Chemiluminescence Immunoassay of Cyclosporine in Whole Blood

T. V. Stabler and A. L. Siegel

We describe a chemiluminescent immunoassay (CLI) for measuring cyclosporine in whole blood. Its sensitivity and accuracy are comparable with those of an RIA method that makes use of the same specific monoclonal antibody. The comparison with the RIA method was excellent: y(RIA) = x(CLI) + 11.24 (r = 0.99). In our procedure the samples are incubated with cyclosporin C-hemisuccinate-aminobutyryl-N-ethylisoluminol, antibody, and paramagnetic particles coated with second antibody. After magnetic separation and washing, the samples are incubated with 200 μL of NaOH (2 mol/L) at 60 °C for 30 min. The chemiluminescence generated by automated serial injections of solutions of microperoxidase and dilute (2 mL/L) H2O2 is measured for 5 s. The data are processed by using a spline fit of log B/Bo log conversion. This method is easy to perform and avoids the hazards and costs associated with isotopic waste disposal. The label is stable for at least three years.

Additional Keyphrases: monoclonal antibodies • antibody-coated magnetizable particles

An increasing number and variety of luminescent immunoassays are becoming available for use in the clinical chemistry laboratory. The primary benefits of this technology include a sensitivity equal to or better than RIA, convenient procedures, label stability, and the elimination of exposure to radioisotopes and costs associated with waste disposal.

Here we describe the preparation of an N-(4-aminobutyryl)-N-ethylisoluminol (ABEI) label for cyclosporin C and its application in a chemiluminescent immunoassay (CLI) for cyclosporine in whole blood. The assay was compared with the CYCLO-Trac™ SP kit (Incstar Corp., Stillwater, MN 55082), which is based on the use of a second specific monoclonal antibody (produced by Sandoz Ltd., Basle, Switzerland) as that available in the Sandoz Sandimmune kit.

Materials and Methods

For the Incstar CYCLO-Trac SP RIA we used a Model 20/20 gamma counter (Iso-Data, Rolling Meadows, IL 60008). Chemiluminescence was measured with the Magic Lite luminometer (Ciba Corning Diagnostics Corp., Houston, TX 77060).

Materials

Cyclosporine and specific monoclonal antibody to cyclosporine were supplied in the Sandoz Sandimmune kits; cyclosporin C was kindly provided by Sandoz. Bovine serum albumin, N,N-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide, microperoxidase (MP-11; EC 1.11.1.7), Tween 20, and ABEI were obtained from Sigma Chemical Co., St. Louis, MO 63178. Goat anti-mouse IgG coated on paramagnetic particles was from Advanced Magnetics, Inc., Cambridge, MA 02138. Thin-layer chromatography plates (Silica Gel-60) were from Merck, Darmstadt, F.R.G.

For the RIA and CLI procedures, we used cyclosporine calibrants (range 0–1600 μg/L) prepared in whole blood with the cyclosporine supplied in the Sandoz Sandimmune kit. All other solutions were prepared in glass-distilled de-ionized water.

Assay buffer: Phosphate-buffered saline (sodium phosphate, 50 mmol/L, pH 8.0, containing, per liter, 9 g of NaCl, 0.1 g of bovine albumin, and 1 g of sodium azide) was stored refrigerated.

Sandimmune specific monoclonal antibody: We diluted the lyophilized antibody material in the kit with 10 mL of assay buffer and stored it refrigerated.

Wash solution: This consisted of (per liter) 9 g of NaCl, 1 g of sodium azide, and 0.5 mL of Tween 20.

Microperoxidase stock solution: This consisted of microperoxidase, 1 g/L, in Tris HCl (0.01 mol/L, pH 7.4). This is stable stored at 4 °C for one month. We prepared fresh microperoxidase working solution daily by mixing 0.1 mL of stock with 9.9 mL of distilled water.

H2O2 solution: Add 0.1 mL of 30% solution to 50 mL of distilled water.

Sodium hydroxide: This was 2 mol of sodium hydroxide per liter of distilled water.

