sensitivity comparable with other HPLC methods for methylxanthines (11, 12) and is suitable for both therapeutic drug monitoring and pharmacokinetics studies.

Because the specificity of the assay, in the absence of any extraction procedure, relies on the chromatographic separation, we have given special care to examining the possibility of co-eluting peaks. Theophylline and caffeine are well resolved from dyphylline and doxofylline, and none of 76 drugs we examined (Table 1) has shown significant interferences. However, paracetamol (acetaminophen) partly overlaps the theophylline peak. According to Dawson et al. (13), use of a 250-mm-long column should resolve the problem.

It has been suggested that strong protein binding theoretically could hamper the use of direct-injection HPLC methods because of disturbances of the absorption equilibrium of the analytes (9). The parallelism between the calibration curve for both the drugs, in buffer and in serum, suggests that they are not so strongly linked to proteins as to interfere with separation on this column.

We saw no significant decrement in column performance or pressure increase after more than 500 injections of samples. We consider this direct-injection HPLC method a simple, quick, accurate, and inexpensive way to measure methylxanthines of therapeutic interest.

References

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Three Commercial Polyclonal Immunoassays for Cyclosporine in Whole Blood Compared: 1. Results with Patients' Specimens

Michael J. Strassman, Gary L. Lensmeyer, Donald A. Wiebe, and Ian H. Carlson

We assessed the performance of three commercially available polyclonal immunoassays for apparent cyclosporine in 120 whole-blood specimens collected from transplant recipients just before their next dose of cyclosporine (CsA). The assays were (a) Abbott's TDx fluorescent polarization immunoassay for CsA and its metabolites in whole blood; (b) the Sandoz radioimmunoassay (RIA); and (c) Incstar's CycloTrac RIA. Mean respective CVs were 3.8%, 9.3%, and 24.3%. Analytical recovery was nearly 100% for concentrations up to 1000 µg/L for Incstar and up to 1500 µg/L for Abbott and Sandoz; linearity was compromised at greater concentrations. We also quantified the parent CsA concentrations by HPLC. Moreover, to follow day-to-day fluctuations in patients' "cyclosporine" concentrations with each method and to assess the impact these differences have on interpretation of the analytical results, we assayed serial specimens from six post-transplant patients. These showed significantly dissimilar, but parallel, results among the methods for any single sample. Occasionally, however, a result would not fit the established trend. Biases observed among the assays can be explained in part by the nonspecific antisera cross-reacting with CsA metabolites. Most important, we demonstrate that patients' results are not reliably interchangeable among the methods.

Polyclonal radioimmunoassays (RIAs) and an assortment of "in-house"-developed HPLC procedures have formerly been the laboratory's only tools for therapeutic monitoring of cyclosporine (CsA). Unfortunately, these assays can be labor-intensive, time-consuming, and (in the case of RIA) present a health risk from radioactive tracers. Sandoz Pharmaceuticals and Incstar Corp. remain the commercial sources for RIAs, and both companies now offer a more-specific monoclonal RIA. The monoclonal assays purportedly measure only the parent drug, with negligible...
cross-reactivity from CsA metabolites. Clinically, the advantages of the specific over the nonspecific assays remain controversial. Recently, Abbott Laboratories developed a semiautomated, nonradioactive polyclonal immunoassay for CsA and its metabolites in whole blood based on fluorescent polarization (FPIA) (I); separate kits are available for use with plasma and whole-blood specimens.

Selecting the most appropriate assay from this myriad of methods can be perplexing. Lack of reliable comparative data creates a dilemma for both physician and laboratory, who seek a method that can help them effectively maintain immunosuppression while minimizing toxic side-effects of CsA. Few investigators have attempted to describe the clinical relevancy of results from the various nonspecific assays (2-5). Cross-reactivity of commercial polyclonal antisera with CsA metabolites distorts the analytical result and brings it into question because of the still-uncertain clinical role of the metabolites. Also to be considered when a "cyclosporine" assay is selected are test cost, time to obtain the result, and expertise required of technical personnel.

We have closely examined three commercially available assays and report here the performance of each. In an effort to minimize inconsistencies associated with isolation of plasma—specifically the hematocrit, temperature, and concentration-dependent distribution of CsA and its metabolites among plasma and cells (6)—we tested only whole-blood specimens. Haven et al. (3) confirmed that results from plasma tested with various immunoassays can differ by as much as 100%. In our studies, we (a) measured the reproducibility, accuracy, and linearity of each immunoassay; (b) performed an interassay comparison of patients' results; and (c) assessed long-term assay-distinct trends established with daily serial CsA concentrations from six hospitalized transplant patients who were being treated with CsA.

