A Modified Fluorescence Polarization Immunoassay Method Incorporating Fat Emulsion (FE-FPIA) to Determine Cyclosporin A Concentrations in Rat Skin

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We have developed a simple, sensitive and reliable assay procedure for cyclosporin A (CyA), a modified fluorescence polarization immunoassay method incorporating fat emulsion (FE-FPIA), to determine the CyA content in rat skin. The conventional fluorescence polarization immunoassay (FPIA) method for CyA using a commercially available FPIA kit, TDX® cyclosporine monoclonal whole blood, was modified. A fat emulsion for intravenous infusion, Intralipos®, was incorporated for dissolving the CyA extracted from the skin tissue, and a mixture of MeOH/purified water was used as the sample pretreatment medium instead of the precipitation reagent in the conventional FPIA kit intended for whole blood samples. These modifications enabled us to produce a reliable and the sensitive assay of CyA in skin tissue. The reproducibility (coefficient of variation), detection limit, and assay time for FE-FPIA were below 2%, 25 ng/ml, and about 24 min/24 samples, respectively, and were comparable with those for the whole blood samples determined by the conventional FPIA. Pre-purification of samples required by the HPLC assay is not needed in the FE-FPIA method. The usefulness of the FE-FPIA method in evaluating the topical pharmacokinetics of CyA in skin is discussed.

Key words cyclosporin A; fluorescence polarization immunoassay; fat emulsion; topical pharmacokinetics

Cyclosporin A (CyA), an undecapeptide produced by Tolypocladium inflatum Gams,1 suppresses the rejection mediated by T-cells in allograft transplantation.2 CyA has been used world wide to prolong graft survival of several human organs such as liver, kidney, and bone marrow following transplantation. In the last decade, clinical studies on the application of CyA to autoimmune diseases have also been attempted.3, 4 Recently, in order to reduce the systemic side-effects of CyA in the treatment of psoriasis, CyA ointments have been developed.5-7 Topical pharmacokinetic studies on CyA in the skin is indispensable for discussing the efficacy-safety balance of ointments containing the immuno suppressant, and topical pharmacokinetic information is expected to facilitate the rational development of CyA ointments for psoriasis. However, the topical pharmacokinetics of CyA in the skin has not yet been investigated.

To assay CyA, HPLC8,9 and fluorescence polarization immunoassay (FPIA)10,11 methods have been developed. The former is capable of separating intact CyA from its metabolites concomitantly present in biological fluid or in tissue samples. However purification of the samples to remove biological components which may interfere with the chromatographic peak of CyA is essential, because the wavelength for detection is restricted to around 200 nm.12 Furthermore, the detection limits of the HPLC method are very much influenced by assay conditions.13 The alternative, the FPIA method, is specific for immuno-intact drugs. TDX® cyclosporine monoclonal whole blood (Abbott Laboratories, IL) is a commercially available assay kit for CyA. A TDX® analyzer, a semi-automated FPIA instrument, employing this assay kit enables us to perform highly sensitive and reliable routine assays relatively quickly. Thus, the FPIA method is commonly used for clinical therapeutic drug monitoring (TDM) of CyA concentrations in patients who have been given CyA after a transplant. However the utility of this conventional FPIA method is restricted to whole blood samples. In order to determine CyA concentrations in skin using the TDX® analyzer, dissolution of CyA extracted from skin by the addition of ethanol was attempted. However, the reproducibility of the conventional FPIA method applied to those ethanol-rich samples was unsatisfactory for quantitative assay.

In this study, we modified the conventional FPIA method for CyA to make it applicable to skin samples by the incorporation of Intralipos®, a fat emulsion for intravenous infusion, to dissolve the CyA extracted from skin tissue. The usefulness of the modified method (FE-FPIA) to evaluate the topical pharmacokinetics of CyA in skin is discussed.

MATERIALS AND METHODS

Materials Sandimun® injection (50 mg CyA/ml), Intralipos® 10%, and 20% Urea Keratinamin were obtained from Novartis Pharma (Tokyo, Japan), The Green Cross Corporation (Osaka, Japan), and Kowa (Nagoya, Japan), respectively. TDX® Cyclosporine-SP- Dainabon® was purchased from Dainabot Co. (Tokyo, Japan). X Systems™ calibrators cyclosporine monoclonal whole blood and X Systems™ controls cyclosporine monoclonal whole blood, which were supplied by Abbott Laboratories (IL, U.S.A.), were used as the standard and control for CyA in human whole blood, respectively. All other reagents were reagent-grade commercial products.