Stock cyclosporin C-hemisuccinate-ABEI (cyclo-C-HS-ABEI): 0.5 μg in 100 mL of ethanol was prepared by diluting from an ethanolic primary stock solution (see below for preparation) of 2 mg in 50 mL. The absorbitivity of the primary stock solution is 20 000 L·mol⁻¹·cm⁻¹ at 280 nm. The light-shielded solution is stable at 4 °C for at least three years.

The working solution of cyclo-C-HS-ABEI, 50 pg per 100 μL of assay buffer, is prepared weekly by adding 0.1 mL of stock solution to 9.4 mL of assay buffer. This volume of working reagent is sufficient for 100 tests. The zero sample tube of our assay produced between 30 000 and 40 000 RLU over 5 s in our luminometer.

Methods

Preparation of cyclosporin C-hemisuccinate (cyclo-C-HS):

We synthesized this compound from cyclosporin C by using succinic anhydride in pyridine, as described by Traber et al. (2). In brief, we dissolved 30 mg of cyclosporin C and 15 mg of succinic anhydride in 0.5 mL of dry pyridine in a 12-mL capped centrifuge tube. After 24 h at room temperature the tube was chilled, and we added dropwise enough cold 0.2 mol/L to precipitate the reactants. The precipitate tube was washed with cold water and dried in a vacuum oven at 39 °C. The hemisuccinate was isolated on thin-layer plates (Silica Gel-60) developed in chloroform/methanol (96/4 by vol; Rf 0.2) and eluted with ethanol.
Preparation of cyclo-C-HS-ABEI: We synthesized cyclo-C-HS-ABEI in two steps similar to the procedure described by Kim et al. (3). In a capped 12-mL centrifuge tube we dissolved 10 mg of cyclo-C-HS and 5 mg each of N-hydroxysuccinimide and DCC in 0.5 mL of dimethylformamide. We incubated this in the dark at room temperature for 24 h, then centrifuged, to remove crystals of urea. We transferred the supernatant solution to a small, capped, flat-bottomed vial containing a magnetic bead. While stirring, we added dropwise a solution of 5 mg of ABEI in 0.5 mL of NaHCO₃ (0.13 mol/L); after 1 h, we acidified the solution to pH 2 with HCl (2 mol/L). We washed the contents of the vial into a separatory funnel with 15 mL of H₂O and extracted twice with ethyl acetate. After washing the organic fraction with 5 mL each of NaHCO₃ (0.13 mol/L) and H₂O, we dried the organic phase over Na₂SO₄, evaporated the solvent under reduced pressure and, dissolved the residue in ethanol. Using thin-layer chromatography plates (Silica Gel-60) developed in chloroform/methanol (80/20 by vol), we isolated the labeled product (Rf 0.5), which exhibits the typical sky-blue fluorescence of ABEI under ultraviolet light. The unreacted ABEI remains at the origin in this system. We eluted the product from the silica gel with ethanol; the compound can be purified by repeating the thin-layer chromatography process if required. We found it useful to run a blank tube through the labeling steps to help identify the appropriate fluorescent product. Kept in a shielded tube, the refrigerated ethanolic solution is stable for at least three years.

Analytical procedures: We performed the RIA procedure for the Incstar CYCLO-Trac SP according to the kit instructions except that we substituted our own standards prepared in whole blood for the serum-based material supplied with the kit.

The CLI procedure is performed as follows: Prepare samples (standards) by methanolic extraction as indicated in the RIA method (4) in microcentrifuge tubes. After vortex-mixing, centrifuge the tubes in the Abbott TDx centrifuge for 1 min. In duplicate 12 x 75 mm glass tubes add 100 µL of extract, 50 µL of diluted antibody, 100 µL of cyclo-C-HS-ABEI, 100 µL of goat anti-mouse antibody-coated paramagnetic particles solution, and 500 µL of assay buffer. Incubate on a shaker for 2 h. Separate the magnetized particles on the magnetic tray for 5 min, decant, and wash twice with 500 µL of wash solution. Add 200 µL of NaOH (2 mol/L) and incubate at 60 °C for 30 min. Place tubes in a Magic Lite luminometer and develop chemiluminescence by using automated serial injection of 150 µL each of working microperoxidase solution and H₂O₂ (2 mL/L). Measure the RLU for 5 s. The instrument performs the reduction of the data according to a spline fit of log B/B₀ conversion.

