An Optimized Method for Measuring Cyclosporin A with $^{125}$I-Labeled Cyclosporin

Robin A. Felder,1 Theodore E. Mifflin,1,3 and Bahar Bastani2

We evaluated the use of the new iodinated ligand for the in vitro measurement of cyclosporin A by radioimmunoassay (RIA). Substitution of the iodinated cyclosporin ($^{125}$I-CyA) for the corresponding tritium-labeled analog ($^3$H-CyA) considerably simplifies and accelerates the currently available RIA, and improves its precision. Analysis of the respective dose–response curves showed that the 50% $E_0$ value was lower for the $^{125}$I-CyA assay than for the $^3$H-CyA assay (37 vs 77 $\mu$/L). Use of whole-blood specimens minimizes interference from temperature and hematocrit. We conclude that the use of $^{125}$I-CyA in a commercially available RIA for whole-blood specimens is accessible to most laboratories and provides rapid, reproducible data for management of transplant patients.

Additional Keyphrases: radioimmunoassay · $^{125}$I and $^3$H tracers compared · variation, source of · renal transplant · immunosuppressive drugs

Cyclosporin A (CyA), a cyclic undecapeptide of fungal origin, acts as a potent immunosuppressant by inhibiting the activation of T-lymphocytes (1). The clinical use of CyA is complicated by the narrow therapeutic “window” between inadequate immunosuppression and toxic effects.

References
For routine analysis, whole-blood samples were allowed to equilibrate at room temperature for 60 min before processing. The blood sample was well mixed by inversion and a 100-μL aliquot was added to 700 μL of buffer B (Tris, 20 mmol/L, pH 8.5, containing 0.3 mL of Tween 20 polyoxyethylene (20) sorbitan monolaurate per liter). The dilute suspension was frozen at −20 °C for 1 h, then thawed. Plasma was obtained after centrifugation (1800 × g) of the remaining whole blood at 25 °C for 15 min.

Methods of analysis: Radioimmunoassay of CyA with the tritiated CyA (3H-CyA) tracer (Cyclosporin RIA kit; Sandoz, Basel, Switzerland) was performed according to the manufacturer's instructions (8). Briefly, unknown samples and controls are first pre-diluted 10-fold with buffer B. Next, aliquots of CyA standards, pre-diluted unknowns, and controls are incubated with sheep anti-CyA antisera and tracer (3H-CyA or 125I-CyA) for 2 h at 22 °C. Bound and free CyA are separated at 4 °C after addition of charcoal suspension and centrifugation. We measured the bound tritium activity with an LS 8600 beta counter (Beckman Instruments, Irvine, CA).

To assay CyA with the 125I-CyA tracer, we substituted the 125I-CyA (Immunonuclear Corp., Stillwater, MN) for the 3H-CyA, then proceeded with the Sandoz assay as above. We measured the radioactivity of the samples with a multiwell gamma counter (Model 1260; LKB Instruments, Bromma, Sweden). The samples were assayed by this method were plasma and hemolysates prepared from whole blood by the freeze–thaw method. Unless otherwise stated, all CyA assays were done with 125I-CyA tracer.

CyA standard: A working standard of CyA was prepared by diluting purified CyA solution (40 μg/mL, in ethanol) from the Sandoz kit with buffer B containing 1 mg of normal human serum protein per milliliter. The 2000 ng/mL standard of CyA was stored at 4 °C and used within 24 h of its preparation.

Data analysis: Results of the 3H-CyA method were analyzed by manually plotting %B0 vs log CyA concentration (ng/mL). Results of the 125I-CyA assays were provided by an integrated software package within the LKB gamma counter. This program performs a linear regression analysis of the log-logit transformation of the data, then interpolates CyA concentrations of individual samples. CyA concentrations were usually expressed as mean ± SD. The significance of any differences was determined by analysis of variance.

Optimization of hemolysate ratio: We determined the optimum ratio of whole blood to buffer B (obtained from the Sandoz RIA kit). First, we added increasing volumes of buffer B to a constant volume of whole blood (obtained from normal subjects or renal transplant patients). Next, an aliquot of CyA standard solution was added to each sample to maintain a constant concentration of CyA, independently of volume. These mixtures were frozen (−20 °C) once, then thawed. The resulting hemolysates were assayed for total CyA by the 125I-CyA tracer.

