Bioavailability versus Bioequivalence: The Cyclosporine Model

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The quest for a fixed-dose immunosuppressive drug continues. Experience with cyclosporine, tacrolimus and mycophenolate mofetil has taught us that there is no correlation between dose and clinical events. These data indicate that the concentration of the drug at the site of action (bioavailability) of each of these agents differs from one patient to the next. In addition, the bioequivalence (concentration of intact drug at the site of action resulting in a measurable response [effect]) may differ among individuals. Technically, it is very difficult to measure the drug concentration at a particular site, especially in organs or tissues that are not directly accessible. Therapeutic drug-blood-level monitoring is a simple indirect method that is used to estimate both bioavailability and bioequivalence. However, the immunosuppressive effect of all these drugs is initiated by binding to receptors on the surface of lymphocytes, which leads to inhibition of cytokine production and proliferation of activated lymphocytes. Thus, it would be more advantageous to monitor the level and effect of these drugs at the site of action (bioequivalence), the lymphocyte. This report describes an assay of this type that was developed for monitoring transplant patients at one center. The assay is based on measuring drug levels in the cytoplasm of lymphocytes. It is quick and easy to perform (20 samples per hour), inexpensive, and reproducible. The between-run Coefficient of Variance (CV) is 5.4 and a within-run CV is 3.1. For this study, blood and lymphocyte drug levels in transplant patients were determined and correlated with graft function and clinical events (biopsy-proven rejection and/ or toxicity).

Keywords: *Bioavailability, Bioequivalence, Immunosuppressive Drug Monitoring*

Bioavailability is defined as the concentration of intact unmetabolised drug at the agent's site of action. Technically, it is very difficult, if not impossible, to measure the drug concentration at a particular site, especially if the organ or tissue is not directly accessible. Even at sites that can be accessed, invasive manipulations are required for routine monitoring. Therapeutic drug-blood-level monitoring is a simple indirect method that is used to estimate bioavailability. For many agents, this parameter is valuable for establishing therapeutic guidelines. However, for many others, and particularly immunosuppressives, blood-level monitoring has not proven highly effective.

In the case of cyclosporine (CYA), even after 20 years of use there is still no agreement on what to measure—the area under the curve (AUC), base line (C0), exposure index (EI) or 2 hours post dose (C2) [1-6]. The controversy is due to many factors, mainly inter- and intrapatient variability in CYA pharmacokinetics, which reflects bioavailability. The factors that influence bioavailability include absorption, metabolism, distribution and elimination. Absorption is mainly determined by the drug formulation, and by physiological and genetic factors. To date, more than 15 different types of gene polymorphism related to CYA absorption rate have been identified. The observed variations in CYA metabolism are due to extensive gene polymorphism linked to hepatic enzymes, especially cytochrome P450. More than 50 different allelic forms of this enzyme have already been identified. Area-under-the-curve (AUC) monitoring can signal

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the need for CYA dose adjustment, and can help the physician identify problems with malabsorption and patient compliance; however, it does not provide information about the influence of high peaks (Cmax) or high EI and does not provide effective therapeutic guidance [7-12]. As a result of variation among individuals, each patient requires his or her own tailored CYA regimen. Tacrolimus (FK506) was the next calcineurin inhibitor developed after CYA, and the situation is similar with this agent.

Mycophenolate mofetil (MMF) and Sirolimus were introduced as fixed-dose drugs with no need for blood-level monitoring. With MMF, it has been shown that 3 g/day offers no added benefit in comparison to 2 g/day or 1.5 g/day [13]. Interestingly, patients who received 1.5 g/day achieved higher trough levels than those who received 2 or 3 g/day [14]. Monitoring of blood trough levels in these patients indicated that concomitant administration of CYA reduces the MMF level, and that concomitant treatment with FK506 results in near doubling of the MMF level [15-18].

Whereas bioavailability is the concentration of intact drug at the agent's site of action, bioequivalence is defined as the concentration at the site that results in a measurable response (effect). In order to determine bioequivalence, the site of action must be identified and must be accessible; there must be a specific, accurate, reproducible assay for measuring the drug concentration at the site; the measurable response must be clearly defined; and there must be an assay for measuring this effect.

