CLIN. CHEM. 33/12, 2225–2229 (1987)

Determination of Cyclosporine Concentrations with Monoclonal Antibodies

Horst F. Schran,[1] Thomas G. Rosano,[2] Alan E. Hassell,[1] and Michael A. Pelli[2]

We measured cyclosporine in whole blood from normal volunteers administered single oral doses of the drug and from two renal-transplant patients on immunosuppressive maintenance therapy, by liquid chromatography (**I**) and by radioimmunoassay with use of nonspecific polyclonal (**II**), specific monoclonal (**III**), and nonspecific monoclonal (**IV**) antibodies. Concentrations determined by **III** were equivalent to **I**, irrespective of cyclosporine dose, concentration, time after dose, or time after transplant. Concentrations determined by **II** and **IV** were consistently higher than those by **I**, owing to cross reactivity with metabolites. Ratios of values by **II** and **IV** to those by **I** increased from <1.5 to about 3–4 between 0.5 and 12 h after a single cyclosporine dose, owing to differences in rates of appearance and disappearance of cyclosporine and cross-reacting metabolites, though for the constant 12-h dose intervals in the two renal-transplant patients at steady state these ratios (most within the range 3–4) were relatively stable. Ratios of concentrations measured by **IV** to those by **II** (mean of 1.2 for single-dose data, most within the range of 1.2 to 1.5 at steady state) were unaffected by time after dose or time after transplant, suggesting that, despite certain cross-reactivity differences between the two nonspecific antibodies, results are proportional throughout therapy. We therefore propose that **III** and **IV** offer alternatives, respectively, to the currently used **I** and **II** for cyclosporine monitoring.

Additional Keyphrases: *therapeutic monitoring · renal-transplant patients · kinetic profiles · liquid chromatography · radioimmunoassay · polyclonal vs monoclonal antibodies*

Monoclonal antibodies (MAbs) show a highly restrictive epitope specificity as compared with polyclonal antisera. Taking advantage of this property, Quesniaux et al. (*1*) developed two types of MAbs, one reported to bind specifically to the immunosuppressive drug, cyclosporine, and the other nonselectively recognizing cyclosporine and several of its metabolites. The metabolite products, which arise via N-demethylation and other oxidative bioconversions of cyclosporine, maintain the cyclic undecapeptide structure of the parent drug (*2*) and therefore cross react with the polyclonal antibodies that are currently used for radioimmunoassay of the drug (*3*). The cross reactivity of metabolites in the polyclonal RIA results in two- to fourfold or higher concentration estimates of cyclosporine in patients' blood or plasma as compared with "high-performance" liquid chromatography (HPLC) methods, and this has contributed to the controversy surrounding the choice of methodology for cyclosporine monitoring (*4*).

The ratio of drug concentration determined by polyclonal RIA and HPLC may differ between patients, owing to inter-subject variability in metabolizing capability (*5*). Even though differences in this ratio may also occur for the same subject during a course of therapy as a result of differential absorption, bioconversion, and clearance rates of the parent drug or metabolites, for measurements taken at equivalent intervals in a stabilized patient at steady state the ratio should be constant (*6*).

Accordingly, as the first step in the evaluation of RIA methods employing the specific and nonspecific cyclosporine MAbs for therapeutic monitoring, we serially determined cyclosporine after single oral administrations of the drug at three dose levels for several subjects and compared these "profiles" with profiles obtained with the polyclonal RIA and HPLC assays. Any between-assay differences in measured concentrations as a function of the time after dosing should provide a sensitive index of selectivity differences between the antibodies and HPLC as well as among the antibodies.

To establish the relevance of these selectivity differences in the clinical setting, we also determined cyclosporine concentrations by HPLC, nonspecific polyclonal RIA, and specific and nonspecific MAb RIAs at various times post-transplant in renal-allograft recipients.

