Arch. Pharm. Res. Vol.18, No.6, pp. 385-390, 1995

Pharmacokinetics of Two Cyclosporine Formulations Using FPIA and HPLC Assay in Volunteers

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(Received July 25, 1995)

The analytical methods for the analysis of cyclosporine (CsA), a fluorescence polarization immunoassay (FPIA) and HPLC method, were compared in a pharmacokinetic study of two CsA soft capsule formulations (Sandimmun®; Sandoz, Implanta®; Hanmi). Sixteen healthy volunteers completed the study and each subject ingested single doses (4×100 mg) of the test and the reference formulations in a two-way crossover design with a one-week drug-free interval between doses. Following each administration, whole blood concentrations of CsA were monitored over a period of 24 hour by both FPIA and HPLC methods. Blood concentrations and pharmacokinetic parameters determined by either analytical method showed large intersubject variation, with the FPIA data showing relatively higher magnitude of intersubject variation than the HPLC data. The blood concentrations determined by FPIA were 1.1-1.3 times higher than those determined by HPLC. There were strong and significant correlations between the two methods ($r>0.83$: $p<0.0001$). Intersubject variation for the $AUC_{inf.}$ and AUC_{24hr} of the test formulation was slightly reduced without statistical significance (paired-*t* test: $p>0.05$. t_{max} was earlier and C_{max} was slightly lower for the test formulation. AUC_{24h}, C_{max}, t_{max} and MRT determined separately from the data obtained by the two methods for the two formulations were examined by analyses of variance (ANOVA) for the bioequivalency evaluation. Results of ANOVA and confidence limits of test/reference ratios of AUC_{24h}, C_{max}, t_{max} and MRT, and statistical tests indicated the bioequivalence of the two formulations (i.e., test/ reference ratio was within 100×20%) except for C_{max} and t_{max}. The mean of C_{max} showed only 7.9% and 11.6% differences but the detection limit were 26.6% and 21.4% as determined by FPIA and HPLC respectively which is slightly over the 20% criteria. The mean of tmax also showed 11.1% and 9.3% differences but the detection limit were 29.2% and 29.6% as determined by FPIA and HPLC respectively. This experiments suggest that the data yielded for the two formulations demonstrated that they were bioequivalent.

Key words : Cyclosporine, Pharmacokinetics, Bioequivalence, FPIA, HPLC

INTRODUCTION

Cyclosporine (CsA) is the most potential immunosuppressive and, therefore, is used to prevent graft rejection in organ transplant recipients. The impact of CsA has been even more dramatic in hepatic, cardiac, and heart-lung transplant patients (Powles R.L. *et al.*, 1980; Calne R.Y. *et al.*, 1979). Another its potential indication is in the treatment of autoimmune diseases without significant myelotoxicity (Bach J.F., 1989). The major adverse effect of CsA is dose-related nephrotoxicity (Calne R.Y. *et al.*, 1978). Its therapeutic index is very narrow, and its individual therapeutic response and the oral bioavailability are both variable. For this reasons, therapeutic drug monitoring plays an important role in treatment with this drug (Leslie M.S., 1989).

CsA is slowly and incompletely absorbed after oral administration and is available for oral administration as a clear, yellow, olive oil solution. Its peak concentrations are reached in about 3.3 hours after oral dosing and the bioavailability ranged from 20 to 50%. Approximately, 90% of CsA in plasma are protein bound, mainly to lipoproteins. About 60 to 70% of CsA in whole blood is contained in erythrocytes (M. Lemaire *et al.*, 1982). Despite their small contribution to blood volume, leukocytes contain 10 to 20% of circulating CsA.

Blood concentrations of CsA appear to decline in a biphasic manner following oral administration. In adults with nomal renal and hepatic funtion the half-life in the initial ($t_{1/2\alpha}$) has been reported to 1 to 2 hours and the terminal phase ($t_{1/2\beta}$) has averaged in 19

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to 21 hours. CsA is extensively metabolized in the liver (Freed *et al.*, 1987; Kahan *et al.*, 1990)

The present study was designed to compare whole blood concentrations of CsA determined by two assay methods, HPLC and fluorescence polarization immunoassay (FPIA), and also to assess the performance of the two methods in the determination of the single dose bioequivalence of two different formulations of CsA (gelatin soft capsules; Implanta® and Sandimmun®) in a group of strictly controlled volunteers.

