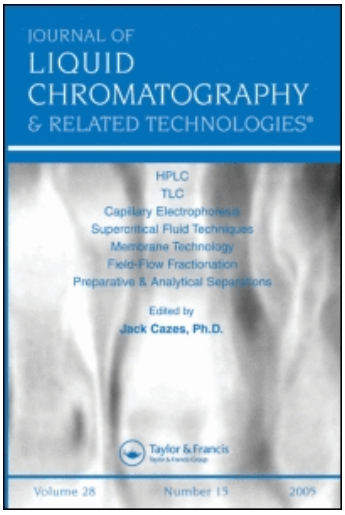

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ANALYTICAL METHODOLOGIES FOR CYCLOSPORINE PHARMACOKINETICS: A COMPARISON OF RADIOIMMUNOASSAY WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Radioimmunoassay (RIA) and high performance liquid chromatography (HPLC) with ultraviolet absorbance detection have been compared as potential analytical methods for cyclosporine pharmacokinetic studies. Cyclosporine concentrations were measured by each method in the serum and bile of cyclosporine-treated humans and in the plasma and urine of cyclosporine-treated dogs. While clearly affording greater assay sensitivity, RIA overestimates concentrations of parent cyclosporine, ostensibly because of cross-reactivity with cyclosporine metabolites. Thus, RIA-measured time course data result in underestimations of primary pharmacokinetic parameters, volume of distribution and clearance. Ratios of measured concentrations (RIA/HPLC) in human bile are significantly greater than those in serum, lending further evidence to the previously-suggested metabolite interference with RIA. Similar results were obtained from the urine of cyclosporine-treated dogs. Moreover, ratios (RIA/HPLC) in dog plasma rise and then decline as

a function of post-administration time, reaching a peak in approximately 8 hours. This might be consistent with the formation and disposition of metabolites. HPLC appears to be specific for parent cyclosporine. Thus, HPLC-measured time-course data afford more reliable estimates of cyclosporine pharmacokinetic parameters, although for certain studies, sensitivity might be less than adequate.

INTRODUCTION

While immunosuppression with cyclosporine (CsA) has led to major advances in the transplantation of solid organs (1,2,3) and bone marrow (4), individual variations in therapeutic responses and serum time course data (5,6) continue to complicate the management of therapy. Moreover, nephrotoxicity remains a major limitation (7). Although therapy might be improved by an increased understanding of CsA input and disposition, results of many pharmacokinetic studies (6,9,10,11,12, 13) might be questionable because of reservations about analytical methodologies for CsA in biological media. Numerous high performance liquid chromatographic (HPLC) methods have been reported (14-32). While such methods might be specific for parent CsA, sensitivity of ultraviolet absorbance detection is less than desirable. Radioimmunoassay (RIA) is a sensitive quantitative method for CsA in biological media. However, results of RIA analyses are thought to exceed true values because of cross-reactivity with CsA metabolites (17,21,22). The application of pharmacokinetics in the management of

CsA therapy can be realized only after the proper analytical method has been selected and validated. This paper presents an evaluation of RIA and HPLC as potential tools for CsA pharmacokinetic studies.

METHODS

Drug Administration and Sample Collection

Adult male mongrel dogs, weighing 14.1 - 19.1 kg and not previously exposed to CsA, were used in these studies. CsA (Sandimmune Injectable - Sandoz) was diluted with lactated Ringer's solution so that each member of the first group received the prescribed dose (10, 15, 20, 30 or 40 mg/kg) by short-term intravenous infusion in a similar volume (10 ml in 5 min). One additional dog received 20 mg/kg diluted to 250ml with lactated Ringer's solution and administered by constant-rate intravenous infusion over a 30 min period. Dogs were fasted overnignt prior to drug administration and food was withheld for an additional 24 hr. Serial blood samples (5 ml) were obtained over a 72 hr post-adminstration period by venipuncture using heparinized glass tubes. Plasma was separated immediately by centrifugation at 3000 rpm for 10 min. As CsA distributes into blood cells as an inverse function of temperature (33,34,35), samples were drawn and centrifuged immediately, permitting only a minimal decline in temperature. Although samples were centrifuged 10 min, separate

