Liquid-Chromatographic Determination of Cyclosporin in Serum with Use of a Rapid Extraction Procedure

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In this procedure, reversed-phase liquid chromatography is used to measure cyclosporin, a fungal metabolite with immunosuppressive activity, in human serum. With a gradient elution, the retention times for cyclosporine and cyclosporin D were 14.1 and 15.7 min, respectively. Ultraviolet absorbance at 215 nm was used to detect cyclosporine; this wavelength improved assay accuracy without decreasing sensitivity, as compared with detection at 205 nm, which is near the absorption maximum. The major advantage of our procedure is the clean-up method, which involves use of disposable extraction columns. This extraction is simple and requires only 10 to 15 min per sample. Results by radioimmunoassay for cyclosporine were unpredictably greater than those measured by the present method. Dosing guidelines for cyclosporine need re-evaluation, based on more specific assay methods.

**Additional Keyphrases:** preliminary pharmacokinetic data • radioimmunoassay compared • drug metabolites in serum • drug assay • sample preparation

Cyclosporine, a fungal metabolite with immunosuppressive activity (1), is a cyclic undecapeptide with a relative molecular mass of 1202.6 (cyclosporine is the recently adopted USAN name replacing "cyclosporin A"). The drug is neutral, rich in hydrophobic amino acids, and insoluble in water and n-hexane but soluble in most organic solvents (2). It is used to prevent rejection of kidney, liver, and heart-lung allografts (3) and to prevent or treat graft-vs-host disease in patients who are receiving bone marrow transplants (4-6).

Although conclusions regarding efficacy would be premature, the drug produces several side effects, in particular dose-related nephrotoxicity (4-8). Cyclosporine-induced nephrotoxicity is usually manifested as reversible increases in serum creatinine. Several investigators have correlated cyclosporine concentration with nephrotoxicity (4, 5, 8, 9). Keown et al. (9) observed that patients with nadir serum concentrations of cyclosporine >400 µg/L were likely to develop renal toxicity. However, these correlations are based on cyclosporine concentrations determined by radioimmunoassay (RIA), a method that measures both cyclosporine and its metabolites (10). Therefore, dosing guidelines based on these data should be re-evaluated by use of specific assays such as "high-pressure" liquid chromatography (HPLC).

The chemical nature of cyclosporine has made the development of analytical techniques difficult. Two HPLC procedures have been reported (11, 12), but interfering substances in human blood necessitate laborious liquid-liquid extractions before analysis. We describe here a HPLC method that involves a rapid clean-up procedure. Cyclosporine is extracted from human serum by use of disposable extraction columns. With this method, single samples can be extracted, dried, injected into the chromatograph, and quantified in about 1 h. Multiple samples (up to 10) can be extracted simultaneously by use of a specially designed vacuum manifold (Baker-10 extraction system), further shortening the total analysis time.

**Materials and Methods**

**Reagents.** Acetonitrile and methanol were HPLC-grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865). Water was de-ionized, distilled in glass, and filtered through a membrane of 0.45-µm pore size. Trifluoroacetic acid was from Sigma Chemical Co., St. Louis, MO 63178.

Cyclosporine and cyclosporin D (CyD) from Dr. J. F. Borel (Sandoz, Basel, Switzerland) were separately dissolved in acetonitrile to make standard solutions having a concentration of 25 mg/L.

**Extraction.** We used disposable extraction columns (3-mL Cyano; J. T. Baker Chemical Co.) to isolate cyclosporine and CyD from serum samples. Cyclosporin D was used as an internal standard; sufficient amounts of CyD were added to each sample serum (usually 2 mL) to yield a concentration of 500 ng per milliliter. Before sample extraction, the extraction column was conditioned by passing through it one 3-mL wash with acetonitrile followed by two 3-mL washes with water. A known volume of sample was applied to and drawn through the column with a vacuum pump maintained at about 58 kPa. We then washed the extraction column three times with water, being careful not to allow the column to dry out. The column was then washed with 1 mL of methanol/water (40:60 by vol), and air was then drawn through the column for 15 s to remove as much liquid as possible. The compounds of interest were eluted with two 1-mL washes of methanol into a glass tube. The solvent was evaporated from the eluate in a stream of nitrogen and the sample was transferred into a 0.5-mL polyethylene centrifuge tube in two 55-µL washes of the initial mobile phase used in the HPLC gradient (see below). The sample was centrifuged with a Beckman Microfuge (about 10 000 × g) for 10 s and 100 µL was injected into the HPLC.

