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Note

Determination of a novel potent immunosuppressant (FK-506) in rat serum and lymph by high-performance liquid chromatography with chemiluminescence detection

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FK-506 (I) a macrolide antibiotic extracted from the fermentation broth of *Streptomyces tsukubaensis* [1], has been shown to have strong immunosuppressive activity against a mixed lymphocyte reaction (MLR) [2]. In addition, intramuscular injection of I at 1 mg/kg per day will prolong graft survival in heterotropic cardiac allotransplantation in rats [3]. The pharmacological mechanism is considered to be a suppression of both interleukin 2 (IL-2) and IL-2 receptor expression on T cells [4]. The pharmacological activity of I is reported to be *ca.* 100 times greater than that of cyclosporin A (CyA) [5,6].

To perform a bioavailability study of I in both humans and dogs, an enzyme immunoassay (EIA) method has been established and used for the determination of plasma levels of I [7]. However, we cannot deny the existence of the cross-reactivity of the antisera with some of the circulating metabolites of I, as suggested from a pharmacokinetic study with CyA [8,9]. A biopharmaceutical study using EIA [8] showed that the amounts of I excreted into both the urine and the bile of dogs is less than 1% of the injected dose. Therefore, the main elimination pathway of I is thought to be the metabolism in the body. Thus, a new assay method specific to I in the plasma is needed to perform basic pharmacokinetic studies in experimental animals. In addition, we have been studying the relation-

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ship between the effect of immunosuppressants and their lymphatic concentrations, especially with CyA. To perform this study with I, a specific assay method for I in the lymph samples is needed.

This paper describes a new specific assay method for the determination of I in rat plasma and lymph by high-performance liquid chromatography (HPLC) using chemiluminescence (CL) detection [10] after pre-labelling with dansyl hydrazine as a fluorescent reagent.

EXPERIMENTAL

Chemicals and reagents

FK-506 (I) was obtained from Fujisawa Pharmaceutical (Osaka, Japan). Dansyl hydrazine and bis(2,4,6-trichlorophenyl) oxalate (TCPO) were of reagent grade from Tokyo Kaseikogyo (Tokyo, Japan). HPLC-grade methanol and hydrogen peroxide (30%) were obtained from Wako (Osaka, Japan). Sep-Pak® C_{18} cartridges were 1.0 ml size and were obtained from Millipore (Bedford, MA, U.S.A.). All the other reagents were of reagent grade.

HPLC equipment

The liquid chromatographic system (Fig. 1) consisted of three pumps: P_1 Hitachi Model L-6000 (Tokyo, Japan); P_2, Gasukurokogyo Model MPS-31; P_3, Gasukurokogyo Model MPS-7DL (Tokyo, Japan). Because P_3 was used as a two-channel single-plunger pump, two air dampers (D) were attached. The detec-

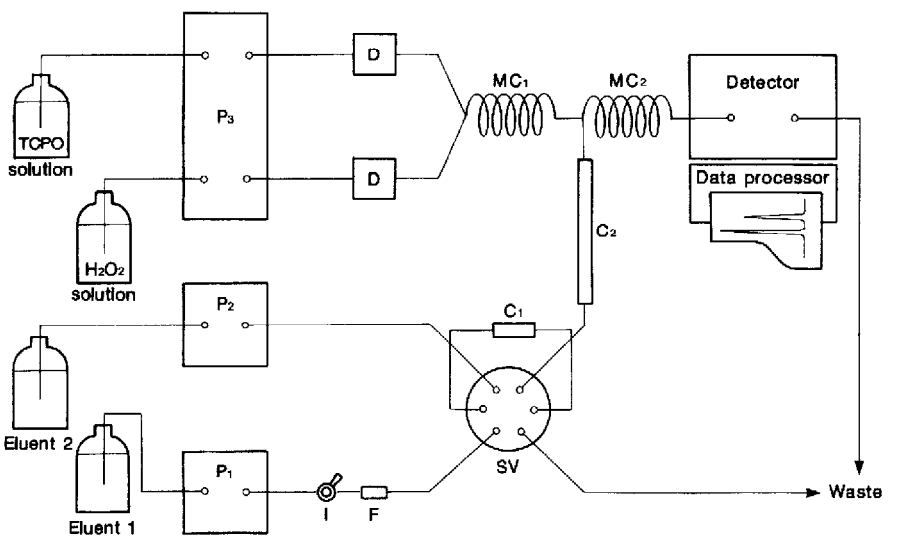

Fig. 1. Schematic diagram of the HPLC system for the determination of I in plasma and lymph with a column-switching system. C_1, pre-column; C_2, analytical column; D, damper; F, line filter; I, automatic sample injector; MC_1 and MC_2, mixing coils; P_1, P_2 and P_3, pumps; SV; switching valve.

