Simplified Liquid-Chromotographic Analysis for Cyclosporin A, and Comparison with Radioimmunoassay

S. G. Carruthers, D. J. Freeman, J. C. Koegler, W. Howson, P. A. Keown, A. Laupacis, and C. R. Stiller

We describe a simplified isocratic “high-performance” liquid-chromatographic method for measuring a new immunosuppressive drug, cyclosporin A, in biological fluids with use of its analogs cyclosporin C and cyclosporin D as internal standards. The method is reproducible and accurate and appears to be specific for cyclosporin A; the detection limit is 31 μg/L. The chromatographic measurements of the concentration of cyclosporin A in serum of patients receiving the drug were invariably lower than those by radioimmunoassay and the difference became more pronounced the greater the period of time after dosing. Because measurements of cyclosporin A in serum standards were almost identical with both techniques, the differences between the two sets of results for patients’ samples suggests that the radioimmunoassay is nonspecific and measures metabolites of cyclosporin A.

Additional Keyphrases: drug assay • immunosuppressive drugs • renal transplant • cyclosporine

Cyclosporin A (CyA), recently renamed cyclosporine, an immunosuppressive agent, is preferentially active against proliferating T cells (1, 2). It is demonstrably effective in the prevention of graft-vs-host disease in man after allogeneic bone marrow transplantation (3, 4) and in prevention of graft rejection after kidney (5, 7) or liver transplantation (8).

Adverse effects include persistent nonspecific immunosuppression (9), lymphoma (10), and hepato- and nephrotoxicity (11, 12). Some of these adverse effects, and the likelihood of graft rejection, may be diminished by monitoring concentrations of CyA in plasma, in conjunction with immunological evaluation of its efficacy (13). Other immunological, pharmacological, and therapeutic aspects of CyA have been recently reviewed (14).

Radioimmunoassay (RIA) of CyA in biological fluids appears to overestimate its actual concentration, presumably because the antibodies cross react with one or more of its metabolites (T. Beveridge, personal communication). A recently published “high-performance” liquid chromatographic (HPLC) technique (15) is complex and does not readily permit the concurrent evaluation of metabolites of CyA.

We describe here a modified HPLC technique that is technically simpler, and we compare CyA measurements by this method with those by RIA. With further development and the availability of various CyA metabolites in pure form, this method may permit identification and concurrent measurement of these metabolites in the same sample of biological fluid.

Materials and Methods

The analytical equipment consisted of a “high-performance” liquid chromatographic system (Waters Scientific, Ltd., Mississauga, Ontario, Canada) controlled by a M-660 solvent programmer. Injection was either manual or by an automatic injection system (Waters Intelligent Sample Processor, WISP-710B). The column was a Beckman Ultrasphere-Octyl 5-μm particle size reversed-phase column, 25 cm × 4.6 mm, maintained at 72 °C in a water bath. The detection system was a Waters M-450 variable wavelength detector. Peaks were recorded and integrated with a Waters data module.

Reagents consisted of glass-distilled diethyl ether, HPLC-grade acetonitrile and methanol, CP hydrochloric acid, ammonium hydroxide, granular purified ammonium sulfate, and cyclosporins A, C, and D (donations from Sandoz Ltd., Basle, Switzerland). Water was further purified by passing filtered, de-ionized water through a C-18 Sep-Pak (Waters).

The primary stock solution of CyA was prepared as 20 mg/L in methanol/water (60/40 by vol). Stock solutions of cyclosporins C and D were 10 mg/L in 50/50 and 60/40 mixtures (by volume) of methanol/water, respectively. Stock solutions of cyclosporins A, C, and D were kept at room temperature to avoid the precipitation of cyclosporin that occurs at refrigeration temperature.

Standard concentrations of CyA in plasma were prepared freshly each day by serial dilution over the concentration range 500, 250, 125, 62.5, and 31.25 μg/L and a blank.

The acid extraction phase was hydrochloric acid, 180 mmol/L. The basic extraction solution was sodium hydroxide, 95 mmol/L. The residue was reconstituted in a 75.8 mmol/L solution of ammonium sulfate in acetonitrile/methanol/water (20/20/60 by vol).

