Liquid-Chromatographic Determination of Cyclosporin A in Blood and Plasma

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We describe a liquid-chromatographic assay for cyclosporin A in blood and plasma. The method is sensitive enough to allow quantitation of the drug at concentrations observed clinically, is isocratic, requires no derivatization, and takes only 10 min of analysis time.

Additional Keyphrases: "high-pressure" liquid chromatography • immunosuppressive agents • temperature-dependent resolution • drug assay • organ allografts and transplants

Cyclosporin A (CyA), an immunosuppressive agent, prolongs survival of various experimental organ grafts and has also been effective clinically in prolonging survival of renal and pancreas allografts (1–3). Its use is currently being investigated in humans with transplants of other organ systems as well.

The drug is a cyclic undecapeptide with a relative molecular mass of 1202. The compound is neutral and is rich in hydrophobic amino acids such as methylenecine. Its structure is given in Figure 1. Cyclosporin A is practically insoluble in water and in n-hexane, but very soluble in many organic solvents (4, 5).

A side effect of CyA, apparently dose-related, is nephrotoxicity (1, 2). In a study (3) involving renal transplant patients who received CyA in combination with prednisone for the first postoperative month, trough concentrations of CyA in blood greater than 400 μg/L were uniformly associated with increases in serum creatinine. In every instance, reducing CyA dosage reduced the trough concentrations, and, concomitantly, serum creatinine. In these patients it is difficult to know whether to attribute symptoms of renal dysfunction to the drug or to poor initial function of the graft.

A specific and reliable method of analysis for CyA in blood or plasma is obviously needed, to define its therapeutic range. Although a radioimmunoassay is available, it is not reliable (2). Concentrations in serum have not yet been correlated with dose.

We describe a procedure that now is routinely used in our laboratory to quantitate CyA in concentrations as low as 25 μg/L, in 2.0 mL of patients' blood or plasma. Unlike a previously reported method, which involves the formation of a β-naphthyl-seleno-substituted tetrahydrofuran derivative (6), this procedure requires no derivative formation; moreover, it requires only about one-third the chromatographic time required in a gradient procedure described earlier (7).

Materials and Methods

Instrumentation. For liquid chromatography we used a high-pressure liquid chromatograph (Model 1084B; Hewlett-Packard, Palo Alto, CA 94304) equipped with a variable (190–660 nm) wavelength detector and an automatic sampling system. The separation is carried out on a 15 cm × 4.6 mm (i.d.) prepacked, microparticulate (5-μm av particle size) reversed-phase column (LC-18; Supelco, Inc., Bellefonte, PA 16823) with the thermostated chromatograph oven maintained at 75 °C. The flow rate of the mobile phase, acetonitrile/distilled water (68.5/31.5 by vol), is 1.4 mL/min. The column effluent is monitored at 202 nm. Attenuations ranging from 24 to 27 are used, depending on the concentration of the samples (or standards) being analyzed. Chart speed is ordinarily 0.5 cm/min.

Reagents. Chemicals used were CyA and cyclosporin D (CyD; Sandoz Inc., East Hanover, NJ 07936); diethyl ether, distilled in glass, preserved with 20 mL of ethanol per liter, and packaged under nitrogen (Burduck & Jackson Labs., Inc., Muskegon, MI 49442); acetonitrile (UV grade, distilled in glass; Burdick & Jackson); n-hexane (nanograde; Mallinckrodt, Inc., St. Louis, MO 63160); and methanol (ACS grade; Fisher Scientific Co., Fair Lawn, NJ 07410).

Distilled water and the acetonitrile were filtered separately under reduced pressure through a prefilter and a 0.4-μm pore size polycarbonate filter (both from Nucleopore Corp., Pleasanton, CA 94566) to remove any particulate matter. Degassing was done on the liquid chromatograph under reduced pressure with heat (100 °C for water and 40 °C for acetonitrile).

Standard solution of CyA: Dissolve 125 mg of CyA in 50 mL of methanol. Prepare the working standard by diluting this stock solution 1000-fold with methanol.