All immunosuppressive drugs act by binding to receptors on the surface of lymphocytes, which leads to inhibition of cytokine production and proliferation of activated lymphocytes. Lymphocytes can easily be isolated from blood samples. Previous work by Masri et al. described an accurate, specific, reproducible assay for measuring the CYA concentration in lymphocytes [19]. This assay measures lymphocyte cytoplasmic drug levels (LCL) and the extent to which proliferation of isolated lymphocytes is inhibited.

The aim of this study was to establish a therapeutic guideline for immunosuppressive agents. These guidelines are based on bioequivalence rather than bioavailability. This would be achieved by the measurement of these agents in the isolated lymphocytes from peripheral blood.

Materials and Methods

The CYA basic (C0) levels of 670 renal and 20 heart transplant patients (mean age 37 years; range 24-65 years) were retrospectively monitored over a 3-year period in total 3856 blood samples from these patients who were receiving either the Equoral® (IVAX, Miami USA) or Neoral® (Novartis, Basel Switzerland) formulation of CYA.

Thirty renal transplant patients had their blood CYA and lymphocyte CYA levels, measured at the following times, (t0, t30, t1, t1:30, t2, t3, t4, t5 t6, t8, t10 and t12) twice. Once while receiving Neoral® and once following their conversion to Equoral® on a 1/1 mg/mg bases. The CYA levels obtained from 720 samples of these 30 patients were used to draw 60, 12-hour bioavailability and bioequivalence pharmacokinetic profiles (curves).

The CYA level in each sample was measured using monoclonal antibodies specific for the parent compound (fluorescence polarization assay, TDx, Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL 60064 USA) Measurements (ng/ml) were recorded at baseline (C0), 1 hour (C1), 1.5 hours (C1/30) and 2 hours (C2) after dosing. The highest level obtained from these measurements was considered in this study to represent the maximum concentration (Cmax) achieved during a 12-hour period.

To assay LCL, two milliliters of blood were collected into an EDTA tubes, and the lymphocytes from each sample were separated off using either density-gradient centrifugation on ficoll-hypaque, or anti-CD45, -CD3 or -CD4 monoclonal antibodies conjugated with magnetic beads. Both white blood cell (WBC) and lymphocyte from each sample were enumerated using a Coulter counter. The cells were then washed once with phosphate-buffered saline and suspended in 150 ul of this solution. The CYA level present in the lymphocytes was determined using a monoclonal-specific assay (TDx Abbott Diagnostics, Abbott Park, IL 60064 USA). An in-house calibration curve was used to determine the CYA/lymphocyte level (LCL) in each sample using the following standards 0,15.62, 31.25, 62.5, 125 and 250 ng/ml [20-22]. Values for LCL at base line (L0), 1 hour post dose (L1), 2 hours post dose (L2) were recorded. Maximum lymphocyte CYA level (Lmax) was determined as it was done for blood CYA Cmax. For measurement of blood CYA levels the normal TDx curve, supplied by Abbott, with the fol-

lowing standards 0, 100, 250, 500, 1000 and 1500 ng/ml was used.

Relationships between mean CYA levels at the different time points (C0, C1, C2 and Cmax) and biopsy-proven rejection, toxicity, normal findings, frequency of rejection episodes serum creatinine level, and lymphocyte count were statistically tested. Similarly, relationships between the above parameters and mean LCL at the different time points (L0, L1, L2 and Lmax) were statistically tested.

One hundred blood samples from 60 renal transplant recipients who were on an MMF based therapy but without CYA were also evaluated. Lymphocytes were isolated from each blood sample as described above, and their MMF levels were determined in each blood and the corresponding lymphocyte sample using the EMIT assay (Dade Behring Inc, Cupertno, CA 95014 USA).