Extraction Procedure

Transfer 1 mL of plasma to a meticulously clean acid- and ether-washed 15-mL glass centrifuge tube. Prepare internal standards of 500 ng of CyC and CyD by adding 50 μL of 10 mg/L CyC and CyD stock solutions to each sample. Vortex-mix for 5 s. Add 1 mL of 180 mmol/L hydrochloric acid to the plasma and vortex-mix for a further 5 s. Add 10 mL of diethyl ether and shake the mixture for 15 min (200 strokes/min) in a horizontal shaker. Centrifuge at 450 × g for 10 min. Discard the aqueous phase. Add 2 mL of 95 mmol/L sodium hydroxide to the organic phase and shake each sample for a further 15 min. Transfer 8 mL of the ether layer to another centrifuge tube and evaporate it under medical-grade nitrogen at 37 °C. Reconstitute the residue with 250 μL of the ammonium sulfate solution. Inject 100-μL samples, in duplicate.

Chromatographic Conditions

The mobile phase consisted of acetonitrile/methanol/water (47/20/33 by vol) and its flow rate was 1.5 mL/min, column pressure 1200 psi (8274 kPa). The detector was set at 210 nm and 0.02 attenuation units full scale. For optimal peak integration with the data module, peak width setting was 45 s and
noise rejection was set at 4.0 μV/s. Under usual operating conditions, the chart speed was 0.25 cm/min.

Results

Examples of actual chromatograms are presented in Figure 1. Figure 1A shows a chromatogram of pooled plasma from individuals who had not taken CyA, Figure 1B a chromatogram of plasma taken from a patient 2 h after an oral dose of CyA, and Figure 1C a chromatogram of plasma containing 500 ng of CyA per milliliter with 500 ng of CyC and 500 ng of CyD per milliliter added as internal standards.

Within the range of concentrations studied, the ratios CyA/CyC and CyA/CyD were linearly related (Figure 2). Within the 250–500 μg/L range, the CV for the assay ranged from 3.6 to 6.0%.

At a noise-rejection setting of 4 μV/s, the signal/noise ratio at the lowest standard studied (31.25 μg/L) was 2.5/1.

Analytical recovery studies were performed by comparing peak areas for CyA, CyC, and CyD with known concentrations of these materials prepared as pure solutions in the reconstitution fluid. The respective average recoveries were 96, 99, and 83%.

We checked for possible interference from endogenous and exogenous substances but saw no peaks that co-eluted with CyA, CyC, or CyD in the numerous pooled plasma samples used for preparing standards, in plasma samples from patients not receiving CyA, in plasma samples from patients receiving other drugs, or in plasma samples deliberately supplemented with various other drugs (analgesics, sedative-hypnotics, antibiotics, corticosteroids, anti-arrhythmic agents, bronchodilator drugs, antidepressants, psychotropic drugs, and anti-hypertensive drugs, including the beta-blockers and diuretics). However, plasma samples of two patients receiving

Fig. 1. Chromatograms of CyA, CyC, and CyD: A, pooled plasma without cyclosporins present; B, plasma from a patient receiving CyA (retention time 20.46 min); C, pooled plasma to which 500 μg of CyC (retention time 18.06 min), CyA (20.53 min), and CyD (27.00 min) was added per liter.

Fig. 2. Standard curves for CyA with either CyD (O) or CyC (●) as internal standards.

Fig. 3. CyA as measured by HPLC and RIA at 2 h (●) and 12 h (O) in plasma of a 33-year-old man after renal transplantation. The line of identity and comparison of standards (X) are presented for reference.
CyA showed peaks slightly before the CyD peak in virtually all their samples, which interfered with proper evaluation of the CyD peak. We have not observed any interference with the CyC peak.

Comparison of HPLC with RIA Measurements

We compared RIA of pure CyA in plasma with HPLC measurements. In addition, we compared RIA and HPLC measurements in plasma samples taken 2 and 12 h after oral drug administration. Examples of these comparisons are presented in Figures 3-5.

