measurement of enzyme activity showed a high correlation to the enzyme protein measurement method, the numerical values for the two methods differed. This is probably explained by a difference in standard and sample (12; and Isaksson and Hultberg, manuscript in preparation): the standard contained placental Hex A or Hex B.

In conclusion, the present investigation describes a simple procedure for carrier detection of GM₂-gangliosidosis and presents two principally different enzyme immunoassay methods for determination of Hex A and Hex B. These methods have been tested on sera from normal subjects, sera with increased total Hex activity, and sera from carriers of TSD and SHD and seem to give the same information. The methods based on enzyme activity are, however, preferred because of their simple performance. Furthermore, the results obtained from the two methods did not indicate the presence of antigen-reactive enzyme protein lacking enzyme activity in any serum analyzed.

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Whole-Blood Cyclosporin G in Renal Transplant Recipients Determined by Two Immunoassays and Liquid Chromatography

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Cyclosporin G (CsG) is less nephrotoxic than cyclosporin A (CsA) and is undergoing clinical trials for use as an immunosuppressive agent after renal transplantation. Three assays for whole-blood CsA—HPLC, RIA (INCSTAR, Cyclo-Trac SP), and FPIA (Abbott TDx)—were adapted for use with CsG and were assessed for analytical suitability and to determine which assay was capable of deriving CsG values rapidly after transplantation. The assays were acceptable in terms of sensitivity, linearity, analytical recovery, and precision. When considering blood samples (n = 107) from renal transplant recipients receiving a low dose of CsG (5 mg/kg per day) and a high dose (10 mg/kg per day), we obtained the following correlation data: RIA = 0.974HPLC + 27.89 (r = 0.9798, S_p = 39.24); FPIA = 0.964HPLC + 33.59 (r = 0.9819, S_p = 36.63); and FPIA = 0.977RIA + 9.50 (r = 0.9894, S_p = 28.12). The FPIA of CsG is recommended as the most rapid method, although it is the most expensive. HPLC, RIA, and FPIA were capable of accurately deriving projected CsG concentrations at various stages of the clinical trial when the low- and high-dose regimes were tapered over a period of 16 weeks.

Indexing Terms: immunosuppressive drugs • monitoring therapy • cyclosporine • clinical trials • radioimmunoassay • fluorescence polarization immunoassay • intermethod comparison

To date, >700 natural, synthetic, and semisynthetic analogs of cyclosporin A (CsA; cyclosporine) have been produced and tested in the search for more selective, safer, and more effective derivatives for use as immunosuppressive agents after organ transplantation (1). A

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2 Nonstandard abbreviations: CsA, cyclosporin A; CsC, cyclosporin C; CsG, cyclosporin G; and FPIA, fluorescence polarization immunoassay.
few cyclosporine analogs have been shown in screening processes to have significant immunosuppressive properties; in general, however, few have been more potent than CsA (1). The analogs considered to have strong immunological activity include CsC, D, G, M, val^2- dihydrocyclosporine D, and dihydrocyclosporine C (2).

Cyclosporin G (OG37 – 325; norvaline^2-cyclosporine; CsG) is a naturally occurring analog of CsA in which norvaline has replaced a-aminobutyric acid at the 2 position. The immunosuppressive properties of CsG have been studied in several in vitro and in vivo models. In vitro experiments with CsG have demonstrated that it is as potent as CsA in the suppression of mixed lymphocyte cultures, cell-mediated lympholysis, and sheep erythrocyte antibody responses (3) and of mitogen-induced cell proliferation (4). In vivo immunological studies have shown that CsG is as effective as CsA in preventing a primary humoral immune response to sheep erythrocytes, delayed-type hypersensitivity reactions to oxazolone, and localized graft vs host responses (3).

In transplantation experiments, CsG was as effective as CsA in prolonging skin allograft survival in rats and mice (3), rat renal allografts (3) and canine liver transplants (5). A dose dependency may exist, CsA and CsG being equally effective at prolonging rat heart and kidney allograft survival at doses of 10 mg/kg per day, but less effective at lower doses of 5 mg/kg per day (6, 7). Thus CsG may emerge as an important immunosuppressive agent for use after organ transplantation.

