Identification and Analysis of Nine Metabolites of Cyclosporine in Whole Blood by Liquid Chromatography. 2: Comparison of Patients' Results

Gary L. Lensenmeyer, Donald A. Wiebe, and Ian H. Carlson

We used a "high-performance" liquid-chromatographic assay [for parent cyclosporine (CsA) and nine metabolites] and a radioimmunoassay to detail the diversity of results among whole-blood samples from patients with transplanted organs. Heterogeneous populations of metabolites in samples collected just before the next dose of CsA were detected by HPLC, with CsA, M1, M17, M1, or M8 predominating; M21, M203-218, MUNDF1, and M18 were detected in lesser amounts. Results by HPLC vs RIA for CsA or for individual metabolites vs CsA (or RIA) were diverse, with correlation coefficients (r) ranging from 0.058 to 0.933. RIA vs HPLC(sum of CsA + metabolites) gave the best comparison (slope = 0.931, y-intercept 14 μg/L, r = 0.933); but the scatter of data about the regression line remained significant (S_x = 132 μg/L). Most important, RIA/HPLC(CsA) vs HPLC(sum of metabolites) was remarkably poor (r = 0.222).

A 12-h pharmacokinetic curve (for drug concentrations in a heart-transplant patient) displayed dissimilar times for peak concentrations of CsA and metabolites; each differed in the proportion (48% to 81% of peak concentration) eliminated from blood over the 12 h. These studies exemplify the utility of a more-inclusive, specific assay to monitor the diverse disposition of cyclosporines in patients and to demonstrate the errors associated with use of the RIA/HPLC ratio technique to predict metabolite concentrations.

Additional Keyphrases: pharmacokinetics • transplants • immunosuppressive drugs • chromatography, reversed-phase

Routine RIA and "high-performance" liquid-chromatographic (HPLC) assays fail to describe completely and specifically the disposition of cyclosporine (CsA) parent drug and individual metabolites in blood/serum/plasma. Current assays are nonspecific or exclude the metabolites entirely (7). Recent evidence demonstrates that metabolites M17, M1, and M21 are immunosuppressive in vitro (2, 3) and suggest that one or more metabolites may be toxic (4). The diverse influences of drug interactions on metabolism of CsA (5), the effect of a 24-h circadian cycle on the metabolism and (or) absorption of CsA (5), and the impact of the type of organ transplanted on the disposition of CsA (7) further necessitate a better insight into the pharmacokinetics of CsA for each patient. However, most assays preclude the selective measurement of CsA and individual metabolites. Often, to estimate the accumulation of "metabolites" in blood, the RIA/HPLC(CsA) ratio technique is used (8). However, for some patients this ratio remains constant even though bilirubin concentrations, one indicator of hepatotoxicity, may continue to rise (9).

A suitable, universally applicable algorithm to interpret analytical results has been difficult to formulate. So far, clinical data have been acquired for a variety of specimen types, with various handling protocols, dosing regimens, and analytical methods, such that overall comparison of these data is nearly impossible (10, 11). We need to better address and control those variables if we are to enhance the usefulness of therapeutic monitoring of CsA.

In the preceding paper (12) we reported a discrete HPLC assay for CsA and individual metabolites. Here we describe the practical utility of this assay to monitor the diverse disposition of CsA in patients. To support our conclusions, we compare RIA and HPLC results for CsA in a series of samples collected daily from two transplant patients. We also document a 12-h pharmacokinetic profile from one patient with a transplanted heart, and evaluate data from "trough" whole-blood samples—samples collected just before the next dose of CsA—by both RIA and the more inclusive HPLC assay.

Materials and Methods

All reagents, standards, and apparatus used here have been described previously (12). The routine RIA, based on the method of Donatsch et al. (13), was performed with a commercially available kit (Sandoz Pharmaceuticals, Basel, Switzerland) according to the manufacturer's instructions, except that we substituted an 125I-labeled CsA tracer (Immunonuclear, Stillwater, MN) for the 3H tracer in the kit. In our laboratory, the between-run precision (n = 36) for this RIA was 9.7%, 4.8%, and 7.3% at CsA concentrations of 91, 339, and 951 μg/L, respectively.