Internal standard solution: Dissolve 25 mg of CyD in 10 mL of methanol. Prepare the working internal standard (25 mg/L) by making two consecutive 10-fold dilutions of this stock solution.

Procedure

Sample preparation and extraction. To eight 35-mL centrifuge tubes (no. 15845-00; Wilkens-Anderson Co., Chicago, IL 60651), add 0 (blank), 20, 40, 100, 200, 400, 800, and 1200 μL of the CyA standard solution. Remove the methanol by evaporation (Evap-o-Mix; Buchler Instruments, Fort Lee, NJ 07024). This series is used to prepare the standard curve.

Fig. 1. Structure of cyclosporins A and D: CyA, R = −CH3CH3; CyD, R = −CH(CH3)2
Add 25 µL of internal standard solution to all standard curve tubes (except the blank) and to each sample tube. Add 2.0 mL of drug-free human whole blood (or plasma) to standard curve tubes, and 2 mL of patient’s blood (or plasma) to the appropriate sample tubes. Rinse each pipette with 2 mL of distilled water, adding the rinse to the appropriate standard or sample tube. From this point on, all tubes are treated identically. Add 14.0 mL of ether by automatic pipette (Repipet; Lab Industries, Berkeley, CA 94710). Stop the centrifuge tubes with ground-glass stoppers, place them horizontally on a mechanical shaker (Eberbach Corp., Ann Arbor, MI 48106), and shake at 180 cycles per minute for 15 min. Centrifuge at 750 × g for 5 min.

Transfer an 11.5-mL aliquot of the ether phase by pipette to a clean 13-mL centrifuge tube (no. 410050; Kontes, Evanston, IL 60204). Dry the tubes (Evapo-Mix) and add 1.0 mL of HCl (0.025 M/L), 2.0 mL of methanol, and 7.0 mL of n-hexane. Shake the samples for 5 min and centrifuge for 5 min as before. Aspirate and discard the hexane layer and add 7.0 mL of fresh n-hexane. Repeat the shaking, centrifuging, and aspirating of the hexane layer as described. Finally, add 1.0 mL of NaOH (25 mmol/L) and 7.0 mL of ether to the remaining aqueous phase. Shake the samples for 10 min and centrifuge for 5 min as before. Transfer as much as possible of the ether layer to a clean 13-mL centrifuge tube, evaporate to dryness (Evapo-Mix), and reconstitute with 100 µL of mobile phase. Transfer the solutions into 0.1-mL microvials (no. 3-3208; Supelco) for automatic injection into the chromatograph.

Chromatography. Inject into the liquid chromatograph approximately 90 µL of the samples reconstituted with the mobile phase, and chromatograph as described above. Measure peak heights manually and calculate the ratio of peak heights of CyA to the internal standard, CyD.

Calculations. Concentrations of CyA are determined from the regression equations relating measured peak-height ratios of the standards to their concentrations. We did not force the regression lines to pass through the origin.

Results and Discussion

Chromatograms

No interfering peaks have been detected in blood or plasma blanks stored for less than three weeks. In samples stored for longer periods, we occasionally noted extraneous peaks. Their occurrence was variable, with respect to retention time and intensity, from sample to sample. Because of this, which we speculate results from the breakdown of endogenous compounds, samples were normally analyzed within two weeks of being drawn. This precluded the determination of between-run precision for this analytical method.

Chromatograms resulting from the analysis of blank blood and patients’ blood are shown in Figure 2. Retention times for CyA and CyD are approximately 5.8 and 7.8 min, respectively. Spectral scans of the CyA peak observed during the analysis of unextracted standards and patients’ blood samples were obtained with the chromatograph in scan mode. These scans were virtually identical. The wavelength of maximum absorption for CyA has been reported to be 195 nm, although the solvent was not specified (7). Because detection at wavelengths less than 202 nm generally produced increased background absorbance in that portion of the chromatogram just preceding the CyA peak, we used 202 nm as the wavelength for analysis.