Results

In this transplant group, we found that regardless of the formulation, 29 % of the patients achieved their C max at C1 (786 ng/ml), 25 % at C2 (678 ng/ml), 25 % at C2 (678 ng/ml) and 46 % at C1/30). Surprisingly, in the same patient while on the same dose of CYA, with time, there was a flip over in Cmax between C1 and C2 in 50 % of the patients ($p<0.001$). Eighty-five (85 %) of those patients who had their Cmax at C1 had also their Lmax at C1 (84.4 pg/cell) while 15 % of them had their Lmax at C2 (55.5 pg/cell). There was no correlation between C2 with rejection rate and nephrotoxicity (Figure 1). The lymphocyte counts from patients who had a C2 of <500, did not differ from those whose C2 levels were between 500-1000 or >1000 ng/ml (Figure 2). Similarly there was no correlation between C0 and lymphocyte count or WBC (Figure 3 A and 3 B). On the other hand, there was an excellent inverse correlation between LCL0, LCL1, LCL2 and LCLmax with lymphocyte cell count. The higher the CYA LCL was the lower lymphocyte counts (Figure 4 A, 4B and 4 C). Similarly was the case for patients receiving

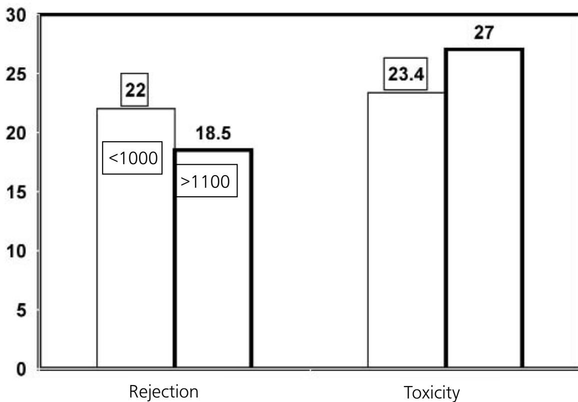


Figure 1. C 2 <1000 and C 2 >1100 vs biopsy proven rejection rates and CYA nephrotoxicity.

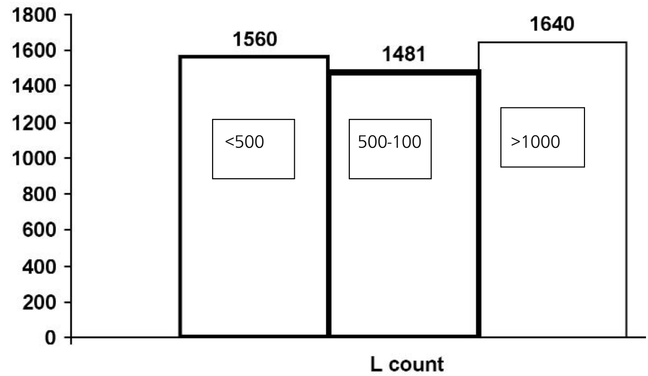
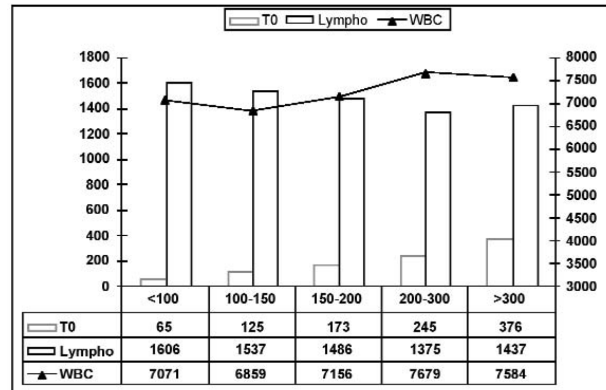


Figure 2. Lymphocyte count vs. C2 levels



	<100	100-150	150-200	200-300	>300
T0	65	125	173	245	376
Lympho	1606	1537	1486	1375	1437
WBC	7071	6859	7156	7679	7584

Figure 3A. C0 (T0) vs lymphocyte count (Lympho) and white blood cell (WBC).

