Conversion of Cardiac and Liver Transplant Recipients From HPLC and FPIA (Polyclonal) to an FPIA (Monoclonal) Technique for Measurement of Blood Cyclosporin A

James H. McBride,* Steaven Kim, Denis O. Rodgerson, and Ana Reyes

Clinical Chemistry Laboratory, Department of Pathology and Laboratory Medicine, UCLA School of Medicine, Los Angeles, California

In an effort to replace HPLC and FPIA (polyclonal) for whole blood determination of Cyclosporin A (CsA), this study examined the application of FPIA (monoclonal) in patients post cardiac and liver transplantation. The assay had a minimum detectable dose of 15 µg/L, an overall recovery of 97% and was linear to 1200 µg/L, and gave inter-assay precision values of < 5% (CV). On comparing FPIA (monoclonal) and HPLC for 59 cardiac transplant recipient blood samples, a correlation of $FPIA (monoclonal) = 1.30 (HPLC) + 36.34$, $r = 0.96$ was obtained. With liver transplant samples ($n = 348$), the correlation was

$FPIA (monoclonal) = 1.21 (HPLC) + 42.15$, $r = 0.98$. Correlation on 131 cardiac transplant recipients gave $FPIA (monoclonal) = 0.31 FPIA (polyclonal) + 43.97$, $r = 0.68$. It is concluded that when converting from HPLC to FPIA (monoclonal) a positive bias of 21%–30% is observed, and in replacing FPIA (polyclonal) with FPIA (monoclonal), a negative bias of 50%–69% is seen with liver and cardiac patients respectively. These data indicate that therapeutic ranges should be re-established or adjustments in CsA dosing would be necessary. J. Clin. Lab. Anal. 12:337–342, 1998. © 1998 Wiley-Liss, Inc.

Key words: transplantation; immunosuppressive drugs; therapeutic drug monitoring; intermethod comparison; fluorescence polarization immunoassay

INTRODUCTION

Appropriate therapeutic drug monitoring (TDM) and, hence, adequate regulation of immunosuppressive therapy are essential aspects in the clinical management of the transplant patient. With the advent of cyclosporine (Cyclosporin A, CsA), both cardiac and liver transplantations have now become routine procedures in most major medical centers throughout the world and although other newer immunosuppressive agents such as tacrolimus (FK-506) are now approved by the FDA, CsA is still widely used.

CsA is a cyclic undecapeptide of fungal origin with unique immunosuppressive properties. Administration of the drug has not been without complications; high doses have resulted in nephrotoxicity and hepatotoxicity and other side effects which, in turn, have presented problems in cardiac and liver transplant recipients. Thus the need to monitor CsA whole blood concentrations is widely accepted due to the drug's narrow therapeutic window, the intermittent variation in bioavailability, and the variable metabolism of the drug by patients and especially by transplant type (1,2).

With the administration of CsA in cardiac and liver transplant recipients, acute renal hemodynamic changes have included reduced renal flow, afferent arteriolar vasoconstriction, a decreased glomerular filtration rate, and increased renal

vascular resistance. Unless CsA dosages are reduced in accordance with values for the drug reported by the laboratory, there is a possibility that hyalinization of renal afferent arterioles may occur (3,4).

With regard to nephrotoxicity and hepatotoxicity, the issue of the metabolites of CsA has been controversial. To date, more than 30 metabolites of the drug have been isolated by liquid chromatography, with fifteen of these being characterized by structure (5). Some metabolites AMIC9 and AM19 are associated with nephrotoxicity in the early post-operative stage in liver transplant recipients (6). Recently it has been reported that the clinical importance of CsA metabolites is questionable and that in most transplant situations, determination of metabolites, particularly in renal transplant recipient's blood in addition to that of the parent drug, is not warranted (7).

Analytical methods for CsA based on HPLC and on immunoassays with polyclonal and monoclonal antibodies have

*Correspondence to: James H. McBride, Clinical Chemistry Laboratory, Department of Pathology and Laboratory Medicine, UCLA School of Medicine, Los Angeles, CA 90095-1732. E-mail: jmcbride@pathology.medsch.ucla.edu

Received 5 March 1998; Accepted 26 April 1998

been developed and provide the means to measure CsA concentrations in whole blood in a variety of transplant types (8). In patients receiving cardiac and liver transplants, it has been common clinical practice to determine CsA by FPIA (Abbott Labs, Chicago, IL) which includes an antibody developed in sheep that, for the purposes of this investigation, will be referred to here as a polyclonal antibody since it reacts with both the parent drug and its main metabolites. Also in major transplant centers, in-house HPLC techniques have been developed which detect CsA parent drug and are capable of separating possible interfering metabolites (9). In an effort to specifically measure CsA by immunoassay as an alternative to HPLC, an FPIA technique utilizing a monoclonal antibody has been developed and applied in the monitoring of patients who have received cardiac and renal transplants (10).

