MATERIALS AND METHODS

Materials

All reagents and instruments used in the study were listed as follows;

Reagents and instrument for determination of blood cyclosporine concentrations (Abbott Laboratories, IL, U.S.A.)

Cyclosporine monoclonal whole blood antibody (mouse monoclonal) solution

Cyclosporine monoclonal whole blood fluorescein tracer solution

Pretreatment solution

Whole blood precipitation reagent/probe wash

Solubilization reagent

The Abbott TDx analyzer

Reagents and instrument for determination of blood tacrolimus concentrations (Abbott Laboratories, IL, U.S.A.)

Anti-tacrolimus (mouse, monoclonal) antibody coated microparticles in TRIS buffer with protein (bovine) stabilizers

Tacrolimus alkaline phosphatase conjugate in TRIS buffer with protein (bovine) stabilizers

4-Methylumbelliferyl phosphate, 1.2 mM, in AMP buffer

Wash solution

Whole blood precipitation reagent

The Abbott IMx analyzer

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Chemicals, reagents and instrument for determination of hyaluronan concentrations

Biotinylated-hyaluronic acid binding protein (prepared at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University)

Biotinylated-hyaluronic acid binding protein solubilization reagent (prepared at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University)

Bovine serum albumin (Sigma Chemical Co., U.S.A.)

Citrate phosphate buffer (prepared at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University)

Coating buffer (prepared at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University)

Hyaluronic acid (Sigma Chemical Co., U.S.A.)

Hydrogen peroxide (Merck, Germany)

O-phenylenediamine (Sigma Chemical Co., U.S.A.)

Peroxidase conjugated anti-biotin monoclonal antibody (Zymed Laboratory, Inc., U.S.A.)

Phosphate buffer saline (prepared at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University)

Phosphate buffer saline-Tween (prepared at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University)

Sulfuric acid (J.T. Baker Neutrasorb, U.S.A.)

Microtiter plate (Nunc, Denmark)

ELISA plate reader: Titertek multiscan Mcc/340 (ICN, Flow, U.S.A.)

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Sample collection

The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University for use of human tissue samples. The blood samples (N=200) were collected from stored samples obtained from the Drug Monitoring Center, Clinical Pharmacology Unit, Department of Pharmacology, Faculty of Medicine, Chiang Mai University. The blood samples were drawn from the renal transplant patients who were followed up at the Division of Nephrology, Department of Medicine, Faculty of Medicine, Chiang Mai University. The primary

purpose for sending the samples was to monitor the cyclosporine or tacrolimus levels after renal transplantation. After the drug level was determined, the rest of blood samples were centrifuged for 5 minutes, then the plasma were collected and stored at $-20^{\circ}C$ until analysis. The demographic of the patients as well as their medical history, laboratory findings such as liver function test, blood urea nitrogen, creatinine, and the result of renal biopsy were recorded.

Exclusion criteria

Blood samples obtained from the patients who had one of the following criteria were excluded from the study.

- Patients who had a history of liver disease or elevated liver enzyme.
- Patients who had inflammatory joint or bone disease.
- Patients who had chronic bronchitis or asthma.
- Patients who had infectious disease.
- Patients who had tumor or cancer.
- Patients who were pregnant.

Determination of blood cyclosporine concentrations

The whole blood samples were analyzed for cyclosporine concentrations by fluorescence polarization immunoassay (FPIA) technique using the Abbott TDx analyzer. The FPIA procedure is an automated method for drug level monitoring routinely performed at the Drug Monitoring Center, Clinical Pharmacology Unit, Department of Pharmacology, Faculty of Medicine, Chiang Mai University.