**Chromatography.** For the chromatographic analysis, we used a Varian Vista HPLC system, which included a Rheodyne injector with a 200-µL sample loop, a 25 × 0.46 cm Beckman Ultrasphere 5-µm ODS analytical column, a Varian UV-50 detector, and a Varian CDS 401 microprocessor. A 4 × 0.4 cm precolumn dry-packed with Vydac C18 reversed-phase packing was used to protect the analytical column from adsorption of sample constituents. The precolumn and analytical column were maintained at 70 °C with a column heater block (Varian Associates, Palo Alto, CA 94303). The column was brought to this temperature from ambient temperature during 45 min to minimize damage to the column. To separate cyclosporine and CyD from other extracted serum compounds, we used a gradient elution at 1 mL/min throughout. The starting mobile phase at time 0 was 1 mL/L trifluoroacetic acid in water (solvent A)/acetonitrile (solvent B), 35:65 by vol. The mobile phase was varied linearly to give final proportions of A/B of 5:95 (by vol) at 15 min. The final mobile phase was held...

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for 5 min, then in 2 min brought back to the starting mobile phase, which was continued for 10 min to establish equilibrium before another sample was injected. Cyclosporine and CyD were quantified by monitoring the effluent at 215 nm. The changing baseline due to the gradient was automatically corrected for by the microprocessor. Chart speed was 0.5 cm/min.

Calculations. Quantification of cyclosporine was based on peak areas. Extraction efficiency for cyclosporine was calculated from the peak area ratio of cyclosporine to CyD. We prepared a standard curve by adding appropriate amounts of the cyclosporine standard solution to a series of glass tubes to produce concentrations of 0, 50, 100, 250, 500, 1000, 2000, and 3000 ng per milliliter. The solvent was evaporated in a stream of nitrogen and 20 μL of acetonitrile was added to each residue. Known volumes of pooled sera from marrow-transplant patients not receiving cyclosporine were added to each tube; each sample was vortex-mixed and transferred into a polycarbonate plastic tube. The tubes were then frozen at −70 °C to simulate storage before sample extraction and injection into the HPLC.

Coefficients of variation were calculated from results for 20 samples of serum supplemented with 100 or 1000 ng of cyclosporine per milliliter.

Results and Discussion

Chromatograms

Figure 1 illustrates representative chromatograms from cyclosporine standards, supplemented serum extracts, and a patient's serum extract. Retention times for cyclosporine and CyD on a new analytical column were 14.1 and 15.7 min, respectively, but the retention times and resolution for both compounds decreased with time because of the high temperatures used in our procedure. The standard curve for cyclosporine-supplemented serum samples was linear between 50 and 3000 μg/L. We performed linear regression on the line relating peak area ratio to cyclosporine concentration. The line was described by the equation \( y = 0.002x + 0.024, r = 0.999 \). The y-intercept was not significantly (p > 0.4) different from the origin. The standard curve did not change during the lifespan of the analytical column.

Analytical Recovery, Precision, and Reproducibility

Cyclosporine and CyD were extracted equally well by the extraction columns at concentrations ranging from 50 to 3000 μg/L. The extraction efficiency ranged from 50 to 70% and was not concentration-dependent. To minimize cost, we tried using the extraction columns a second time and saw no change in either extraction efficiency or the resulting chromatograms.

We saw no interfering peaks when we assayed serum samples from patients who were being treated with tobramycin, ticarcillin, vancomycin, amphotericin B, steroids, methotrexate, or trimethoprim–sulfamethoxazole. Lipemic and icteric samples were also assayed without difficulty. This is important because marrow transplant patients frequently receive intravenous fat emulsions during periods of poor oral uptake or are icteric because of treatment-related hepatic damage.

Although the ultraviolet absorbance maximum of cyclosporine is 195 nm and other HPLC procedures have monitored cyclosporine concentrations at wavelengths ranging from 202 to 210 nm, we find that the number of interfering peaks at these wavelengths makes quantification of cyclosporine difficult. Although the area of the cyclosporine peak at 215 nm is only about 70% of the area at 205 nm, there are fewer interfering substances at the higher wavelength. Furthermore, as indicated below, the accuracy of the assay was improved when cyclosporine was monitored at 215 nm.