The marked effect of column temperature on the resolution and peak shapes of CyA and CyD is shown in Figure 3. Although only small changes in retention time were observed as temperature was increased, peak shape and resolution improved significantly, and were optimized at 75 °C. It has been suggested (5) that the conformation of CyA is markedly affected by intramolecular hydrogen bonding. It is therefore conceivable that temperature-related conformational changes could alter solubility, thus influencing chromatographic behavior of the cyclosporins.

Standard Curves

The relationship between the peak-height ratio of CyA to the internal standard (CyD) and the concentration of CyA in blood or plasma was linear over the range 25–1500 µg/L. The intercepts averaged (n = 23) −0.0087 peak-height ratio units, and the slopes averaged 5.57 L/mg.

The use of peak-area ratios occasionally created problems related to the proper assignment of baseline or slope sensitivities for peak integration. Apparently, the tendency of the baseline to deflect negatively immediately after injection causes an uncertainty in baseline assignment, which greatly complicates peak integration by the tangent skin algorithm. In addition, sample-related differences in the chromatograms sometimes cause difficulty in the assignment of the optimum slope sensitivity. Thus peak-height ratios are used in preparing the standard curves and quantitating the CyA in the samples.

Analytical Recovery, Precision, and Reproducibility

Recovery of CyA was determined by comparing the peak-height ratios after extraction of plasma and blood samples containing known amounts with those peak-height ratios measured in unextracted samples supplemented with known amounts of CyA. For purposes of this calculation, we added
the internal standard to the samples just before injection into the chromatograph.

Recoveries of CyA from plasma and blood were calculated to be 74 and 49%, respectively. Omission of the n-hexane wash did not alter this, but it resulted in a large number of interfering peaks. Percent recovery from plasma and blood was found not to be a function of CyA concentration. Pooled blood (n = 5) and plasma (n = 5) controls containing CyA in concentrations ranging from 130 to 1300 μg/L (blood) and 110 to 120 μg/L (plasma) were stored at room temperature and analyzed over a 17-day period. No significant change in CyA concentration was observed, suggesting that stability is not a problem over this period. Stock solutions of CyA and CyD in methanol were found to be stable when stored at −25 °C for at least nine months.

Coefficients of variation for the simultaneous analysis (n = 10) of blood samples containing 625 μg of CyA per liter were 6.5%. Coefficients of variation of the slopes of the standard curves (n = 23) used in weekly routine analysis of CyA in the blood of renal transplant patients were 9.2%, exhibiting acceptable reproducibility. These were prepared over a period of 30 weeks, and involved five different columns. Useful column life is somewhat shortened by the elevated oven temperature, averaging approximately 100 hours of analysis time.

Measured Values of Cyclosporin A

We have measured concentrations of cyclosporin A in the blood of transplant patients undergoing chronic CyA therapy with oral prednisone therapy was common for all; other coadministered drugs included trimethoprim, sulfamethoxazole, furosemide, hydrodiuril, and propranolol. Approximately 1000 blood samples have been analyzed for CyA to date, and no interfering peaks have been noted in any of these.

Blood samples were drawn for analysis under steady-state conditions just before dosing, to determine the minimum concentration of CyA during a dosing interval. In a few patients, samples were also collected frequently over the dosing interval to characterize extremes in concentration, and to allow an assessment of the pharmacokinetic profile of the drug. The blood concentration vs time during a 24-h interval for one patient is presented in Figure 4.

Because only limited data relating the effectiveness of CyA to its concentration in biological fluids are available, we do not know yet whether blood or plasma is the preferred fluid. In four patients from whom we have analyzed simultaneous blood and plasma samples (n = 20), the concentrations in blood were significantly higher than in plasma, by a ratio of 1.92 (SD 0.81).

The sensitivity of the present technique appears adequate for monitoring minimum (trough) plasma or blood CyA concentrations in patients receiving the drug in the dosage range used in this study (3). The linearity and reproducibility of the method also permit an assessment of the concentration-time course of CyA over a dosing interval. Careful monitoring of CyA concentrations in transplant patients will undoubtedly assist in defining the therapeutic range of this promising new agent from patients.

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References