tor was a Soma Model S-3400 CL detector (Tokyo, Japan), connected to a Shimadzu C-R4A Chromatopac® (Kyoto, Japan). For column-switching, a motor-actuated six-port column-switching valve (SV) (Kyoto Chromato, Kyoto, Japan) controlled via a laboratory-made interface was used. The liquid chromatograph and the valve were controlled by a Shimadzu system controller (Model SCL-6A). Samples were injected using a Shimadzu automatic injector (I) (Model SIL-6A) with a 100-μl microsyringe. A 1-μm line filter (F) (Cosmosil® column prefilter, Nacalai Tesque, Kyoto, Japan) was placed between the auto-injector and the pre-column. The pre-column (C_1) (20 mm × 4.6 mm I.D., Gasukurokogyo) was dry-packed with Wakogel® C_{18} (Wako, 10 μm particle size). The analytical column (C_2) was a Chemcosorb® C_{18} (250 mm × 4.6 mm I.D., 5 μm particle size, Chemco, Osaka, Japan), maintained at 60°C with a Hitachi column oven (Model 655A-52). A mixing coil (MC_2) made from stainless-steel tubing (70 cm × 1.58 mm O.D. × 0.1 mm I.D.) was placed between the analytical column and the flow-cell in the detector. To promote mixing of the two reagent solutions, another mixing coil (MC_1) made from PTFE tubing (15 m × 1.5 mm O.D. × 0.25 mm I.D.) was placed between pump P_3 and mixing coil MC_2.

Mobile phases and reagent solutions

The compositions of the two mobile phases were as follows: mobile phase 1, methanol–water (70:30, v/v) and mobile phase 2, methanol–water (90:10, v/v). These mobile phases were filtered and degassed by vacuum and sonication. A 0.05% (w/v) solution of dansyl hydrazine was prepared by dissolving 5 mg of dansyl hydrazine in 10 ml of acetonitrile and stored at 4°C until use. A 0.5% (w/v) solution of sodium pyruvate was prepared by dissolving 50 mg of sodium pyruvate in 10 ml of acetonitrile. Hydrochloric acid–acetonitrile solution consisted of 180 μl of concentrated hydrochloric acid (37%, w/v) per 100 ml of acetonitrile. Methanol solutions (50, 70 and 80%, v/v) were prepared by mixing methanol with distilled water in the ratios 1:1 for 50%, 7:3 for 70% and 8:2 for 80% solution. A 0.2% (w/v) solution of TCPO was prepared by dissolving 1.0 g of TCPO in 500 ml of ethyl acetate according to the method of Mohan and Turro [11]. A 1.8% (v/v) solution of hydrogen peroxide was prepared by adding 30 ml of hydrogen peroxide solution to 500 ml of acetonitrile. A stock solution of I in methanol was stored in amber bottles at 4°C and used to prepare calibration standards. The calibration curve samples were prepared by adding known amounts of I to plasma or lymph from rats. These were then treated as described in the following extraction procedure.

Clean-up for plasma and lymph samples

To 100 μl of rat plasma or lymph in a conical glass centrifuge tube (15 ml) were added 4 ml of water and 100 μl of isoamyl alcohol. After the addition of 5 ml of ethyl acetate, the contents of the tube were mixed on a reciprocating shaker for 10 min and centrifuged at 800 *g* for 5 min. I was extracted into the ethyl acetate

phase. Exactly 4 ml of the supernatant organic phase were transferred to another clean tube and evaporated to dryness at 40°C under a stream of nitrogen. To the residue were added 1 ml of hydrochloric acid–acetonitrile solution and 100 μl of dansyl hydrazine solution. After incubation for 5 min at 60°C, 100 μl of sodium pyruvate solution were added, and the tube was warmed for 10 min at 25°C to accelerate the degradation of excess reagent. To the resulting mixture were added 3 ml of water and 4 ml of *n*-hexane–ethyl acetate (9:1, v/v). The tube contents were mixed on a reciprocating shaker for 5 min, then centrifuged at 800 *g* for 5 min. Then 3 ml of the organic phase were transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved with 150 μl of 50% methanol solution. The mixture was added to a Sep-Pak cartridge and 8 ml of 70% methanol solution were passed through the cartridge followed by 5 ml of 80% methanol solution. The eluates were collected and evaporated to dryness at 40°C under a stream of nitrogen. The resulting residue was dissolved in 200 μl of mobile phase (70% methanol solution), and a 50-μl aliquot was injected into the HPLC system.

