Comparison of Cyclosporin G (Nva²-Cyclosporin) Concentrations Measured in Whole Blood by Monoclonal Fluorescence Polarization Immunoassay, Monoclonal Radioimmunoassay, and HPLC

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Two commercial monoclonal immunoassays for monitoring cyclosporin A were used to measure whole-blood concentrations of the immunosuppressant cyclosporin G (CsG) in renal transplant patients. We performed a three-way comparison of these two immunoassays and HPLC. Although the two immunoassays agreed favorably, both the monoclonal fluorescence polarization immunoassay and the monoclonal RIA yielded higher CsG results for patients' specimens than did the liquid-chromatographic assay. The experimental data indicate that the observed differences are most likely due to the cross-reactivity of CsG metabolites in the immunoassays.

Indexing Terms: intermethod comparison  · monitoring therapy  · immunosuppressant drugs  · variation, source of  · monoclonal antibodies

Cyclosporin A (CsA; cyclosporine; Sandimmune®) is currently the most widely used immunosuppressant drug for preventing rejection in organ transplant recipients. The narrow therapeutic range for CsA, along with the extensive intra- and interpatient variability in pharmacokinetics of the drug, have made it essential to monitor the blood concentrations of this drug in transplant recipients. Consensus reports from several groups or conferences (1–3) have recommended that whole blood be used as the specimen of choice and also that the analytical methods used to monitor CsA in blood be specific for the parent compound. The proposed reference method, which is used in many clinical laboratories for quantifying CsA, has been HPLC. Although HPLC is the most selective method and satisfies the consensus recommendations for therapeutic drug monitoring, the highly technical nature and potentially slow turnaround time eliminate HPLC as a usable analytical method in some institutions. The most widely used alternative methods have been commercially available RIA and fluorescence polarization immunoassay (FPIA) kits. Both of these immunoassays use monoclonal antibodies that are deemed to be highly selective for the parent compound CsA. The choice of a range of methods for monitoring CsA has prompted a number of investigations of the comparative performance of RIA, HPLC, and FPIA methods. Although much more selective for the parent compound than were early polyclonal immunoassays, the monoclonal specific RIA and FPIA demonstrate a positive bias in the quantitative determination of CsA in blood because they show cross-reactivities with metabolites of CsA. Regression data from several studies indicate good correlation between methods, but demonstrate some biases ranging from 1.1 to 1.6 in comparison with HPLC (4–8). Comparison of the two immunoassays has indicated a closer agreement of results (9–11), although for samples from liver transplant recipients there may be substantial disagreement between FPIA and RIA (12).

Despite the potent immunosuppressive properties of the drug, the clinical use of CsA is complicated by several negative side effects, primary among these being renal dysfunction. Over the last several years, other CsA analogs that may be useful as immunosuppressants have received attention. One of these candidates is the compound cyclosporin G (OG 37–325; CsG), a structural analog of CsA, in which norvaline is substituted for α-aminobutyric acid in position 2. Studies on urine samples show that CsG is metabolized via hydroxylation and demethylation, as is CsA (13). CsG is as potent as CsA in preventing allograft rejection in several animal models, but reportedly does not produce the reduced renal function observed with the use of CsA. Because CsG may also prove to be an effective immunosuppressant, with a wider therapeutic range than CsA, clinical studies are currently under way to investigate its efficacy in human transplant recipients.

In the current human trials evaluating the efficacy of CsG for immunosuppression in renal transplantation, the assay recommended as the standard technique for monitoring blood concentrations of CsG has been a commercial RIA of CsA that includes a monoclonal antibody with high specificity for the parent drug. This choice was based on the observation, both by the pharmaceutical manufacturer and in independent unpublished and published reports (14), that commercial RIAs designed for the analysis of CsA appear to have similar reactivity with CsG and that calibration curves for each of the two compounds are basically identical. However, we cannot assume that the same high specificity for CsG exists. The two parent drugs CsG and CsA may be detected equivalently in the assay, but the significance or contribution of CsG metabolites in patients' specimens has not been characterized. These metabolites may react to a greater or lesser degree with the monoclonal antibody used in the assay. Comparison of results

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³ Nonstandard abbreviations: CsA, cyclosporin A; CsG, cyclosporin G; and FPIA, fluorescence polarization immunoassay.

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obtained by immunoassay with those of a CsG-specific method such as HPLC would be of value.

Recently an HPLC method was reported (15) that was successfully used to monitor blood concentrations of CsG in ongoing human renal transplantation trials. Here we report our comparison of results for whole-blood CsG concentrations measured with the commercial RIA and with this published HPLC method. Additionally, because of the increasing use of the monoclonal-based specific FPIA method for CsA, we have also included this commercial assay in this comparative study of CsG results.

