Improved Liquid-Chromatographic Determination of Cyclosporine, with Concomitant Detection of a Cell-Bound Metabolite

Gary L. Lensmeyer1 and Barry L. Fields2

This unique extraction and isocratic "high-performance" liquid chromatographic method for measuring cyclosporine (CsA) in blood involves a Zorbax® cyanopropyl analytical column maintained at 58 °C, with detection at 214 nm, and recycling of the water:acetonitrile mobile phase for improved long-term column stability and efficiency. Routinely, 1.0 mL of serum, plasma, or whole blood is diluted with water:acetonitrile (70:30) and applied to a disposable solid-phase cyanopropyl column to rapidly extract the drug and the internal standard cyclosporin D (CsD). Analytical recovery for this step averages 90% with whole blood and 98% with serum and plasma. Between-run CVs were 6.5 and 2.6% for means of 104 and 1128 μg/L, respectively. The standard curve is linear up to 1600 μg/L. The minimum detection limit is 10 to 15 μg/L. No interferences from endogenous substances or other drugs were found. In addition, a compound cross reacting with the Sandoz radioimmunoassay antibody was isolated from patients' samples with the present procedure and was tentatively identified as a CsA metabolite(s). It appears to be highly partitioned on blood cells, very little being detected in the serum or plasma. In a comparison with RIA, correlation coefficients were 0.828 and 0.852 for serum and whole blood, respectively. Results from a 12-h pharmacokinetic study in which different sample types were analyzed by RIA and liquid chromatography further exemplified major discrepancies between types of CsA determinations.

The inherent complexity and difficulty in obtaining consistently accurate and precise measurements for CsA in blood has evoked the plaint 'Is it possible?' (1). Analytical and interpretative guidelines are not yet clear, because many factors contribute to the elusive analytical behavior of this unique immunosuppressive polypeptide and its many metabolites that possess undetermined immunosuppressant and toxic activity in humans. Clinical use of CsA concentrations as measured in blood with present methodologies does not assure adequate immunosuppression or the avoidance of toxic side effects, but serves only as a rough guide to therapy (2). Definite therapeutic and toxic ranges have not been established, nor has it been clearly established whether serum, plasma, or whole blood is appropriate for analysis (3, 4). The temperature at which blood samples are equilibrated before and during centrifugation has been deemed critical to the distribution of CsA between plasma and the cellular components of blood, but contradictory conclusions are reported as to the best sample-handling technique to control this phenomenon (5, 6). All of this compounds the confusing picture of CsA monitoring.

RIA and "high-performance" liquid-chromatographic (HPLC) procedures are competing analytically to establish the concentration range of CsA that indicates adequate immunosuppression without hepatotoxicity, nephrotoxicity (7), or neurotoxicity (8). The RIA method (Sandoz Pharmaceuticals, Basel, Switzerland) (9) lacks specificity because of the different cross reactivity exhibited by each CsA metabolite; RIA results are therefore more subject to interindividual variability. Recently, the structures of some CsA metabolites have been elucidated after their extraction from urine (10). But the identification, quantification, immunosuppressant activity, and potential toxicity of these or other metabolites were not reported for blood samples.

Many difficulties have been reported with HPLC methods for CsA assay. Some procedures require time-consuming multistep extractions (11, 12); in some the extraction efficiency is poor, ranging from 49 to 74% (11, 13); some have unsatisfactory minimum detection limits as high as 100 μg/L (15, 18); and (or) in some there is rapid deterioration of the costly reversed-phase analytical columns which, in some cases, are functional for 125 h or less (11, 13). This last is accentuated by the high column temperature (70 to 75 °C) that often is necessary for satisfactory resolution and sensitivity with octyl and octadecyl reversed-phase packings. Time-consuming mobile-phase gradient procedures (13, 14) and complicated column-switching techniques utilizing multiple columns and mobile phase solutions (16, 17) also have been applied in this difficult analysis. In all cases, only CsA was measured; metabolites were not.

We describe here an HPLC procedure that circumvents the problem of analytical column deterioration, instability, and low sensitivity by using the less-retentive Zorbax cyanopropyl packing to chromatograph the lipophilic CsA. Lower operating temperatures help to achieve better column longevity, good peak symmetry, and baseline resolution of CsA and the internal standard. By recycling the mobile phase, the useful life of the column is extended and the cost of solvents is minimized. The 2- to 3-min sample extraction on a disposable cyanopropyl bonded-phase column reproducibly retains both the drug and a CsA-related metabolite(s) and affords excellent recovery. The simplicity of the system lends itself to the rapid turnover of results required of a clinical laboratory.