Materials and Methods

**Immunoassays.** The three commercial polyclonal immunoassays we evaluated were as follows: (a) Abbott Laboratories' (Abbott Park, IL) TDx FPIA (sheep anti-CsA antisera) for cyclosporine and metabolites in whole blood with TDx instrumentation; (b) Incstar's (Stillwater, MN) CYCLO-Trac RIA with rabbit anticyclosporine antisera and an 125I-labeled tracer, counted in a Model 1260 Multi-gamma II counter (Turku, Finland); and (c) Sandoz' (Basle, Switzerland) RIA with sheep antisera and a tritiated tracer, counted in a Tri-Carb liquid scintillation spectrophotometer (Packard Instruments, Downers Grove, IL). Quenching by hemoglobin was minimized by preparing calibrators in whole blood with hemoglobin concentrations approximately the same as those in the transplant-patients' specimens. All assays were performed according to manufacturers' instructions, in duplicate.

**HPLC assay.** We used a previously published HPLC procedure (7) for parent CsA, with slight modifications. To improve analytical recovery of CsA, we substituted a 500-mg BondElut Cyanopropyl cartridge solid-phase extraction column (Analytichem International, Harbor City, CA) for the 100-mg size and washed the final residue (reconstituted in mobile phase) with heptane before injecting it onto the analytical HPLC column (8). Overall, CVs were 6.9% and 4.5% at CsA concentrations of 102 and 460 μg/L, respectively.

**Control materials.** The commercial materials we used for precision studies were the "low," "medium," and "high" concentrations of CsA in a simulated blood matrix, available in the commercial Abbott TDx kit, to evaluate the TDx method; and Lyphocheck I and II controls (BioRad, Anaheim, CA), commercially prepared with CsA in whole blood, to evaluate the Sandoz, Incstar, and HPLC methods.

**Procedures.** First, we determined the precision of the three immunoassays using the above-described controls. We also determined the analytical recovery of CsA in each immunoassay, assaying specimens of drug-free whole blood supplemented with pure CsA (from Sandoz Pharmaceuticals, East Hanover, NJ), added in dry form, to give concentrations of 0, 250, 500, 1000, and 2000 μg/L.

To assess variations in test results among the three immunoassays and the HPLC, we obtained by venipuncture whole-blood specimens from 210 post-transplant patients just before their next dose of CsA ("trough" values), added EDTA anticoagulant, and assayed with all methods. The specimens were selected to provide data over a clinically relevant concentration range. Most patients were concomitantly receiving prednisone, azathioprine, and OKT3 in addition to CsA.

To assess day-to-day trends in patients' results among the four procedures, we collected, as before, specimens from six patients: a 51-year-old man with a transplanted liver; a 42-year-old woman and a 51-year-old man, each with a transplanted heart; a 60-year-old man with a transplanted kidney; and a 35-year-old man and a 39-year-old man, each with a transplanted kidney and a transplanted pancreas. Each patient was monitored for at least 23 days.

**Results**

Table I lists precision data from the three polyclonal immunoassays. Average overall CVs were 3.8%, 9.3%, and

<table>
<thead>
<tr>
<th>Method</th>
<th>Control</th>
<th>CsA, μg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbott FPIA</strong></td>
<td><strong>Abbot</strong></td>
<td><strong>Low</strong></td>
<td>360</td>
<td>10.1</td>
<td>2.8</td>
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<tr>
<td>(n=60 each)</td>
<td></td>
<td><strong>Medium</strong></td>
<td>1266</td>
<td>49.3</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>High</strong></td>
<td>1770</td>
<td>84.9</td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Incstar RIA</strong></td>
<td><strong>Lyphocheck</strong></td>
<td><strong>I</strong></td>
<td>162</td>
<td>14.6</td>
<td>9.0</td>
</tr>
<tr>
<td>(n=36 each)</td>
<td></td>
<td><strong>II</strong></td>
<td>905</td>
<td>86.9</td>
<td>9.6</td>
</tr>
<tr>
<td><strong>Sandoz RIA</strong></td>
<td><strong>Lyphocheck</strong></td>
<td><strong>I</strong></td>
<td>100</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>(n=20 each)</td>
<td></td>
<td><strong>II</strong></td>
<td>775</td>
<td>105.4</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Table 1. Overall Precision Data for the Three Commercial Immunoassays for "Cyclosporine"
24.3% for Abbott FP1A, Incstar, and Sandoz methods, respectively. The precision of the Abbott assay was determined with use of TDx controls; other control products may give different results. We did recovery studies (Table 2) to check the accuracy of calibration (kit calibrators) and assure that losses of CsA were negligible during pretreatment (dilution/deproteination) of samples. With all three assays we could account for nearly 100% of CsA added to whole blood, within their range of linearity. With the Incstar RIA, recovery and linearity were compromised at CsA concentrations >1000 µg/L; for the Abbott and Sandoz methods, this limit was higher, 1500 µg/L. Regression analysis of data points for concentrations up to 1500 µg/L (1000 µg/L for Incstar) gave the following equations: Abbott FP1A = (1.10 × actual CsA concn) + 6.7 µg/L; Incstar RIA = (0.923 × actual CsA concn) + 30.5 µg/L; and Sandoz RIA = (0.963 × actual CsA concn) + 17.6 µg/L.