In the field of organ transplantation, cost containment is now a major issue with clinicians challenging the laboratory to provide a single properly standardized CsA assay which will meet the majority of clinical needs. Further, laboratories have called for establishment of CsA assays that have been thoroughly validated and which not only meet clinical requirements but also are useful in future pharmacokinetic studies (11).

The purpose of this study was to consider the conversion of cardiac and liver transplant recipients from CsA as measured by HPLC and FPIA (polyclonal antibody) to a FPIA (monoclonal antibody) technique. With this approach, decreased turnaround time in the reporting of laboratory data could be achieved with a realized cost savings, while accomplishing standardization with other national transplant centers.

MATERIALS AND METHODS

Clinical Specimens

All blood samples were collected into tubes containing EDTA as anticoagulant. A total of 190 samples were obtained from cardiac transplant recipients and 348 samples were obtained from liver transplant recipients and studied over a period of ten weeks. Any samples not analyzed on receipt were maintained at 4°C with all assays performed within 72 hr.

Precision studies and quality control were conducted using whole blood CsA samples supplied by Abbott Labs for FPIA polyclonal and monoclonal assays and in-house controls for HPLC.

CsA by HPLC

In this study, a CsA HPLC procedure was a modification of a method previously described (9). The procedure was as follows: 1ml of patient's sample or control was treated with 3ml of an internal standard solution containing cyclosporin D (CsD; 100µg/L) in methanol, acetonitrile, and water (50/30/20, by vol) and zinc sulfate (50g/L).

After vortex-mixing for 1 min, the samples were centri-

fuged ($4,000 \times g$ for 5 min) and the supernatants transferred to Bond Elut C₁₈ bonded-phase extraction columns supplied by Analytichem International (Harbor City, CA). Extraction columns were pretreated with 95% ethanol and used immediately without becoming dry.

Contaminants on the extraction columns were eluted with 5ml of a solution containing acetonitrile and water (50/50 by vol) and cyclosporines eluted with 0.4 ml of 950ml/L ethanol. After addition of 0.2ml of de-ionized water to each eluate, lipid compounds were removed by a 0.7ml hexane wash which was removed and 100µL of the sample were assayed for CsA by isocratic chromatography. The chromatographic step was performed at 70°C with a solvent flow rate of 0.8ml/min on a reversed-phase C₁₈ column (Waters Inc., Milford, MA). The mobile phase used was acetonitrile/water (70/30, by vol) and the absorbance of the column eluate was monitored at 210nm. Concentrations of CsA were derived by calculation of the peak height for CsA and CsD and interpolation from a standard curve. Using this technique, CsA values between 10 and 25µg/L were reported to the transplant units as "< 25µg/L" and values < 10µg/L were considered "none detected." With frequent changes of the guard column on the HPLC system, up to 1,500 samples could be analyzed before changing the analytical column. The entire clinical study was conducted using the same analytical column conditions and when not in use, the column was maintained at 70°C with the passage of mobile phase at a rate of 0.2ml/min.

CsA by FPIA

The FPIA monoclonal kit for CsA was obtained from Abbott Laboratories and the assay was conducted similarly to the whole-blood polyclonal FPIA as previously described (12). In both assays, 150µL of whole blood were treated with 50µL of solubilizing agent containing surfactant and a protein-precipitating reagent (zinc sulfate in methanol), centrifuged, then the supernatant was analyzed by fluorescence polarization using the Abbott TDx (13). For both procedures, the TDx analyzer system provides calibrators and controls, pretreatment solution, CsA antisera (sheep and mouse) and a CsA-fluorescein tracer. The crossreactivity of CsA metabolites in the FPIA monoclonal assay was studied by adding metabolites of CsA notably AM1(M17), AM1_c(M18), AM4N(M21), AM9(M1) and AM19(M8) in the concentration range 200 to 500µg/L to a pool of whole blood obtained from liver transplant recipients having a CsA concentration of 300µg/L as determined by HPLC.

CsA Standardization

CsA standards (25, 50, 100, 150, 200, 300, 400, 500, 750, and 1,000µg/L) were prepared by addition of 25mg of CsA to 950ml/L ethanol to give a 250mg/L stock standard solution. This solution was further diluted in ethanol to obtain a concentration of 2.5mg/L and amounts of this standard were added

to donor whole blood to give the stated concentrations of CsA. As a stabilizer, Tween 20 surfactant (0.5ml/L) was also added to the prepared standards which were verified by HPLC.