studies in this laboratory have shown that centrifugation at 3000 rpm affects complete separation of blood cells within 30 sec leaving plasma temperatures in excess of 31 degrees. Smith, et al. recently incubated whole blood from CsA-treated patients at 20 degrees and reported an approximate 20% decline in plasma concentrations in 20 min (34). In the present studies blood cells were in contact with plasma less than 1 min after samples were drawn (including sample handling and the first 30 sec of centrifugation) and plasma temperatures remained well above 31 degrees. Thus no significant distribution of CsA into blood cells was anticipated. From another group of 5 dogs receiving 20 mg/kg CsA by 30 min intravenous infusion, total urine was collected at intervals for up to 6 days. The volume was recorded at each collection. Plasma and urine were stored at -70 degrees until assay.

Two patients being maintained on oral CsA following liver transplantation were used in this study. Each patient received CsA (4mg/kg) by constant-rate intravenous infusion over a 4 hr period. Serial blood samples (5 ml) were obtained over 12 hr post-administration periods by venipuncture using heparinized glass tubes. Plasma was separated as previously described. Cumulative bile samples were collected from each patient through a T-tube which had been inserted during surgery. Plasma and bile samples were stored at -70 degrees until assay.

Radioimmunoassay

CsA concentrations in each sample were measured by RIA using cyclosporine RIA kits (Sandoz, Ltd., Bazel, Switzerland). Kit instructions were followed in detail.

High Performance Liquid Chromatography

CsA concentrations in each sample were measured by HPLC. Analytical samples of CsA and internal standard cyclosporin D (CsD) were supplied by Sandoz, Inc. (East Hanover, NJ). All solvents and reagents were analytical grade. Instrumentation consisted of a U6K injector, a model 6000A solvent delivery system, a μ-Bondapak C-18 column, and a model 481 variable wavelength UV absorbance detector (214 nm) [Waters Associates, Milford, MA]. Column temperature was maintained at 70 degrees using a circulating water pump-heater and a column heating jacket (Alltech Associates, Arlington Heights, IL). A mobile phase of acetonitrile/methanol/water (60:15:25) was passed through the column at a rate of 1.0 ml/min.

Prior to analysis CsA was removed from the biological matrix by solvent extraction. To each 20 ml screw-cap tube was added 100 μL internal standard (CsD, 12.5 μg/ml in methanol). To each calibration sample was added CsA (5 or 20 μg/ml in methanol) to simulate a concentration range of 25 to 1000 ng/ml. Methanol was evaporated to dryness under nitrogen. To each calibration sample was added 1 ml blank dog plasma (urine,

bile) and to each remaining tube was added 1 ml of plasma (urine, bile) containing an unknown quantity of CsA. Each tube was vortexed 45 sec. After the addition of 10 ml water-saturated ether, each tube was shaken 20 min, centrifuged 5 min at 3000 rpm, and the aqueous phase discarded. Following the addition of 2 ml hydrochloric acid (0.025N), each tube shaken 20 min, centrifuged 10 min at 3000 rpm and the aqueous phase discarded. Two ml sodium hydroxide (0.025 N) was added, each tube was shaken 20 min and centrifuged 10 min at 3000 rpm. The ether layer was transferred to a silanized 15 ml conical tube and evaporated to dryness under nitrogen. Following reconstitution in 100 µl methanol and vortex mixing for 60 sec, 25 µL was applied to the column. Peak height ratios (CsA/CsD) were used to calculate CsA concentrations. Calibration curves were prepared in plasma (urine, bile) over a concentration range of 50-1000 ng/ml. Samples exceeding 1000 ng/ml were diluted and reanalyzed. Between-day precision over a 5-day period was assessed by the daily analysis in duplicate of plasma samples containing CsA concentrations fo 50, 100, 250, 500, 750 and 1000 ng/ml. Extraction recovery for plasma over the same concentration range, was measured daily for 5 days by ratios of calibration curve slopes (extracted spiked plasma/CsA standards in methanol simulating 100% recovery).