Hematocrit study: To investigate the influence of hematocrit, we prepared a series of samples containing various proportions of erythrocytes as follows. Whole-blood samples from each of two transplant patients receiving CyA were centrifuged and the plasma removed. Blood samples obtained from normal, healthy volunteers were used as a source of additional erythrocytes, which we added to (a) two patients' plasmas containing cyclosporin or (b) normal plasma to which CyA standard solution was added. The resulting hematocrits ranged from 13 to 47%, as measured with a Beckman Microfuge II. After a 90-min incubation at 37 °C, we measured CyA in both plasma (obtained by centrifugation at 25 °C for 15 min) and whole blood by the 125I-CyA procedure.

Quality control: We prepared a series of controls by adding sufficient quantities of purified CyA to heparinized plasma from persons not receiving CyA. Aliquots were stored at −70 °C until needed.

Results

Using identical concentrations of CyA for calibration, we obtained a similar but not equivalent response with the two tracers (Figure 1). Doubling the antisera concentration gave 125I-CyA and 3H-CyA curves that looked very similar. Log-logit transformations of both standard curves yielded linear responses (data not shown). The 50% B0 value for the 125I-CyA curve, 37 ng/mL, was lower than the value observed for 3H-CyA, 77 ng/mL. Use of 125I-CyA therefore provided increased sensitivity for CyA, as reflected by the lower values of 50% B0 (and ED50) compared with those obtained with 3H-CyA.

Replotting the 125I-CyA and 3H-CyA standard curves according to Scatchard (9) showed a marked difference between the binding of the two tracers (Figure 2). Incompleteness of the binding reaction was not responsible for the shape of the 125I-CyA curve, because prolonging the initial incubation step to 16 h did not significantly alter the shape (Figure 2). Doubling the antisera concentration shifted the position of both curves, but did not alter the relative shapes (data not shown).

The precision of the CyA assay with both tracers is reported in Table 1. The CV with the 125I-CyA tracer was half that with 3H-CyA. This improved precision was also observed when the two standard curves were compared (Figure 1). Figure 3 illustrates the results for a patient's plasma serially diluted with buffer B. The upper limit of linearity for the CyA assay with the 125I-CyA tracer was estimated at 400 ng/mL. The lower limit of detection (defined by 85% of the B0 value) was 10 ng/mL for 125I-CyA, 17 ng/mL for 3H-CyA. Figure 4 reports the comparison between CyA concentrations in 26 plasma samples from four renal

Fig. 1. Cyclosporin A dose–response curves with 3H-CyA (□) and 125I-CyA (○) used in Sandoz routine procedure

Dose–response of 125I-CyA with (○) CyA antisera at double the routine concentration. Both routine responses curves represent the mean ± SD at each CyA concentration for six separate runs. A single run (in triplicate) was performed with the antisera doubled in concentration.

CLINICAL CHEMISTRY, Vol. 32, No. 7, 1986
transplant patients as measured by both methods. Mahoney and Orf (10), using the same two tracers, measured the concentration of CyA in serum and found a comparable correlation (slope = 1.16, y-intercept = 11.09, r = 0.98, x = 131I-CyA).

Using whole blood from the four patients who had received renal transplants, we investigated the effect of temperature on CyA distribution. Concentrations of CyA in plasma were measured at 4, 25, and 37 °C at regular intervals up to 8 h. In this study, samples appeared to achieve steady-state equilibrium within 60 min of changing the temperature to 25 °C (data not shown). A large variation was observed within the series for each patient, regardless of incubation temperature.