Materials and Methods

We determined profiles of cyclosporine concentration in whole blood for 10 normal, healthy men (ages, 18–43 y; mean, 31 y) who received a single dose of 350, 700, and 1400 mg of cyclosporine (Sandimmune Oral Solution; Sandoz Ltd., Basel, Switzerland). The study design was a replicated 3 × 3 Latin square, with a one-week "washout" interval between doses. Heparinized blood was collected immediately prior to dosing and 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, and 96 h after each dose. Complete sets of blood specimens were obtained from all 10 subjects. The 390 blood specimens were analyzed by HPLC (*7*) and by the polyclonal RIA (*3*). The results of these analyses have been previously reported (*6*).

We also analyzed all blood specimens with the specific and nonspecific MAb RIAs (Sandimmune-Kit, Sandoz Ltd.). Before analysis it was verified that no decomposition of cyclosporine or metabolites in blood had occurred during long-term (about three years) storage at −20 °C, as determined by re-analysis of selected specimens spanning a wide concentration range by the polyclonal RIA (data not shown). An interim analysis of these specimens after one year of storage had revealed no decomposition (*8*).

Cyclosporine analyses by MAb RIAs were preceded by sample pretreatment, involving a methanol precipitation step. To 950 μL of methanol we added 50 μL of the blood specimen. The tube was capped, the contents vortex-mixed, then centrifuged, and 50 μL of the supernatant fluid (equivalent to 2.5 μL of unknown specimen) was added to the RIA incubation mixture. The incubation mixture consisted of reagents identical to those used in the polyclonal RIA with the exception of the antiserum.

We prepared the cyclosporine standards according to the two-step dilution procedure described in the protocol for the polyclonal RIA, but using methanol instead of buffer for the second dilution step. For each subject we constructed a

[1] Drug Safety and Metabolism Division, Sandoz Research Institute, East Hanover, NJ 07936.
[2] Departments of Clinical Chemistry and Biochemistry, Albany Medical Center, New Scotland Avenue, Albany, NY 12208.
Received July 24, 1987; accepted September 3, 1987.

standard curve from data on appropriate dilutions of predose (blank) whole blood supplemented with cyclosporine.

All determinations were done in duplicate and calibrators containing known amounts of cyclosporine were co-analyzed in a blind sample protocol to test the reproducibility and accuracy of the MAb analyses.

We also analyzed whole blood obtained from two renal-allograft recipients who had received cyclosporine as the primary immunosuppressive agent. Cyclosporine (5 mg/kg per day in two doses given at 12-h intervals) was initiated when the patient had achieved good renal function. The cyclosporine dose was adjusted to maintain a trough (i.e., minimum) cyclosporine concentration in blood in the range of 150 to 400 μg/L as determined by the polyclonal RIA method. Concomitant therapy with methylprednisolone was given initially as a 300-mg bolus and then adjusted to 20 mg/day by 30 days post-transplant and 10 mg/day by 60 days. Azathioprine was initiated preoperatively and immediately tapered to 0.75 to 1.0 mg/kg and adjusted if the leukocyte count fell to 5×10^9/L. Whole blood for cyclosporine monitoring was collected just before the evening dose (trough) and was obtained daily while the patient was in the hospital, then less frequently on an outpatient basis in the absence of complications. For the present study, we analyzed blood samples during the first three months post-transplant by HPLC, polyclonal RIA, and the two MAb RIAs. The HPLC method used for analysis of patients' blood specimens has been previously described (*9*). The analyses for cyclosporine by the RIA methods were performed on whole blood as described above for the human-volunteer studies.

For the comparisons of the analytical methods we employed scatterplots, descriptive statistics, and paired *t*-tests, and performed regression analysis on concentration data and ratios at different dosages, times post-dose, or time post-transplant.