RESULTS

Sample chromatograms from the analysis of plasma containing CsA are presented in Figure 1. Between-day coefficients of variation over a 5-day period at selected plasma CsA concentrations were 18.4% (50 ng/ml), 10.9% (100 ng/ml), 8.9% (250 ng/ml), 4.8% (500 ng/ml), 6.4% (750 ng/ml), and 5.6% (1000 ng/ml). Between-day extraction recoveries for an identical time period were 83.9±7.7% (mean ±SD, N=5). As precisions and extraction recoveries declined at lower concentrations (recovery at 50 ng/ml was ≤75%), sensitivity for this assay was limited to 50 ng/ml.

Plasma CsA concentration time course data from dogs receiving rapid intravenous doses are presented in Figure 2 along with ratios of RIA/HPLC plotted as a function of time. CsA concentrations in all samples were measurable by RIA. Samples obtained after 24 hr were below the limits of the HPLC detector. As evidenced by the time course data and observed in linear regression analysis, also presented in Figure 2, concentrations measured by RIA were substantially higher than those by HPLC. Ratios of RIA/HPLC, shown in Figure 2, tend to increase with time, peak at approximately 8 hr, and then decline. Secondary peaks, characteristic of enterohepatic recycling, were observed in time course data. Such peaks were more dramatic in HPLC findings, resulting in periodic troughs in ratio curves.

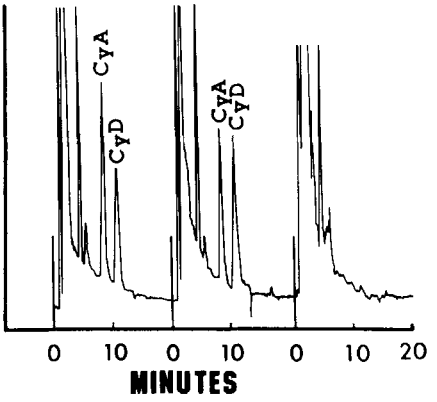

FIGURE 1. HPLC chromatograms from the analysis of: (a) plasma from a CsA-treated dog, (b) a calibration sample (200 ng/ml) and (c) blank plasma (0.01 aufs).

All rapidly-dosed dogs of these studies suffered toxic effects, the most common being vomiting and diarrhea. While present data do not conclusively attribute such toxicities to CsA, it was observed that a dose of 30 mg/kg produced more severe toxicities and following 40 mg/kg the dog was lethargic for several hours. Subsequent studies revealed that further dilution of the formulation followed by a slower infusion rate greatly reduced the severity of toxicities. A dose of 20 mg/kg in 250 ml infused over a 30 min period produced no significant toxicities. Absence of gastrointestinal toxicities in this dog might have permitted increased reabsorption of parent CsA, accounting for the longer HPLC-measurable time course.

TABLE 1

Urine Data From CsA-treated Dogs

Dog	Collection Interval (hr)	Volume (ml)	HPLC (ng/ml)	RIA (ng/ml)	RIA/HPLC
1	0-25	275	430.3	8093	18.8
	25-49	450	73.8	2259	30.5
	40-58	150	61.5	1918	30.9
	58-74	525	28.7	730	25.2
	74-96	675	24.6	476	19.0
2	0-48	600	394	4978	12.6
	48-97	425	39.6	744	18.6
	97-119	435	25.4	361	14.4
	119-143	245	0	239	
3	0-24	225	118.8	5097	42.8
	24-48	230	68.7	1813	26.3
	48-72	165	40.6	761	18.6
	72-120	315	41.8	405	9.6
4	0-10	7	196.7	3902	19.8
	10-24	250	418	2847	6.8
	24-50	14	49.2	2074	42.3
	50-75	250	49.2	1533	31.3
5	0-35	350	245.9	4878	19.8
	35-50	19	73.8	2757	37.3
	50-72	375	49.2	1428	29.2
	72-81	125	32.8	535	16.3

Table 1 presents urine data from dogs receiving 20 mg/kg CsA by 30 min infusion. Ratios (RIA/HPLC) far exceed those in plasma. While no consistency in the ratios as a function of time was observed, variations in urine volumes and collection intervals might mask such trends.

