STANDARDIZATION OF A DILUTION METHOD FOR CYCLOSPORIN (C2) ESTIMATION IN RENAL TRANSPLANT PATIENTS

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ABSTRACT

Accurate monitoring of blood cyclosporin C2 levels is vital to prevent over immunosuppression and acute renal toxicity in patients who receive organ transplant. The matrix used to dilute patients' C2 samples prior to the assay affected the final measured values. Hence there was a need to develop a method of dilution that would accurately estimate C2 levels when cyclosporin levels were beyond the calibration range of the method employed. Whole blood, cyclosporin free hemolysate and cell and protein free supernatant obtained after pretreatment of normal blood were used to dilute patients' C2 samples. C2 was measured in 188 patients using the supernatant method of dilution. C2 was correlated with Co and dose of cyclosporin received by the patient. The use of cell and protein free supernatant obtained after pretreatment of normal blood as a C2 diluent detected higher levels of C2 in the sample. Measured C2 correlated significantly with Co and the cyclosporin dose received by the patient. The uniformly aqueous cell and protein free supernatant ensures uniform dilution of the patients' C2 sample and measures higher cyclosporin levels.

KEY WORDS

Dilution method, Cyclosporin, Renal transplant.

INTRODUCTION

Whole blood cyclosporin levels measured two hours post dose (C2), is now the accepted parameter for monitoring the cyclosporin dose in transplant patients (1, 2). Hence, accurate measurement of C2 is essential to optimize immunosuppression therapy. The commonly available non-HPLC based commercial assays have been developed for monitoring trough concentrations (Co). Blood samples for C2 estimation from patients with higher levels of cyclosporin need to be diluted before assaying. Accurate monitoring of C2 levels by maintaining strict C2 dilution protocols in the laboratory is vital to prevent over immunosuppression and acute toxicity leading to impaired renal function. Although every commercially available kit gives instructions on dilution protocols, it allows the use of a commercially available diluent or whole blood from normal individuals as diluents. There are no studies

cited for the comparison of the different matrices for C2 dilution on measured C2 levels. In the absence of a single well established dilution protocol, we have found that the levels of C2 measured were affected by the method of dilution. This is of particular concern in renal transplantation where the nephrotoxic effects of cyclosporin are difficult to distinguish from organ rejection due to inadequate immunosuppression (3).

The purpose of this study was to investigate whether the matrix used to dilute C2 samples would affect the final concentration of cyclosporin assayed. The diluents used were whole blood, cyclosporin free hemolysate and cell and protein free supernatant made from EDTA whole blood collected from normal individuals. Using the latter method of C2 dilution, we estimated C2 levels in 188 patients receiving varied doses of cyclosporin in combination with other drugs. Estimated C2 levels were correlated with Co levels, dose of cyclosporin and patient outcomes.

PATIENTS AND METHODS

Patients

Between February 2003 and December 2003, 188 men or non pregnant, non lactating women, aged 18-67 years who had undergone a single renal transplant participated in this study. Patients who were newly

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transplanted and at various stages of post transplant periods were recruited into this study. Patients received twice daily Neoral cyclosporin capsule regimen in addition to other medications. Fluids and meals taken between the Co and C2 sampling were to be similar in content and amount to the patients' usual dietary regimen. Patients were requested to stay back in the hospital after drawing the morning trough Co sample. Cyclosporin was administered to the patient in the presence of the laboratory or nursing staff and C2 samples were drawn exactly 2 hours later. The sample size was 188 individual patients in whom both Co and C2 were estimated. The study protocol was approved by the Institutional Review Board of Kamineni Hospitals Ltd.

Controls

Healthy volunteers who were not on any medication, donated blood that was collected in EDTA vacutainers and used to dilute C2 samples and prepare cell and protein free supernatants for dilution.

Samples

2 ml of venous blood was drawn from patients into potassium EDTA containing vacutainers for Co and C2 samples. Laboratory staff received instructions on correct labeling of Co and C2 samples. Packed cell volume (PCV) of the samples were measured using an automated cell counter (Swelab).