The solid-phase extraction and HPLC separation of CsA and individual metabolites (M1, M8, M17, M18, M21, M25, M26, M203-218, and MUNDF1) were as described earlier. In brief: the cyclosporines are extracted from whole blood with a Bond Elut® cyanopropyl (CN) sorbent cartridge and chromatographed on a Zorbax® CN analytical column with a mobile phase of water/acetonitrile/tetrahydrofuran/acetic acid/n-butylamine (600/390/20/0.16/0.10, by vol) [see System A, Table 1, in ref. 12]. The between-run precision for individual cyclosporines ranged from 7.1% to 9.6% at 200 μg/L.

We carried out the following three studies:

Study A. Heparinized whole-blood samples from two patients—one with a transplanted liver, the other with a transplanted heart—were collected 24 h post-dose and again before the next dose of CsA over a 15- and 23-day period, respectively, and assayed for parent CsA by HPLC and by RIA.

Study B. Heparinized whole-blood samples from a patient with a transplanted heart who was taking a steady-state dose of 400 mg of CsA (7.0 mg/kg, every 12 h) were collected hourly for 12 h and assayed by the more inclusive HPLC procedure (CsA + metabolites) and by RIA.

Study C. Heparinized whole-blood samples from patients with transplanted liver (n = 9), kidney (n = 36), bone marrow (n = 8), or heart (n = 3) were collected just before the next dose of CsA and assayed with the more inclusive HPLC procedure and by RIA.

Clinical Laboratories and Department of Pathology and Laboratory Medicine, University of Wisconsin, 600 Highland Ave., Madison, WI 53792.
Received May 27, 1987; accepted July 27, 1987.
Results

Study A. Analytical results for "trough" whole-blood samples collected over several days from a liver-transplant patient and a heart-transplant patient are shown in Figure 1. Clinically, the cardiac transplant patient displayed mild congestive heart failure (days 1 to 4), which resolved; concentrations of liver enzymes were slightly increased, and values for serum creatinine ranged from 12 to 35 mg/L. The liver-transplant patient had slightly increased concentrations of liver enzymes (post transplant), which gradually decreased; serum creatinine ranged from 14 to 28 mg/L, and there was no evidence of cardiac failure. The RIA result ranged from 120% to 400% and from 108% to 1500% of the parent CsA value for the liver- and heart-transplant patients, respectively. Most important, irrespective of the type of transplant, the value from one assay could not be used to accurately predict the result of the other assay, nor could an algorithm be derived that would reliably estimate the amount of parent CsA (with or without metabolites) from the results of the other assay.

Fig. 1. Comparison of RIA results vs HPLC(parent CsA) results for patients with transplanted (left) liver and (right) heart, over 23 and 15 days, respectively

Fig. 2. 14-h pharmacokinetic curve for a patient receiving 400 mg of CsA orally (7.0 mg/kg, every 12 h) at time 0 and 12 h later (arrow): shown are results for CsA and major individual metabolites by HPLC, the sum of the cyclosporines by HPLC, and CsA by RIA
Study B. A 14-h pharmacokinetic profile (Figure 2) demonstrates the disposition of CsA and metabolites M1, M8, M17, M21, and MUNDF1 detected in the whole-blood samples of a patient with a transplanted heart. For each specimen we plotted, vs time, individual results for the parent CsA and each metabolite (by HPLC), the sum of these cyclosporines by HPLC, and the RIA results. For this patient, the concentration of parent CsA is about 68% of the RIA value; the sum of parent CsA and metabolites (by HPLC) is about 138% (range 112% to 154%) of the RIA value. Predominant metabolites include M17, M1, and M8. The "trough" sample at 12 h showed the concentration of M17 > CsA > M1 > M8; M21 was <16 μg/L. The concentration of M17, M1, and CsA decreased by 49%, 65%, and 81%, respectively, from the time of peak concentration to just before next dose. The concentration of M8 changed very little during these 12 h.

Study C. RIA and HPLC data for whole-blood samples from 56 patients were variable. Our comparisons of the data include: parent RIA vs CsA(HPLC) (Figure 3A), HPLC(sum of CsA + metabolites) vs HPLC(CsA) (Figure 3B), HPLC(sum of metabolites) vs RIA metabolites (Figure 3C), and RIA vs HPLC(sum of CsA + metabolites) (Figure 3D). The "RIA metabolites" value was obtained by subtracting the CsA(HPLC) result from the RIA result. Comparisons of M17 vs CsA(HPLC), M17 vs the sum of CsA + metabolites(HPLC), and M17 vs RIA (Figure 4) also showed no significant relationships. When M1, M8, M21, N203-218, and MUNDF1 were compared individually with parent CsA (Figure 5), results did not correlate well. Also, results from

---

**Fig. 3. Comparisons of analytical results for patients:** (A) RIA vs HPLC (parent CsA); (B) HPLC(sum of CsA + metabolites) vs HPLC(CsA); (C) HPLC(sum of metabolites) vs RIA(metabolites); (D) RIA vs HPLC(sum of CsA + metabolites)

S_yx represents the data scatter along the regression line and is equal to 1 SD in the y-direction at the mean concentration.