HPLC procedure

The column-switching system for the HPLC analysis of dansyl hydrazone of I is shown in Fig. 1. Two mobile phases were used. The pretreated sample was injected on the pre-column (C_1) and the dansyl hydrazone derivative of I was first adsorbed to the pre-column with mobile phase 1 during 5 min. Thereafter, the line was switched to mobile phase 2, which is more hydrophobic than mobile phase 1. The dansyl hydrazone derivative of I was transferred to an analytical column with mobile phase 2. The flow-rates of the pumps P_1 and P_2 were 1.0 ml/min. The eluate from the column and the reagent solutions were mixed by passage through the mixing coil connected to the flow-cell. The flow-rate of the reagent solutions was 0.5 ml/min. The HPLC system was ready for a new cycle 20 min after an injection. Levels were estimated by the chromatographic technique of comparing peaks obtained from rat plasma or lymph with the curve obtained from plasma or lymph to which were added known amounts of I. A set of six or seven calibration standards was run with each series of unknown samples.

RESULTS UND DISCUSSION

The dansyl hydrazine reagent reacts with the carbonyl group of I to form a fluorescent product. The derivative of I has an excitation maximum at 350 nm and an emission maximum at 510 nm. The chromatograms obtained from the same amount of this derivative with a spectrofluorometric detector and the CL detector were compared and it was clear that the peak area obtained with the spectrofluorometer was only *ca.* one hundredth of that obtained with the CL detector. This result supports the usefulness of the CL detector in measuring I in rat body fluids.

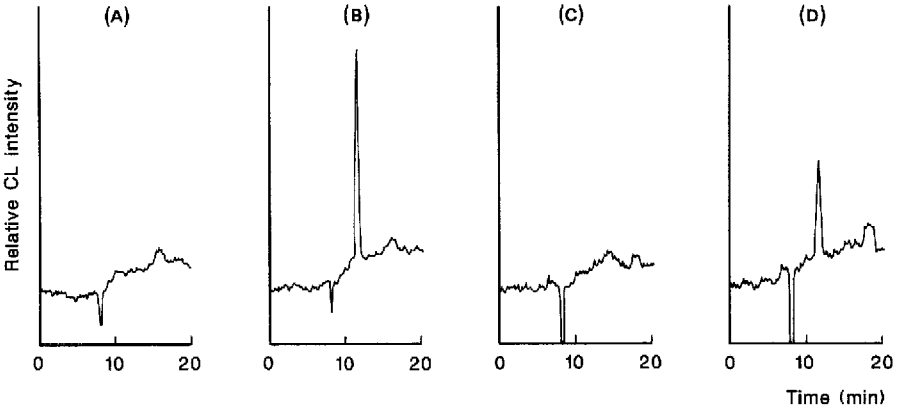

Fig. 2. Chromatograms of rat plasma and lymph samples extracted as described and analysed with the CL HPLC system. (A) Sample of drug-free rat plasma; (B) rat plasma sample spiked with I, 100 ng/ml of plasma; (C) sample of drug-free rat lymph; (D) rat lymph sample spiked with I, 100 ng/ml of lymph.

Fig. 2 shows the chromatograms of 100 μl of blank plasma sample (A) and blank lymph sample (C). No interfering peak was detected in either, or in chromatograms of plasma or lymph samples obtained just before the administration of I. Fig. 2 also shows the chromatograms of 100 μl of rat plasma (B) or lymph (D) containing I at the concentration of 100 ng/ml. The retention time of the dansyl hydrazone derivative of I is 11.8 min. As an internal standard was not available, the concentration of I in rat plasma or lymph was determined from the calibration curve of peak area of the dansyl hydrazone derivative of I using rat plasma or lymph spiked with known amounts of I.