Statistical Analysis

Throughout the study, unweighed regression analysis was used to compare results by HPLC and those obtained by FPIA (monoclonal) for CsA on 59 blood samples received from cardiac transplant recipients and 348 liver transplant recipients, all covering the analytical range of 10 to 1,400 ng/mL. As liver transplant physicians requested that the group of 348 patients be divided into CsA values of < 150ng/ml and > 150ng/ml (150ng/ml had been established as the upper limit of the therapeutic drug level), correlations were also established for these groups.

Correlations were also derived for 131 cardiac transplant blood samples between FPIA (polyclonal) and the FPIA (monoclonal) technique. When studying FPIA (polyclonal) and FPIA (monoclonal) correlations, the liver transplant group was further subdivided to study values obtained from pediatric patients ($n = 63$), and both pediatric and adult liver transplant CsA results were combined ($n = 300$).

RESULTS

Analytical Performance

In-house CsA standards were prepared in a whole blood matrix and analyzed by HPLC and both FPIA for CsA methods using the calibrators supplied in the kits. When comparing FPIA (polyclonal) calibrators with in-house CsA standards, an overall recovery of 97% was obtained and intra-assay precision gave $\pm 2CV$ of 7.1% and 5.3% ($n = 20$) at CsA concentrations of 122 and 424 $\mu\text{g/L}$, while inter-assay precision gave $\pm 2CV$ of 9.3% and 5.8% ($n = 25$). Recovery of the FPIA (monoclonal) calibrators gave an overall recovery of 97% when compared to in-house standards and intra-assay precision resulted in $\pm 2CV$ of 6.4% and 5.0% ($n = 20$) at CsA concentrations of 122 and 424 $\mu\text{g/L}$, while inter-assay precision gave $\pm 2CV$ of 9.5% and 5.7% ($n = 25$) respectively.

Sensitivity and Linearity

The sensitivity (detection limit) of the HPLC method was 20 $\mu\text{g/L}$ and the standard curve was linear over the range 20–1,200 $\mu\text{g/L}$ of CsA. The FPIA (polyclonal) assay was linear over the range 15–1,200 $\mu\text{g/L}$ and sensitive to a level of 15 $\mu\text{g/L}$ while the FPIA (monoclonal) assay had similar sensitivity and linearity characteristics. In all studies, linearity was established using a pool of patient whole blood having a concentration of CsA up to 1,500 $\mu\text{g/L}$ as verified by HPLC. Linearity dilutions were performed using whole blood obtained from healthy donors. Analysis of variance of the calculated concentrations corrected by dilution factor demonstrated that the dilution curves for each of the

CsA methods were related in linear manner over the range between the initial concentrations and the highest diluted sample ($r = 1.00$) in all studies).

Crossreactivity Studies

In this study, crossreactivity in the FPIA monoclonal antibody assay with cyclosporine metabolites was 25.2% with AM9(M1) and 10.6% with AM1(M17). Other metabolites AM1_c(M18), AM4N(M21), and AM19(M8) did not react significantly in the assay. For the FPIA polyclonal assay, metabolites AM9(M1), AM1(M17), AM1_c(M18), AM4N(M21), AM19(M8), AM14N(M25), AM1A^c(M203-218), and MUNFI have been reported to crossreact 19%, 103.5%, 78.8%, 66.9%, 13.7%, 62.6%, 60.5%, and 27.0%, respectively (7).

Comparison of Methods

On comparing HPLC to FPIA (monoclonal) for 59 cardiac transplant recipients, linear regression analysis yielded FPIA (monoclonal) = 1.30 (HPLC) + 36.34, $r = 0.96$ and the results are shown in Figure 1. In the case of 348 samples analyzed by both HPLC and FPIA (monoclonal) techniques, a correlation equation of FPIA (monoclonal) = 1.21 (HPLC) + 42.15, $r = 0.98$ for liver transplant recipients was obtained and the results are shown in Figure 2.

On subdivision of the liver transplant data into values of < 150ng/ml and > 150ng/ml, the following correlations resulted: for values < 150ng/ml, FPIA (monoclonal) = 1.53

Fig. 1. Correlation study between HPLC and FPIA (monoclonal) methods for cyclosporin A in whole blood from 59 cardiac transplant recipients.

Fig. 2. Correlation study between HPLC and FPIA (monoclonal) methods for cyclosporin A whole blood from 348 liver transplant recipient samples.

(HPLC) + 8.88, $r = 0.88$ ($n = 184$), and for values > 150 ng/mL, FPIA (monoclonal) = 1.17 (HPLC) + 56.85 , $r = 0.98$ ($n = 164$). These data are given in Figures 3 and 4.