Clinical trials are now underway at various centers using CsG as an immunosuppressive agent after kidney transplantation. In this study, three treatment groups were studied: CsG (5 mg/kg per day), CsG (10 mg/kg per day), and CsA (10 mg/kg per day). Whole-blood samples were analyzed for CsG by HPLC, RIA, and fluorescence polarization immunooassay (FPIA). We studied the three methods for the analysis of CsG for their analytical suitability and to establish which assay was capable of rapidly deriving CsG data to ensure proper clinical management of kidney transplant recipients receiving CsG.

Materials and Methods
Clinical Specimens

Blood samples were collected into tubes containing EDTA as anticoagulant. In the clinical trial, six patients received a low oral dose of CsG (5 mg/kg per day), which was tapered to 4 mg/kg per day beginning at week 4 after transplantation and further reduced to 2.5 mg/kg per day by week 8. This protocol called for achieving target whole-blood CsG trough values of 150 μg/L (range 100–200 μg/L), 115 μg/L (range 75–150 μg/L), and 75 μg/L (range 50–100 μg/L), initially, and at weeks 4 to 8 and 8 to 16, respectively. These target values for low- and high-dose regimens of CsG measured by RIA were recommended by Sandoz Pharmaceuticals (East Hanover, NJ) to be used in a 4-month, multicenter, randomized dose-ranging study in patients receiving a renal transplant.

A group of six patients also received CsA (10 mg/kg per day), which was tapered to 8 mg/kg per day at the beginning of week 4 after transplantation and then to 5 mg/kg per day starting at week 8. CsA was measured in these patients’ samples by HPLC.

All transplant patients were also receiving azathioprine and prednisone, and any patients receiving drugs thought to interfere with measurements of CsG blood concentrations were excluded from the study. These drugs included nephrotoxic drugs such as ketoconazole, amphotericin B, aminoglycosides, melphalan, erythromycin, trimethoprim, or sulfamethoxazole (oral). Also excluded were patients receiving within 4 weeks before transplantation, drugs such as barbiturates, phenytoin, phenylbutazone, rifampin, isoniazid, and sulfamethoxazole plus trimethoprim (intravenous), which are known to alter CsA blood concentrations.

Trough values of CsG in whole blood were drawn at the following intervals: (a) three times per week after the first dose of study medication for the duration of hospitalization, (b) twice weekly for the first 2 weeks after discharge from the hospital, and (c) once a week for 4 weeks and then every other week until the end of the clinical study at 16 weeks.

CsG Calibrators

CsG calibrators—25, 50, 100, 300, 500, 750, 1200, and 1500 μg/L—were prepared by adding 25 mg of CsG to ethanol (950 mL/L) to give a 250 mg/L stock solution. The stock solution was further diluted in ethanol to give a concentration of 2.5 mg/L and aliquots of this solution were added to whole blood obtained from blood donors to give the necessary concentrations of CsG. The prepared calibrators also included Tween 20 surfactant (0.5 mL/L) as a stabilizing agent. The concentration of each calibrator was verified by HPLC, RIA, and FPIA.

Assays

CsG by HPLC. The HPLC procedure was a modification of a method previously described (8). The modified procedure was as follows: we vortex-mixed for 30 s a 1 mL of patient’s sample or control with 3 mL of an internal standard reagent containing CsC (100 μg/L) in methanol, acetonitrile, and water (50/30/20, by vol) and zinc sulfate (50 g/L). The samples were then centrifuged at 4000 x g for 5 min and the supernates were transferred to Bond Elut C18 bonded-phase extraction columns (Analytichem International, Harbor City, CA). The extraction columns were primed with 950 mL/L ethanol before use. Contaminants were removed by eluting the columns with 5 mL of a solution containing acetonitrile and water (50/50, by vol), and the cyclosporins were eluted

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with 0.4 mL of 950 mL/L ethanol. Upon addition of 0.2 mL of deionized water to each eluate, we mixed the samples with 0.7 mL of hexane to remove lipids. The hexane layer was removed and 30 μL of each sample was assayed by isocratic chromatography at 70 °C with a flow rate of 0.8 mL/min on a reversed-phase C18 column (Waters, Inc., Milford, MA). The mobile phase was acetonitrile/water (70/30, by vol) and the absorbance of the eluate was monitored at 210 nm. The CsG concentration of each sample or control was obtained by calculating the ratio of the peak heights for CsG and CsC and interpolating from a set of standards analyzed each day in the assay.