Materials and Methods

Apparatus. We used a modular isocratic HPLC system conformed to recycle the mobile phase (Figure 1): a Model 100A pump (Beckman Instruments, Arlington Heights, IL), a Model 7125 sample-injection valve with 50-μL loop (Rheodyne, Cotati, CA), a Model 441 single-wavelength (214 nm) spectrophotometer (Waters Associates, Milford, MA), a column heater maintained at 58 °C (Rainin Instrument Co., Woburn, MA), and a strip-chart recorder (Omniscribe, Houston, TX). We dry-packed a mobile-phase silica saturating column (25 cm × 4.6 mm) with 37- to 53-μm particle size silica gel (Whatman Chemical Separations, Clifton, NJ) and connected it between the pump and the injector. A 2-μm
(pore size) prefilters (Upchurch Scientific, Oak Harbor, WA) were placed before the guard column. The 25 cm × 4.6 mm Zorbax® cyanopropyl (5- to 6-μm particle size) analytical column and the 5 cm × 4.6 mm guard column dry packed with Permaphase ETH® (30-μm particle size) were purchased from Du Pont Instruments, Wilmington, DE. Bond Elut® disposable solid-phase extraction columns (1-mL capacity, containing 100 mg of cyanopropyl phase packing) and the Vac Elut® extraction apparatus were from Analyti-Chem International, Harbor City, CA.

Reagents. All reagents are "HPLC" grade, including acetonitrile and methanol from Fisher Scientific Co., Fair Lawn, NJ, and glacial acetic acid from J.T. Baker Chemical Co., Phillipsburg, NJ. Distilled, de-ionized water was prepared with the "Milli-Q" water purification system (Millipore Corp., Bedford, MA). The diluent is a 350 μg/L solution of cyclosporin D in water/acetonitrile (70/30 by vol); it is used to dilute samples, for hemolysis of whole blood, and in adding internal standard. Three solutions prepared by volume are used for the extraction: A is water/acetonitrile, 80/20; B is acetic acid (0.5 mol/L)/acetonitrile, 80/20; and C is acetic acid (0.5 mol/L)/acetonitrile, 60/40. All solutions are stored in reagent-dispensing bottles (Glenco Scientific Inc., Houston, TX) for rapid and precise application to the extraction column.

Standards. The cyclosporin A (CsA), C (CsC), and D (CsD) in pure form were obtained courtesy of Sandoz Pharmaceuticals, East Hanover, NJ.

Individual stock solutions (100 mg/L in methanol) of each standard are stored at room temperature. Working CsA standards of 300, 600, and 1600 μg/L are made by diluting the stock with methanol. They are stable at room temperature for at least four months. For a routine analysis, pipet 0.5 mL of each working standard into separate 16 × 100 mm round-bottom test tubes, evaporate the methanol under reduced pressure, and add 1.0 mL of heparinized drug-free whole blood, serum, or plasma. Vortex-mix the contents and let the tube sit for 10 minutes before analysis. Final concentrations are 150, 300, and 800 μg/L, respectively. Standards must be prepared in a matrix identical to the unknown samples to maintain accuracy.

The resolution mixture, used to verify chromatographic integrity, contains 2.0, 1.5, and 2.5 mg of CsA, CsC, and CsD, respectively, per liter of mobile phase. This mixture is stable for at least two months at room temperature.

Mobile phase. A 4000-mL batch of mobile phase, water/acetonitrile, 51/49 (by vol), is filtered and degassed with the Millipore filter system (Type FS, 3.0 μm). Discard the batch after one month of daily recycling when the resolution mixture shows poor separation or when a high background absorbance develops. Before the first use of a new Zorbax cyanopropyl column, pump 500 mL of acetonitrile/water (95/5 by vol) through the column to remove the hexane/isopropanol solvent used during factory testing.

Procedure

Extraction. Add 1.0 mL of heparinized whole blood, serum, or plasma and 2.0 mL of the diluent solution containing the internal standard to a 16 × 100 mm disposable test tube, mix, and leave at room temperature for 5 minutes. An automatic pipet with polypropylene tip is useful for precise transfer of whole-blood samples. Centrifuge (2500 rpm) the tube for 5 minutes. Precipitation of protein can occur if the diluted sample is kept needlessly long in a warm centrifuge or if an hemolytate is not extracted within 30 minutes after centrifugation.