The relationship between the peak area of the hydrazone of I and the concentration of I in plasma or lymph was linear over the range 0–1000 ng/ml ($r = 0.999$ for plasma and $r = 0.998$ for lymph). As an internal standard is not available, these standard curves were prepared daily. The reliabilities in slope from ten daily standard curves were 8.4% (coefficient of variation, C.V.) for plasma and 9.2% for lymph samples, respectively. The intercepts were not significantly different from zero.

The reproducibility of the procedure was evaluated by extracting and analysing replicate plasma or lymph samples containing 5, 10, 20, 50, 100, 200, 500 and 1000 ng of I per ml of the sample fluid. The C.V. ranged from 8.4% (5 ng/ml) to 1.7% (1 μg/ml).

Plasma or lymph aliquots (100 μl) were spiked with known amounts of I to a concentration of 200 ng/ml. The samples were treated as described, and the peak areas for the dansyl hydrazone derivative of I were compared with the peak areas obtained from the same amount of drug spiked into a pH 7.4 phosphate buffer (200 ng/ml) that was also derivatized and extracted. The peak-area ratios were used to estimate the percentage recovery. The recoveries of I were 84.7 and 45.0%

from plasma and lymph, respectively. The recovery of I from the lymph sample was *ca.* 50% of that from the plasma sample.

As the noise level on the chromatogram from the lymph sample was greater than that from the plasma sample, and the recovery of I from the lymph sample is less than that from the plasma sample, the lower limits of sensitivity of the method are 5 ng/ml for plasma and 20 ng/ml for lymph.

The pre-column was not used in the initial stage of this study. As the eluate coming from the analytical column was introduced into the CL detector after mixing with CL reagents, the peak at the void volume had an extensive absorption on the chromatogram. This peak was so large that a high background affected the chromatogram throughout the analysis, and so it was difficult to obtain greater sensitivity than with a conventional spectrofluorometric detector. To improve the sensitivity, a pre-column packed with C_{18} material was introduced. Column-switching and a less hydrophobic mobile phase (mobile phase 1) were used to elute unchanged fluorescent materials from the pre-column and removed them from the system. Therefore, the CL level of the background eluted with a more hydrophobic mobile phase (mobile phase 2) was much lower and a high sensitivity was obtained.

In the case of CyA, a radioimmunoassay method had been used in the initial stage of the study on the relationship between the pharmacokinetics and the immunosuppressive activity or side effects of CyA [12]. However, after the chemical assay procedure had been developed, the HPLC method has been widely used in both basic and clinical studies [13]. As in the case of CyA, the first reported method for the determination of I in the biological fluids, especially plasma, was a biological one, an EIA method [7]. The sensitivity of this EIA method is very high: the reported minimum detection limit of I in dog plasma is 0.1 ng/ml when 1 ml of the plasma was used for extraction. However, the main drawback of this method is thought to be the cross-reactivity of the antisera with some of the circulating metabolites of I, though the metabolic pattern of I has not yet been clearly established. The lack of sensitivity in the analytical method has a considerable effect on bioavailability studies of drugs, especially a drug that undergoes a hepatic first-pass effect.

In general, the detection limit of an HPLC assay depends on both the sample volume and the efficiency of the detector. By a simple prediction, when 1 ml of the rat plasma was used for the extraction of I, the minimum detection limit of I in 1 ml of rat plasma is estimated to be 0.5 ng/ml, which is of the same order of magnitude as in the EIA procedure. In addition, when the residue in the assay tube is dissolved in 100 μl of the mobile phase at the final step, the detection limit of the dansyl hydrazone derivative of I is thought to decrease to *ca.* 2 ng/ml. Therefore, the sensitivity of this HPLC method is of the same order as that of the EIA method.

As a preliminary experiment, normal-phase adsorption chromatography on a microparticulate silica column was studied. However, the peak obtained after the

void volume of the column was so large that the reversed-phase mode was selected for further experiments. In addition, the reversed-phase mode gave a clear chromatogram when the samples extracted from rat plasma and lymph were analysed with the HPLC system.

We have been performing basic pharmacokinetic studies on I in rats after several modes of administration: intravenous bolus, intravenous infusion and infusion into the portal vein. Since the introduction of this CL HPLC assay procedure, the method has been used successfully for the analysis of more than 1000 plasma and lymph samples at the dose level of 1 mg/kg, which is thought to correspond to the clinical dose in human renal transplant patients.

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