Determination of Cyclosporine in Plasma: Specific Radioimmunoassay with a Monoclonal Antibody and Liquid Chromatography Compared

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A radioimmunoassay of cyclosporine (Sandimmune®) involving use of a mouse monoclonal antibody was tested to monitor specifically the parent drug in plasma. The cyclosporine concentrations obtained by RIA were compared with those obtained by the HPLC method. For the RIA method, the within- and between-assay CVs are <6%, the limit of detection is about 10 μg/L for a 50-μL sample of plasma. For the HPLC method, the within- and between-assay CVs are <20%, the limit of detection is about 15 μg/L for a 1-mL sample of plasma. The concentrations by RIA correlated well with those by HPLC in samples from patients receiving bone-marrow (n = 39), heart (n = 52), or liver (n = 51) transplants. In all indications, the ratio of values by RIA to those by HPLC for these samples remained stable and close to 1 during the drug-monitoring period, i.e., for up to 202 days. Therefore, the specific RIA can be used instead of HPLC to measure the parent drug in plasma.

Additional Keyphrases: chromatography, reversed-phase · organ transplants · monitoring therapy

Cyclosporine, the active ingredient of Sandimmune® (Sandoz Ltd., Basel, Switzerland), is a hydrophobic cyclic undecapeptide used clinically to prevent rejection of allografts (1–3) and more recently to treat autoimmune diseases (4–6). However, the drug has a narrow therapeutic range and displays large within- and between-patient variabilities in its pharmacokinetics. Therefore, it is necessary to monitor its concentrations in patients during treatment.

It is difficult to define a single therapeutic range for monitoring purposes, because just what cyclosporine concentrations are measured depends on the specificity of the analytical method used. Radioimmunoassay (RIA) with polyclonal antiserum measures both the parent drug and some of its metabolites (7–8), whereas "high-performance" liquid chromatography (HPLC) methods specifically detect the parent drug (9–12).

Because our current knowledge of this drug suggests that its immunosuppressive activity as well as its side effects are almost entirely attributable to the parent drug (13), it would be preferable to monitor patients by using a specific method. HPLC methods are tedious for routine use, and most medical centers prefer to monitor cyclosporine by RIA.

To monitor the unchanged cyclosporine, Sandoz Ltd. developed a RIA with a specific monoclonal antibody (4, 14), which was tested for measuring the parent drug in plasma samples from transplant patients. Here, we report the results obtained with this antibody and compare them with those measured by HPLC to assess the RIA’s specificity for the parent drug in plasma.

Materials and Methods

HPLC Reagents

The reagents used for HPLC were all "HPLC grade," and consisted of methanol, diethyl ether (Carlo Erba, Milan, Italy), acetonitrile (Rathburn, Walkerburn, U.K.), and n-hexane (Merck, Darmstadt, F.R.G.). De-ionized water was prepared with the "Milli Q" water-purification system (Millipore, Molsheim, France). Hydrochloric acid and sodium hydroxide were analytical grade. The human cyclosporine-free plasma used to prepare the standards and the drug-supplemented control samples was obtained from the French blood-transfusion service (Centre National de Transfusion Sanguine, Paris, France). Cyclosporine and cyclosporin D were supplied by Sandoz Ltd.

The stock solution of cyclosporin D, the internal standard, was prepared at a concentration of 2 mg/L in methanol. This solution was diluted in methanol/water (80/20 by vol) to give a working solution containing 500 μg/L. The solutions of cyclosporine and cyclosporin D in methanol or in methanol/water were stored at 4 °C.

RIA Reagents

The reagents used for RIA were contained in the Sandimmune kit (Sandoz Ltd., Basel, Switzerland). This consists of the Tris buffer concentrate solution (pH 7.5), the [3H]hydrocortisone tracer solution [1.8 μCi (66.6 kBq) in 1 mL of aqueous ethanol], and the lyophilized specific monoclonal antibody, prepared for use according to the manufacturer’s instructions. Methanol, the cyclosporine-free human plasma, and the cyclosporine standards were the same as the HPLC reagents.

HPLC Procedures

The HPLC method involved isocratic reversed-phase liquid chromatography followed by an ultraviolet detection, as follows. Mix 1 mL of each plasma sample (cyclosporine standards, supplemented control samples, clinical specimens) with 0.5 mL (0.25 μg) of the solution of internal standard, cyclosporin D, followed by 3 mL of a 100 mmol/L sodium hydroxide solution and 10 mL of diethyl ether. After shaking, centrifuge the samples and transfer an 8.5-mL aliquot of the diethyl ether layer to a glass tube containing 5 mL of the sodium hydroxide solution. Again shake each sample, centrifuge, and remove a 1.0-mL aliquot of the diethyl ether layer into a conical glass tube and evaporate it under nitrogen.