Attach the Bond Elut cyanopropyl extraction column to the Vac Elut apparatus and set the vacuum gauge at 10 to 12 inches of mercury (flow rate about 1.7 mL/min). Prime the column with one reservoir volume (about 1.0 mL) of acetonitrile; repeat this step and follow with two reservoir volumes of solution A. Do not let the column go dry between applications. Apply the diluted sample in two 1-mL portions just as the meniscus of solution A approaches the top of the packing; an automatic pipet is convenient for this transfer. After the last of the sample passes the top of packing, add two 1-mL portions of solution B. To ensure a clean extract, minimize any mixing of residual sample with this wash. After the last of the wash passes the top of packing, add 0.25 mL of solution C. Maintain the reduced pressure for 30 seconds to remove residual liquid from the column.

Elution. Suspend the column in a 16 × 100 mm disposable centrifuge test tube, add 0.4 mL of acetonitrile, leave for 2 minutes, then force the liquid through with a rubber bulb. Evaporate the eluate under a stream of air at room temperature. The Bond Elut column can be reused for two more extractions. The analytical recovery does not change, but resistance to flow increases with each successive use. The CsA is stable in the dry extracts for at least three weeks when sealed and stored at −20°C.

CsA was found to be stable for at least one week in patients’ heparinized blood specimens stored at 4°C when whole blood was analyzed by the HPLC procedure. Samples of whole blood should not be frozen, because substances form upon thawing that can plug the extraction column.

Chromatography. Reconstitute the dry extract with 150 μL of mobile phase. Vortex-mix for 15 seconds, centrifuge at 2500 rpm for 2 minutes, and fill the 50-μL sample loop. Inject the sample at a mobile phase flow rate of 1.5 mL/min, with detector range set at 0.02 A full-scale at 214 nm and recorder chart speed at 1 cm/min.

Results

Inject the resolution mixture daily before sample analysis, to verify the integrity of the system (Figure 2A). Figures 2B and 2C show chromatograms of CsA-supplemented and drug-free whole-blood extracts, respectively, and that for a patient’s sample is shown in Figure 2D. Identification and quantification are based upon relative retention time and relative peak-height techniques, respectively.

Analytical day-to-day precision, analytical recovery, and linearity are listed in Table 1. The sensitivity limit (signals equal to twice the baseline noise) ranges from 10 to 15 μg/L. The within-run precision (n = 12, mean = 522 μg/L) showed a CV of 1.7%. For the absolute recovery studies, various
1. Whole blood is used.

2. Test sample:
   - 1000 peaks were added.
   - Plasma recovery was plotted.

3.added the heparinized whole blood, serum, and plasma and were extracted; we compared results with those for unextracted standards in mobile phase. We plotted relative peak height ratio vs concentration for CsA, and used least squares linear regression analysis to assess linearity. Performance data for both serum and plasma samples are similar to the data for whole blood, but the recovery is better (98% vs 90%).

**Interferences.** Drug-free whole blood, serum, and plasma samples showed no peaks that would interfere with the CsA or the internal standard.

Various drugs were checked for potential interference at concentrations of 1000 mg/L or greater. The following compounds emerged from the column before CsA and did not interfere: acetaminophen, salicylic acid, prednisone, methyprednisolone, hydrocortisone, azathioprine, 8-hydroxyamoxapine, amoxapine, doxepin, desmethyllofoxepin, imipramine, desipramine, nortriptyline, amitriptyline, lidocaine, propanidid, N-acetylprocainamide, propranolol, disopyramide, N-desalkylisopropamide, quinidine, dihydroquinidine, ethosuximide, primidone, phenobarbital, nirvanol, mesantoin, phenytoin, pentobarbital, carbamazepine, diazepam, N-desmethyl Diazepam, chloridiazepoxide, N-desmethylchloridiazepoxide, demoxepam, oxazepam, N-desalkylflurazepam, hydrochlorothiazide, chlorothiazide, chlorothalidone, hydroflumethiazide, polythiazide, furosemide, atenolol, metoprolol, verapamil, clonidine, nadolol, morphone, codeine, meperidine, captorpril, prazosin, minoxidil, hydralazine, methyl dopa, antithymocyte globulin (ATG), cyclophosphamide, cytARBine, gentamicin, tobramycin, vancomycin, digoxin, insulin (10 units/mL), nitroglycerin, ampicillin, trimethoprim, and sulfamethoxazole.