MATERIALS AND METHODS

Drugs-Cyclosporine soft capsule

Test formulation: Implanta® (Hanmi), Lot No. Pilot CF 100-1 (1993. 11. 12)

Reference formulation: Sandimmun® (Sandoz), Lot No. 329MFD0593 (1993. 11. 1)

Informed consent

All subjects participating in this study were provided with detailed information on the possible side effects of CsA and other hazards that might be encountered. Each subject gave a written informed consent prior to his/her entry in the study.

Subject

The subjects were selected from healthy male and female volunteers, aged 21~25 years, with standard weight-to-height ratio. In order to be eligible to participate in the study, each volunteers was required to be an abstainer from drug or alcohol abuse and to be free of cardiovascular, hepatic, renal or gastrointestinal diseases, as assessed by physical examination and review of medical history. They were also required to have their blood pressure and results of clinical laboratory test (blood chemistry, hematology and urinalysis) within the normal ranges. Sixteen subjects were intially entered and completed the study. All subjects avoided using other drugs for at least one week prior to the study and until after its completion. They also refrained from alcoholic beverages and caffeine containing food and beverages 48 h prior to each dosing and until the collection of the last blood sample.

Study design and blood samples

The administration of the two formulation of CsA to the subjects followed a balanced two-way crossover design with a week drug free interval between the two administrations. Subjects were assigned randomly to receive the test (Implanta®; 4×100 mg gelatin soft capsules, Hanmi) or the reference (Sandimmun®; 4×100 mg gelatin soft capsules, Sandoz) for the first dose of CsA and then the respective alternative formulation for the second dose. After an overnight fast, each subject was ingested CsA soft capsules with 200 ml of water. Fluid and food intakes were controlled for 10 h following each dose. Beverage and standard lunch was supplied at 1.0 and 3.0 h, respectively, after each dose. Beverage and foods containing caffeine were not permitted over the entire course of the study. Blood samples (8 ml each) were collected by venipuncture using the three way cock equipted with 10 ml 20 I.U. heparin solution, and stored in heparinized glass tubes at 0 (predose), 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 and 24.0 h postdose. The blood samples were stored at − 20°C until analysis.

Analysis of blood samples

Analysis of CsA by FPIA : Reagents for the assay are provided as fully reconstituted reagent kits (Abbott Diagnostics), which included a solubilization solution containing surfactant in buffer, a precipitating reagent (zinc sulfate in methanol and ethylene glycol), CsA antiserum (mouse monoclonal) in buffer with protein stabilizers, and CsA fluorescein tracer in buffer containing surfactant and protein stabilizer. The assay is calibrated with CsA whole-blood standards (concentration 0-1500 μg/l; Abbott Diagnostics).

The assay procedure requires pretreatment of the whole blood before analysis, which involves the following steps : mix 150 μl of the sample to be analyzed (calibrators, controls, and subject's specimens) with 50 μl of solubilization reagent and 300 μl of precipitation reagent. After mixing, centrifuge the specimens for 5 min at 9500×g. Decant the supernate (a minimum of 150 μl is required for analysis) into the sample cartridges and load these in the TDx. All subsequent steps for analysis are performed by the instrument.

Analysis of CsA by HPLC : To each whole blood sample (0.5 ml), 250 μl of saturated sodium chloride solution and 1.0 ml of diethyl ether were added. After being vortex-mixed for 2 min, the samples were centrifuged at 3000 rpm for 10 min and the supernatant (0.7 ml) were transferred to silica Sep-Pak® extraction column (3 ml, 40 μm APD, 60 Å, J.T. Baker Co. Ltd.). Before use, the extraction columns were primed with dichlormethan (3 ml) and n-hexane (3 ml). On removal of contaminants with 3 ml of n-hexane, the CsA were eluted with 3 ml of methanol. After evaporation under nitrogen, each sample was reconstituted with 200 μl of mobile phase, and 100 μl of each sample was assayed by isocratic chromatography at 75°C at a flow rate of 1.0 ml/min on a reversed-phase C_{18} column (Waters Inc., Milford, MA).