Results

Accuracy and reproducibility data for the MAb RIA analyses are summarized in Table 1. We obtained acceptable within- and between-run reproducibility in the course of the analyses, similar to or better than the variability reported for HPLC and polyclonal RIA (*10, 11*). For both laboratories, assay accuracy was such that any single-use duplicate determination would be within ±23% of the actual concentration at the 95% confidence level. Curves for the mean cyclosporine concentration in the blood of the 10 normal subjects administered single oral doses of the drug are shown in Figure 1. The curves obtained by HPLC and by the specific MAb RIA are nearly identical. The curves obtained either by nonspecific MAb RIA or polyclonal RIA indicate an overestimation of the parent-drug concentration as determined by HPLC. Figure 1 also reveals a small, though consistent, overestimation of cyclosporine concentrations by the nonspecific monoclonal as compared with the

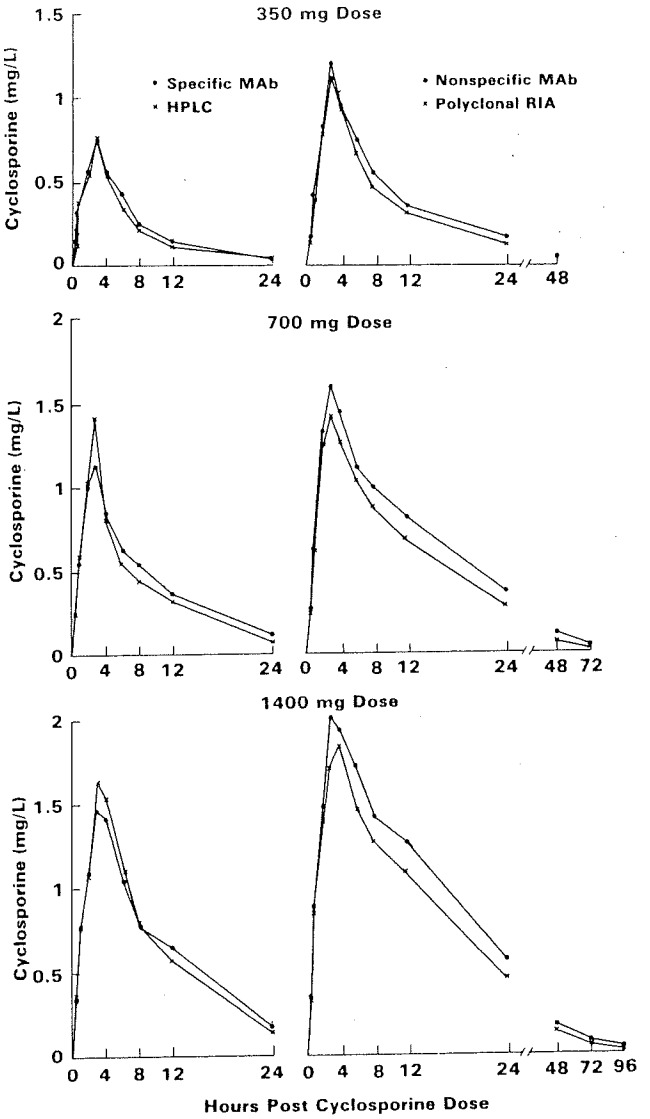

Fig. 1. Mean cyclosporine concentrations in blood (n = 10 subjects) after administration of single oral doses (350, 700, 1400 mg) to normal healthy male volunteers, as measured by HPLC, specific MAb, nonspecific MAb, and polyclonal RIAs

Values at $t > 24$ h shown only if majority of individual concentrations exceeded the minimum detectable concentration

Table 1. Accuracy and Reproducibility of Cyclosporine Analyses by MAbs

MAb	Actual concn, ng/mL	Concn found, ng/mL		Reproducibility (CV, %)	
		Grand mean[a]	Range[a]	Within-assay	Between-assay
Specific	100[b]	114	93–127	4.8	8.7
	100	104	91–117	4.2	5.8
Specific	400	411	337–478	1.9	11.0
	400	393	347–444	3.0	5.9
Nonspecific	100	120	109–127	1.3	4.6
	100	106	94–126	4.9	8.9
Nonspecific	400	447	425–466	1.8	2.7
	400	387	344–426	2.4	4.9

[a] For n = 10 (lab. no. 1) and n = 18 (lab. no. 2) assay runs with duplicate determinations. [b] The first-listed values are for lab. no. 1; the second-listed, for lab. no. 2.