CLINICAL CHEMISTRY, Vol. 33, No. 10, 1987 1853
M17, M1, or M8 vs "RIA metabolites" [Figure 4 (right) and Figure 6] were unremarkable and suggest that mathematical manipulations of RIA data to determine a value representative of prevailing metabolites are inappropriate.

Discussion

Our data demonstrate that the RIA/HPLC ratio technique, often used to assess metabolite concentrations (14), is unreliable. For those samples tested in this study, the ratios of results from RIA/HPLC(CsA only) ranged from 1.2 to 20 (mean 4.39) with CV = 75%. The ratios of RIA/HPLC(sum CsA + metabolites) were less diverse (range 0.44 to 1.55, mean 0.96, CV = 24%); however, the heterogeneous populations of metabolites could not be accurately assessed with this mathematical manipulation. Most important, the poor correlation ($r = 0.222$, slope $= 20.7$, $y$-intercept $= 526 \mu g/L$, $S_{y/x} = 294 \mu g/L$) observed for RIA/HPLC(CsA) vs HPLC(sum of metabolites) (y vs x, respectively) further illustrates the error associated with the RIA/HPLC ratio technique.

The relative quantities of CsA and metabolites present in the 56 whole-blood samples varied significantly. Either CsA, M1, M8, or M17 predominated; M18 was minimal; and M25 and M26 were below the limits of detection. In 30% of the blood samples, concentrations of M8 > M17; 9% had concentrations of M1 > M17; 35% had concentrations of M1 and M8 each > M17; and 35% had concentrations of CsA > M17. Overall, concentrations ($\mu g/L$) of CsA ranged from 15 to 627, M17 from 23 to 717, M1 from <15 to 144, and M8 from <15 to 507; M21 was detected in 28% of the

Fig. 4. Comparisons of patients' results: (left) M17 vs CsA(HPLC); (middle) M17 vs sum CsA + metabolites(HPLC); (right) M17 vs RIA

Fig. 5. Comparisons of individual metabolite results vs CsA results for whole-blood samples from patients: (A) M1 vs CsA; (B) M8 vs CsA; (C) M21 vs CsA; (D) M203-218 vs CsA; (E) MUNDF1 vs CsA
samples, but only at concentrations less than 31 μg/L. M203-218 was detected in 41% of the samples, at concentrations ranging from <15 to 269 μg/L, and MUNDF1 was present in 50% of the samples, at <15 to 127 μg/L. These data substantiate the diversity in the relative amounts and types of cyclosporines in measured whole-blood samples collected 24-h post-dose. Results of the pharmacokinetic study further illustrate these dynamics and emphasize the importance of day-to-day consistency in time of dosing and specimen collection for uniform, interpretable results. We predicated our data on whole blood to minimize variability associated with sample handling for serum/plasma. Whether whole blood is the better sample remains to be determined.

In conclusion, we demonstrate that the analytical method must be specific to address accurately the diverse patterns of disposition of CsA and metabolites, irrespective of influences from sample type, handling protocol, dosing regimen, drug interactions, or type of organ transplanted. RIA was not useful for characterizing and quantifying the predominant metabolite(s) in a patient’s whole blood. To concomitantly profile CsA and each metabolite with one assay would make available to the clinician additional data for more effective dosing to maintain immunosuppression, possibly to lessen the chance of toxicity, and perhaps to lower the cost of CsA therapy. Early detection of metabolic differences of CsA in patients would permit adjustment of dosage in a more timely fashion, thereby minimizing episodes of rejection or toxicity. At present, interpretation of individual metabolite concentrations in blood remains unclear and further in-depth investigation is required. A specific HPLC procedure, inclusive of metabolites, may be a better tool for describing the interrelationships of CsA and metabolites in therapy and toxicity.

We thank R. Kim, D. deVos, J. Horwill, G. McManamy, J. Hunziker, J. Werbe, Linda Machmuller, and L. Liston for performing the RIA determinations, and G. Mittnacht for contributions to and preparation of both parts of the manuscript.

References