In practice, values between 10 and 25 μg/L were reported as <25 μg/L, and values <10 μg/L were reported as “none detected.” With this method and frequent change of the guard column, all the samples in this study were processed on the same analytical column. Further, between daily assays, the column was maintained at 70 °C, with mobile phase being passed through it at a flow rate of 0.2 mL/min.

CsG by RIA. The CYCLO-Trac SP kit for CsA was obtained from INCSTAR Corp. (Stillwater, MN). The RIA contains a primary monoclonal antibody against CsA obtained from Sandoz Corp. In-house studies demonstrated that the antibody cross-reacted 100% with CsG over the assay range 0–1500 μg/L; consequently, we used the kit as such to assay CsG in whole-blood samples.

CsG by FPIA. The FPIA monoclonal kit for CsA was obtained from Abbott Laboratories (N. Chicago, IL). Whole-blood CsG calibrators ranging from 0 to 1500 μg/L, prepared as for the RIA, were substituted for CsA calibrators. As with the RIA, in-house studies showed that the antibody cross-reacted 100% with CsG over the range 0–1500 μg/L. The assay was carried out by using the Abbott TDx analyzer similarly to the whole-blood CsA polyclonal assay previously described (9).

Assay Evaluation

Detection limit and linearity. For HPLC, RIA, and FPIA, assay sensitivity (detection limit) was assessed by assaying 20 blood-donor whole-blood samples with zero concentrations of CsG and calculating the mean ±2 SD for the samples. By interpolation from each calibration curve we determined the detection limits for HPLC, RIA, and FPIA. For the immunoassays, linearity was established by using a pool of patients’ whole-blood samples containing a high concentration of CsG as verified by HPLC. Dilutions of samples were performed with whole blood from normal healthy donors.

Precision studies. For both intra- and interassay precision studies with HPLC, RIA, and FPIA we used a low and high concentration of CsG in whole blood supplied by Sandoz Corp.

Statistical analysis. Unweighted regression analysis was used to compare results by HPLC with those by RIA and FPIA. Initially, we used 107 patients’ results in the comparison and then subdivided the results in accordance with CsG dose. For the latter comparison, we considered 47 patients’ samples in the low-dose regime (5 mg/kg per day) and 60 samples in the high-dose protocol (10 mg/kg per day).

Results

Analytical Performance

Calibration of CsG assays. We prepared in-house CsG calibrators in a matrix of whole blood from blood donors and analyzed them initially by HPLC. The calibrators were then substituted into the RIA and FPIA kits for CsA supplied by each vendor. For HPLC the mean recovery was 102%, whereas RIA and FPIA gave mean recoveries of 97% and 107%, respectively.

Sensitivity and linearity. The sensitivity (detection limit) of the HPLC method was 10 μg/L and the calibration curve was linear over the range 20–1500 μg/L. RIA was sensitive to 10 μg/L and linear from 15 to 1200 μg/L, whereas the FPIA was sensitive to 15 μg/L and linear from 15 to 1200 μg/L. The analysis of variance of the calculated concentrations corrected by the dilution factor demonstrated that the various dilution curves for each of the CsG methods were linearly related over the whole range between initial concentration and the highest diluted sample (r = 0.99 in all cases).

Precision. Precision studies were conducted at two concentrations of CsG for each method (Table 1). HPLC gave higher interassay CV values than either RIA or FPIA.

Comparison of methods. Table 2 summarizes the comparison of CsG concentrations determined by all three methods in whole blood from 107 samples obtained after renal transplantation. We also subdivided the data in accordance with low- and high-dose CsG, considering 47 and 60 samples for each dose, respectively. For samples in the low-dose category, HPLC gave a CsG range of 33–258 μg/L (x̄ = 162 μg/L), RIA 42–342 μg/L (x̄ = 179 μg/L), and FPIA 24–353 μg/L (x̄ = 179 μg/L). For the high-dose samples, HPLC gave a CsG range of 66–1261 μg/L (x̄ = 355 μg/L), RIA 84–1199 μg/L (x̄ = 359 μg/L), and FPIA 93–1136 μg/L (x̄ = 365 μg/L).