Acetonitrile/methanol/water (55/15/30, by vol) was

the mobile phase, and the absorbance of the eluate was monitored at 210 nm. For elution during routine analysis, a isocratic running for 18 min with the mobile phase was performed followed by a 6 min column clean-up with 100% acetonitrile (1.5 ml/min). The column was reequilibrated within about 20 min. Standard curves, generated daily for determination of test sample, remained linear ($r>0.998$) in the concentration range 0.125~2.0 μg/ml of blood. The measured retention time for CsA was about 11.3 min and the sensitivity of the assay was 50 ng/ml.

Analysis of data and statistics

The basic pharmacokinetic parameters for CsA (AUC, C_{max} and t_{max}) were determined from the blood concentration-time data obtained by FPIA and HPLC methods. For each set of data, the maximum blood concentration (C_{max}) and time to reach maximum blood concentration (t_{max}) were obtained directly from the blood concentration-time data. The area under the blood concentration-time curve of the analyte (AUC^t_o) up to the last time (t_{last}=24 h) was determined by using the trapezoidal rule.

The mean residential time (MRT) was calculated using the equation :

$$MRT=\frac{AUMC}{AUC}$$

$$AUMC=\int_o^\infty t \cdot C\, dt$$

where AUMC is the area under the first moment time curve from 0 to infinity. Comparison of the blood concentrations, AUC^t_o and C_{max} within the same formulation determined by the FPIA and HPLC methods was performed by paired t-tests and regression analysis. For the bioequivalency evaluation, pharmacokinetic parameters (AUC_{24h}, t_{max}, C_{max}, and MRT) were examined by three-way analysis of variance (ANOVA) in which the effects of formulation, period and subjects were examined. For this, the computer program of bioequivalence which was developed by Korea National Research Institute of Health and Safety was utilized. The power of each ANOVA to detect a 20% difference between the test and the reference was calculated by the method of Lamda test. The confidence limits of test : reference ratios were also computed. Comparison of the pharmacokinetic parameters between the formulations was performed by paired *t*-test. The level of confidence was set at 95% (α=0.05) for all the statistical test (Midha *et al.*, 1990).

RESULTS AND DISCUSSION

The distribution between plasma, erythrocytes, and mononuclear cells depends on the temperature at which the blood was seperated, the equlibration time before the separation, and the concentration of CsA present (Jacobus W.O. *et al.*, 1985 ; Gary L. Lensmeyer *et al.*, 1989 ; Randall W. Yatscoff *et al.*, 1984). Thus, CsA measurements in plasma separated from the red blood cells at less than 37°C - as it is performed for practical reasons in virtually all routine laboratories - do not reflect the true plasma concentrations *in vivo*. Therefore, values of clearance, volume of distribution, and systemic availability obtained from such plasma concentration measurements are inadequate. In order to overcome the problem of temperature-dependent partitioning, we assessed the concentrations of CsA in whole blood.

The clinical tolerability of both CsA formulations were good in general. The signs of burning sensations in the hands and feet were reported for the first several hours in many of the volunteers.

Both of the formulations of CsA were readly absorbed from gastrointestinal tract of the volunteers as indicated by the data obtained with either FPIA or HPLC. CsA was measurable at the first sampling time (0.5 h) in all volunteers following either of the formulations. The mean tmax values for the test and the reference were, respectively, 2.25 and 2.53 h as determined by FPIA, and 2.13 and 2.34 h as determined by HPLC. After reaching C_{max}, blood concentrations of CsA declined polyexponentially on most of the volunteers.

Fig. 1 (Panel A : FPIA; Panel B : HPLC) shows the semilogarithmic plot of the arithmetic means of the blood concentrations and the interindividual comparison of CsA over time after separate administrations of the two formulations, respectively.

Intermethod comparison of blood concentrations and pharmacokinetic parameters within the same formulation

Direct comparison of the blood concentrations of CsA by paired *t*-tests indicate that the concentrations determined by FPIA are significantly higher than those determined by HPLC for both test and reference ($p<0.0001$).