polyclonal RIA. Prior studies of the polyclonal RIA have shown that cross reactivity with metabolites contributes to the large overestimate as compared with HPLC, and the smaller difference in concentration determinations between the polyclonal and nonspecific MAb RIA may also be due to cross reactivity differences (*10, 11*). The cross reactivity spectra previously elaborated in vitro for the polyclonal (*3*) and monoclonal (*1*) antibodies are consistent with our findings. Thus the nonspecific polyclonal and monoclonal antisera both cross react strongly with cyclosporine metabolites no. 1, 8, 17, and 18, although to differing degrees, whereas the specific monoclonal antiserum shows only slight or no cross reactivity with these compounds (*1, 3*).

Figure 2 compares concentration profiles obtained after a single oral dose of the drug, as measured by HPLC and polyclonal RIA in the present study, with total radioactivity measured in normal volunteers after a single oral dose of radiolabeled cyclosporine (*2*). The radioactivity curve, which represents concentrations of cyclosporine plus all metabolites, substantially exceeds the values (dose normalized) measured by the polyclonal RIA and, as expected, those measured by HPLC.

Scatterplots of individual data on concentration in whole blood for the 10 subjects (Figure 3) reveal a linear correlation for HPLC vs specific MAb RIA (Figure 3*A*) and for polyclonal RIA vs nonspecific MAb RIA (Figure 3*B*). The increasing scatter of concentrations <100 μg/L may be due to the fact that these approach the limit of detection of the analytical methods as used for the normal volunteer study, of 20–40 μg/L, 2 mL and 2–2.5 μL aliquots being used for

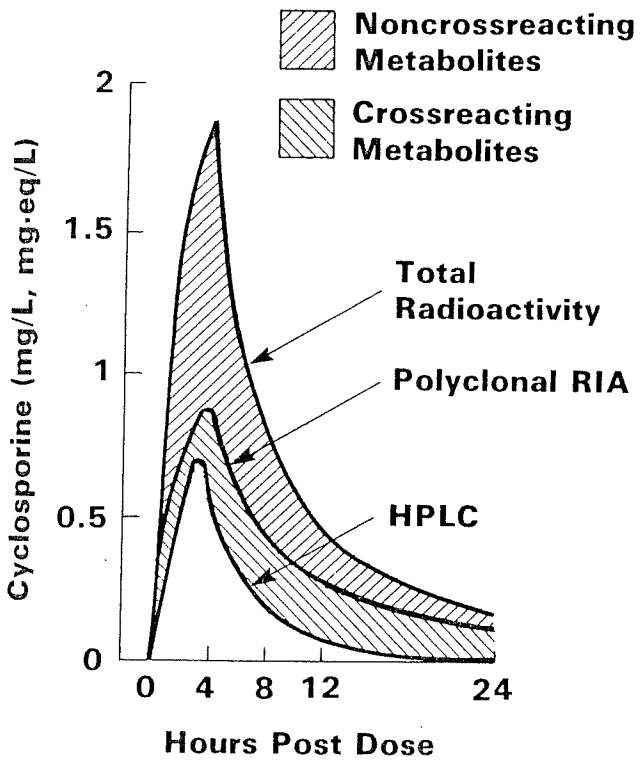

Fig. 2. Mean cyclosporine concentrations in blood (n = 10 subjects) after a 300-mg dose of cyclosporine, as measured by HPLC and polyclonal RIA (350-mg data of present study were adjusted by the factor 300/350) and mean concentrations of total radioactivity in blood (n = 6 subjects) after a 300-mg dose of [^{3}H]cyclosporine (data from ref. *2*)

Adjustment of concentrations is justified by dose proportionality of cyclosporine seen in this dose range (*6*)

HPLC and RIA, respectively (*3, 6*). Inspection of residuals for the linear regression plot in Figure 3*A* reveals that its deviation from unity slope, and therefore the significantly positive intercept, is caused by concentrations >1600 μg/L. These concentrations gave a response B/B_0 of <0.1; that is, they were outside the most-reliable portion of the RIA standard curve. Regression analysis on the concentration data between 0 and 1600 μg/L yielded a slope of 0.96 ±0.01.