<p>| Table 1. Intra- and Interassay Precision of CsG Measurements in Whole Blood by HPLC, RIA, and FPIA |
|--------------------------------|-------|-------|-------|</p>
<table>
<thead>
<tr>
<th>CsG conc, μg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td>Intraassay (n = 10 each)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>147</td>
<td>5.6</td>
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</tr>
<tr>
<td></td>
<td>453</td>
<td>35.2</td>
<td>7.7</td>
</tr>
<tr>
<td>RIA</td>
<td>120</td>
<td>4.7</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>406</td>
<td>15.9</td>
<td>3.9</td>
</tr>
<tr>
<td>FPIA</td>
<td>148</td>
<td>4.2</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>510</td>
<td>15.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Interassay (n = 20 each)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>150</td>
<td>10.7</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>42.1</td>
<td>8.6</td>
</tr>
<tr>
<td>RIA</td>
<td>120</td>
<td>6.0</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>620</td>
<td>43.5</td>
<td>7.0</td>
</tr>
<tr>
<td>FPIA</td>
<td>150</td>
<td>6.7</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>516</td>
<td>19.5</td>
<td>3.8</td>
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</table>

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Clinical Results

A typical CsG profile (as analyzed by HPLC, RIA, and FPIA) for a renal transplant recipient receiving low-dose CsG is given in Figure 1a. For the clinical trial it was anticipated that whole-blood trough concentration should be ~150 µg/L (range 100–200 µg/L) at week 4 after transplantation. This was confirmed (Figure 1a) by all three methods, which all gave comparable results. At weeks 4 to 8 posttransplantation (when the drug dose was reduced to 4 mg/kg per day), it was anticipated that CsG concentrations should be ~115 µg/L (range 75–150 µg/L). This too was achieved, and again the three methods gave clinically acceptable results (Figure 1a). By weeks 8 to 16, the CsG dose was reduced to 2.5 mg/kg per day and the whole-blood concentrations should have been 75 µg/L (range 50–100 µg/L); however, the three assays gave results ranging from 105 to 125 µg/L, slightly higher than expected.

Figure 1b presents a typical profile for a renal transplant recipient receiving a high dose of CsG (10 mg/kg per day) who was tapered to a dose of 8 mg/kg per day beginning 4 weeks posttransplantation and to 5 mg/kg per day starting at 8 weeks after surgery. By week 4 in the protocol, this patient should have had a value of 225 µg/L CsG (range 150–300 µg/L); however, this was not quite achieved; values obtained by the three methods were ~380 µg/L. By weeks 4 to 8 after surgery, the target value was 150 µg/L (range 100–200 µg/L); in this study, the mean value was 100 µg/L for the three methods at the eighth week. The anticipated concentration of CsG by weeks 8 to 16 should have been 140 µg/L (range 75–250 µg/L). At week 16, a CsG range of 150 to 200 µg/L was obtained for the three analytical methods (Figure 1b).

Discussion

CsG is a member of a class of compounds produced by the fungus Tolypocladium inflatum GAMS that have marked immunologic activity. CsA, the most extensively studied compound of the group, has been very successfully applied in clinical use as an immunosuppressant in organ transplantation. The major problem with the clinical use of CsA is its propensity to produce nephrotoxicity in patients at a wide range of doses. CsG is very similar in structure to CsA and has been reported to lack nephrotoxicity in a rat model (3). Further, CsG has been shown to be as potent as CsA in preventing rejection of transplanted livers in a canine model (5).

In this study, CsG was administered at two oral doses and HPLC, RIA, and FPIA were assessed for their capability to provide accurate and precise determinations of CsG after surgery. We also wanted to know whether any of the methods would be useful for rapid pharmacokinetic studies in patients receiving a bolus of CsG after renal transplantation.