CsA concentration time course data from human plasma and bile are presented in Table 2. At the adminis-

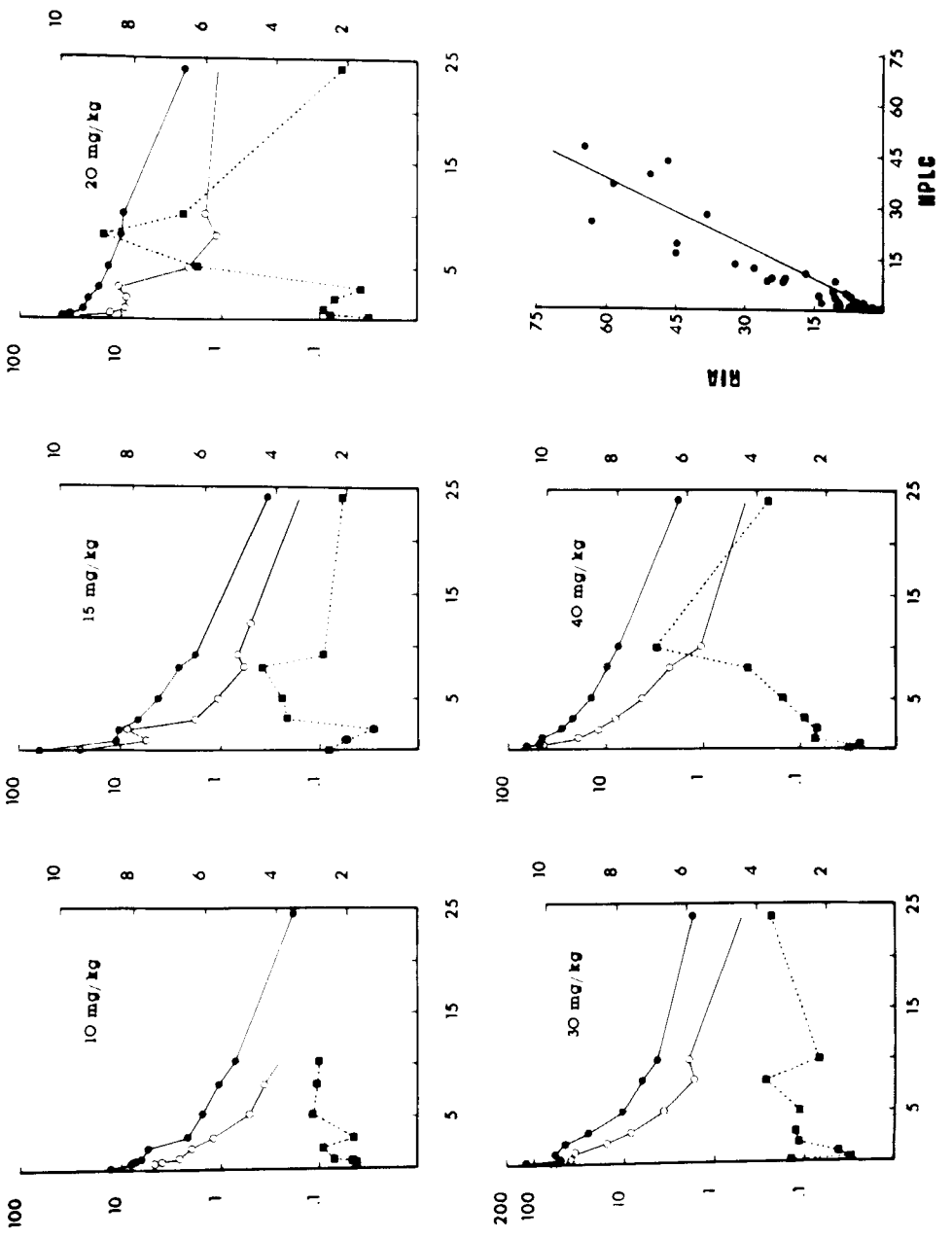

FIGURE 2. Plasma CsA concentration time course data measured by RIA (●) and HPLC (○) compared with time course of RIA/HPLC ratios (■). [Left ordinate = semi-log plot of concentrations in μg/ml. Right ordinate = ratios of RIA/HPLC.] Linear regression analysis comparing RIA with HPLC measurements μg/ml is presented at the lower right (slope = 1.79) (38).

TABLE 2

Data From CsA-treated Patients (Pt1, Pt2)

PLASMA

t (hr)	HPLC (ng/ml)		RIA (ng/ml)		RIA/HPLC	
	Pt1	Pt2	Pt1	Pt2	Pt1	Pt2
1	171.3	101.9	222	167	1.3	1.6
2	185.2	120.4	377	235	2.0	2.0
3	189.8	120.4	369	277	1.9	2.3
end infusion	143.5	199.1	387	380	2.7	1.9
1	46.3	51.0	486	238	10.5	4.7
2			441	195		
4			415	140		
6			332			
12			254	55		

BILE

t (hr)	HPLC (ng/ml)		RIA (ng/ml)		RIA/HPLC	
	Pt1	Pt2	Pt1	Pt2	Pt1	Pt2
1	122	306.9	3881	3798	31.8	12.4
2	752	480.0	4326	3745	5.8	7.8
3	894		4752		5.3	
end infusion	736	500	4384	4000	6.0	8.0
1	833	1061	4359	6261	5.2	5.9
2	918	1098	4499	5056	4.9	4.6
4	240	455	2776	4732	11.6	10.4
6	134	431	2925	5276	21.8	12.2
12	756	366	4562	5503	6.0	15.0

tered doses CsA concentrations in plasma were not detectable by HPLC for more than one hour after termination of infusion. Concentrations were measurable by RIA in all samples. As anticipated, CsA concentrations in the bile were much higher than those in plasma. Moreover, data presented in Table 2 show that ratios of RIA/

HPLC were far larger in bile than in plasma. As the liver is the major route of CsA clearance and CsA metabolites have been identified in bile (36,37), increased metabolite interference might be anticipated in the RIA analysis of bile samples.