As expected, in the comparison data for nonspecific MAb RIA vs HPLC (Figure 3*C*), a slope substantially different from unity and a large positive intercept were obtained. Table 2 summarizes the mean, range, and variance of all concentration data by the four assay methods. Even though the concentrations span a wide range, paired *t*-test comparisons revealed statistically significant (P <0.001) differences between all methods except for the comparison between HPLC and specific MAb RIA (P = 0.13).

Because of the probably transient nature of the cross-reacting metabolites that cause the overestimation with the nonspecific MAb RIA or polyclonal RIA as compared with HPLC, we evaluated the effect of time post-dose on the methods comparisons (Figure 4). It had no relevant effect on the concentration ratios for specific MAb RIA vs HPLC or nonspecific MAb RIA vs polyclonal RIA, although such an effect was clearly present with the ratio for nonspecific MAb RIA vs HPLC. Similarly, the dose, and therefore the concentration, had no effect on the ratio for specific MAb RIA vs HPLC or the ratio for nonspecific MAb RIA vs polyclonal RIA. For the latter comparison at 12 h after the cyclosporine dose the mean ratios (and ranges) for the 10 subjects dosed with 350, 700, and 1400 mg were 1.19 (0.97–1.52), 1.19 (0.97–1.53), and 1.19 (1.06–1.45).

The cyclosporine concentrations in blood determined in two renal-transplant recipients up to two months post-transplant are shown in Figure 5, which also summarizes the cyclosporine dose and serum creatinine concentrations during this time period. As was the case for the normal subjects, HPLC profiles are nearly superimposable with those obtained by the specific MAb RIA, while the profiles for the polyclonal RIA and the nonspecific MAb RIA show higher concentration estimates, owing to contributions from the metabolites. Figure 3*D* shows a scatterplot of the specific MAb RIA vs HPLC data. A slope near unity and zero intercept attests to the good correlation of results by these two methods for patients' blood samples.

Table 2 also compares the mean, range, and variance of the 46 cyclosporine estimates for the two renal-transplant patients' assays. Similar to the data on normal volunteers, statistically significant differences exist between all the methods except for the comparison between the HPLC and specific monoclonal RIA method (P >0.30). An analysis of the concentration ratios of nonspecific MAb RIA vs polyclonal RIA, HPLC vs specific MAb RIA, and nonspecific MAb RIA vs specific MAb showed no significant change or trend during the period of therapy for either patient.

Discussion

Cyclosporine monitoring, either by HPLC or polyclonal RIA, is now recognized as a useful adjunct to patient monitoring, and therapeutic ranges have been elaborated based on these two methods (*10, 11*). It is therefore desirable that new monitoring techniques be evaluated in parallel with these established methods. The cyclosporine MAbs were developed by Quesniaux et al. (*1*) to overcome difficulties caused by variations in binding specificity and avidity

