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Homogenous Enzyme Immunoassay for Cyclosporine in Whole Blood Using the EMIT®2000 Cyclosporine Specific Assay With the COBAS MIRA-Plus Analyzer

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We describe the evaluation of the EMIT®2000 cyclosporine specific assay kit, an enzyme-multiplied immunoassay for cyclosporine in whole blood, with a COBAS MIRA-plus analyzer. The enzyme used for the assay was glucose-6-phosphate dehydrogenase (EC 1.1.1.49 G6PDH) from Leuconostoc mesenteroides; the monoclonal antibody is fairly specific for cyclosporine, and is not reactive with most metabolites. The assay principle is based on competitive immunoassay with G6PDH-labeled cyclosporine and cyclosporine in sample to the anticyclosporine mouse monoclonal antibody binding site. The within-assay coefficient of variation (CV) of this method was 2.7–4.2% (n = 10) at the levels of 56.2–339.7 μg/L. Day-to-day CVs ranged from 4.2–8.1% at the levels of 47.2–350.2 μg/L. The within-day CVs ranged from 2.0–6.4% at the levels of 43.3–330.5 μg/L. The functional detection limit was 24.9 μg/L. Samples treated with pretreatment reagent were stable at least 5 hr. Calibration was stable at least 10 days. The analytical recovery was 81–109%. The correlation between values obtained with the EMIT®2000 cyclosporine specific assay kit (y) and fluorescence polarization immunoassay (FPIA) (TDxFLx) (x) was: y = 0.880x – 13.053 μg/L (r = 0.984, Sy/x = 15.968, n = 71) with a mean difference of 31.42 ± 19.89 μg/L ((TDxFLx – EMIT®2000) ± SD); for the FPIA (AxSYM) (x): y = 0.989 – 4.144 μg/L (r = 0.981, Sy/x = 17.478, n = 71) with a mean difference of 5.56 ± 17.38 μg/L ((AxSYM – EMIT®2000) ± SD); and for the radioimmunoassay (RIA, CYCLO-Trac SP) (x): y = 0.893 – 6.764 μg/L (r = 0.993, Sy/x = 10.582, n = 71) with a mean difference of 22.18 ± 14.98 μg/L ((RIA – EMIT®2000) ± SD) using the Bland-Altman technique. J. Clin. Lab. Anal. 15:319–323, 2001.

Key words: cyclosporine; homogenous enzyme immunoassay; glucose-6-phosphate dehydrogenase; method comparison

INTRODUCTION

Cyclosporine is a cyclical polypeptide of 11 amino acids with a molecular weight of 1202.6. It has been used as a powerful immunosuppressive agent to prevent the rejection of organic transplantation and to treat certain autoimmune diseases (1). The action of this drug inhibits the generation of lymphokines from helper T cells, such as interleukin-2, and γ-interferon (2,3). On the other hand, the major side effects of this drug, related to dosing period and/or given dose, have been associated with a reduction in glomerular filtration rate and hypertension (4). Cyclosporine has a narrow therapeutic range and displays large inter- and intrapatient differences in its pharmacokinetics. Therefore, trough level monitoring in whole blood is required for effective clinical management of patients.

Analytical methods for cyclosporine in whole blood include high-performance liquid chromatography (HPLC), fluorescence polarization immunoassay (FPIA), radioimmunoassay (RIA), and enzyme-multiplied immunoassay technique (EMIT) (5–9). The HPLC method has the advantage of detecting only native cyclosporine and cyclosporine metabolites, but it usually involves time-consuming processes for analysis, and requires special equipment and maintenance. The monoclonal antibody used in FPIA [TDxFLx or AxSYM (Dinabott Co., Ltd., Tokyo, Japan)] and in the RIA CYCLO-Trac SP kit (Dade Behring Co. Ltd., Tokyo, Japan) has been reported to cross-react with cyclosporine metabolites, and these methods require a specific analyzer (6,7,10). On the other hand, the cross-reactivity of cyclosporine metabolites by the EMIT method is comparatively low, and common analyzers can be used for the measurement of cyclosporine. Pretreatment of the sample is simple when compared with other methods (7,11,12).

