Use of 125^I-Labeled-Histamine–Cyclosporin C for Monitoring Serum Cyclosporine Concentrations in Transplantation Patients

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We synthesized an 125^I-labeled-histamine–cyclosporin C tracer, to obviate the use of tritiated tracer in radioimmunoassay of cyclosporine. With this tracer, the assay results varied linearly with concentration up to at least 800 µg/L. The within-assay CV was 6.6% at 39 µg/L, 4.2% at 100 µg/L, and 7.0% at 300 µg/L (n = 15). The between-assay CV was 10.0, 6.4, and 7.8% for the same respective concentrations. Comparison with an assay involving tritiated tracer (x) showed good agreement of results: y = 3.81 + 0.927x (r = 0.975, n = 604). Analytical recovery ranged from 100 to 106%. We also compared another commercially available radiolabeled tracer ("125^Iodocyclosporin"); Immunonuclear Corp). Our tracer appeared to be more specific for cyclosporine, as determined by assaying chromatographic fractions of bile extract from a patient being treated with cyclosporine. Results with use of our tracer compared favorably with those obtained with the tritiated tracer, and our assay has the advantages of gamma counting vs liquid-scintillation counting.

Additional Keyphrases: radioimmunoassay  •  iodinated and tritiated tracers compared  •  liquid chromatography  •  cyclosporins

Cyclosporine (cyclosporin A, CsA), a cyclic peptide containing 11 amino acid residues (I), is a powerful immunosuppressant in humans (2). Since its introduction in the late 1970s, immunosuppressive protocols in renal, cardiac, hepatic, pancreatic, and bone-marrow transplantation have undergone a major change (3, 4). More effective than standard drug therapies in preventing rejection, it is, however, not without major side-effects and toxicity, especially as manifested in renal failure and hepatotoxicity. A minimal (trough) concentration of 200 µg/L in serum has been proposed as the threshold for drug toxicity, but trough values lower than 100 µg/L may be associated with insufficient immunosuppression and excessive graft rejection (5-7). The bioavailability of cyclosporine varies with the patient, and dosing must be individualized (8). A growing number of patients are receiving this medication, so a simple, accurate, and precise method for cyclosporine, applicable to the routine clinical laboratory, is necessary for their proper management.

Cyclosporine can be assayed by "high-pressure" liquid chromatography (HPLC) (9, 10) or radioimmunoassay (11). The radioimmunoassay used currently, the kit method produced by the manufacturer of the drug (Sandoz), involves a tritiated tracer, which is subject to all the disadvantages of liquid scintillation counting. Development of a gamma-emitting tracer would greatly simplify the analytical proce-

dure by eliminating the problems of separation with charcoal, use of liquid scintillation fluid, quenching, and fluor disposal. Here we report a radioimmunoassay for determination of cyclosporine in serum, for which we developed, in-house, an 125^I-labeled-histamine–cyclosporin C (125^I-HCsc) tracer.

Materials

Blood samples were allowed to stand at room temperature for at least 1 h before separation of serum, to ensure consistent distribution of the drug between erythrocytes and serum (12).

To study the specificity of the assay, we obtained bile from the surgical drainage of a patient who was receiving intravenous CsA because of small-bowel transplantation. The bile presumably would contain various metabolites of the drug, which is primarily metabolized in the liver.

CsA radioimmunoassay kits, CsA, and cyclosporin C (CsC) were supplied by Sandoz Pharmaceuticals, East Hanover, NJ 07936. "125^Iodocyclosporin" was supplied by the Immuno Nuclear Corp., Stillwater, MN 55082.

We prepared serum standards with cyclosporine concentrations of 0, 25, 50, 100, 200, 400, and 800 µg/L by diluting stock standard (40 mg/L) with cyclosporine-free serum. Stored in aliquots at −20 °C, the standards were stable for four months.

Na125^I was from Amersham Corp., Arlington Heights, IL 60005.

Benzene, dioxane, ethanol, methanol, ethyl acetate, pyridine, 4-dimethylaminopyridine, polyethylene glycol 6000, succinic acid anhydride, Chloramine T, and sodium metabisulfite were supplied by BDH Chemicals, Toronto, Ontario, Canada. Histamine, sodium azide, tri-n-butylamine, diethyl ether, acetonitrile, and isobutylchloroformate were from Fisher Scientific, Toronto, Ontario. Bovine gamma globulin was supplied by Sigma Chemical Co., St. Louis, MO 63178. TWEEN 20 (polyoxyethylene sorbitan monolaurate) was supplied by Merck–Schuchardt, Munich, F.R.G.

We used two buffer solutions. Buffer A was 50 mmol/L Tris buffer, pH 8.5, containing, as preservative, 1 g of sodium azide per liter. Buffer B was the same as Buffer A but with 0.3 mL of TWEEN 20 added per liter.