Sample pretreatment

Cyclosporin calibrators, quality control materials, Co and diluted or undiluted C2 samples from patients and samples from healthy volunteers were processed using a commercially available pretreatment reagent which lyses the erythrocytes, extracts the cyclosporin and precipitates the blood proteins. Samples were centrifuged and the clear supernatant was used for the assay.

Assay

Cyclosporin was assayed on the COBAS INTEGRA 400 using reagents based on the Enzyme Multiplied Immunoassay Technique (EMIT). C2 samples with more than 500 ng/ml required dilution prior to the assay, since the highest concentration of calibrator available in this method was 500 ng/ml only.

C2 Dilution protocol

3 different C2 dilution protocols (methods A, B and C) were compared in 10 patients receiving varied doses of cyclosporin (75-450 mg/day)

I. Dilution of C2 sample before addition of pretreatment solution

Method A: C2 blood samples of 10 patients were

diluted 1:5 using EDTA whole blood from a single control blood sample, such that the final concentration of cyclosporin was approximately 500 ng/ml or less in the diluted sample.

Method B: C2 samples from the same 10 patients were diluted appropriately with the diluent provided by the manufacturer (cyclosporin free hemolysate) such that the final concentration of cyclosporin in the diluted sample was approximately 500ng/ml or less.

II. Dilution of C2 samples after the pre-treatment procedure

Method C: Blood samples (C2) from the 10 patients and from controls were processed identically with pretreatment solution and supernatants separated. Cell and protein free supernatant obtained after pretreatment of control blood samples was used to dilute cell and protein free supernatant obtained after pretreatment of patients' C2 sample such that the final concentration of cyclosporin in the diluted supernatant was approximately 500 ng/ml or less.

Using the method C, cyclosporin (C2) was estimated in 188 patients over a period between February 2003 to December 2003.

Statistical Analysis

Relationships between parameters were studied by a Pearson's correlation matrix and significance was calculated using the Fischer's 2-test. All analysis were performed with two tailed tests, with $p=0.005$ as the level of significance.

RESULTS

One hundred and eighty eight subjects completed the study. Demographic characteristics of these patients are listed in Table 1. C2 dilution protocols (Methods A, B and C) were compared in 10 patients receiving varied doses of cyclosporin (75 - 450 mg/day). On an average, method C gave the highest measurement; followed by method B. Method A gave the lowest values. Method C values were 11.57% higher than method A and 10.43% greater than method B (Fig. 1).

When whole blood was used to dilute C2 samples (method A), the measured cyclosporin values correlated well with the dose ($r=0.707$, $p<0.05$). The PCV of the normal individuals' blood used to dilute the C2 samples did not statistically affect measured C2 values (results not shown).

When cyclosporin free hemolysate which was commercially supplied was used as a diluent (method B), measured cyclosporin values were 6.135 ± 4.51 greater than method A in 60% of cases and 6.22 ± 1.53 less than method A in 40% of cases. Hence there was not much difference in the values obtained

Table 1. Details of study population (n=188) in whom Co and C2 were analyzed

NO.	Mean±S.D	Min - Max
Age (year)	38.79±12.6	18-67
Sex		
Men	136	
Women	52	
Cyclosporin dose (mg/day)	181.98±93.84	75-450 mg/day
Period of transplant (years)	3.64±1.8	8 days-6 years

Fig. 1. Comparison of methods of C2 dilution (A, B and C) on individual patient samples

Table 2. C2 levels were measured using method C for dilution which used cell and protein free supernatant as C2 diluent. Measured C2 was correlated with Co and dose of cyclosporin received by the patients, using a Pearson's correlation matrix

Groups	Group A	Group B	Group C
Dose (mg/day)	24-100	125-250	275-450
Number of patients	36	105	47
Correlation of Co and C2 (p)	$r = 0.740$ ($p < 0.01$)	$r = 0.701$ ($p < 0.01$)	$r = 0.545$ ($p < 0.05$)
Correlation of dose and C0 (p)	$r = 0.450$ ($p < 0.01$)	$r = 0.287$ ($p < 0.05$)	$r = 0.345$ ($p < 0.05$)
Correlation of dose and C2 (p)	$r = 0.475$ ($p < 0.01$)	$r = 0.448$ ($p < 0.01$)	$r = 0.232$ (NS)

between methods A and B. C2 values measured by method B correlated well with the dose of cyclosporin received by the patient. ($r=0.837$, $P<0.01$).