**Technical Considerations**

Analytical success with this procedure depends on close attention to important technical details if one is to achieve better long-term column stability and efficiency than reported previously. The recycling of the mobile phase and the use of a silica saturating column and a lower column temperature all protect the column packing from rapid degradation. HPLC procedures that require temperatures above 60 °C to decrease the characteristic band broadening of CsA and CsD observed on reversed-phase packings shorten the useful life of an expensive column. The solubility of silica in the mobile phase increases as the temperature of the analytical column increases. To compensate for this temperature-dependent dissolution process, the mobile-phase silica saturating column should be maintained when possible at the same temperature as the analytical column. This will be additional insurance for longer column life.

The oven used to heat the columns must maintain a constant temperature and minimize temperature gradients for the entire length of guard and analytical columns. Otherwise, the CsA peak will be asymmetrical and exhibit undesirable band broadening. Wrapping the entire length of columns and the oven's transfer tubing with aluminum foil effectively ensures that the heating-block type oven can transfer heat more evenly.

The efficiency of the analytical column decreases slightly after extended use. To compensate for this, the column temperature can be decreased by 2–3 °C and the flow rate can be decreased 0.2 to 0.3 mL/min to improve resolution. The composition of the mobile phase should not be varied because of the potential for interference due to the nonproportional shifting of CsA, CsD, and endogenous peaks.

The HPLC prefiltre prevents irreversible plugging of the guard and analytical columns. On occasion, an extract from a serum (or plasma) or an old sample of whole blood will contain substances that plug the filter. Periodic backflushing of the filter (usually once every two or three days) ensures a low back pressure and acceptable chromatography.

Initially, we observed very low analytical recovery of CsA and CsD from aqueous solutions, blood, serum, and plasma during extraction. When the sample was diluted with a solution containing acetonitrile and then applied to the Bond Elut column, the absolute recovery improved markedly (as illustrated in Figure 3) and the amount of potentially interfering materials decreased. Similarly, the wash solutions effectively remove interferences and quantitatively retain the drug only when some acetonitrile is contained in the solution that is maintained at an acidic pH.

The manufacturer of the column allows a ±10% variabili-
in the packing volume of their product. When we varied the packing volume in the column by ±20%, with a range of 80 to 120 mg, we observed no discrepancy in CsA recovery. In addition, the recovery remained consistent with expected temperature fluctuations that occur at ambient conditions.

Comparison of RIA and HPLC

CsA results obtained via the HPLC procedure and the Sandoz RIA for specimens of whole blood and serum were compared, to ascertain discrepancies or trends apart from those described by other investigators (12, 19, 20). Data from the whole-blood samples (Figure 4A) demonstrated: n = 46, mean (RIA) = 996 μg/L; mean (HPLC) = 465 μg/L, slope = 1.226, y-intercept = 425 μg/L, $S_{xy} = 332 \mu g/L$, and $r = 0.652$. Data from serum samples showed (Figure 4B): n = 77, mean (RIA) = 474 μg/L, mean (HPLC) = 217 μg/L, slope = 0.9679, y-intercept = 266 μg/L, $S_{xy} = 152 \mu g/L$, and $r = 0.828$.

A 12-h pharmacokinetic curve (Figure 5) demonstrates the disposition of CsA in serum and whole-blood specimens from a patient with a cardiac transplant, who was maintained at steady-state CsA dose of 300 mg (4 mg/kg, orally, every 12 h). To minimize redistribution of the CsA from the serum onto cells during the study, we centrifuged the blood sample for 5 min at room temperature immediately after collection at the patient's bedside. The fibrin clot formed in the serum was broken by adding three glass beads to the tube and centrifuging again for 3 min. All samples were analyzed by both RIA and HPLC. The results demonstrated that in this individual the RIA value for serum was approximately 40% of the RIA value for whole blood and the HPLC result for serum was approximately 65% of the HPLC value for whole blood. For whole blood, the RIA results exceeded the HPLC value by an average of 260%; for serum, the RIA result exceeded the HPLC value by about 200%. 

![Graph of pharmacokinetic curve](image)

Fig. 5. Twelve-hour pharmacokinetic curve for a patient receiving 300 mg (4 mg/kg body wt.) of CsA, orally, at time 0
These data reflect the nonspecificity of the RIA that is the result of cross reaction of compounds other than CsA with the RIA antibody, presumably CsA metabolites. The different degree of cross reactivity for each of the known metabolites and the intra- and inter-individual pattern changes seen with the disposition of CsA and metabolites generate a wide range of variables in the RIA procedure. The significant utility of present RIA and HPLC methods will be clearer once the identification, quantification, and assessment of immunosuppressive activity and toxicity for each CsA metabolite in blood are established.