Over the range of concentrations studied, measurements of CyA concentration in plasma samples to which pure drug had been added were essentially identical by either RIA or the HPLC technique, although HPLC measurements of pure drug in plasma tended to be slightly greater than RIA measurements of pure drug (Figures 3 and 4). However, HPLC measurements of CyA concentration in plasma of patients who had actually received the drug were invariably less than those by RIA. Moreover, there was considerable variation in the relationship between HPLC and RIA measurements at each of the two study times, both within-patient and between-patients.

Figure 3 presents the comparison of plasma concentrations of CyA measured by both RIA and HPLC techniques. The samples were obtained two to 27 days after renal transplantation in a 33-year-old man. His course was complicated by refractory rejection, with graft loss occurring four weeks post-transplant.

Figure 4 shows results of these measurements in a 28-year-old woman one to 33 days after renal transplantation. During this time, the patient experienced one acute rejection episode, which was reversed with intravenous methylprednisolone therapy, and her graft function subsequently stabilized.

Figure 5 compares the ratios of CyA plasma concentrations measured by HPLC with those measured by RIA at each study time of 2 h and 12 h during the course of treatment in the man described above.

Discussion

The extremely lipophilic and neutral nature of CyA, CyC, and CyD has allowed us to use an acid-base extraction technique to remove the ionizable acids and bases that had interfered with the assay when extraction was undertaken under neutral, acidic, or basic conditions alone.

With this extraction procedure, solvent programming (15)

was unnecessary and the chromatography was instead performed under isocratic conditions. We do, however, find it helpful to purge the system occasionally with acetone-trile to prevent accumulation on the column of (presumed) endogenous lipophilic materials.

Our results confirm the findings of Beveridge and his associates that the RIA overestimates the plasma concentrations of CyA determined by the more specific HPLC method, presumably because some, at least, of the metabolites of CyA cross react with the antibody. Such a discrepancy would be relatively unimportant if the results determined by each technique bore a constant relationship to each other, but we find, on the contrary, that differences in the ratios of concentrations within the same individual occur when samples are taken at different times after dosing and that there are considerable differences in ratios between individuals.

Preliminary evidence indicates that some of the variation results from differences in the elimination kinetics of CyA and its metabolites, and from a greater influence of renal dysfunction on the excretion of CyA metabolites than on the clearance of the parent CyA itself. We suspect also that concurrent administration of certain drugs may influence the ratio of CyA concentrations measured by HPLC to those measured by RIA; i.e., the metabolism of CyA may be accelerated or retarded, thereby altering the relative fraction of parent compound. Further studies are needed to explore these possibilities. The development of a therapeutic concentration range for parent CyA in plasma requires much greater experience in relating the measurements to patient outcomes. A comparison of the pharmacokinetic profiles developed by using both HPLC and RIA measurements will provide useful insights into these problems until specific assays for individual CyA metabolites become available.

Some chromatographic peaks eluting within the first 15 min (Figure 1B) probably represent CyA metabolites because these or similar peaks are commonly seen in patients receiving CyA but are not seen in blank plasmas or plasma samples to which standard amounts of CyA, CyC, and CyD have been added (Figures 1, A and C). The purification of individual CyA metabolites may permit future qualitative identification of these peaks and their quantitative measurement, analyses that would be especially useful if one or more CyA metabolites are found to exert significant therapeutic or toxic effects.

Although there were variations in the relationships between CyA measurements by HPLC and by RIA at each of the study times, we have not observed any obvious trends with time (Figure 5). In particular, there was no convincing evidence of accumulation of parent CyA or its (presumed) metabolites.
over the study period. Similar observations in other patients support this conclusion.

The HPLC method described here is suitable for the measurement of CyA in plasma, whole blood, urine, and cerebrospinal fluid. The preparative phase remains relatively time-consuming but is not technically exacting. The application of radial column technology should reduce the retention times further and enhance the efficiency of the analysis.

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References