The methods considered were analytically acceptable for the measurement of CsG in whole blood. The HPLC assay was suitable sensitive, accurate, and precise. Furthermore, a laboratory performing CsA analysis by HPLC could also substitute CsC as an internal standard and with the same mobile phase and analytical column codeetermine CsG concentrations for clinical use. Thus the turnaround time for CsG assays would be of the same order of magnitude as for CsA parent drug determinations. In this study, CsG usually had a retention

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**Table 2. Linear-Regression Analysis for the Comparison of Three CsG Assays**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Regression Equation</th>
<th>r</th>
<th>s_\text{xy}</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>0.974 HPLC + 27.89</td>
<td>0.9798</td>
<td>39.24</td>
</tr>
<tr>
<td>RIA</td>
<td>0.964 HPLC + 33.59</td>
<td>0.9819</td>
<td>36.66</td>
</tr>
<tr>
<td>FPIA</td>
<td>0.977 RIA + 9.50</td>
<td>0.9894</td>
<td>28.12</td>
</tr>
<tr>
<td>Low-dose samples, 5 mg/kg per day (n = 47)</td>
<td>RIA</td>
<td>1.064 HPLC + 7.05</td>
<td>0.9607</td>
</tr>
<tr>
<td>FPIA</td>
<td>1.101 HPLC + 0.79</td>
<td>0.9889</td>
<td>25.29</td>
</tr>
<tr>
<td>High-dose samples, 10 mg/kg per day (n = 60)</td>
<td>RIA</td>
<td>0.945 HPLC + 42.42</td>
<td>0.9782</td>
</tr>
<tr>
<td>FPIA</td>
<td>0.919 HPLC + 56.67</td>
<td>0.9821</td>
<td>39.65</td>
</tr>
</tbody>
</table>

---

Fig. 1. CsG profiles after renal transplantation for (a) a patient receiving a low dose (5 mg/kg per day) of drug (which was tapered to 4 and 2.5 mg/kg per day) and (b) a patient receiving a high dose (10 mg/kg per day) of drug (which was tapered to 8 and 5 mg/kg per day)
time of 8.0 min, whereas the internal standard (CsC) eluted at 5.0 min and CsA at 6.0 min.

CsG may also be measured by RIA and FPIA with the use of available reagents for assay of CsA, due to the cross-reactivity of the monoclonal antibodies with CsG. In this report both CsA and CsG gave concentration-response curves that were superimposable. However, in these immunoassays, we prepared CsG calibrators in-house for routine use, using donor whole blood. Considering that standardization problems with CsA determinations have arisen in the past, we recommend that laboratories undertaking such CsG studies prepare in-house calibrators (10, 11).

In this study, reasonable correlation was obtained when comparing RIA and FPIA with HPLC for 107 patients' samples. FPIA and RIA both gave superior correlation data in terms of regression-line slope, intercept, and standard error of the estimate, possibly because both techniques incorporate the same monoclonal antibody developed against CsA. When both low- and high-dose CsG groups were compared, findings were not significantly different from when combined data from the two groups were analyzed.

These results are interesting, given our recent studies comparing HPLC, RIA, and FPIA for the measurement of CsA in whole blood (11). In that report we obtained the following data for adult renal transplant recipients (n = 50) receiving CsA: RIA = 1.396 HPLC − 10.76 (r = 0.985, Ŝxy = 36.04) and FPIA = 1.307 HPLC + 2.06 (r = 0.989, Ŝxy = 30.82). For pediatric renal transplant recipients (n = 50) receiving CsA, we obtained RIA = 1.396 HPLC − 32.14 (r = 0.980, Ŝxy = 33.30) and FPIA = 1.314 HPLC − 6.48 (r = 0.963, Ŝxy = 28.05). However, the present study for CsG gave better correlation data for comparisons of HPLC, RIA, and FPIA (see Table 2) and indicated that interference from CsG metabolites may be less of a problem in the immunoassays considered. To date, little is known of the immunosuppressive properties of CsG metabolites or of their activities in various assays.

Concerning turnaround time, FPIA is the most rapid of these methods for CsG determination, with results available before HPLC and RIA. Both immunoassays were less labor-intensive than HPLC, which has long been regarded as the gold standard for CsA analysis. Further, FPIA would be the method of choice when a laboratory is requested to conduct rapid CsG pharmacokinetic studies after administration of the drug.

In terms of cost, HPLC is certainly the most labor-intensive technique. For CsG determination in an average of 50 patients' samples per day, we calculated that HPLC would require 13.6 h of technologist time and an overall reagents and consumables cost of $2.76 per specimen. For FPIA 4 h of labor would be necessary, with a reagents and consumables cost of $10.44 per sample. RIA required 4 h of labor time for 50 samples, with a reagents and consumables cost of $5.14 per sample.

References
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