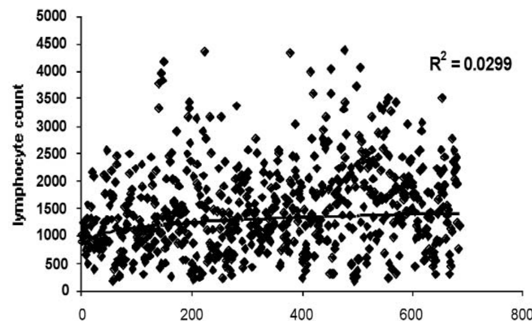


Figure 3B. Correlation between C0, X axis and lymphocyte count Y axis

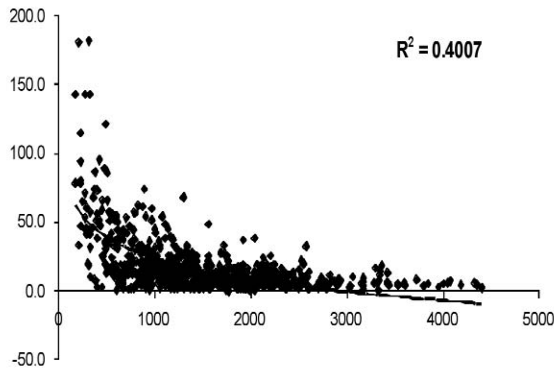
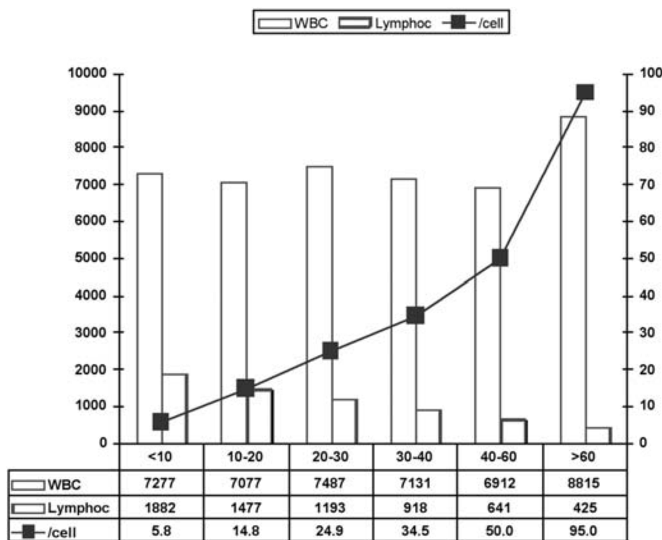


Figure 4A. Correlation between L0, X axis and lymphocyte count Y axis

MMF (Figure 5). Biopsy proven rejection rate was 10 % in patients who had an Lmax of ≥40 picogram/lymphocyte (pg/cell), 21% in patients having 20-40 pg/cell and 33% in patients having ≤ 20 pg/cell. There was a significant difference in the serum creatinine (P<0.04) level (1.04 ± 0.2 mg/dl) of patients with an Lmax of ≤ 20 pg/cell and patients with Lmax ≥ 40 (1.4± 0.2 mg/dl). Biopsy proven toxicity was 10% in patients having an Lmax of ≥ 40 pg/cell 31% for patients with L max of 20-40 pg/cell and 33% for patient with an L max of ≤ 20 pg/cell respectively. The full 12-hour lymphocyte pharmacokinetics of both Neoral and Equoral was similar, resulting in a similar decrease of the lymphocyte count. The maximum decrease was achieved at 5-6 hours post dose (Figure 6 A and 6 B).



	<10	10-20	20-30	30-40	40-60	>60
WBC	7277	7077	7487	7131	6912	8815
Lymphoc	1882	1477	1193	918	641	425
/cell	5.8	14.8	24.9	34.5	50.0	95.0

Figure 4B. Lymphocyte count (Lympho) vs L0 (/cell) vs WBC

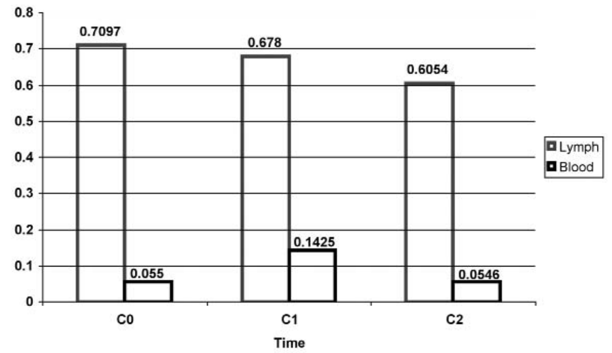
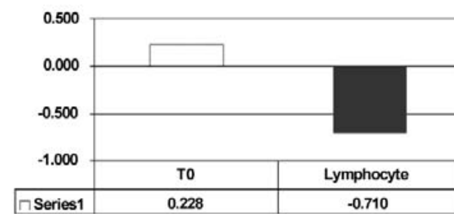
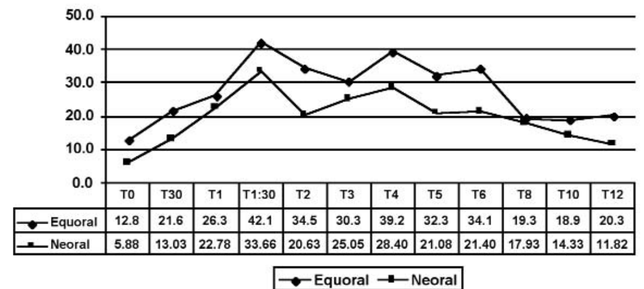