Detection of Cell-Bound Metabolite

We observed a significant nonendogenous peak at 5.4 min on chromatograms of extracts of whole blood from patients receiving CsA while chromatograms from the corresponding serum or plasma showed a negligible peak at this elution time. This phenomenon seems not to have been described hitherto. Initially the within-run precision of the response for the unknown compound was assessed by analyzing replicate samples of one patient’s whole blood, yielding a CV = 4.1% (n = 10).

Using immediate centrifugation as our sample-handling technique, we collected whole blood, serum, and plasma samples in tandem from numerous patients receiving the drug, at a time when the drug concentration would be lowest. This technique minimized potential in vitro redistribution of the unknown compound onto cells. The specimens were extracted and chromatographed, and individual fractions, collected at 30-s intervals from the HPLC column (Figure 6), were submitted to RIA (Sandoz) to detect compounds that would cross react with the commercial antibody. Figure 6 illustrates the RIA results for fractions collected sequentially from the whole-blood extract of one patient. The unknown compound(s) in fractions 8 and 9 cross reacted with the Sandoz CsA antibody for this patient and the other patients. Little or no chromatographic response or cross reactivity was demonstrated in the patients' serum or plasma specimens for those fractions. The chromatographic response for the unknown in serum and plasma averaged 0.07 (7%) of the response in whole blood. These data suggest that the unknown is a CsA metabolite(s), bound primarily to blood cells. We observed that the quantity of the metabolite(s) increased with time relative to the amount of CsA. Figure 7 represents the relative amount of the metabolite(s) in the same samples from the patient illustrated in Figure 5. After 12 h the metabolite(s) remained primarily in the cellular portion of blood.

Discussion

The procedure described here circumvents many of the recognized problems associated with HPLC procedures for CsA. The extraction is performed rapidly on a solid-phase cyanopropyl column and affords excellent analytical recovery of CsA and the internal standard, Cd. The usual problems of instability and low sensitivity associated with the chromatography of CsA on reversed-phase columns are minimized by use of the less-retentive Zorbax cyanopropyl column, by recycling the mobile phase, and by operating at a relatively low temperature (58 °C). Mobile-phase gradients or elaborate column-switching techniques were not necessary for this procedure. Three different Zorbax cyanopropyl columns were evaluated and found to reliably reproduce the chromatographic separation by the method described above. In our laboratory, column longevity has exceeded five months and the cost per test has been slightly less for the HPLC method than for the RIA. Comparison of analytical results from patients' specimens assayed via the RIA and HPLC methods reaffirm the nonspecificity and consistently higher values seen with RIA for all sample types. This difference reflects the presence in blood of metabolites that are immunoreactive with the commercial RIA antibody. Within any given individual, the rate of metabolism and the spectrum of resulting metabolites change with the duration of therapy. Induction of hepatic enzyme pathways and enterohepatic recirculation have been considered to be the major causes of this time-based difference. Interindividual variation in the rate of metabolism and in the relative proportions of each metabolite produced compounds the difficulty in obtaining and interpreting data on concentrations in blood. Thus, a pharmacokinetic profile will appear different between individuals at any point in therapy and
will differ within a single individual over time. The subject becomes even more complex with the recognition that different tests (RIA vs HPLC) measure different parameters and the sample source (whole blood, serum, or plasma) present different amounts of CsA and metabolites for analysis.

Finally, we report the separation of a CsA metabolite(s) which is reproducibly extracted and chromatographed. Moreover, the metabolite(s) is highly partitioned onto cellular components of blood and is present in negligible amounts in serum and plasma. It accounts for a significant portion of the immunoreactive compounds other than CsA detected by the commercial RIA antibody for whole-blood specimens. Its identity and pharmacological role remain to be determined.

This assay has provided our laboratory with a relatively easy, reliable method for determining CsA concentration in patients' samples without the need for bulk processing. The issue of whether serum, plasma, or whole blood samples and HPLC or RIA procedures are used awaits further study of the pharmacology of the CsA metabolites, their distribution in blood, and the appropriate sample handling technique. It is difficult at this time to argue in favor of one analytical procedure over the other without this information. At present it appears to be a matter of choice as to which analysis is used, predicated upon the biases of the user and the availability of technical expertise and instrumentation.

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