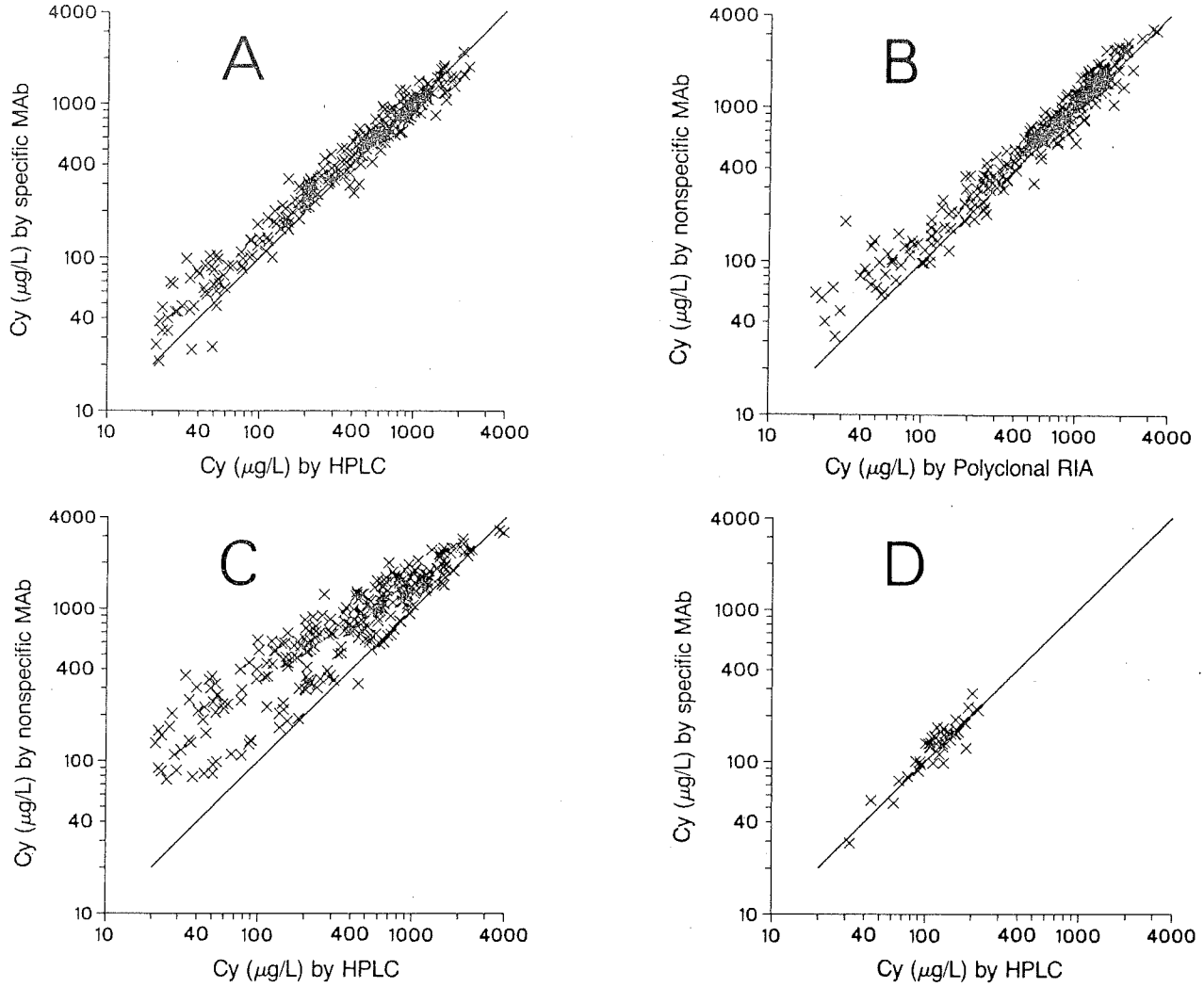

Fig. 3. Scatterplots of cyclosporine concentrations in blood of normal volunteers (*A, B, C*) and renal-transplant patients (*D*) as measured by HPLC and RIAs

Solid line is line of identify. Parameters for linear regression equations were:

Plot	Slope and std. error		Intercept (μg/L) and significance		r
A	0.87	0.01	76	$P < 0.001$	0.97
B	1.09	0.02	22	NS $P > 0.05$	0.96
C	1.19	0.03	222	$P < 0.001$	0.92
D	0.98	0.08	12	NS $P > 0.05$	0.88

Table 2. Methodologic Comparison of Serial Cyclosporine Monitoring in 10 Normal Volunteers and Two Renal-Allograft Recipients

Assay	Mean	Range	SD	n	
	Concn, μg/L				
Normal volunteers					
Polyclonal RIA	601	0–3255	613	390	
Nonspecific MAb RIA	674	0–3268	683	387	
Specific MAb RIA	426	0–3308	494	390	$P = 0.13$[a]
HPLC	434	0–3830	553	373	
Patients					
Polyclonal RIA	320	16–532	120	46	
Nonspecific MAb RIA	473	64–1072	197	46	
Specific MAb RIA	137	29–278	46	46	$P > 0.3$
HPLC	128	32–220	42	46	

[a] In the pairwise comparisons, all differences in the means were statistically significant ($P < 0.001$) except where indicated.