To minimize interference from endogenous protein-bound fluorescence compounds, a pretreatment sample preparation was preformed as follows. Pipetted 150 μl of blood samples into the centrifuge tubes. Added 50 μl of solubilization reagent and 300 μl of whole blood precipitation reagent/probe wash into the sample in order to solubilize cells and precipitate protein, followed by centrifugation for 15 minutes (at 2,500 rpm) to obtain a clear supernatant. Thereafter, the assay was conducted according to the manufacturer's protocol without modification. Three controls (low: 150 ng/ml; medium: 400 ng/ml; and high: 800 ng/ml) were run with the carousel of samples. The coefficient of variation between measurement was

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8.63%, 9.67% and 3.52% for the low, medium and high cyclosporine levels, respectively. The results were shown below:

Standard concentration (ng/ml)	Reading (ng/ml±SD)	Coefficient of Variation (%)
120.00 – 180.00	136.46±11.78	8.63
360.00 – 440.00	389.99±37.70	9.67
680.00 – 920.00	755.05±26.61	3.52

Principle of fluorescence polarization immunoassay

The Abbott TDx System uses a competitive binding immunoassay methodology[29], to allow tracer-labeled antigen and patients antigen to compete for binding sites on the antibody molecules. The components in this binding reaction are the antibody, the patient antigen, and the antigen labeled with fluorescein (tracer-antigen complex). When competitive binding occurs, the more antigen-antibody complex then becomes a part of very large antibody molecule, and consequently the less tracer-antigen complex that remains in solution.

While the tungsten halogen lamp in the TDx System emits light of different wavelengths or colors in a random spatial orientation. An interface filter, located in front of the light source, allows blue light (481-489 nm) to pass through. The light is then passed through a liquid-crystal polarizer to produce a beam of plane polarized blue light. The plane polarized blue light excites the tracer, or fluorophore, and raises it to an excited state. After excitation, the fluorophore returns to steady state and green light (525-550 nm) is emitted from the fluorophore. If the fluorophore is bound to a very large antibody molecule and does not rotate freely, the emitted green light will be in the same plane as the blue excitation light and polarization is retained. Conversely, if the fluorophore is free to rotate because the small free tracer molecule is not bound, the emitted green light will be in a different plane than the blue excitation light and polarization is lost. Because of the rotational properties of molecules in solution, the degree of polarization is directly proportional to the size of the molecule, that is, polarization increases as molecular size increases. Therefore, if a patient sample contains a low concentration of antigen, after a competitive binding reaction reaches steady state, there will be a high concentration of

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bound tracer in the reaction mixture and polarization will be high. Conversely, if there is a high concentration of antigen in the sample being tested, after the competitive binding reaction reaches the steady state, there will be a low concentration of bound tracer in the reaction mixture and polarization will be low. The precise relationship between polarization and concentration of the unlabeled drug in the sample is established by measuring the polarization values of calibrators with known concentrations of the drug.

Determination of blood tacrolimus concentrations

The samples were analyzed for tacrolimus concentrations by microparticle enzyme immunoassay (MEIA) technique using the Abbott IMx analyzer. The MEIA procedure is an automated method for tacrolimus level monitoring routinely performed at Drug Monitoring Center, Clinical Pharmacology Unit, Department of Pharmacology, Faculty of Medicine, Chiang Mai University. The pretreatment sample preparation was performed as follows. Pipetted 150 μl of blood samples into the centrifuge tubes. Added tacrolimus whole blood precipitation reagent into the sample in order to precipitate protein, followed by centrifugation for 15 minutes at 2,500 rpm. Then immediately decanted the supernatant into the well. The assay was conducted according to the manufacturer's protocol without modification, and 3 controls (low: 5 ng/ml; medium: 11 ng/ml; and high: 22 ng/ml) were run with the carousel of samples. The coefficient of variation between measurement was 16.5%, 9.35% and 10.5% for the tacrolimus levels of low, medium and high, respectively. The results were shown below:

Standard concentration (ng/ml)	Reading (ng/ml±SD)	Coefficient of Variation (%)
3.0 – 7.0	5.13±0.85	16.5
7.7 – 14.3	10.49±0.98	9.35
15.4 – 28.6	24.79±2.60	10.50

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Principle of microparticle enzyme immunoassay

The Abbott IMx System uses a competitive binding immunoassay methodology[30] to allow tracer-enzyme antigen and patients antigen to compete for binding sites on the antibody molecules. The components in this binding reaction are the antibody, the patient antigen, and the antigen labeled with enzyme (enzyme-antigen complex). When competitive binding occurs, the more antigen-antibody complex then the less enzyme-antigen complex that remains in solution. While adding the fluorogenic substrate, the enzyme-antigen will catalyze the substrate and then emit the light. The MEIA optics measure the rate at which the fluorescent product is generated. The rate, at which fluorescent product is generated, is proportional to the concentration of antigen in the test sample.