When cell free supernatants from normal individuals were used as C2 diluent (method C), cyclosporin values measured were the highest. Cyclosporin levels correlated well with dose ($r=0.758$, $p<0.005$). In 80% of the cases, values of cyclosporin measured by method C was $14.73 \pm 10.35\%$ greater than method A and $9.85 \pm 5.34\%$ greater than method B.

Using method C, 188 patients' samples were analyzed between February 2003 to December 2003 for C2 levels (Table 2). Patients were divided into 3 groups based on the dose of cyclosporin received in the past one month. The first group, (group A, $n=36$) were receiving between 25-100 mg/day. These patients were on lower maintenance dose and had received the renal transplants at least 8 months before or earlier. The second group (group B, $n=105$) were receiving between 125-250 mg of cyclosporin / day, and were on tapering doses. The period of post transplant was between 2-8 months. The third group (group C, $n=47$) were patients receiving between 275-450 mg/day. These patients were on initial high loading doses 3 days to 2 months following a renal transplant. Among the 188 patient studied, 2 patients expired during the period of study, from reasons not related to rejection of the allograft.

Measured C2 levels correlated well with measured Co levels as well as with the cyclosporin dose when patients received less than 250 mg/day (groups A and B). In these patients, C2 was the appropriate dose monitoring tool, rather than Co.

In patients who were recently transplanted and were receiving higher doses of cyclosporin (275-450 mg/day) measured C2 levels correlated well with Co. The dose of cyclosporin received correlated better with Co than with C2.

In order to check the linearity of measurement of C2 values with dose, multiple measurements of C0 and C2 in the same patient was done at different time intervals following renal transplant when the patient was receiving decreasing dose of cyclosporin. Levels of C2 measured in 3 patients using dilution method C, rose or fell as did the dose, ($r=0.99$) (Table 3). The 3 patients in whom multiple measurements were done had cyclosporin levels distributed across the reportable range of the assay.

Table 3. Multiple measurements of Co and C2 from individual patients on tapering doses of cyclosporin. C2 estimation was carried out using protein and cell free supernatant as C2 diluent.

Dose (mg/day)	C2 (ng/ml)	C0 (ng/ml)
Patient 1		
200	939	166
175	836	122
150	750	143
Patient 2		
175	1028	125
275	1184	290
350	1324	226
Patient 3		
375	1880	504
325	1661	492
275	1268	271

Within run variation of the method C type of C2 dilution was estimated using 5 samples with C2 levels of 1200, 1985, 1421, 986, 1180 ng/ml Coefficient of variation (%CV) varied between 3.06 to 6.48%.

DISCUSSION

Most commercially available immunoassays were developed for measurement of Co concentrations of cyclosporin (4). C2 concentrations are outside the calibration range of most of these immunoassays. Hence C2 samples must be diluted before assaying.

In our study, C2 values measured using the method of dilution selected (method-C) correlated well with the

dose of cyclosporin given to the patient, as well as with Co levels. This method of dilution gave the highest measurement of cyclosporin. Hence, dose dependent acute toxicity of cyclosporin can be accurately detected. Also, since the matrix of dilution in this method is a homogenous liquid supernatant rather than a suspension of cells as in whole blood or cell fragments as in hemolysate, the dilution of the C2 sample is more uniform, leading to higher values being measured.

Cyclosporin controls and calibrators use hemolysates that are different matrices and may differ in their behaviour when compared to whole blood samples. Hence this study was carried out using patients' samples which also contain other drugs commonly co-administered to renal transplant patients.

Multiple Co and C2 measurements from individual patients showed C2 levels correlated well with the dose of cyclosporin received. An interesting observation in this study was, that while C2 correlated well with the dose of cyclosporin in the patients who were receiving low doses and tapering doses of the drug (groups A and B), C0 was a better parameter to monitor the dose of cyclosporin in newly transplanted patients.

In conclusion, this study shows that using cell and protein free fluid obtained after pretreatment of normal blood samples is a better diluent for C2 samples than whole blood or cyclosporin free hemolysate and yields higher cyclosporin levels.

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