When all the blood concentrations within the same formulation determined by FPIA were regressed on those determined by HPLC, the relationships obtained were FPIA=1.13 (HPLC)+55.61 (R=0.95) and FPIA=1.11 (HPLC)+100.73 (R=0.93), for the test and the reference, respectively (Fig. 2). The FPIA and HPLC procedures employed in this study were sensitive enough to monitor CsA concentrations in whole blood over the entire 24 h study period in all volunteer after each administration.

In the present study, the strong significant linear correlation between the blood concentrations det-

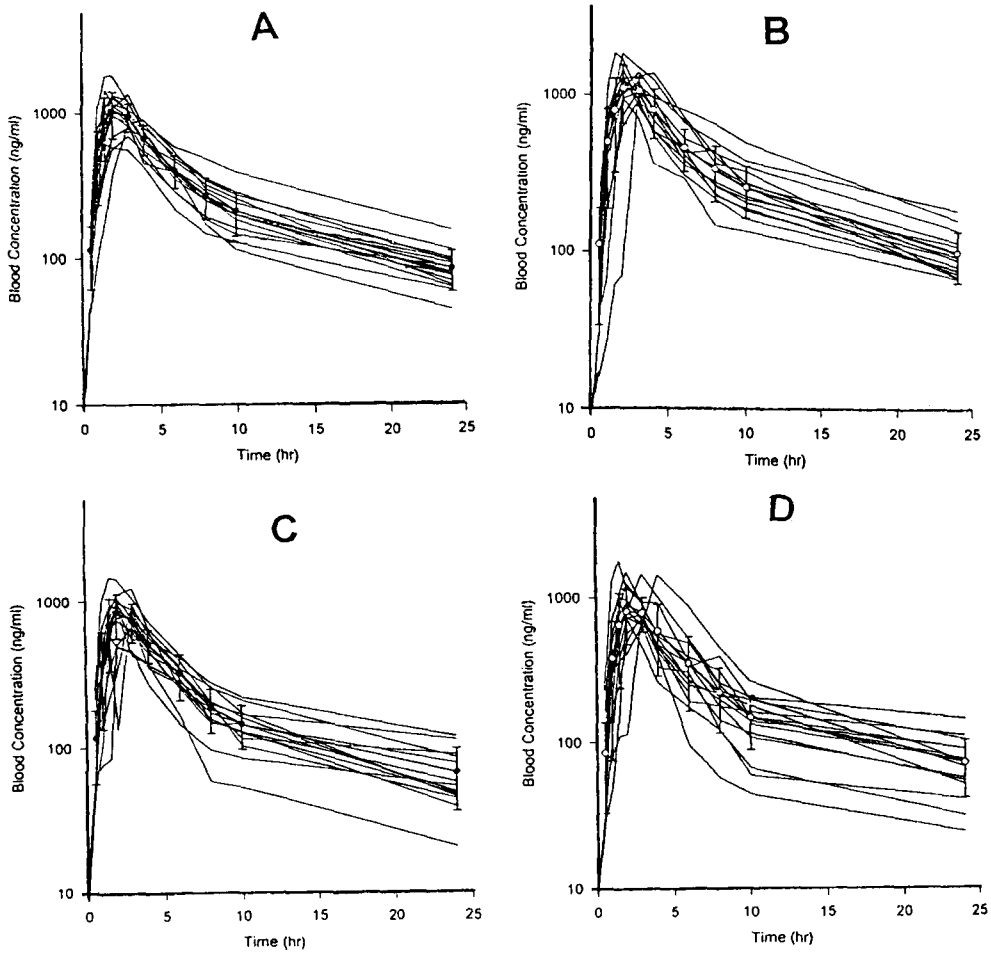

Fig. 1. Mean blood concentrations and interindividual comparison of CsA in healthy volunteers (n=16) following separate administration of 4×100 mg soft capsules of the test (●), and reference (○) as determined by FPIA (A, B) and HPLC (C, D)