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Here we report a homogenous enzyme immunoassay for the detection of whole-blood cyclosporine using an EMIT®2000 cyclosporine specific assay with a COBAS MIRA-plus analyzer. In the assay system, cyclosporine-specific anticyclosporine mouse monoclonal antibody reacts competitively with glucose-6-phosphate dehydrogenase (G6PDH)-labeled cyclosporine and various levels of cyclosporine in the sample. The ratio of bound and free forms cause a change in the activity of the enzyme with certain amounts of the anticyclosporine mouse monoclonal antibody. Comparison with other methods was also performed with whole blood from patients.

MATERIALS AND METHODS

Apparatus

The EMIT®2000 cyclosporine specific assay was carried out with COBAS MIRA-plus (Roche Diagnostics Co., Ltd., Tokyo, Japan), and the FPIA assay with the TDxFLx and AxSYM analyzer (Dinabott). HPLC was performed according to the procedure of Kovarik et al. (13), with minor modifications.

Reagents

The EMIT®2000 cyclosporine specific assay kit was obtained from Dade Behring Co., Ltd., Tokyo, Japan. The first reagent, A, was a Tris buffer solution containing anticyclosporine mouse monoclonal antibody, glucose-6-phosphate, and NAD^+. The second reagent, B, was a Tris buffer solution containing cyclosporine labeled with G6PDH.

Calibration was performed with cyclosporine whole-blood standards (concentrations of 0, 50, 100, 200, 350, and 500 μg/L) (Dade Behring, Marburg, Germany).

The new EMIT®2000 cyclosporine pretreatment reagent (EMIT-NPT) contains cupric sulfate, methanol, ethylene glycol, Tris buffer, sodium azide (<0.05%), streptomycin (<0.005%), and surfactant (11).

Sample Pretreatment

The procedure of pretreatment with whole blood consists of lyses of cells, solubilization of cyclosporine, and precipitation of proteins. The control and patient samples (100 μL), and the calibrator were each transferred to microcentrifuge tubes containing 300 μL of EMIT-NPT reagent. Tubes were capped and mixed for 1 min. After incubation for 2 min at room temperature, each tube was centrifuged at 9,600 × g for 4 min; the supernatant was then transferred to the sample cup and analyzed with the COBAS MIRA-plus.

Assay Procedure

The procedure was performed by kinetic assay at 37°C with the COBAS MIRA-plus centrifugal analyzer. To the calibrator, control, and patient samples (27.5 μL), a diluent of 47.5 μL was added and mixed with 155 μL of reagent A, and kept at 37°C for 100 sec; then 75 μL of reagent B and 20 μL of diluent were added. The change of absorbance was measured at 340 nm 275–550 sec after start of reaction.

Method for Comparison

Cyclosporine monoclonal whole-blood assay and AxSYM Cyclosporine II Dinapak, based on the FPIA method, were purchased from Dinabott. The Cyclo-Trac SP whole-blood cyclosporine RIA kit was from Dade Behring Co., Ltd. HPLC was carried out according to the procedure of Kovarik et al. (13) using a Phenomenex LUNA 5 μm C18, 250 × 4.6 mm column (Shimadzu Co., Ltd., Kyoto, Japan), at 75°C. The flow rate was 1.0 mL/min during the first 35 min, and then 1.5 mL/min for 20 min. The spectrophotometer was set at 215 nm. Cyclosporine and its metabolites were separated by gradient elution with isopropyl alcohol.

Samples

Whole-blood samples were collected in test tubes containing EDTA as the anticoagulant. A total of 71 samples were taken from patients who had received immunosuppressive therapy with cyclosporine after transplantation of kidney or bone marrow, or for autoimmune diseases.

RESULTS

Evaluation of EMIT®2000 Cyclosporine Specific Assay

A representative calibration curve for cyclosporine is shown in Fig. 1. Cyclosporine calibrators were analyzed in duplicate, and the data was managed with four-point logit-log mathematical equation.

