its own metabolism, in which hydroxylated or N-demethylated derivatives, or both, are the primary metabolites. Such decreases in metabolic activity may lead to increased CyA/metabolite ratios, suggesting that measurement of metabolites may be useful clinically in this situation. Met 17 did not cause nephrotoxicity in spontaneously hypertensive rats (27), and indirect evidence also suggests that these metabolites do not cause nephrotoxicity (28). Fabre et al. (29) have demonstrated that CyA is rapidly transformed in rabbit hepatocytes to various monohydroxylated, dihydroxylated, and dihydroxylated N-demethylated metabolites. These metabolites, like CyA, have an affinity for intracellular binding components and are selectively retained for long periods inside the cells.

Metabolites Met 17 and Met 1 have been measured in the blood of renal-transplant (10) and heart-transplant patients (26). Concentrations of Met 17 were found to be as much as four- to fivefold those of the parent compound in some heart-transplant patients during chronic therapy with CyA (26). Further studies are needed to confirm in vivo immunosuppressive activity of these metabolites (10, 27). The rate of metabolism of CyA is of importance in determining the duration of action of the drug. Pharmacokinetic studies based on radioimmunoassay may be difficult to interpret (30) because of the nonspecificity of the methodology. Assay of metabolites of CyA in transplant patients may provide insight into the role of these transformation products in immunosuppressive therapy.

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References
Accumulation of Glycolic Acid and Glyoxylic Acid in Serum in Cases of Transient Hyperglycinemia after Transurethral Surgery

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Experimental data are presented here proving the accumulation of glycine in serum after transurethral prostatectomy and increased production of glycine metabolites: serine, alanine, glyoxylic acid, and glycolic acid. The presence of the metabolites glyoxylic acid and glycolic acid was demonstrated by gas-liquid chromatography and mass spectrometry. Glycine, glyoxylic acid, and glycolic acid possess neurological activity, so we examined the pathophysiology of the transurethral prostatectomy syndrome in view of the transient accumulation of these compounds in serum.

Additional Keyphrases: glycine and its metabolites · prostatectomy syndrome

The transurethral prostatectomy syndrome is characterized by the association of biological and neurological disorders. These disorders could be the consequence of the intravascular absorption of a large volume of the irrigating fluid, with a concomitant dilutional hyponatremia. During transurethral surgery, the isotonic glycine solution used as an irrigating solution may be absorbed through the venous sinuses and into the retroperitoneal or perivesical space. Thus, after the surgical procedure, hyponatremia and a variable increase in glycinemia could be observed. The glycine temporarily accumulated in serum in this way is metabolized, and there is an increase in alanine and serine.

In addition, direct metabolites of glycine such as glyoxylic acid and glycolic acid may be produced. Glycolic acid results from the enzymatic deamination of glycine and is reduced to glycolate by glyoxylate reductase (NADP+(EC 1.1.1.79)). Moreover, glycolate can be transformed to glyoxylate by (L)-2-hydroxyacid oxidase (EC 1.1.3.15) (I).

We set out to prove that these two metabolites accumulate in the blood, resulting in hyperglycinemia, after transurethral surgery. In addition, we discuss the relation between the production of these compounds and the transurethral prostatectomy syndrome, in view of their neurological activity.

Materials and Methods

Patients

In plasma from a group of 20 men (mean age 71, range ± 8 y) who underwent transurethral prostatectomy, we measured amino acids by gas-liquid chromatography, using a methodology previously described (2). This was done before surgery (T0), immediately after (T1), and 6 h (T2), 12 h (T3), 24 h (T4), and 48 h (T5) later.

Among these patients, one patient had visual disturbance for 30 min during the immediate postoperative time. We