Dissolve each residue in 250 μL of hydrochloric acid (100 mmol/L)/methanol (20/80 by vol) and wash with 5 mL of n-hexane. After shaking, centrifuge each tube and discard the n-hexane layer. Inject 120 μL of the hydrochloric acid/
methanol layer onto a 4.6 × 75 mm Ultrasphere 3-μm particle ODS analytical column protected by a Beckman in-line filter with 2-μm frit (Beckman Instruments, Berkeley, CA). Maintain the analytical column and filter at 75 °C with a column heater block. To minimize damage to the column, gradually (over 60 min) bring it to this temperature from room temperature. Use isocratic elution with acetonitrile/water (72/28 by vol) at a flow rate of 1 mL/min to separate the cyclosporine and the cyclosporin D from other extracted plasma compounds. Monitor the absorbance at 214 nm of the column eluate with a fixed-wavelength detector. Under these conditions, the retention times for cyclosporine and cyclosporin D are 4.4 and 6.0 min, respectively.

To quantify the cyclosporine content of each unknown sample, we used the internal standard method based on the peak-area ratios. Each clinical specimen was analyzed once only. Three plasma control samples (with added cyclosporine concentrations of 30, 100, and 300 μg/L) were included in each analysis batch.

**RIA Procedure**

In the RIA method, the specific monoclonal antibody contained in the Sandimmune kit was used as previously described. All reagents were added to polystyrene assay tubes according to the pipetting scheme described in the leaflet accompanying the kit. In brief, the procedure is as follows. Mix 50 μL of each plasma sample (cyclosporine standards, drug-supplemented control samples, clinical specimens) with 950 μL of methanol and immediately cap all tubes. After shaking, centrifuge each tube and remove a 50-μL aliquot of the methanol supernate into a tube containing 500 μL of the diluted Tris buffer concentrate (50 mmol/L, pH 7.5) and 100 μL of [3H]dihydrocyclosporine tracer. To each tube, except the nonspecific binding tube, add 50 μL of the antibody solution. After shaking, incubate all tubes for 16 h at 4 °C, then for 15 min in an ice bath. Then add 500 μL of the stirred charcoal suspension to each tube except the total-radioactivity tube, keeping the samples in the ice bath. Shake the tubes and leave them in the ice bath for 15 min, then centrifuge and collect all the radioactive supernate from each tube, and quantitate the result by liquid scintillation counting.

We performed a logit-log linearization of calibration curves for quantifying the cyclosporine concentration of control samples and clinical specimens. Three plasma control samples (with added cyclosporine in final concentrations of 30, 100, and 400 μg/L) were included in each analysis batch. Each clinical specimen was analyzed in triplicate.

**Clinical Plasma Specimens**

Whole-blood samples were drawn, with EDTA as anticoagulant, from patients who were undergoing immunosuppressive therapy with cyclosporine after a bone-marrow allograft or a liver- or heart-transplant. Plasma was separated from erythrocytes at 22 °C. Each plasma sample was stored at −20 °C until analysis. These samples were collected at the time the concentration (Cmin) was smallest, after an oral administration of cyclosporine, from patients during the post-operative period at times ranging from one to 202 days.

**Regression Analysis**

For regression analysis of RIA and HPLC data we used a nonparametric linear regression, assuming that the variables were from arbitrary continuous distribution and that both variables were subject to error (16). With this procedure, the regression line parameters, slope, and intercept were estimated, together with their confidence interval at the 95% significance level.

**Results**

**HPLC Performance**

Under the chromatographic conditions, no interfering peak was detected at cyclosporine and cyclosporin D retention times in the plasma blank samples.

The relationship between the peak area ratio of cyclosporine to the internal standard (cyclosporin D) and the cyclosporine concentration was linear up to 500 μg/L in plasma, and the correlation coefficient was 0.9996.

Analytical recovery of cyclosporine from plasma was 99% over the range 15.6 to 500 μg/L.

The within-day variability of the method was evaluated by analyzing standard samples with cyclosporine concentrations of 31.3, 62.5, 250, and 500 μg/L, in sextuplicate. The coefficients of variation (CVs) were 18.5, 12.9, 5.4, and 3.4%, respectively.

The day-to-day variability of the method was evaluated from three control samples, with cyclosporine concentrations of 30, 100 and 300 μg/L, included in each HPLC analysis batch (n = 8). The CVs were 19.7, 11.6, and 8.5%, respectively.

The limit of detection (signals equal to three times the baseline noise) was about 15 μg/L.

**RIA Performance**

The between-assay variability (CV, %) of calibration curve variables (slope, intercept, percent of specific binding) was <7%, showing a good reproducibility of standard curves throughout the study, from April 1987 to June 1987 (n = 6).

The mean correlation was very good up to 500 μg/L (r = 0.9993), demonstrating the validity of the logit-log linearization used in the treatment of calibration curves.

We evaluated the within-day variability of the method by analyzing standard samples with cyclosporine concentrations of 31.3, 62.5, 250, and 500 μg/L, in octuplicate. The CVs were 5.7, 4.1, 3.6, and 2.7%, respectively.

The day-to-day variability of the method was evaluated with three control samples containing cyclosporine concentrations of 30, 100, and 400 μg/L included in each RIA analysis batch (n = 6). The CVs were 5.4, 5.0, and 2.0%, respectively.

The limit of detection, defined according to Rodbard's equation (17), was about 10 μg/L.