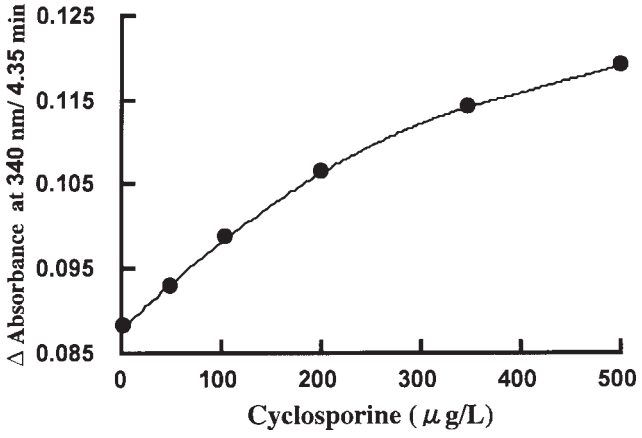

Fig. 1. Typical calibration curve for cyclosporine assay. The cyclosporine calibrators were 0, 50, 100, 200, 350, and 500 μg/L. The calibration curve was constructed by concentration vs. Δ absorbance at 340 nm with a four-parameter logistic function.

TABLE 1. Precision of cyclosporine assay

	Control 1	Control 2	Control 3
Within-assay (n = 10)			
Mean (μg/L)	56.20	148.00	339.70
SD (μg/L)	2.35	3.94	9.92
CV %	4.18	2.66	2.92
Day-to-day assay (n = 10)			
Mean (μg/L)	47.15	156.05	350.20
SD (μg/L)	3.84	6.51	19.06
CV %	8.14	4.17	5.44
Within-day assay (n = 10)			
Mean (μg/L)	43.25	148.35	330.50
SD (μg/L)	2.75	2.92	10.15
CV %	6.36	1.97	3.07

To determine the detection limit, absorbance of the zero calibrator was measured 10 times, and the mean value ± SD was calculated. The detection limit was defined as the cyclosporine concentration corresponding to an absorbance equal to the mean of zero calibrator value + 3SD, and the detection limit obtained was 20 μg/L. Furthermore, the functional detection limit was examined with cyclosporine calibrators in which the 50 μg/L calibrator was diluted by serial dilution with the zero calibrator, and was defined as the concentration of which the mean CV is ≈10%, and the functional detection limit obtained was 24.9 μg/L.

The within-assay, day-to-day, and within-day assay precision for three different whole-blood controls were tested as shown in Table 1. Day-to-day assay precision was carried out for 10 days over a 1-month period at 3- or 4-day intervals, and calibration was performed each day. Within-day assay precision was carried out for 10 hr at 1-hr intervals. Day-to-day and within-day precision was obtained in duplicate assay (the mean value is shown in Table 1). The within-assay CVs ranged from 2.7–4.2% at levels of 56.2–339.7 μg/L, day-to-day CVs were 4.2–8.1% at 47.2–350.2 μg/L, and within-day CVs were 2.0–6.4% at 43.3–330.5 μg/L.

In the linearity test for calibrator and whole-blood samples, dilution curves using the highest calibrator and whole-blood samples using serial dilution of zero calibrator were linear up to at least 500 μg/L cyclosporine concentrations.

The analytical recovery of cyclosporine (50, 100, and 200 μg/L) added to two different whole-blood controls ranged from 81.4–109.2%, and was performed in triplicate assay. The mean analytical recovery was 94.4%.

To examine pretreated sample stability, pretreated samples were stored at room temperature for 5 hr and were assayed at 1-hr intervals. The pretreated samples in covered cups were stable for at least 5 hr at room temperature.

We also examined the stability of calibration using three different whole-blood controls and a reagent kit with two different lot numbers for 1 month at 3-day intervals. The assay was performed in duplicate (mean value shown in Fig. 2). Calibration was stable for 10 days.