Method-comparison data from patients' specimens are illustrated in Figure 1. Linear-regression analysis documents substantial slope and intercept differences among the methods. Moreover, the $S_{xy}$ (1 SD of the regression line at the mean concentration measured) was 94.4 µg/L for Incstar RIA vs Abbott FP1A; 115.4 µg/L for Sandoz vs Abbott FP1A; 128.7 µg/L for HPLC vs Abbott FP1A; and 94.8 µg/L for Sandoz RIA vs Incstar RIA.

Trend comparisons (Figure 2) of daily serial specimens from six patients demonstrate that the four assays afford results that differ but roughly parallel one another. Relative differences are not consistently proportional for a patient's whole-blood specimens. Occasionally, a result from one assay deviated substantially from the established intermethod trend. Usually, Abbott's FP1A measured more CsA-related material than did the other methods. For any single sample, intermethod "cyclosporine" results usually followed the pattern Abbott FP1A > Incstar RIA > Sandoz RIA > HPLC (parent CsA).

### Discussion

Abbott Laboratories' FP1A for CsA and metabolites in whole blood offers distinct advantages over the Incstar and Sandoz polyclonal RIA's. Most notably, CVs were <4% with the TDx controls over the clinically significant concentration range. All three assays displayed acceptable analytical recovery (range 94% to 110.9%) of parent CsA from whole blood. Our initial recovery studies with the Abbott FP1A suggested a calibration problem: recovery ranged from 130% to 140%. We suspect incomplete solubilization of CsA in the simulated blood matrix used by the commercial supplier in preparing the calibrators. Recovery of CsA from whole blood with the current formulation is acceptable, albeit slightly high (110.9%), and it appears to be influenced by this sample matrix. When we supplemented CsA in drug-free calibrator matrix (contained in the TDx kit) instead of in whole blood, recovery was essentially 100% (101.9%). The linearity of the Abbott and Sandoz methods appears compromised at concentrations >1500 µg/L; the Incstar RIA loses linearity at concentrations >1000 µg/L.

As shown in our companion paper (9), commercial antisera in the three immunoassay kits cross-react variably with CsA metabolites and are inherently nonspecific. Whether distortion of the analytical result by metabolites adversely influences clinical interpretation of the results is unclear. Assays specific for parent CsA purportedly are more "analytically correct" (10); however, the immunosuppressive and toxic activities of metabolites are not yet definitively known, and some of the metabolites may contribute to a patient's clinical outcome. In the haste to switch to the more specific methods, the potential usefulness of the polyclonal assays must not be overlooked. Sole reliance on assays specific for parent CsA may not be in the best interest of the patient. For example, Kunzendorf et al. (11) observed that increased concentrations of metabolites M17 and M1 contribute to immunosuppression and lower the incidence of rejection episodes.

### Table 2. Analytical Recovery of CsA—Added to Drug-Free Whole Blood—in Three Commercial Immunoassays for "Cyclosporine"

<table>
<thead>
<tr>
<th>Added concn of CsA, µg/L</th>
<th>Abbott FP1A</th>
<th>Incstar RIA</th>
<th>Sandoz RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Result, µg/L</td>
<td>Recovery, %</td>
<td>Result, µg/L</td>
<td>Recovery, %</td>
</tr>
<tr>
<td>0</td>
<td>&quot;low&quot;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>250</td>
<td>283</td>
<td>113</td>
<td>253</td>
</tr>
<tr>
<td>500</td>
<td>531</td>
<td>106</td>
<td>505</td>
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<tr>
<td>1000</td>
<td>1155.6</td>
<td>115.6</td>
<td>950</td>
</tr>
<tr>
<td>1500</td>
<td>1633</td>
<td>109</td>
<td>1189</td>
</tr>
<tr>
<td>2000</td>
<td>&quot;hi&quot;</td>
<td>1617</td>
<td>81</td>
</tr>
</tbody>
</table>

n = 2 each
metabolite produced in patients during their course of therapy; moreover, the type of transplant may directly influence drug pharmacokinetics. Past experiences demonstrate that interassay comparisons of results—for instance, use of ratios of RIA/HPLC results to ascertain metabolite concentrations—are unreliable (12). Santiago-Delpin and Cervoni (4) observed >50% discrepancy in results with the Incstar and Sandoz RIAs for 15% (range 5% to 20%) of the patients’ serum specimens they tested. Investigators have attributed an increased RIA/HPLC ratio (>2.8) to acute tubular necrosis in renal-transplant patients (13) and have observed a positive correlation between increasing RIA/HPLC ratios and enzyme activity in liver-transplant patients (3). In the following paper (9) we describe in depth the diverse cross-reactivity of commercial antisera with CsA metabolites and how this contributes to the analytical result.

References