Figure 4C. Correlation between lymphocte count and CYA levels in the lymphocytes (Lymph) and blood at C0 C1 and C2.



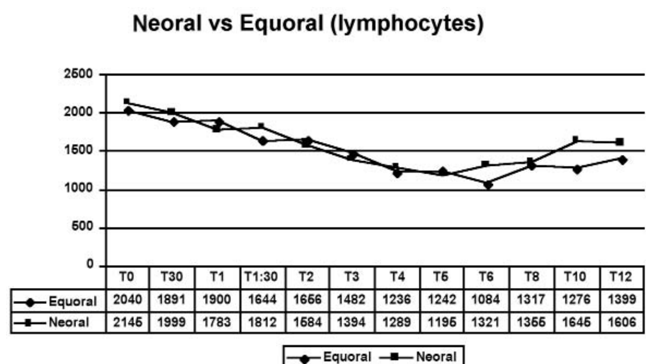
	T0	Lymphocyte
Series1	0.228	-0.710

Figure 5. Correlation between lymphocyte count with MMF blood levels (T0) and lymphocyte levels Lymphocyte.



	T0	T30	T1	T1:30	T2	T3	T4	T5	T6	T8	T10	T12
Equoral	12.8	21.6	26.3	42.1	34.5	30.3	39.2	32.3	34.1	19.3	18.9	20.3
Neoral	5.88	13.03	22.78	33.66	20.63	25.05	28.40	21.08	21.40	17.93	14.33	11.82

Figure 6A. Lymphocyte 12 hour pharmacokinetic profiles of Neoral vs Equoral.



	T0	T30	T1	T1:30	T2	T3	T4	T6	T6	T8	T10	T12
Equoral	2040	1891	1900	1644	1656	1482	1236	1242	1084	1317	1276	1399
Neoral	2145	1999	1783	1812	1684	1394	1289	1196	1321	1365	1645	1606

Figure 6B. Lymphocyte count proflle over the 12 hour period of Neoral and Equoral

Discussion

The absence of correlation between lymphocyte level and blood level indicates that some patient could achieve a "therapeutic lymphocyte level" LCL with low C0 while others with much higher C0 do not achieve adequate LCL. Patient with biopsy proven graft rejection had low L max (≤20 picogram/lymphocyte) although C2 measurement has been shown to have a good correlation with AUC however it is not a reliable predictor of the peak concentration Cmax. The flip over of peak levels between C1 and C2 indicates that the C2 is the least reproducible parameter in the pharmacokinetic profile as few minutes deviation from the exact timing of C2 will lead to a large error in concentration [10]. The absence of correlation between CYA lymphocyte level and blood level indicates that some patient could achieve a LCL with low C0 or C2 while others with much higher C0 or C2 do not achieve adequate LCL. Bioequivalence is a logical alternative to blood level (bioavailability) monitoring and its use at our center has resulted in a cost effective dose adjustments protocols. We have established a CYA LCL monitoring program for our transplant patients. Our one center study has resulted in a total dose reduction by nearly 1/3. Moreover, we have nearly eliminated the biopsy program. We are currently using biopsy in less than 5% of the patients as compared to 35% prior to the use of the protocol. Toxicity and rejection episodes were also reduced by more than 80%. The assay is easy to perform, fast, as up to 30 samples can be assayed within 1 hour. The assay is inexpensive only 1.2 times more costly than the currently available whole blood assays and is reproducible with a between run CV of 5.4 and within run CV of 3.1. The assay also correlate with the immunological status of the patient [23,24]. Finally, with bioequivalence it becomes easier to evaluate different formulation of the same drug and it provides a relevant parameter for the degree of immunosuppresion effects "lymphocyte count" of the various agents available.

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