Instrument Used for FPIA and FE-FPIA A TDX® analyzer (Dainabot Co.) was used for determining the CyA content of the sample solutions in both the FPIA and the FE-

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FPIA methods.

Determination of CyA Content by the FPIA Method
Determination of the CyA content in samples were performed by the conventional FPIA method according to the TDX® analyzer manual.

Standard CyA Solution for FE-FPIA
Standard CyA solution was prepared as follows: 4 ml CyA solution in ethanol (5000 ng/ml) was diluted to 20 ml with a mixture of Intralipos® 10%–purified water (1:1 by volume) to obtain the stock CyA solution (1000 ng/ml) containing approximately 4% fat emulsion. The stock solution was diluted to give final CyA concentrations of 125, 250, and 500 ng/ml using the mixture of Intralipos® 10%–purified water/ethanol (2:2:1 by volume). One hundred and fifty microliters aliquots of the resulting standard CyA solutions were used to prepare a calibration curve for the FE-FPIA method.

Standard CyA in Skin for FE-FPIA
An aliquot of the ethanolic CyA solution (5000 ng/ml) was added to excised rat abdominal skin tissue (1.13 cm²), the CyA content of which was 5000, 2500, 1250, 250 or 125 ng/ml, in a screw-capped test-tube. After storage of the tube in a refrigerator overnight, the ethanol was evaporated. Then, the skin tissue in the tube was kept at 5°C for another day. After the addition of 4 ml phosphate buffered saline (pH 7.4, PBS), the skin tissue was cut into pieces with scissors. CyA in the skin tissue was extracted by the addition of chloroform (5 ml) with shaking (320 str/min × 15 min) and centrifugation (3000 rpm × 10 min). Two milliliters of the chloroform layer was withdrawn and the solvent was evaporated at 50°C. Then 0.2 ml ethanol and 0.8 ml of the mixture of Intralipos® 10%–purified water (1:1 by volume) were added to dissolve the residue. One hundred and fifty microliters aliquots of the resultant solution were used as standard CyA in skin tissue for the FE-FPIA method.

Assay Procedure for CyA Samples Containing Intralipos® by FE-FPIA
One hundred and fifty microliters of each CyA sample solution containing Intralipos® was transferred to a micro-centrifuge tube. Then 300 μl MeOH–purified water (7:3 by volume) was added followed by 50 μl solubilization reagent. After centrifugation of the mixture at 13000 rpm for 5 min, more than 50 μl of the supernatant was transferred to the sample well of the TDX® analyzer and the CyA content assayed automatically in the usual manner.

Animals
Male Wistar rats, 9 to 10 weeks old, were used in the study.

Oral Administration of CyA to Rats
The rats were allowed free access to water and a standard laboratory diet during the duration of the study. Sandimmun® injection (50 mg CyA/ml) was diluted 10 times with PBS. CyA (30 mg/kg/d) was administered to the rats intragastrically via a gastric sonde twice a day for 12 d. Rats were sacrificed 12 h after the last dose, and the abdominal skin tissue was excised. After removal of the stratum corneum layer and subcutaneous fat using tape-stripping and scissors, respectively, skin tissue (1.13 cm²) was prepared and the CyA extracted as described above.

Transdermal Administration of CyA to Rats
The abdominal hair of rats was removed 2 d before the experiment. CyA ointment containing 1% CyA was prepared by the addition of Sandimmun® injection to 20% Urea Keratinamine, and then applied to the abdominal skin (15.5 cm²) at a dose of 1.5 g/rabbit under urethane anesthesia (1000 mg/kg, i.p.). A percutaneous absorption experiment was performed over 12 h. At appropriate time intervals, 250 μl venous blood was withdrawn from the jugular vein and used to assay CyA by the FPIA method. At the end of the experiment, the rats were sacrificed by decapitation, and the ointment remaining on the skin surface was removed carefully together with the stratum corneum by tape stripping. After careful dissection of the subcutaneous fat, viable skin tissue (1.13 cm²) was excised and prepared for assay of the CyA content by the FE-FPIA method.