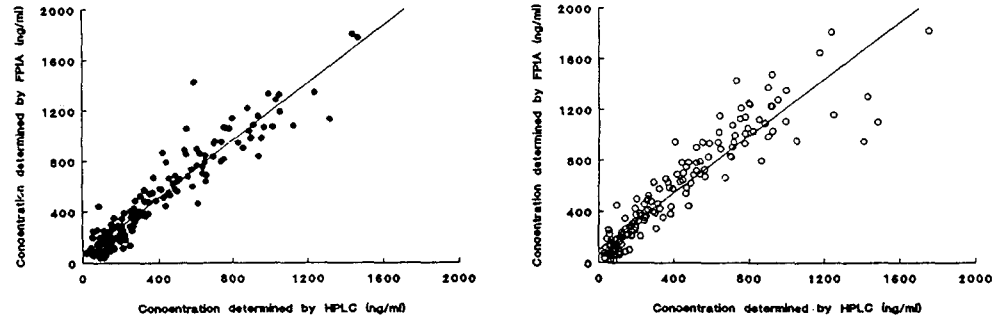

Fig. 2. Regression plots of blood concentrations of CsA as determined by FPIA and HPLC following oral administration of test (A; ●) and reference (B; ○) formulations. The regression equations are FPIA=1.13(HPLC)+55.61 (R=0.95, N=159) and FPIA=1.11 (HPLC)+100.73 (R=0.93, N=158) for the test and reference formulations, respectively.

ermined by FPIA and those determined by HPLC probably indicates that the FPIA values contaminated by cross reactions are not concentration dependent in any way. The slopes and intercepts of these plots were entirely consistent with the intermethod regression plots in that an average 20 and 10% over estimation (Winkler *et al.*, 1992 ; Yatscoff *et al.*, 1990; Mcbride *et al.*, 1992) by the FPIA method was detected for the test and the reference, respectively. Therefore, it was not surprising that despite significant difference in the determination of absolute blood concentrations, the FPIA procedure essentially gave the same answer as HPLC in this study.

Intersubject variation in pharmacokinetic parameters

$AUC_{24}h$ was calculated by the computer program "boomer". C_{max} and t_{max} values were obtained from the raw data. Table I lists mean values and CVs (coefficients of variation) of the pharmacokinetic parameters for CsA. Large intersubject variations (large CV) were noted for all the parameters analyzed by FPIA and HPLC. In general, data obtained by HPLC showed larger variation than those obtained by FPIA for each pharmacokinetic parameters (AUC_{24h}, C_{max}). Pharmacokinetic parameters between two formulations were separately assessed by paired *t*-test. As can be seen in Table I, no parameter showed significance in difference and the intersubject variations in all the parameters were comparable (CV within ±10% difference of each other) between the two formulations. But intersubject CVs of the primary parameter (AUC_{24h}, C_{max}) of CsA ranged 23.6 to 28.4% for the test formulation compared with 26.0 to 32.0% for the reference formulation except C_{max} and t_{max} in the case of FPIA (Table I). Fig. 1 also showed intersubject variation of the test was reduced compared with the reference.

Table I. Pharmacokinetic parameters of the test and the reference formulations of CsA analyzed by FPIA and HPLC methods

Formulation	AUC_{24h} (μg/ml/hr)	C_{max} (μg/ml)	t_{max} (hr)	$MRT_{inf.}$ (hr)
FPIA Data				
Test	7.09	1.11	2.25	10.04
(CV, %)[a]	(24.0)	(27.0)	(29.3)	(15.2)
Reference	8.15	1.26	2.53	10.14
(CV, %)	(26.0)	(20.6)	(26.5)	(12.0)
Bioavailability of the Test, %[b]	87.0	88.1	88.9	
paired *t*-test	NS	NS	NS	NS
HPLC Data				
Test	5.37	0.95	2.13	10.18
(CV, %)	(27.2)	(28.4)	(26.3)	(28.9)
Reference	5.61	1.03	2.34	10.11
(CV, %)	(31.6)	(32.0)	(30.8)	(20.1)
Bioavailability of the Test, %	95.7	92.2	91.0	
paired *t*-test	NS	NS	NS	NS

[a] Coefficients of variation that are the ratio of standard deviation of a sample to its mean.
[b] The test: reference ratio of the mean values of the parameter in the same column.
[c] Not significantly different by paired *t*-test ($p>0.05$)