If a patient sample contains a low concentration of antigen, after a competitive binding reaction reaches steady state, there will be a high concentration of bound enzyme in the reaction mixture and the rate of fluorescent product will be high. Conversely, if there is a high concentration of antigen in the sample being tested, after the competitive binding reaction reaches the steady state, there will be a low concentration of bound enzyme in the reaction mixture and the rate of fluorescent product will be low. The precise relationship between the emitted light and concentration of the unlabeled drug in the sample is established by measuring the values of calibrators with known concentrations of the drug.

Determination of HA concentrations

The procedure was modified from Yingsang's method[31]. HA content in individual samples was assayed in triplicate. This involved competitive for bound to biotinylated HABP (B-HABP) between HA in the wells of a microtiter plate, and HA in standard dilution ranged from 10-10,000 ng/ml (in a serial two-fold dilutions) or in samples. Then detected bound B-HABP with peroxidase conjugated monoclonal antibiotin. Finally peroxidase substrate reacts with enzyme peroxidase to form color, which measured at 492/690 nm. The step of method is shown in Figure 6. The inhibition step between HA and B-HABP had been employed in small polypropylene tubes. Samples containing unknown amounts of HA and standard (using known concentrations of highly purified HA (Haelon®) in PBS pH 7.4, containing 6% (w/v) BSA were

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pipetted into small tubes with B-HABP (equal volume of 175 μl/tube) in 0.1 M Tris-HCl pH 8.6 and 0.05% Tween. A vortex mixture was used prior to incubation at room temperature for one hour. Aliquots of 100 μl (triplicate) were applied to HA coated and BSA-blocked plates and incubated at room temperature for 60 minutes. The plate wells were washed three times with PBS-Tween 20 (0.5%v/v), and 2 μl of peroxidase conjugated monoclonal antibiotin was added to each well. Plates were incubated for 60 minutes at room temperature and wash 3 times with PBS-Tween, then dried; peroxidase substrate was added to each well (100 μl) and kept in the dark in order to allow development of the color. The reaction was terminated by the addition of 50 μl of 4 M H_2SO_4. The chromogen was read for absorption at 492/690 nm in a plate reader (Titertek multiscan Mcc/340). The averaged value from triplicate results and the percentage inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{100 - [\text{A492 (B-HABP+sample)} - \text{A492 (BSA)}]}{[\text{A492 (B-HABP+BSA)} - \text{A492 (BSA)}]} \times 100$$

A standard curve for HA assay was constructed using a computed software (Datasoft II), and by using the absorbance unit, the level of HA in the samples were calculated automatically with 4-parameter curve fit.

Coating microtiter plate with HA

The procedure for coating the plate with HA was modified from Goldberg's method[32]. Microtiter plate (Maxisorp) were coated with HA (100 μg/ml) in alkaline carbonate buffer 100 μg/well and allowed to adhere passively to the plastic surface at 4°C overnight. The solution was flicked out and the plate was dried, the unreacted adsorption sites were blocked by the addition of 150 μl/well of 1% BSA in PBS pH 7.4. Incubation was at room temperature for 60 minutes. The wells were washed three times with PBS-Tween 20 (150 μl/well) and the plate was air-dried. The coated HA-plates were wrapped in polythene film and stored at 4°C until used.