RESULTS AND DISCUSSION

Validation of FE-FPIA
Intralipos®, an aqueous intravenous fat emulsion, which consists of purified soybean oil, glycerin for injection, purified egg yolk lecithin, and water for injection, is a suitable solvent for dissolving CyA, because the solubility of CyA in its soybean oil component is extremely high. In order to develop an assay method for CyA in tissues, for example the skin, using a conventional FPIA instrument, the TDX® analyzer, we introduced a dissolution step with Intralipos® for the tissue extract containing CyA.

As the samples in the conventional FPIA method are whole blood, solubilization and precipitation reagents are used to pretreat the samples. However, in the case of the FE-FPIA method, a white precipitate or turbidity appeared after the addition of these reagents to the dissolved samples containing fat emulsion. Although the detection limit for these turbid samples was almost equal to that for standard whole blood samples, the turbid samples resulted in lower assay reproducibility (data not shown). Perhaps, an interaction between the precipitation reagent and the antibody added to the protein-free samples may have caused additional turbidity during assay in the TDX® analyzer. In order to clarify the supernatant of the sample mixture, a mixture of MeOH–purified water (7:3 by volume) was used in the FE-FPIA method, instead of the precipitation reagent in the TDX® Cyclosporine-SP·Dainapack®. The MeOH–purified water mixture was not expected to interact with the antibody, because the precipitation reagent in the TDX® Cyclosporine-SP·Dainapack® consists of 80% (v/v) MeOH. A negligibly small amount of oil droplets was observed after centrifugation of the CyA sample solution containing Intralipos®, following addition of the new pretreatment medium, the MeOH–purified water mixture, but this did not adversely affect assay sensitivity or reproducibility.

Figure 1 shows the standard curves for CyA assayed by conventional FPIA and FE-FPIA methods. When the mNet P values determined by the TDX® analyzer were plotted against the log CyA concentration, both standard curves exhibited satisfactory linearity guaranteeing quantitative detection down to 25 ng/ml. Table 1 summarizes the reproducibility data for the conventional FPIA and FE-FPIA methods. The coefficient of variation (C.V.), indicating the reproducibility of the conventional FPIA method, was around 2.0% when X Systems™ controls cyclosporine monoclonal whole blood was used as a control. The C.V. for the FE-FPIA method was less than 2.0%, suggesting satisfactory reproducibility compared with that of the conventional FPIA method. Both the detection limit and assay reproducibility of
the FE-FPIA method were similar to those of the conventional FPIA method for whole blood samples. The running time for the assay using the FE-FPIA method was about 24 min/24 samples. Thus, the FE-FPIA method offers a reliable quantitative assay for biological samples containing CyA by using Intralipos® and the MeOH–purified water mixture to dissolve the medium and pretreatment medium, respectively.

**Determination of CyA in Skin by the FE-FPIA Method**

Rat abdominal skin tissue was used to evaluate the FE-FPIA method for determining the CyA content of skin after topical CyA application. Although HPLC has been applied to assay CyA in biological tissues, in order to remove the biological components interfering with the CyA peak during HPLC chromatography, complicated pretreatment procedures such as solid-phase extraction are required to obtain acceptable detection limits and good assay reproducibility. However, in the present FE-FPIA method, chloroform-extraction is the only step before the final dissolution. The standard curve for determining the CyA content in skin samples by the FE-FPIA method was in reasonable agreement with that for the calibration standard in Fig. 1 and the C.V. was acceptable (Fig. 2 and Table 2). This means that the recovery of CyA from skin using chloroform-extraction followed by dissolution with Intralipos® is practically 100%. As the physicochemical characteristics of the emulsion, such as the size distribution of the oil droplets, are highly controlled in commercially available aqueous intravenous fat emulsion products, the use of Intralipos® as a lipid emulsion dissolving medium in the FE-FPIA method minimizes the inter-institutional variation in determining the CyA content of biological samples.

Recently, CyA ointments have been developed for psoriatic patients. However, the topical pharmacokinetics of CyA in the skin, information which is indispensable for discussing the efficacy-safety balance of the topically administered immuno-suppressing agent, has not been investigated in developing CyA ointment for psoriasis. The CyA concentrations in rat abdominal skin and in rat whole blood determined by the FE-FPIA method and the conventional FPIA method, respectively, after oral administration of CyA for 12d are listed in Table 3, together with the data after trans-
of CyA in Intralipos®, and Lipo-Microdialysis, a high performance microdialysis method for lipophilic substances using Intralipos®, will enable more accurate pharmacokinetic analysis of CyA after application of CyA ointments to treat psoriasis.

REFERENCES