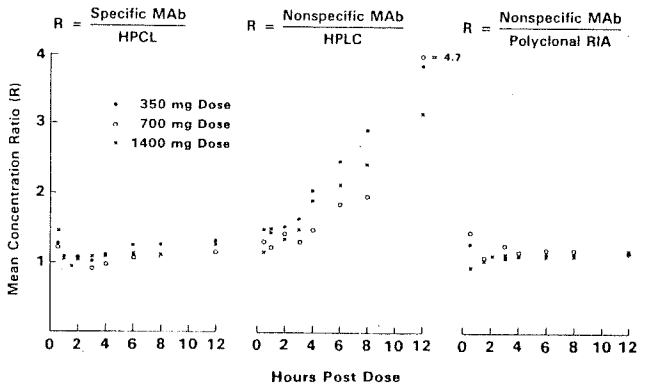

Fig. 4. Effect of time after cyclosporine dose on mean concentration ratios

Data at $t > 12$ h are not included because of higher frequency of concentrations = 0

observed in different bleedings of polyclonal antiserum, to provide restricted recognition patterns for parent drug and metabolites, and to develop monitoring methods that yield data comparable to current methods (*1*). The success of their effort is demonstrated by the correlation of cyclosporine concentration estimates between the respective MAbs and

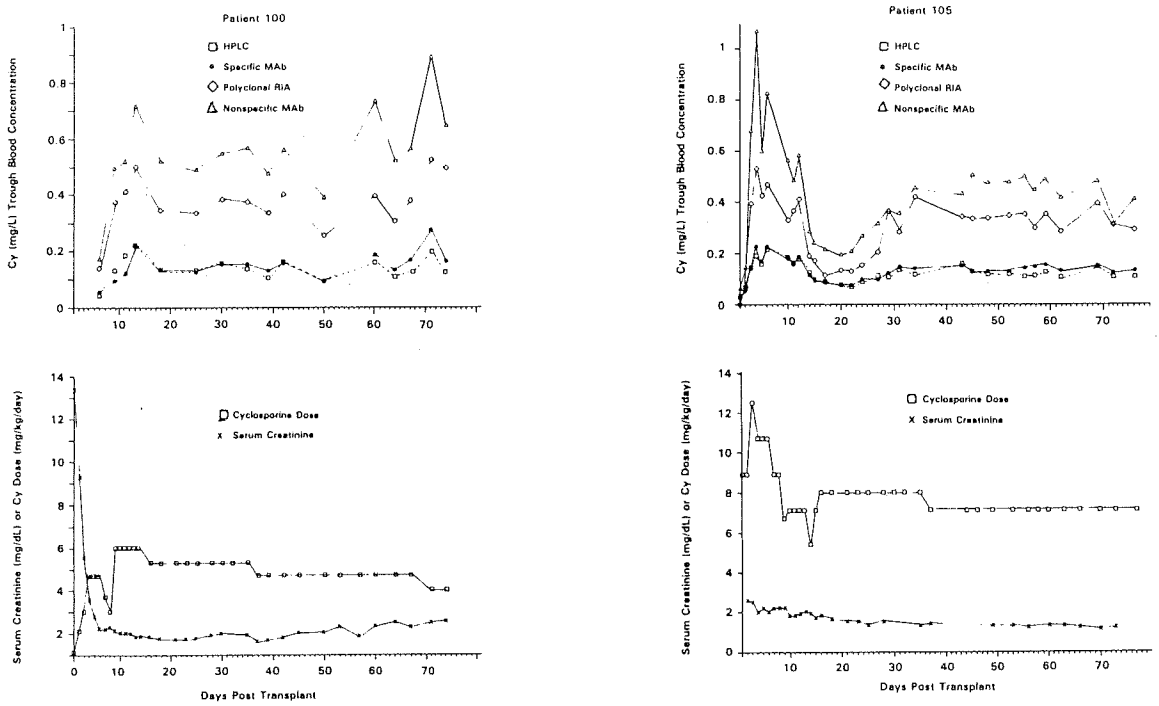

Fig. 5. Cyclosporine monitoring for two renal-allograft recipients by RIA and HPLC methods
Cyclosporine dose and results for serum creatinine monitoring are displayed in the lower panels