Accuracy and precision in measuring cyclosporine concentration were determined at concentrations of 100 and 1000 μg/L. These concentrations are similar to those obtained in vivo after administration of therapeutic dosages of cyclosporine. The CV for serum samples monitored at 215 nm was 14.1 and 7.1% for concentrations of 100 and 1000 μg/L, respectively. Although the CV for samples monitored at 205 nm was similar (14.8 and 9.3%) at 100 and 1000 μg/L, respectively, indicating equal precision, the accuracy was greater at 215 nm than at 205 nm. Serum samples containing 100 μg/L gave mean calculated values \( n = 10 \) at 205 and 215 nm of 162 and 99 μg/L, respectively. Serum with a concentration of 1000 μg/L gave corresponding mean calculated values \( n = 10 \) of 784 and 1004 μg/L, respectively. These data indicate that detection at 205 nm introduced substantial bias; at low concentrations (100 μg/L), measured concentrations exceeded actual concentrations, whereas at high concentrations (1000 μg/L) measured concentrations were less than actual concentrations.

Summary and Preliminary Pharmacokinetic Data

The principal advantage of our HPLC method is the rapid and simple extraction procedure as compared with published extraction procedures (11, 12) that involve liquid–liquid extractions with diethyl ether followed by shaking for 10–20 min.
Furthermore, the procedure by Sawchuk and Cartier (11) involves two clean-up washes with hexane before the final extraction with ether. We tried several modifications of our clean-up procedure—prior protein precipitation, washes with acidic or basic buffers, hexane washes, extraction columns with different packings, and use of other types of extraction columns—without seeing substantial improvement in the chromatograms; in some cases, these modifications actually worsened the chromatograms. Two other clean-up procedures that use disposable extraction columns have been reported (13, 14). However, these reports are in abstract form, thereby precluding direct comparison of their chromatograms with our chromatograms.

A disadvantage of our HPLC method is that column temperature must be 70 °C. We have found, in accord with Sawchuk and Cartier (11), that use of a lower column temperature results in poorer resolution and peak shape. The useful column life was lengthened by heating the column slowly, changing the packing in the precolumn frequently, and centrifuging each sample before injection. Despite these measures, a column can be used for only about 125 h. Another disadvantage of our method is that the chromatography time is about three times longer than the time reported by Sawchuk and Cartier (11) but is similar to the time reported by Niederberger et al. (12).

We are now comparing cyclosporine concentrations determined by RIA with those determined by our procedure. Figure 2 gives data for concentration vs time on one patient with normal renal and hepatic function. Cyclosporine was given as a 1-h intravenous infusion at a dosage of 1.5 mg/kg of body weight. As expected from the extensive metabolism of cyclosporine and the cross reactivity of the RIA between cyclosporine and cyclosporine metabolites, values determined by RIA consistently exceed those determined by HPLC, by from 1.19- to 2.57- (mean 1.77- fold). This difference was not related to cyclosporine concentration, but only a few samples were available for comparison.

Pharmacokinetic parameters determined from concentration-time data can change according to the specificity of the assay (15). This is important because dosing guidelines are usually based on these parameters. For example, the data in Figure 2 indicate that total plasma clearance increased from 1.49 to 2.53 mL/min per kg of body weight when calculations were based on concentrations measured by HPLC rather than by RIA. Similarly, the steady-state volume of distribution changed from 1.58 to 2.69 L/kg when results determined by HPLC were used. The volume of the central compartment, hybrid rate constants, and intercompartmental rate constants also changed, but these changes were unimportant.

There is controversy concerning the most appropriate biological fluid to assay for cyclosporine. Plasma (10-12), serum (4, 8-10), and whole blood (11) have been used, but plasma (and presumably serum) has been referred to as "inappropriate" because of extensive binding to plasma proteins (16). However, in vitro data obtained with radiolabeled cyclosporine show that equilibration between plasma and erythrocytes occurs in 20 min and that the major plasma-binding component is the lipoprotein fraction rather than albumin (17). Furthermore, monitoring of serum or plasma concentrations of several other highly protein-bound drugs has resulted in more rational use of those drugs in clinical practice (18). Therefore, we believe that more studies are needed before firm conclusions regarding specimen appropriateness can be made.

Note added in proof: Since acceptance of our manuscript, we have developed an isocratic procedure in which a Spherisorb 5-μm ODS-2 analytical column is used. The mobile phase consists of water/acetonitrile, 27/73 by vol.

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