When studying a correlation between FPIA (polyclonal)

Fig. 3. Correlation study between HPLC and FPIA (monoclonal) methods for cyclosporin A in whole blood in liver transplant recipient samples ($n = 184$) where HPLC gave values < 150 µg/mL.

Fig. 4. Correlation study between HPLC and FPIA (monoclonal) methods for cyclosporin A in whole blood in liver transplant recipient samples ($n = 164$) where HPLC gave values > 150 µg/mL.

and FPIA (monoclonal) on 131 cardiac transplant recipient blood for CsA concentrations, an overall correlation of FPIA (monoclonal) = 0.31 FPIA (polyclonal) + 43.97 , $r = 0.68$ ($n = 131$) was obtained and the results are shown in Figure 5.

Fig. 5. Correlation study between FPIA (polyclonal) and FPIA (monoclonal) methods for cyclosporin A in whole blood obtained from 131 cardiac transplant recipients.

Comparison of pediatric liver transplant CsA values gave a correlation equation of $\text{FPIA (monoclonal)} = 0.55 \text{ FPIA (polyclonal)} + 38.88$, $r = 0.92$ ($n = 63$) and both adult and pediatric CsA values were combined resulting in an overall correlation of $\text{FPIA (monoclonal)} = 0.50 \text{ FPIA (polyclonal)} - 13.88$, $r = 0.78$ ($n = 300$) as shown in Figures 6 and 7.

DISCUSSION

Transplant medical centers are required to provide accurate, precise, and timely determinations of CsA in the management of cardiac and liver transplant recipients. But in the current environment of reduction in staffing and laboratory economies, not all laboratories can afford to offer HPLC for specific CsA determinations as well as the immunoassays available for the drug. In current routine therapeutic drug monitoring, polyclonal and monoclonal immunoassays are commonly used for CsA determinations and many centers still offer HPLC. Oellerich et al. (7) have indicated that due to differences in specificity of the various antibodies used and problems with calibration (14,15), CsA measured in the same patient's whole blood sample by some current immunoassays can vary by as much as 100%. The presence of CsA metabolites can undoubtedly lead to analytical problems and as metabolites contribute little to overall immunosuppression, using less-than-specific assays in therapeutic drug monitoring can cause underdosing of transplant patients who may have high blood metabolite concentrations.

Fig. 6. Correlation study between FPIA (polyclonal) and FPIA (monoclonal) methods for cyclosporin A in whole blood obtained from 63 pediatric liver transplant recipients.

Fig. 7. Combined correlation study of cyclosporin A in adult and pediatric liver transplant recipients whole blood samples ($n = 300$) measured by both FPIA (polyclonal) and FPIA (monoclonal).

It is highly recommended that transplant centers employ well-validated, analytical methods to ensure successful pharmacokinetic studies performed during preclinical and clinical trials of immunosuppressive agents (16). Not all laboratories can now afford to offer CsA determinations by HPLC and immunoassays and as such, this study was undertaken to present guidelines when considering conversion of cardiac and liver transplant recipients from blood CsA measured by HPLC and FPIA by polyclonal antibody to an FPIA using a monoclonal method. Further, the study could aid in guiding those laboratories and transplant physicians who are required to perform CsA determination in cardiac and liver transplant patients by the Abbott FPIA monoclonal technique in accordance with clinical trials under the guidance of the National Institute of Health in the USA.

This study reports that when making a change from either HPLC or FPIA (polyclonal), the Abbott FPIA (monoclonal) presents an assay that is precise and linear though not free from interferences from metabolites of CsA, particularly AM9(M1) which crossreacts 25.2% and AM1(M17) which crossreacts 10.6% with the monoclonal antibody used. When changing from HPLC to FPIA (monoclonal), there is a positive bias of approximately 30% for cardiac transplant patients and a positive bias of 21% for liver transplant recipients. These high bias values arise from the aspect of CsA metabolite interference and indicate a lack of specificity on the part of the monoclonal antibody used in the FPIA procedure. It is therefore important if considering converting from HPLC to the

Abbott FPIA (monoclonal) that the laboratory consult with the transplant physicians who would be required to readjust dosing regimes of CsA or re-establish therapeutic levels for the drug.

Laboratories converting to the FPIA (monoclonal) assay from the FPIA (polyclonal) should be aware that in this study, a negative bias of 53% was determined in those liver transplant patients whose values were maintained at $< 150\mu\text{g/L}$ whereas a negative bias of 17% was observed in those patients whose CsA values ranged from 150 to $800\mu\text{g/L}$. This is important since many CsA values of liver transplant patients are maintained at level $< 150\mu\text{g/L}$ at six months post-operative.