Bioavailability

Table I also shows the pharmacokinetic characteristics and the relative bioavailability of the test calculated from the test: reference ratios (%) for the mean AUC_{24h}, C_{max}, and t_{max} values as obtained from the data generated by FPIA and HPLC. t_{max} was earlier [2.25±0.66 versus 2.53±0.67 (FPIA); 2.13±0.56 versus 2.34±0.72 (HPLC)] and C_{max} was slightly lower [1.11±0.30 versus 1.26±0.26 (FPIA); 0.95±0.27 versus 1.03±0.33 (HPLC)] for the test formulation. For the primary parameters, the relative bioavailability of test on reference was found to be in the range 87-88% (FPIA) or 92-96% (HPLC). For the mean t_{max}, test: reference ratios were 88.9% (FPIA) and 91.0% (HPLC).

Bioequivalence

The results of three-way ANOVA analysis of the pharmacokinetic parameters indicated that there were no significant effects of formulation, period or sequence on any pharmacokinetic parameter for both of the data obtained by HPLC and FPIA.

Table II shows the statistics of CsA bioequivalences study between the test and the reference formulations, as obtained by FPIA and HPLC. The statistical detection limit to have a 80% power between test and reference for AUC_{24h}, C_{max}, and t_{max} values were, respectively, 19.3, 21.4, and 29.2% by FPIA and 20.6, 26.6, and 29.6% by HPLC. C_{max} and t_{max} values slight-

Table II. Statistics of CsA bioequivalence study between the test and the reference formulations

range of criteria	% difference	F-test[a]	detection limit	confidence limit[c]
Parameters	<20%	F<4.75[b]	<20%	-20%~20%
FPIA Data				
AUC_{24h} (μg/ml/hr)	12.9%	1.274	18.8%	-0.6%~26.3%
C_{max} (μg/ml)	11.6%	0.010	21.4%	-3.6%~26.8%
t_{max} (hr)	11.1%	0.434	29.2%	-9.7%~31.9%
$MRT_{int.}$ (hr)	1.0%	0.118	9.5%	-6.7%~ 6.8%
HPLC Data				
AUC_{24h} (μg/ml/hr)	3.9%	3.987	19.3%	-9.9%~17.7%
C_{max} (μg/ml)	7.9%	1.016	26.6%	-11.1%~26.8%
t_{max} (hr)	9.3%	0.150	29.6%	-11.7%~30.4%
$MRT_{int.}$ (hr)	0.7%	5.432	18.4%	-11.7%~13.1%

[a] F-value between test and reference formulations by ANOVA
[b] F[1,12] value at=0.05
[c] Confidence limit of% difference (delta%)

ly outranged over the limit of 20%. This would be explained by the frequency of blood sampling than by the formulation difference in this case. This is supported by the small differences of the mean of C_{max} and t_{max} between the formulations (Table II). Table II includes the confidence limits for the test: reference ratios of mean AUC_{24h}, C_{max}, t_{max}, and MRT (mean residential time). The confidence limits for the parameters as determined by FPIA, except for $MRT_{inf.}$, outranged over the bioequivalence limits of 100±20%. In the case of HPLC, the confidence limits for all parameters were within the bioequivalence limits of 100± 20%, except C_{max} and t_{max}.

In conclusion, the bioequivalence of two CsA formulations was established based on all three of the parameters (AUC_{24h}, C_{max}, and t_{max}) as determined by FPIA and HPLC. Bioequivalence was demonstrated AUC_{24h}. In case of C_{max} and t_{max}, they showed small differences but the detection limits were slightly over the 20% limit. However, the C_{max} and t_{max} values were obtained from the raw data and the accurate determination of C_{max} and t_{max} are more dependent on the frequency of blood sampling than on the formulation differences or the methodological accuracies (K.K. Midha *et al.*, 1990), in this case. All the pharmacokinetic parameters showed the difference of less than 20%, and demonstrated non-significance in difference between two formulations by ANOVA.

In the present study, the pharmacokinetic characteristics and the bioavailability of the two soft capsule formulations gave the very similar answer and therefore the bioequivalence of two formulations was established. The intersubject variations for the test formulation was slightly lower than the reference formulation (Fig. 1, Table I).

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