**Measurement of Cyclosporine in Clinical Plasma Specimens**

Cyclosporine concentrations in plasma, determined with use of the specific monoclonal antibody, were linearly correlated with those obtained by HPLC (P <0.001) in all the transplant recipients (Figure 1).

In the recipients of bone marrow, heart, and liver transplants the cyclosporine concentration measurements (RIA vs HPLC) clustered closely around the line of identity. Table 1 shows the correlation parameters.

The RIA/HPLC assay ratios were closely scattered around unity and showed no tendency to increase or decrease during the months after organ transplantation in the various transplant recipients (Figure 2). Table 2 gives the mean
TABLE 1. SLOPE, INTERCEPT, AND CORRELATION COEFFICIENT FOR VARIOUS TRANSPLANT RECIPIENTS

<table>
<thead>
<tr>
<th>Transplant</th>
<th>Bone Marrow</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>1.19</td>
<td>1.10</td>
<td>1.06</td>
</tr>
<tr>
<td>y-intercept</td>
<td>(1.00/1.52)</td>
<td>(1.00/1.20)</td>
<td>(1.00/1.15)</td>
</tr>
<tr>
<td>r</td>
<td>0.929</td>
<td>0.967</td>
<td>0.975</td>
</tr>
</tbody>
</table>

Table 2. RATIOs OF RESULTS BY RIA AND HPLC ASSAYS FOR VARIOUS TRANSPLANT RECIPIENTS

<table>
<thead>
<tr>
<th>Transplant</th>
<th>Bone Marrow</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean RIA/HPLC ratio</td>
<td>1.03</td>
<td>1.02</td>
<td>0.96</td>
</tr>
<tr>
<td>SD</td>
<td>(0.43/1.69)</td>
<td>(0.55/1.53)</td>
<td>(0.43/1.42)</td>
</tr>
<tr>
<td>Median</td>
<td>1.00</td>
<td>1.03</td>
<td>0.96</td>
</tr>
<tr>
<td>n</td>
<td>39</td>
<td>52</td>
<td>51</td>
</tr>
</tbody>
</table>

*Values in parentheses show the 95% confidence interval.

The line of unity is shown in each panel, for comparison

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Fig. 1. Comparison of cyclosporine concentrations determined by the specific monoclonal RIA and HPLC for plasma of patients receiving bone-marrow, heart, or liver transplant. The line of identity is shown in each panel, for comparison.

Table 1. Slope, Intercept, and Correlation Coefficient for Various Transplant Recipients

Table 2. Ratios of Results by RIA and HPLC Assays for Various Transplant Recipients

Values in parentheses show the range of the mean RIA/HPLC ratio.
(with the range) and the median of these RIA/HPLC assay ratios for the various transplant recipients.

Discussion

In all the transplant recipients tested, the cyclosporine concentrations in plasma as determined with use of the specific monoclonal antibody contained in the Sandimmune kit are similar to those obtained by the HPLC assay. The slight scattering of a few measurements is probably ascribable to methodological variations and to the imprecision of both methods for the low concentrations of cyclosporine, but this is not clinically significant. Thus, this antibody does not notably cross-react with the metabolites of cyclosporine. Furthermore, the stability of the ratio RIA/HPLC in all transplant indications up to 202 days of drug monitoring confirms the high specificity of the assay for the parent drug despite the possible changes in the amount of metabolites.

Similar conclusions have been previously reported for whole-blood samples from recipients of renal, heart, or liver transplants or bone-marrow graft (15), and from normal volunteers (16). Very similar results were reported for whole-blood and plasma samples from renal-transplant patients (19). In these comparative studies, the HPLC comparison methods differed from ours. Evidently, whatever HPLC method is used, the cyclosporine concentrations are equal to those obtained by the specific monoclonal antibody.

Hereafter, all monitoring centers will be able to measure the unchanged cyclosporine in plasma and in blood samples for patients undergoing bone-marrow, renal, heart, or liver transplantation.

Measurement of cyclosporine in plasma samples is difficult, because cyclosporine exhibits a temperature- and time-dependent binding to erythrocytes (20). The concentration of the parent drug is about four- to fivefold lower (21), and two- to threefold lower (12) when the plasma is separated from erythrocytes at 22 or 37 °C, respectively. The unchanged cyclosporine concentrations are then too low to allow accurate routine measurement of cyclosporine in plasma. Therefore, as has been concluded (22), whole blood seems to be the matrix of choice for cyclosporine monitoring.

To allow measurement of the relative amount of metabolites, the Sandimmune kit also contains a nonspecific monoclonal antibody, which can be used to measure both the unchanged cyclosporine and some of its metabolites. Thus, it can be used to monitor the variations in the patient's metabolism during the immediate post-transplantation period, especially in liver- and heart-transplant patients.

In conclusion, the specific monoclonal antibody contained in the Sandimmune kit provides an easy means of monitoring cyclosporine in patients receiving heart, renal, or liver transplant, or bone-marrow allograft. This specific RIA can be used instead of HPLC to measure the parent drug in plasma and in whole blood.

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References