In the case of cardiac transplants, a negative bias of 31% was seen when comparing CsA measurements by FPIA (polyclonal) and FPIA (monoclonal). Pediatric liver transplants also showed a negative bias of 54% in a comparison of these assays and, in the combined liver transplant study of 300 patient samples, a negative bias of 50% was observed. These data clearly indicate that in situations relating to cardiac and liver transplants there should be serious consideration of readjustment of therapeutic ranges, or, that drug dosage adjustment is necessary to avoid CsA side effects.

In conclusion, it was established by this study of CsA blood levels in cardiac and liver transplant recipients that conversion to a monoclonal assay using FPIA is possible; however, it is essential that laboratories communicate with transplant physicians before any transformation is considered. If this does not result, there might well be serious overestimation of CsA when converting from HPLC to FPIA (monoclonal), and underestimation of the drug concentrations when converting to FPIA (monoclonal) from FPIA (polyclonal). This study will help those medical centers who are interested in the conversion of time-consuming and technically demanding HPLC techniques to a more rapid analysis for CsA with decreased turnaround time.

REFERENCES

1. Shaw LM, Bowers L, Demers L, et al.: Critical issues in cyclosporine monitoring: Report of the task force on cyclosporine monitoring [Special Report]. *Clin Chem* 33:1269–1288, 1987.
2. Vine W, Bowers LD: Cyclosporine: Structure, pharmacokinetics, and therapeutic drug monitoring. *Crit Rev Clin Lab Sci* 25:275–311, 1987.
3. English J, Evan A, Houghton DC, Bennett WM: Cyclosporine-induced acute renal dysfunction in the rat. *Transplantation* 44:135–141, 1987.
4. Solez K, Rocusen LC, Marcussen N, et al.: Morphology of ischemic acute renal failure, normal function and cyclosporine toxicity in cyclosporine-treated renal allograft recipients. *Kidney Int* 43:1058–1067, 1993.
5. Christians U, Sewing KF: Alternative CsA metabolic pathways and toxicity. *Clin Biochem* 28:547–59, 1995.
6. Christians U, Kolhaw R, Budniak J, et al.: Cyclosporine metabolite pattern in blood and urine of liver graft recipient: Association of cyclosporine metabolites with nephrotoxicity. *Eur J Clin Pharmacol* 41:285–290, 1991.
7. Oellerich M, Armstrong VM, Kahan B, et al.: Lake Louise Conference on CsA monitoring in organ transplantation: Report of the consensus panel. *Ther Drug Monit* 17:642–654, 1995.
8. McBride JH, Kim SS, Rodgerson DO, Reyes AF, Ota MK: Measurement of cyclosporine by liquid chromatography and three immunoassays in blood from liver, cardiac, and renal transplant recipients. *Clin Chem* 38:2300–2306, 1992.
9. Kabra PM, Wall JH, Dimson P: Automated solid-phase extraction and liquid chromatography for assay of cyclosporine in whole blood. *Clin Chem* 33:2272–2274, 1987.
10. Schroeder T, Vine W, Ruckrigl D, Pesce A, Bhat G, First R: Evaluation of the new cyclosporine monoclonal antibody fluorescent polarization immunoassay in cardiac and renal transplants [Abstract]. *Clin Chem* 37:990, 1991.
11. Shaw LM, Kaplan B, Kaufman D: Toxic effects of immunosuppressive drugs: Mechanisms and strategies for controlling them. *Clin Chem* 42:1316–1321, 1996.
12. Wang P, Meucci V, Simpson E, et al.: A monoclonal antibody fluorescent polarization immunoassay for cyclosporine. *Transplant Proc* 22:1186–1188, 1990.
13. Jolley ME, Stroupe SD, Schwenzer KS, et al.: Fluorescence polarization immunoassay III: an automated system for therapeutic drug determinations. *Clin Chem* 27:1575–1579, 1981.
14. McBride JH, Rodgerson DO, Park SS, Reyes AF: Measurement of cyclosporine in plasma from patients with various transplants: HPLC and radioimmunoassay with a specific monoclonal antibody compared. *Clin Chem* 35:1726–1730, 1989.
15. Krepil J, McPhillips M: Calibration of 125I immunoassay measuring cyclosporin A. *Clin Chem* 35:181–182, 1989.
16. Shaw LM, Annesley TM, Kaplan B, Brayman K: Analytic requirements for immunosuppressive drugs in clinical trials. *Ther Drug Monit* 17:577–583, 1995.