We examined the correlation between cyclosporine values

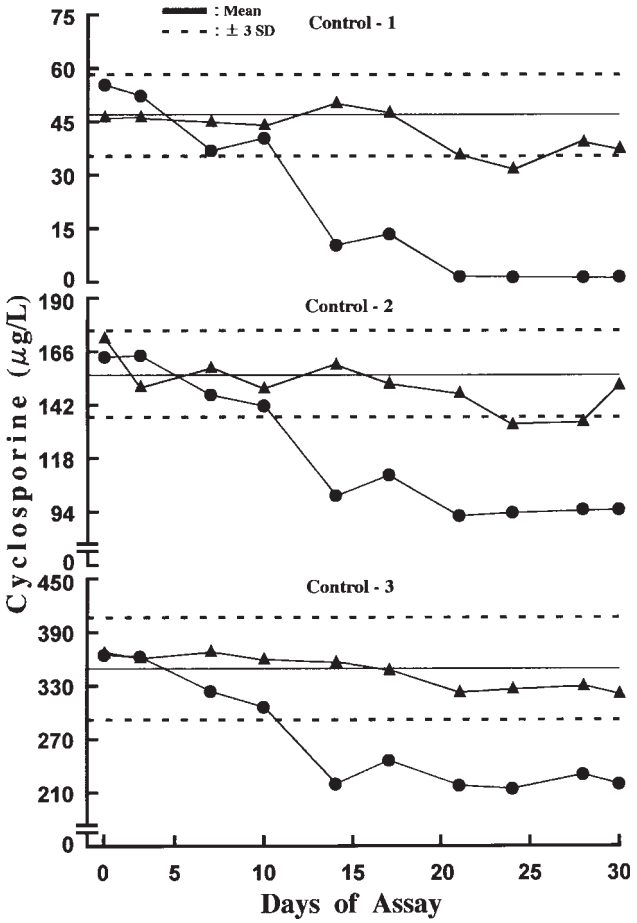

Fig. 2. Stability of calibration for cyclosporine assay. The mean values (solid line) as well as SD (±3 SD) of the mean values (dotted line) are shown parallel to the x-axis; the two different reagent lots are expressed as ● and ▲.

obtained by the EMIT®2000 cyclosporine specific assay (y) and conventional methods (x). Correlation was obtained by linear regression analysis, and differences between the values of EMIT®2000 cyclosporine specific assay and conventional methods were obtained by the methods of Bland and Altman (14). Using 71 samples, the mean values for cyclosporine concentration were 121.3 μg/L for the EMIT®2000 cyclosporine specific assay, 143.4 μg/L for the RIA, 152.7 μg/L for the FPIA (TDxFLx), and 126.8 μg/L for the FPIA (AxSYM). The relationship and regression line between the EMIT®2000 cyclosporine specific assay and RIA and FPIA (TDxFLx and AxSYM) are shown in Fig. 3A, C, and E. The degree of agreement between methods was assessed using the Bland-Altman graphical technique (Fig. 3B, D, and F). The mean bias was 22.18 (SD = 14.98, with 95% confidence interval of 18.64–25.73) for RIA – EMIT®2000 cyclosporine specific assay; for the FPIA (TDxFLx) – EMIT®2000 cyclosporine specific assay, 31.42 (SD = 19.89, with 95% confidence interval of 26.71–36.13); and for the FPIA (AxSYM) – EMIT®2000 cyclosporine specific assay, 5.56 (SD = 17.38,