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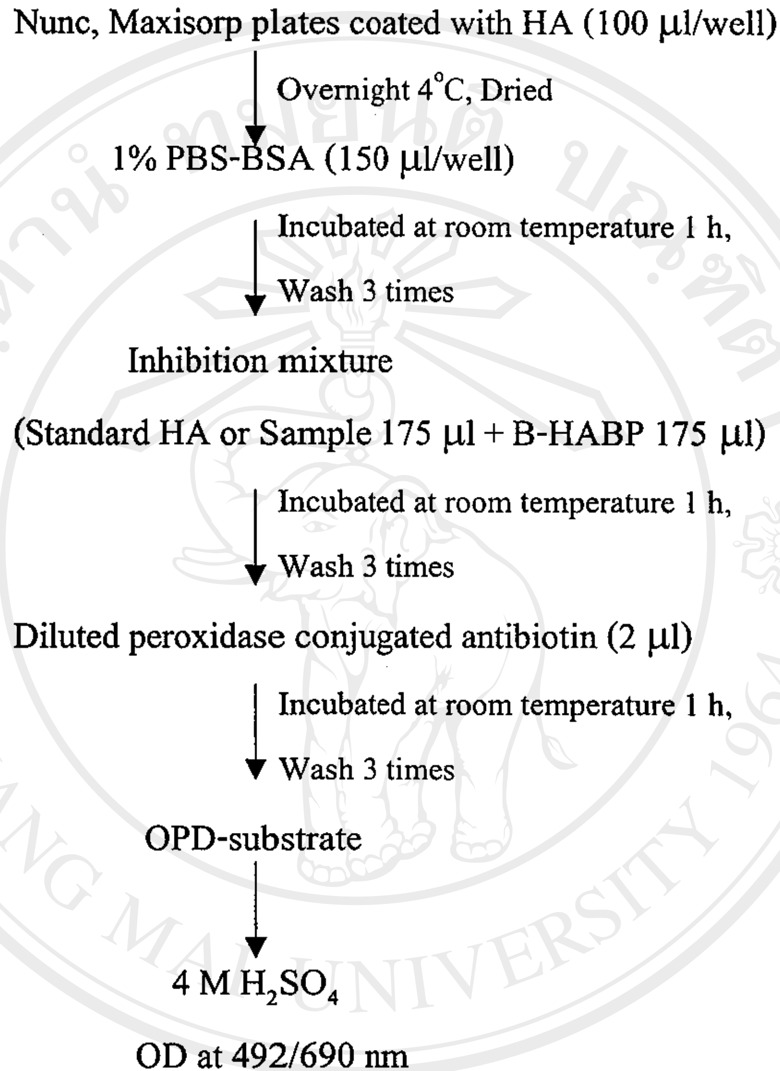

Figure 6. The step of method developed for HA measurement[10].

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Data analysis

1. Correlation of hyaluronan level and immunosuppressive drug levels

The patient's drug levels were divided into 3 groups according to the duration of drug administration as follows;

A. Cyclosporine-treated patients

Group 1: duration of drug administration less than 2 months (< 60 days)

(therapeutic drug level was 250 ng/ml)

Group 2: duration of drug administration 2-6 months (60-180 days)

(therapeutic drug level ranged from 150-250 ng/ml)

Group 3: duration of drug administration more than 6 months (> 180 days)

(therapeutic drug level ranged from 100-150 ng/ml)

B. Tacrolimus-treated patients

Group 4: duration of drug administration less than 2 months (< 60 days)

(therapeutic drug level ranged from 15-20 ng/ml)

Group 5: duration of drug administration 2-6 months (60-180 days)

(therapeutic drug level ranged from 10-15 ng/ml)

Group 6: duration of drug administration more than 6 months (> 180 days)

(therapeutic drug level ranged from 8-10 ng/ml)

The HA level and the patient's drug levels were presented as mean ± SD. The data in each group were tested for the differences by using Kruskal-Wallis Test and the P value of < 0.05 indicated statistical significance. The HA level and drug levels were depicted as scatterplots and the relationship between the two measures were determined using Spearman correlation coefficient.

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2. Correlation of hyaluronan level and graft status

The patient's data were divided into 3 groups according to the graft status as follows;

- acceptable graft (the rejection was not shown on the renal biopsy)
- threatened graft rejection (the sign of graft rejection was shown on the renal biopsy)
- post-rejection graft with successful therapy (the presence of history of graft rejection)

The HA level was presented as mean ± SD. The data in each group were tested for the differences by using Kruskal-Wallis Test and the P value of < 0.05 indicated statistical significance.

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