HPLC or polyclonal RIA in the blood specimens of normal volunteers given single oral doses of the drug. Figure 2, however, clearly shows that, after a single oral dose of cyclosporine, cross-reacting metabolites appear, as well as a considerable amount of circulating metabolites that do not cross react appreciably. Perhaps one or more of these metabolites might accumulate to such an extent upon multiple dosing of the drug that cross-reactivity-based differences could become significant and result in discrepancies between the analytical methods. Therefore, substantial equivalence between any two analytical methods must be confirmed in a broad array of patients and clinical situations. Excellent method comparability is demonstrated in our study of serial monitoring of two renal-transplant patients who experienced good renal and hepatic function throughout the study period. It has been shown, however, that the polyclonal RIA vs HPLC ratio can be particularly high in some patients with compromised liver and biliary function—the major excretory mechanism for cyclosporine (*12*). Parallel-method studies in cases of impaired liver function, nephrotoxicity, and graft rejection will be necessary to confirm the comparability of the current and new monitoring methods.

The application of MAbs for specific measure of cyclosporine represents an important technical and practical advance in therapeutic monitoring of this potent immunosuppressive agent. Additionally, the combination of the specific and nonspecific MAb RIA methods provides a powerful tool to investigate the influence of metabolism on immunosuppressive therapy and its side effects. We conclude that the good agreement between the analytical methods examined here warrants investigational use of these monitoring methods in a wide array of clinical situations.

References

1. Quesniaux V, Tees R, Schreier MH, et al. Potential of monoclonal antibodies to improve therapeutic monitoring of cyclosporine. Clin Chem 1987;33:32–7.

2. Maurer G, Lemaire M. Biotransformation of cyclosporine and blood distribution of its metabolites. Trans Proc 1986;6(Suppl 5):25–34.

3. Donatsch P, Abisch E, Homberger M, et al. A radioimmunoassay to measure cyclosporin A in plasma and serum samples. J Immunoassay 1981;2:19–32.

4. Robinson CA, Ketchum CH. Monitoring of cyclosporin A: is it possible? Ther Drug Monitor 1983;5:371–2.

5. Rosano TG, Freed BM, Cerilli J, et al. Immunosuppressive metabolites of cyclosporine in the blood of renal allograft recipients. Transplantation 1986;42:262–7.

6. Robinson WT, Schran HF, Barry EP. Methods to measure cyclosporine levels—high pressure liquid chromatography, radioimmunoassay, and correlation. Transpl Proc 1983;15(suppl 1 and 2):2403–8.

7. Smith HT, Robinson WT. Semi-automated high performance liquid chromatographic method for the determination of cyclosporine in plasma and blood using column switching. J Chromatogr 1984;305:353–62.

8. Schran HF, Hassell AE, Raskova J, et al. Acquired immune deficiency syndrome: no evidence of the presence of cyclosporine. Am J Med 1984;77:797–804.

9. Rosano TG, Freed GM, Pell MA, Lempert N. Cyclosporine metabolites in human blood and tissue. Transplant Proc 1986;18(Suppl 5):35–40.

10. Shaw LM, Bowers L, Demers L, et al. Critical issues in cyclosporine monitoring; report of the task force on cyclosporine monitoring. Clin Chem 1987;33:1269–88.

11. Schran HF, Robinson WT, Abisch E, et al. Bioanalytical considerations/ciclosporin. Prog Allergy 1986;38:73–92.

12. Burckart G, Starzl T, Williams L, et al. Cyclosporine monitoring and pharmacokinetics in pediatric liver transplant patients. Transplant Proc 1985;17:1172–5.