DISCUSSION

RIA very clearly affords greater assay sensitivity although present data strengthen previously-reported concerns about specificity (17,21,22). All present data strongly suggest that large RIA-measured CsA concentrations are in fact due to cross-reactivity with metabolites. The rise and decline in RIA/HPLC ratios as a function of post-administration time in the dogs might be consistent with metabolite formation and disposition. Moreover, excessively large RIA/HPLC ratios in human bile, relative to plasma, were not surprising, as the liver is the principal organ responsible for CsA clearance. However, such large ratios in dog urine were not anticipated and tend to suggest that renal clearance of CsA metabolites might be significant.

As a tool for pharmacokinetic studies RIA can only measure the time course of CsA plus detectable metabolites. Primary pharmacokinetic parameters, clearance (Cl) and volume of distribution (V) are calculated as follows:

$$Cl = \frac{Dose}{(AUC)}$$

$$V_{area} = \frac{Dose}{\lambda_1(AUC)}$$

where AUC is the area under the CsA concentration time course curve in the reference fluid (blood, plasma) of the subject and λ_1 is the negative slope of the terminal linear phase. The secondary parameter biological half-life is then determined as:

$$t_{1/2} = \frac{0.693}{\lambda_1}$$

Clearly, RIA-measured data would overestimate AUC, resulting in underestimations of Cl and V_{area}. While a correct biological half-life might be determined using RIA-measured data, this parameter is probably less important than the primary parameters as a change in distribution or clearance might alter half life.