To study the effect of dilution on observed values for CyA in whole blood, we used both patients' samples and normal whole blood with added CyA. Figure 5 shows the results of increasing the dilution ratio. When known quantities of

![Figure 2: Scatchard plot of dose-response curves with 3H-CyA (O) and 125I-CyA (D) in routine CyA assay; (A), modified assay incorporating 15-h incubation of antiserum monitored with 125I-CyA](image)

![Figure 3: Linearity of response for assay with 125I-CyA tracer. Mean ± SD of triplicate measurements for each dilution](image)

![Figure 4: Comparison of CyA concentrations measured with 3H-CyA or with 125I-CyA: y = 1.675x + 6.08 (n = 26, r = 0.984, SEE = 14.5 ng/mL)](image)

**Table 1. Precision of These and Other Reported Assays for CyA**

<table>
<thead>
<tr>
<th>CyA concentration, ng/mL</th>
<th>Reported here</th>
<th>Reported elsewhere</th>
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</thead>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td><strong>Within-run</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[125I]CyA</td>
<td>51.0</td>
<td>2.1</td>
</tr>
<tr>
<td>[3H]CyA</td>
<td>54.7</td>
<td>7.2</td>
</tr>
<tr>
<td>316.0</td>
<td>21.4</td>
<td>6.8</td>
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<tr>
<td><strong>Between-run</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[125I]CyA</td>
<td>51.0</td>
<td>2.6</td>
</tr>
<tr>
<td>[3H]CyA</td>
<td>54.7</td>
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</tr>
<tr>
<td>316.0</td>
<td>36.6</td>
<td>11.5</td>
</tr>
</tbody>
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*For [125I]CyA assays, our n = 36; for [3H]CyA assays, our n = 24.*

Figures show linear and non-linear relationships between CyA concentration and other parameters.

![Cyclosporin [3H], ng/mL](image)
survey conducted by the Central Indiana Regional Blood Center reported using HPLC to quantify CyA. In contrast, RIA measures the sum of the parent compound and its metabolites, some of which are possibly active as immunosuppressive agents or toxins. The RIA procedure requires less time and capital outlay and is therefore performed by more transplant centers than is HPLC. We designed this study to present both an optimized specimen-handling protocol and an optimized RIA procedure for CyA measurement, given the need for a consensus in CyA analysis (13).

Radioiodinated cyclosporin appears to be a useful ligand when CyA is measured by competitive-binding analysis. The dose–response curve shifts to the left, however, when 125I-CyA is substituted for 3H-CyA, analogous to the shift observed when the heterologous tracer 125I-labeled progesterone was substituted for its tritium analog (14). Although the 125I-CyA dose curve appears only slightly different from the 3H-CyA curve, there are several important differences.

First, because of the antibody’s weaker binding to 125I-CyA than to 3H-CyA, we have lowered the range of measured CyA standards (from 25–400 ng/mL to 6.3–250 ng/mL). Therefore, although 125I-CyA provides greater sensitivity than 3H-CyA, the dynamic range of the assay is slightly reduced (Figure 1). This loss can be partly compensated by using the anti-CyA antisera at twice the concentration used in the 3H-CyA assay (Figure 1).

A second major difference is seen in the Scatchard plot of 125I-CyA binding (Figure 2). The prominent left hyperbolic (or “hooked”) binding curve suggests either (a) incomplete equilibrium between unlabeled ligand and antibody or (b) a substantially lower binding affinity of the sheep anti-CyA antibody for 125I-CyA than for 3H-CyA. Because increasing the first incubation time produced only minor changes to the 125I-CyA Scatchard plot (Figure 2), we attribute the altered binding curve to a difference in binding affinities, in agreement with an analysis (15) demonstrating that hyperbolic Scatchard plots can be obtained when an antibody displays preferential binding between a tracer and its corresponding analyte.

Donatsch et al. (5), measuring the binding between a group of cyclosporine metabolites and the initial anti-CyA antiserum, observed a wide range of cross reactivities. More recent work has shown a particular CyA metabolite (“no. 18") contains modifications only within the side-chain (16) and has a low cross reactivity (11%) with the current anti-CyA antibody (6). Because the iodinated CyA (17) and the CyA metabolite no. 18 share substantial structure similarity in the side-chain region, iodination of CyA is probably responsible for the diminished binding affinity measured earlier (Figure 2).

We calculated linearity and detection limits similar to those reported for 3H-CyA (6, 8), but we obtained different regression statistics for our patient comparison study than those described for 125I-CyA (10). Presumably, our use of plasma samples instead of serum (10) is responsible for these differences.