Thin-layer chromatographic plates were "Polygram Sil G" (Brinkmann Instruments Inc., Westbury, NY 11590). The solvent system was benzene/ethanol/acetic acid (75/24/1 by vol). We also used a pipettor and dispenser (Micromed Systems Inc., Philadelphia, PA 19105), a liquid scintillation counter (Model LS 3801; Beckman Instruments, Fullerton, CA), and a gamma counter (Model 1612; Nuclear Enterprises, Sighthill, Edinburgh, U.K.). For HPLC we used a Model M-45 pump (Water Associates, Milford, MA), an "ultraspheer-octyl" 5-µm (av particle size) reversed-phase column (Beckman Instruments) and a fraction collector (LKB, Bromma, Sweden).
Methods

Cyclosporin-C hemisuccinate was synthesized from CsC with succinic acid anhydride in a mixture of pyridine and 4-dimethyl-aminopyridine as described by Traber et al. (13). 125I-HCS C was synthesized as described by Wong et al. (14). The 125I-HCSC tracer used in radioimmunoassay was prepared in Buffer B containing 1 g of human serum albumin per liter.

Radioimmunoassay using 125I-HCSC: We followed the reagent preparation protocol described in the Sandoz cyclosporin RIA-kit except that the antiserum was further diluted threefold. We pipetted 10 µL of standard or patients' samples, 500 µL of Buffer B, 100 µL of 125I-HCSC tracer, and 100 µL of antiserum, in this order, into 12 × 75 mm polystyrene tubes, vortex-mixed, and incubated the tubes at room temperature for 1 h. We then added 100 µL of gamma globulin (10 mg/mL, in Buffer B) and 1.0 mL of PEG solution (300 g/L, in distilled water) and again vortex-mixed. All tubes were then centrifuged (except those to be counted for total counts) for 10 min at 1000 × g. The supernate was discarded and the radioactivity of the pellet was counted in a gamma counter. The results were calculated by the linear interpolation method.

Samples for linearity, precision, and recovery studies were prepared by adding known amounts of cyclosporine to drug-free serum.

We extracted 100 µL of the bile with 5 mL of diethyl ether. The ether extract was then evaporated and the residue, reconstituted in methanol, was chromatographed by HPLC according to the procedure of Carruthers et al. (9). The eluate was collected in 0.75-mL fractions, which were radioimmunoassayed similar to serum samples, with use of the three tracers to compare relative immunoreactivity.

Results

Thin-layer chromatography. In the purification of 125I-labeled CsC, two major peaks of radioactivity were obtained on thin-layer chromatography. The first peak (Rr 0.48) was probably a moniodohistamine derivative. It contained 80% of the total radioactivity and showed very high immunological activity. We believe the second peak (Rr 0.68) to be a diiodohistamine derivative; its maximum binding was <10%, even in the presence of excess antibody. The overall yield for 125I-HCSC was 20% of the theoretical value; the specific activity was approximately 1000 Ci/g. Figure 1 shows the presumed structure of the 125I-HCSC derivative.

When the [125I]iodocyclosporin tracer reported by Mahoney and Orf (15) was chromatographed with the same chromatographic system, it had an Rf value of 0.8.

Kinetics: We studied the kinetics of the antigen–antibody reaction at room temperature and 37 °C for various antigen concentrations. For all concentrations, the reaction was nearly at equilibrium when the mixture had been incubated at room temperature for 1 h.

Standard curve: A representative standard curve for the present method is shown in Figure 2. Using a Scatchard plot, we calculated the affinity constant and the antibody concentration to be 1.6 × 10⁶ L/mol and 1.0 × 10⁻⁹ mol/L, respectively.

Linearity: A sample containing exogenously added CsA was diluted with drug-free serum to give dilutions of 1/4, 1/2, 1/4, 1/8, 1/16, and 1/32 and assayed. Figure 3 summarizes the assay results.

Precision: Within-assay precision was examined by measuring serum CsA at low, medium, and high concentrations. The respective coefficients of variation (CVs) were 6.6% (n = 15), 4.2% (n = 15), and 7.0% (n = 15). Between-assay
precision was also examined by measuring the cyclosporine concentrations in the same control sera assayed by eight different technologists during 42 different working days. The respective CVs were 10.6% (n = 42), 6.4% (n = 42), and 7.8% (n = 42) and the respective mean values (±2SD) were 38.5 ± 7.7, 106 ± 13.8 and 276 ± 43 μg/L.

**Analytical recovery:** These results are shown in Table 1. In general, recovery ranged between 100 to 106% of the expected value.

**Inter-method comparison:** We radioimmunoassayed 604 samples, using both the 3H tracer and 125I-HCsC. The linear regression equation was (125I-HCsC) = 3.81 + 0.927(3H tracer); p < 0.001, r = 0.976, and n = 604. The correlation is depicted in Figure 4.