$$t_{1/2} = 0.693 \frac{V_{area}}{Cl}$$

While specificity appears to have been achieved, sensitivity remains the major limitation of HPLC. An assessment of CsA pharmacokinetics in patients, especially following low doses, might be difficult. Although higher concentrations than those of the present study have been reported in humans (8), an adequate characterization of the time course curve using HPLC will often be restricted because of assay sensitivity. As shown in Figure 3, however, the time course of CsA in

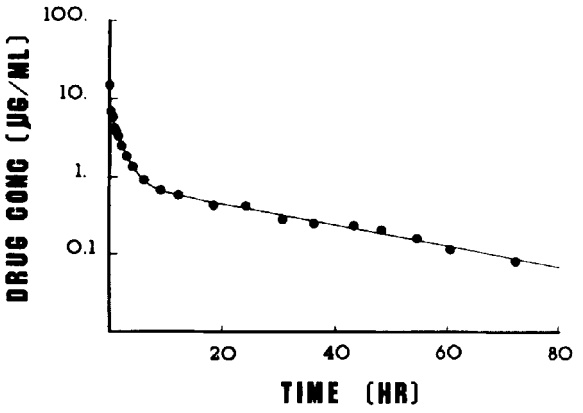

FIGURE 3. CsA plasma concentration time course data from a dog following the administration of 20 mg/kg by 30-min constant-rate infusion.

the plasma of a dog receiving 20 mg/kg by 30 min constant-rate infusion can be followed for 72 hr with excellent characterization of the entire curve. Thus, while HPLC might be suitable for animal studies, the lower doses administered to humans will too often leave a major portion of the time-course curve falling below assay sensitivity.

CsA therapy is often monitored using trough concentrations and these frequently fall below the sensitivity of HPLC. While troughs can usually be detected by RIA, these represent parent drug plus detectable metabolites. Although several metabolites have been identified (36,37), the therapeutic activity and toxicity of each has not been determined, and thus the utility of RIA

data in therapeutic monitoring remains in question. Safe and effective CsA therapy will require an increased knowledge of CsA pharmacokinetics and an effective system for therapeutic monitoring. This can only be accomplished after the development of analytical methodology which is specific for parent CsA in biological media and sufficiently sensitive to afford a realistic characterization of the time course of CsA in biological systems.

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REFERENCES

1. Merton, R. M., White, D. J. G. and Thru, S., N. Engl. J. Med., 310, 148, 1984.

2. Griffith, B. P., Hardesty, R. L. and Deeb, G. M., Ann. Surg., 196, 324, 1983.

3. Iwatsui, S., Starzl, T. E. and Shaw, B. W., Transplantation, 36, 641, 1983.

4. Deeg, J., Exp. Hematol., 10 (Suppl. 11), 48, 1982.

5. Kahan, B. D., Van Buren, C. T., Lin, S., Yoshinari, O., Agastino, G., LeGrue, S. J., Bioleau, M., Payne, W. D. and Kerman, R. H., Transplantation, 34, 36, 1982.

6. Kahan, B. D., Reid, M. and Newberger, J., Transplant. Proc., 15, 446, 1983.

7. Klintmalm, G., Bohman, S. O., Sundelin, B. and Wilczek, H., Lancet, 2, 950, 1984.

8. Beveridge, T., Gratwohl, A., Michot, F., Niederberger, W., Nuesch, E., Nussbaumer, K., Schaub, P., and Speck, B., Curr. Therap. Res., 30, 5, 1981.