Substituting radio-iodinated CyA in the current immunoassay improved both its precision (Table 1) and speed. The improved precision probably results from eliminating the error introduced by the sample manipulation required for liquid scintillation counting. A substantial reduction in assay time (4–5 h compared with overnight for the 3H-CyA assay) also can be realized, so that patients’ results can be reported on the day of sample collection. Use of a second antibody could further increase assay speed (18).

**Fig. 5.** Determination of optimum dilution ratio for measurement of CyA concentration

Results are mean ± SD for triplicate assays. 100% recovery = 112 ng/mL

**Fig. 6.** Effect of hematocrit on CyA concentrations measured in plasma and whole blood

A, B: distribution of metabolized CyA in two renal transplant patients; C: pure CyA added to sample from normal subject. Results are mean ± SD for triplicate assays of each sample

series exhibited any significant response to fluctuations in the hematocrit (p >0.05). Changes in plasma CyA concentrations could not be accurately predicted by changes in hematocrit, as indicated by a lack of correlation between patient curves "A" and "B".

**Discussion**

Due to a lack of consensus, CyA is routinely quantified by both RIA (5) and HPLC (6, 7). For the measurement of unmetabolized CyA, HPLC appears to be the method of choice; however sample throughput is slow and expensive equipment is required. In addition, several antibiotics (nitrofurantoin, sulfaethoxazole) appear to interfere in these assays (personal communication, Dr. Terry Phillips, Dept. of Pediatrics, Georgetown Univ., Washington, DC). Because of these difficulties, only 22% of the respondents to a recent
Previous studies (19–21) have examined the influence of time and temperature upon CyA distribution in serum, plasma, and whole-blood mixtures. During a time/temperature study with four patients' samples, we observed large variations within individual patients' samples. Our results suggest that CyA re-equilibrates within 60 min after a temperature shift, although several samples were not measurably influenced by temperature changes. To obtain consistent plasma CyA concentrations, we concluded that the best approach was to equilibrate the sample at room temperature for at least 60 min, then centrifuge also at room temperature.

Because the 125I-CyA method is not influenced by the presence of hemoglobin at <20 mg/mL, CyA concentrations can be measured directly in diluted whole blood. On the basis of the data in Figure 5, we determined that, for quantitative recovery, the minimum volume ratio of whole blood to buffer B is 1:7. Whether CyA metabolite partitioning is reflected by the calculated ratio of [whole blood CyA]:plasma CyA remains to be clarified. This index could reflect the extent of CyA metabolism (21, 22).

Earlier investigations have shown CyA is extensively metabolized to at least 10 different species (16); i.e., <1% of intact CyA was recovered in urine after 96 h (23). As a result, appreciable amounts of CyA metabolites probably accumulate in most patients on long-term CyA dosing regimens (22). Because the antibody currently used in the RIA method cross reacts to various extents (7–32%) with these compounds (5, 8), metabolite concentrations measured with the RIA (3H-CyA) technique have probably been underestimated (3, 12, 24, 25).

In contrast, a more nearly accurate determination of total CyA species with 125I-CyA is possible because CyA metabolites displace more of this tracer from the antibody than they do 3H-CyA. For a given concentration of CyA species (CyA plus CyA metabolites), this increased displacement will result in a lower %Bu value being measured, which corresponds to a higher CyA concentration. The decreased affinity of the sheep antibody for the iodinated tracer should therefore provide a more nearly accurate measurement of the concentration of total CyA species.

In conclusion we suggest the most precise, rapid, and inexpensive method to measure CyA is a RIA method in which the tritiated tracer is replaced by 125I-CyA. The corresponding sample-of-choice appears to be whole blood. Accuracy of the modified assay can be maintained by periodic comparison of results with those by HPLC or by use of a control with known CyA content.

We thank Dr. David Bruns for providing valuable suggestions during this project and Dr. John Savory for reading the manuscript and providing helpful comments. We appreciate the careful attention of Ruth Bray and Kathy Burns who performed the myriad CyA assays. Finally, we thank Jean Bennett for secretarial assistance in preparation of this manuscript.

References