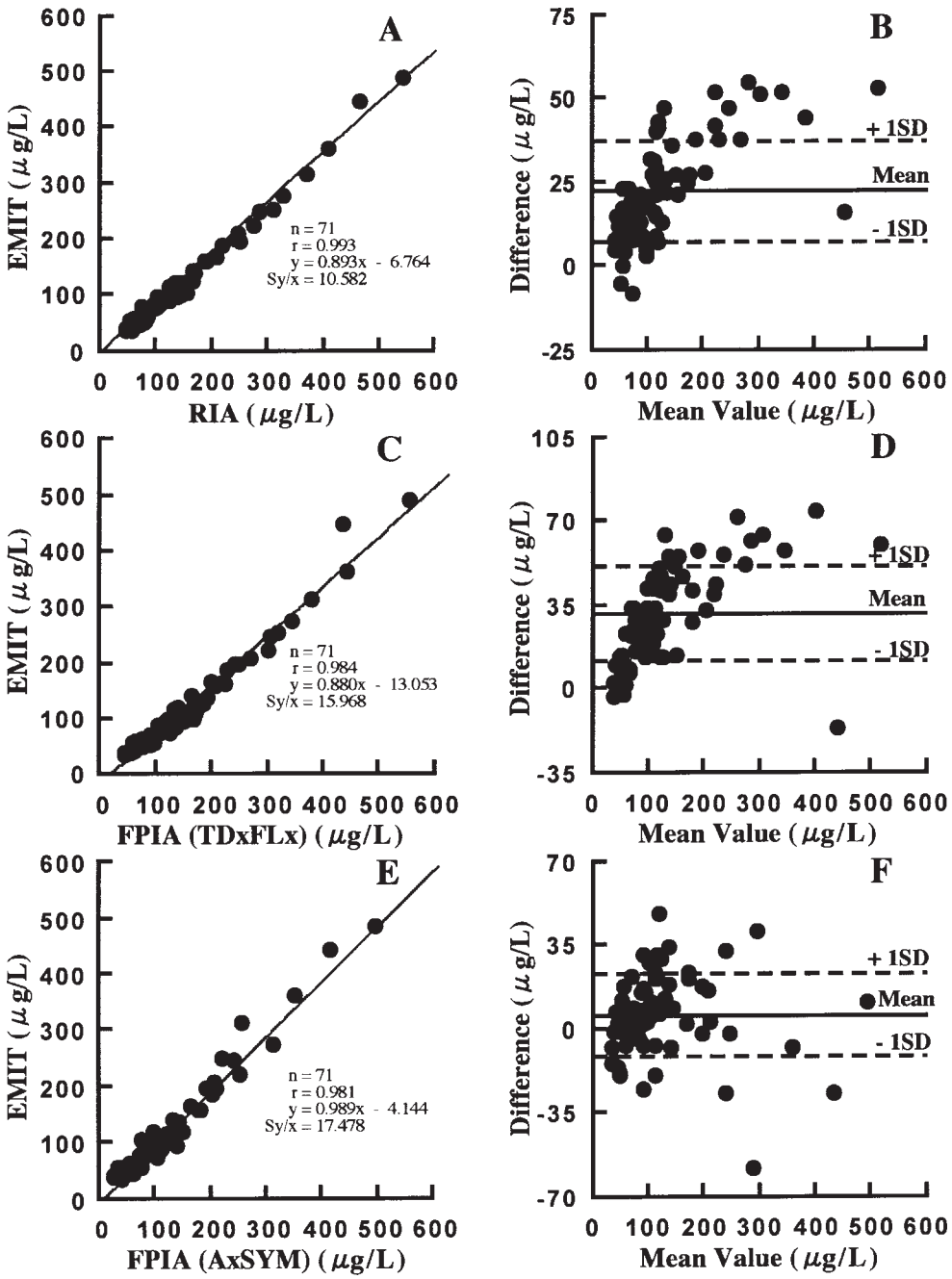

Fig. 3. Correlation of cyclosporine concentrations in whole blood with 71 samples from patients. **A**, **C**, and **E:** Linear regression analysis: (A) EMIT®2000 cyclosporine specific assay (y) vs. RIA (x), (C) EMIT®2000 cyclosporine specific assay (y) vs. FPIA (TDxFLx) (x), and (E) EMIT®2000 cyclosporine specific assay (y) vs. FPIA (AxSYM) (x), respectively. **B**, **D**, and **F:** Bland-Altman plot showing the differences between the cyclosporine values of the EMIT®2000 cyclosporine specific assay and (B) RIA, (D) FPIA (TDxFLx), and (F) FPIA (AxSYM), respectively. The mean value of the two methods is plotted against the difference between the two values. The mean differences (±SD) between the two methods were (B) 22.18 ± 14.98, (D) 31.42 ± 19.89, and (F) 5.56 ± 17.38, respectively. The mean difference (solid line) and the SD (±SD) of the mean difference (dotted line) are shown parallel to the x-axis.