9. Follath, F., Wenk, M. and Vozeh, S., Clin. Pharmacol. Ther., 34, 638, 1983.

10. Wood, A. J., Maurer, G., Niederberger, W. and Beveridge, T., Transplant. Proc., 15, 2409, 1983.

11. Newberger, J. and Kahan, B. D., Transplant. Proc., 15, 2413, 1983.

12. Kennedy, M. S., Yee, G. C., Deeg, H. J., Storb, R. and Thomas, E. D., Transplant. Proc., 15, 2416, 1983.

13. Nooter, K., Schultz, F. and Sonneveld, P., Res. Commun. Chem. Pathol. Pharmacol., 43, 407, 1984.

14. Niederberger, W., Schaub, P. and Beveridge, T., J. Chromatogr., 182, 454, 1980.

15. Sawchuk, R. J. and Cartier, L., Clin. Chem., 27, 1368, 1981.

16. Allwood, M. C. and Lawrence, R., J. Clin. Hosp. Pharm., 6, 195, 1981.

17. Abisch, E., Beveridge, T. and Gratwohl, A., Pharm. Weekbl., 4, 84, 1982.

18. Yee, G. C., Gmur, D. J. and Kennedy, M. S., Clin. Chem., 28, 2269, 1982.

19. Leyland-Jones, B., Clark, A., Kreis, W., Dinsmore, R., O'Reilly, R. and Young, C. W., Res. Commun. Chem. Pathol. Pharmacol., 37, 431, 1982.

20. Nussbaumer, K., Niederberger, W. and Keller, H. P., J. High Resol. Chromatogr. Commun., 5, 424, 1982.

21 Carruthers, S. G., Freeman, D. J., Koegler, J. C., Howson, W., Keown, P. A., Laupacis, A. and Stiller, C. R., Clin. Chem., 29, 180, 1983.

22. Robinson, W. T., Schran, H. F. and Barry, E. P., Transplant. Proc., 15, 2403, 1983.

23. Smith, H. T. and Robinson, W. T., J. Chromatogr., 305, 353, 1984.

24. Lensmeyer, G. L. and Fields, B. L., Clin. Chem. 31, 196, 1985.

25. Kabra, P. M., Wall, J. K., Blanckaert, N., Clin. Chem., 31, 1717, 1985.

26. Bowers, L. D. and Mathews, S. E., J. Chromatogr., 333, 231, 1985.

27. Garraffo, R. and Lapalus, P., J. Chromatogr., 337, 416, 1985.

28. Hamilton, G., Roth, E., Wallisch, E. and Tichy, F., J. Chromatogr., 341, 411, 1985.

29. Gmur, D. J., Yee, G. C. and Kennedy, M. S., J. Chromatogr., 344, 422, 1985.

30. Joosthuizen, M. M., Jacobs, D. R. and Myburgh, J. A., Ann. Clin. Biochem., 22, 402, 1985.

31. Takada, K.,Shibata, N., Yoshimura, H., Yoshikawa, H. and Muranishi, S., Res. Commun. Chem. Pathol. Pharmacol., 48, 369, 1985.

32. Shihabi, Z. K., Scard, J. and David, R. M., J. Liq. Chromatogr., 8, 2641, 1985.

33. Niederberger, W., Lemaire, M., Maurer, G., Nussbaumer, K. and Wagner, O., Transplant. Proc., 15, 2419, 1983.

34. Smith, J., Hows, J. M. and Gordon-Smith, E. C., Transplant. Proc., 15, 2422, 1983.

35. Atkinson, K., Boland, J., Britton, K. and Biggs, J., Transplant. Proc., 15, 2430, 1983.

36. Maurer, G., Loosli, H. R., Schreir, E. and Keller, B., Drug Metab. Dispos., 12, 120, 1984.

37. Hartman, N. R., Trimble, L. A., Vederas, J. C. Jardine, I., Biochem. Biophys. Res. Commun., 133, 964, 1985.

38. Buice, R. G., Gurley, B. J., Stentz, F. B., Sidhu, P., McClellan, T. and Williams, J. W., Transplantation, 40, 483, 1985.