Materials and Methods

Patients' specimens. Specimens were obtained from renal transplant recipients who were enrolled in an institutional-approved clinical study involving the immunosuppressant CsG in combination with prednisone and azathioprine. Whole blood was collected into evacuated collection tubes containing EDTA as the anticoagulant. Specimens that were transported from one evaluation site to another were portioned into 1.5-mL polypropylene tubes, sealed with an O-ring fitted cap, and kept in short-term storage at −20 °C before transport by overnight delivery. To assure specimen integrity during storage, we reanalyzed by HPLC multiple specimens that had been stored frozen for several months and found no change in concentration of CsG.

Immunoassays. The two immunoassays evaluated in the present study were the CYCLO-Trac SP whole-blood monoclonal-based RIA kit (INCSStar Corp., Stillwater, MN) and the cyclosporine monoclonal whole-blood FPIA (Abbott Laboratories, North Chicago, IL). The two immunoassays were performed according to manufacturers' protocols, with use of the calibrators supplied with each kit. For experiments evaluating the cross-reactivity of CsG in the FPIA, we used CsG obtained from Sandoz Pharmaceutical Corp. (East Hanover, NJ).

HPLC for CsG. Analysis of whole-blood specimens by HPLC was performed according to the method of Annesley et al. (15). Briefly, extracts of whole blood were prepared by liquid–liquid extraction with methyl-t-butyl ether. Chromatography of the extracts was performed with a Beckman Ultrasphere octyl (C8) microbore column (Beckman Instruments, San Ramon, CA) maintained at 75 °C. Acetonitrile/methanol/water was the mobile phase, and CsA was the internal standard.

Recovery studies. Gravimetrically prepared stock solutions of CsG or CsA in methanol were used to create whole-blood specimens with target drug concentrations of 200, 400, and 600 μg/L. These were analyzed by FPIA by using a calibration curve established from the CsA whole-blood standards supplied by the manufacturer. Analysis was also performed by HPLC with CsG or CsA whole-blood calibrators.

Statistical analysis. The results obtained from the comparison of HPLC, RIA, and FPIA were evaluated by both Deming regression analysis and simple unweighted regression analysis.

Results and Discussion

In this study 106 specimens from 14 renal transplant patients being treated with CsG were analyzed for CsG by the three methods. The results of these comparisons are shown in Table 1 and Figure 1. The regression data indicate that all of the methods correlate well, but that biases exist between the two immunoassays and HPLC. The slope and intercept of the best-fit regression lines comparing RIA and HPLC showed that the RIA results were 37% or 49% greater than those obtained by HPLC, depending on which regression formula was used. Similarly, the FPIA results averaged 38% or 51% greater than those by HPLC. The two immunoassays, on the other hand, showed excellent agreement of values for CsG concentrations in whole blood (Table 1). Previous published studies comparing immunoassays and HPLC for monitoring CsA have not always specified the types of regression analyses used. For this reason we used two regression formulas, with the goal of allowing appropriate comparisons with future studies involving the measurement of CsG in blood.

The accepted equal immunoreactivity of CsG and CsA in the INCStar RIA has allowed the use of this assay in clinical trials involving therapy with CsG. Consequently, we have not included calibrator cross-over studies for HPLC and RIA in the data presented here. However, for the FPIA, we performed recovery studies for CsG and CsA to address two specific concerns: whether CsG immunoreactivity was essentially equivalent to that of CsA in the commercial FPIA kit (if the immunoreactivities were similar, then the supplied CsA calibrators could be acceptable as comparable substitutes for prepared CsG standards), and whether any differences in values obtained between the immunoassay and HPLC could be attributed to differences in

| Table 1. Comparison of CsG Results for Whole-Blood Specimens |
|---------------|---------------|---------------|------------|
|      | Regression line | x          | y          | r         | s_yp |
| Simple unweighted regression |
| FPIA  | HPLC            | y = 1.38x + 8.4 | 193.5       | 276.3     | 0.939 | 59.9 |
| RIA   | HPLC            | y = 1.37x + 3.5 | 193.5       | 269.0     | 0.939 | 59.1 |
| FPIA  | RIA             | y = 1.00x + 6.9 | 269.0       | 276.3     | 0.992 | 22.3 |
| Deming regression |
| FPIA  | HPLC            | y = 1.51x – 15.9 | 193.5       | 276.3     | 0.939 | 34.1 |
| RIA   | HPLC            | y = 1.49x – 20.2 | 193.5       | 269.0     | 0.939 | 33.8 |
| FPIA  | RIA             | y = 1.01x + 4.7 | 269.0       | 276.3     | 0.992 | 15.7 |