Results

A semi-log plot of the standard curve for the CLI method for cyclosporine is shown in Figure 1. The slope of the curve suggests adequate sensitivity for the range of concentrations of 25 to 1600 µg/L; the usual therapeutic dosage range is 100 to 350 µg/L, but sensitivity and precision at lower concentrations (e.g., <50 µg/L) are required when other immunosuppressants are used in addition to cyclosporine (5).

Correlation with the RIA method: In a series of assays of whole blood for 100 organ-transplant patients (most were renal-transplant recipients), the correlation with results of the RIA method (Figure 2) was excellent: \( y(\text{RIA}) = 1.01x(\text{CLI}) + 11.24 \) (r = 0.99).

Sensitivity: The minimum detectable concentration of cyclosporine (mean + 3 SD at zero concentration) was 6.6 µg/L. The reported sensitivity of the CYCLO-Trac RIA is 13.8 µg/L (3).

Precision: Table 1 presents the performance of CLI within and between runs, respectively. These results are as good or even better than the reported precision data for the RIA method (3).
Table 1. Precision Data for Assay of Four Samples

<table>
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<th>Intra-assay variation</th>
<th>Mean, μg/L</th>
<th>SD, μg/L</th>
<th>CV, %</th>
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<td></td>
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n = 10 each.

Discussion

The sensitivity, accuracy, and precision of the CLI compared favorably with those of the CYCLO-Trac SP RIA. Both procedures take about the same time to perform. The effort involved in the preparation of the CLI reagents is slight and can be accomplished by any competent technologist. The magnetic solid-phase separation and wash steps are rapidly performed. The automatic dispensing and mixing of reagents in the analytical tubes by the luminometer were highly reproducible. A shielded refrigerated ethanolic solution of cyclo-C-HS-ABEI (5 mg/20 mL) is stable for years. Our protocol is designed to consume only half the antibody required in the Sandimmune procedure and thus permits the analysis of 400 samples from a 200-tube kit.

We prepared our calibrants in whole blood because we, like others, are convinced that the matrix of standards and samples in an RIA should be similar to avoid systematic errors of bias (6, 7).

No doubt other more-sensitive luminescent or fluorescent systems could serve as labels (8). Methods for other assays, e.g., with acridinium derivatives (9), europium (10), fluorescence polarization, and enzymes used in conjunction with luminescence-producing substrates (11), are rapidly being introduced into the clinical laboratory. A good many of these materials are expensive and proprietary. We chose to work with the isoluminol labels introduced by Schroeder and Yeager (12) because the materials are inexpensive and readily available, and the synthesis procedures can be performed in any laboratory. We are particularly impressed by the stability of the products.

The CLI we have described not only compares well with the RIA but also can reduce the hazards of isotope exposure and the increasing costs of waste disposal.

References


Screening for Drugs of Abuse: Effect of Heat-Treating Urine for Safe Handling of Samples

Kim Wolff, Mohamed A. Shanab, Marion J. Sanderson, and Alastair W. M. Hay

Heating urine samples from high-risk patients for 1 h at 56 °C is no longer believed to completely inactivate human immunodeficiency virus (HIV; AIDS virus). To protect staff who are handling infectious samples such as those from drug-addiction units, heating at a higher temperature may be necessary. We report the stability to heat treatment (at 60, 70, and 100 °C) at pH 5.1 and 7.6 of some commonly abused drugs, namely, methadone, pethidine, amphetamine, the cocaine metabolite, benzoylecgonine, and the dextropropoxyphene metabolite nordextropropoxyphene. Heat-treating urine at 60 °C for 1.5 h or 70 °C for 1 h did not significantly affect the measured concentrations of these drugs. However, heat treatment at 100 °C for 1 h reduced the recovery of all the drugs. Benzoylecgonine and amphetamine were most susceptible to the different forms of heat treatment.

Additional keyphrases: laboratory safety - drugs of abuse - heat treatment of urine

The prevalence of the human immunodeficiency virus (HIV) and the fact that it has been found in blood, vaginal fluids, semen, and other biological fluids such as urine and