with a 95% confidence interval of 1.45–9.67). The cyclosporine values obtained by five methods for five samples chosen from the 71 samples were compared (Table 2). The cyclosporine metabolites in samples confirmed by HPLC are shown in Table 2 as (+) or (–). In all of the samples AM1, a cyclosporine metabolite, was confirmed, and in one sample AM9 was confirmed. The cyclosporine values obtained by the EMIT®2000 cyclosporine specific assay had better agreement with HPLC findings than those obtained by FPIA and RIA.

TABLE 2. Comparison of cyclosporine values with HPLC and another method

	Cyclosporine (μg/L)					Metabolites	
Specimen	EMIT	TDxFLx	AxSYM	RIA	HPLC	AM1	AM9
1	448	432	421	464	410	+	–
2	190	223	208	218	208	+	–
3	364	438	356	408	352	+	–
4	317	375	259	369	327	+	–
5	127	182	146	163	146	+	+

DISCUSSION

In the EMIT®2000 cyclosporine specific assay, a pretreatment reagent (EMIT-NPT) is used that results in a more simplified procedure than the methanol extraction method. The EMIT-NPT reagent is pipetted to a microcentrifuge tube before the whole-blood sample was added, because the addition of EMIT-NPT to whole blood affects the precision of this assay. The precision when whole blood was added to EMIT-NPT is shown in Table 1. The precision when EMIT-NPT is added to a whole-blood sample was 6.9% (CV) at the level of 55.6 μg/L (mean), 2.0% (CV) at 167 μg/L (mean), and 7.1% (CV) at 416 μg/L (mean) for within-assay (n = 10). These values may be caused by the slow reaction of the EMIT-NPT reagent with the whole-blood sample. Therefore, for good precision the whole-blood sample must be added to the EMIT-NPT reagent in the microcentrifuge tube.

Cyclosporine concentrations in whole blood from 71 samples were compared with EMIT®2000 cyclosporine specific assay, TDxFLx, AxSYM, and RIA. Negative bias of 11–12% for the EMIT®2000 cyclosporine specific assay was observed when compared with TDxFLx and RIA. The mean assay value was highest in TDxFLx, followed in descending order by RIA, AxSYM, and EMIT®2000 cyclosporine specific assay. In the EMIT®2000 cyclosporine specific assay, the cross-reactivity with cyclosporine metabolites AM9 (M1), AM19 (M8), and AM1 (M17), which are hydroxylation metabolites of cyclosporine, and AM4N (M21), which is a demethylated metabolite of cyclosporine, were 7.3%, 3.0%, <0.3%, and <0.3%, respectively (12). The cross-reactivity of other conventional methods has been reported to be 0–15.3% (6,7,10). The cross-reactivity of these cyclosporine metabolites for AM9 and AM1 were 15.3% and 8.2% by TDxFLx analyzer, 10.9% and 6.5% by AxSYM analyzer, and 1.7% and 0.7% (IC_{50}) by RIA, respectively. We also compared the values of cyclosporine and cyclosporine metabolites obtained by the TDxFLx analyzer, AxSYM analyzer, and RIA using HPLC values as a reference for the cyclosporine values. Cyclosporine values by the EMIT®2000 cyclosporine specific assay and AxSYM analyzer were almost the same as those of HPLC. The EMIT®2000 cyclosporine specific assay appears to selectively measure the cyclosporine, and to show less cross-reactivity for cyclosporine metabolites than do conventional methods.

There remain some problems with precision at low and high concentrations, and with stability of calibration in the EMIT®2000 cyclosporine specific assay. On the other hand, the EMIT®2000 cyclosporine specific assay showed less cross-reactivity for cyclosporine metabolites, and could be applied to most random-access analyzers. In conclusion, the EMIT®2000 cyclosporine specific assay may be suitable for routine clinical use in the laboratory, especially for monitoring drugs to prevent rejection of organ transplants and for treating certain autoimmune diseases.

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