**Specificity:** We used HPLC followed by radioimmunoassay of the column effluent to compare the profile of specificity of radioimmunoassay, using 3H tracer and both of the radiiodine-labeled tracers. The results are shown in Figure 5. There were at least five peaks representing apparent metabolites in the pattern for the bile extract. The concentrations of immunoreactive cyclosporin metabolites (fractions 1–30) observed on using the 125I-HCsC were lower than those obtained with use of the 3H tracer and [125I]iodocyclosporin. Perhaps more significantly, use of the tracer ([125I]iodocyclosporin) of Mahoney and Orf (15) resulted in much higher apparent concentrations of cyclosporine than did the former two, sometimes 10 times more. On the other hand, all three tracers responded identically to the parent drug (peak 6 in Figure 5) and to cyclosporin D (peak 7), the internal standard we used in HPLC.

### Table 1. Analytical Recovery Study

<table>
<thead>
<tr>
<th>Cyclosporine added, μg/L</th>
<th>Cyclosporine recovered, μg/L ± SD</th>
<th>Recovery %</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>72.0</td>
<td>6</td>
<td>72 ± 15</td>
<td>100</td>
</tr>
<tr>
<td>105</td>
<td>6</td>
<td>111 ± 17</td>
<td>108</td>
</tr>
<tr>
<td>170</td>
<td>6</td>
<td>178 ± 13</td>
<td>105</td>
</tr>
<tr>
<td>208</td>
<td>6</td>
<td>209 ± 25</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 4. Comparison of results for 604 samples assayed by the 3H method and by the method reported here

\[
y = 3.81 + 0.927x, r = 0.976, S_y = 2.6
\]

**Discussion**

Our results with respect to linearity, precision, accuracy, and specificity indicate that our assay has excellent analytical performance. Taylor (16) reported a method for preparing radioiodinated cyclosporin A. Recently, Mahoney and Orf (15) prepared an iodo-cyclosporin by addition of 125I to the unsaturated double bond of the amino acid residue in position 1. By substituting this tracer in the Sandoz 3H RIA kit, they found that ([125I]iodocyclosporin tracer) = 11.9 + 1.16(3H-tracer), with a correlation coefficient of 0.98. This tracer is now commercially available from Immuno Nuclear Corporation. The 125I-HCsC derivative we prepared in this study is different from the one prepared by Mahoney and Orf. Both tracers are iodinated, but the sites of attachment of the iodine are not the same. As shown in Figure 1, the 125I-labeled histamine tracer differs structurally from the [125I]iodocyclosporin.

Our tracer is prepared by coupling cyclosporin C to 125I-labeled histamine through a succinate bridge. Therefore, the physical properties of the two tracers would be different. This is evident in the difference in Rf values on thin-layer chromatography. In terms of antibody binding, in addition to the structural difference of the tracers, the structure of the antigen against which the antiserum was produced should also be considered. The antiserum provided in the...
Sandoz kit was raised against cyclosporin C attached to guinea pig IgG via the succinate bridge. One therefore would expect the antiserum to bind differently to the two tracers.

When we compared the tracer of Mahoney and Orf with the tritiated tracer in our laboratory, the former gave slightly higher results: \((^{125}\text{I})\text{iodocyclosporin tracer} = 17.3 + 0.96(9\text{H tracer})\), with \(n = 83\), \(r = 0.95\), and \(S_{xy} = 19.4\). In addition, the apparent immunoactivity of cyclosporine metabolites fractionated by HPLC of the bile specimen was much higher than that with either the tritiated tracer or our tracer (Figure 5). This may be due to the fact that \(^{125}\text{I}\)iodocyclosporin is less compatible with Sandoz's antiserum than are either the tritiated and \(^{125}\text{I}\)HCeC tracers. Results for serum cyclosporine by radioimmunoassay are known to be higher than those obtained with HPLC. This is ascribed to cross reactivity of drug metabolites. Apparently, use of the tracer of Mahoney and Orf could further increase the discrepancy between radioimmunoassay and HPLC. This, however, could provide better specificity, because the metabolites appear to be less immunoreactive with the antiserum.

While our \(^{125}\text{I}\)HCeC provides acceptable analytical performance and better specificity, as demonstrated by the above data, it also has several other attractive features. The advantages of a gamma-emitting tracer over beta-emitting tracer have already been mentioned. In addition, because of the high specific activity of the tracer, less antiserum is needed in an assay. As shown in Figure 2, one vial of Sandoz antiserum could be further diluted threefold, enough for 300 tests. Thus the cost per tube can be decreased. For the tracer reported by Mahoney and Orf, the manufacturer recommended no change in reagent requirement. In our laboratory, turnaround time and throughput of the assay were improved as well. Using an automatic pipettor–dispenser, a multi-well gamma counter, and a microcomputer, a technologist can easily assay 100 samples in less than 3 h. With use of the tritiated tracer, 4 to 5 h are required to process one batch of no more than 60 samples.

The data presented here indicate that the \(^{125}\text{I}\)HCeC tracer can be applied to the clinical laboratory. The tracer can enhance the assay with better sensitivity, specificity, and savings in reagent consumption as well as technologist time. We currently are also attempting to produce a specific antiserum for use in radioimmunoassay of cyclosporine, further to improve the assay.

We thank Dr. T. T. Nguyen (International Diagnostic Services) for technical consultation.

References