CLINICAL CHEMISTRY, Vol. 39, No. 6, 1993 1051
calibration assignments. As shown in Table 2, the concentrations assigned to these whole-blood samples agreed well with the assayed values by FPIA for samples supplemented with CsA and those supplemented with CsG. The recovery of CsA by FPIA was 104%, which is comparable with the recovery obtained by FPIA for prepared whole-blood CsA calibrators in other studies (4, 16). The observed recovery of CsG in the FPIA was 110%. Comparing the recovery of CsG by FPIA with that of CsA by the same method indicates that CsG may have a slightly greater immunoreactivity (~106%) than that exhibited by CsA. More significant is the fact that the recoveries of CsG and CsA could not account for the large difference observed between the FPIA and HPLC. The similar recovery results for CsA in HPLC and FPIA are a simple cross-validation of the calibrators used in the FPIA kit. The similar recovery data for CsG and CsA in the FPIA supports the premise that the immunoreactivity of CsG and CsA in the FPIA method are very similar.

From these data we conclude that the differences observed between the two immunoassays and HPLC most probably result from cross-reactivity of the antibodies with CsG metabolites. This belief, as well as the relative degree of bias observed with the two immunoassays for monitoring CsG, is supported by several independent observations: First, the average bias in CsG concentrations observed with the RIA or FPIA in our study is consistent with the magnitude of differences reported for CsA quantification in several studies (6, 8, 17). The closer agreement of results between RIA and FPIA for CsG quantification in our study is also similar to the reported correlation of these two assays for monitoring CsA in blood (9, 17). Second, cross-reactivity of CsA metabolites with monoclonal-based immunoassays has been reported, and the relative cross-reactivity of major CsA metabolites have been evaluated (11). Third, in a recent study, Copeland and Yatscoff (13) showed that CsG is metabolized primarily through the same hydroxylation and demethylation reactions as occurs physiologically for CsA. Given the strong similarity in structure of the two immunosuppressants, the similar cross-reactivity of the parent drugs CsG and CsA with immunoassays, and their similar fates of metabolism and excretion, we believe that our results support the hypothesis that CsG metabolites exhibit a similar pattern of cross-reactivity in immunoassays.

Previous consensus guidelines have recommended that certain criteria be used for therapeutic monitoring of CsA. These include the collection of trough samples for measuring drug concentrations, the use of whole blood as the specimen of choice, and the use of an analytical method that selectively monitors the parent drug. One can logically ask whether the properties of CsG and its metabolites would encourage the use of the same guidelines. Early studies provide evidence of a differential distribution of CsG in blood (18, 19) that is temperature-dependent (19). More recent results (13) suggest that the metabolites of CsG have little immunosuppressive activity relative to the parent drug. Therefore, the present information indicates that, like CsA, monitoring of the parent drug CsG in whole blood will be recommended. The method recognized as the most selective for CsG would be HPLC. However, the technical nature of HPLC, plus the lower throughput, has led to the investigation of potential alternatives such as RIA and FPIA assays. If these immunoassays are to be applied properly to the measurement of CsG for therapeutic drug monitoring, users will need to understand that cross-reactivity with metabolites of CsG does occur and that CsG concentrations in blood will be overestimated. The potential difference, although significant from a technical viewpoint, should not affect the

### Table 2. Recovery/Immunoreactivity of CsG and CsA by FPIA

<table>
<thead>
<tr>
<th>Drug</th>
<th>HPLC conc, µg/L</th>
<th>% recovery</th>
<th>FPIA conc, µg/L</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsG</td>
<td>201</td>
<td>100</td>
<td>220</td>
<td>109</td>
</tr>
<tr>
<td>CsG</td>
<td>405</td>
<td>101</td>
<td>445</td>
<td>111</td>
</tr>
<tr>
<td>CsG</td>
<td>613</td>
<td>102</td>
<td>658</td>
<td>109</td>
</tr>
<tr>
<td>CsA</td>
<td>204</td>
<td>102</td>
<td>206</td>
<td>104</td>
</tr>
<tr>
<td>CsA</td>
<td>402</td>
<td>101</td>
<td>408</td>
<td>102</td>
</tr>
<tr>
<td>CsA</td>
<td>612</td>
<td>102</td>
<td>628</td>
<td>105</td>
</tr>
</tbody>
</table>

*Average value from minimum of three analyses performed on separate days.
use of these assays for therapeutic decisions during CsG therapy; however, adjustments in the targeted therapeutic range will be required.

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References