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FACTORS AFFECTING TAUTOMERIC PHENOMENON OF A NOVEL POTENT IMMUNOSUPPRESSANT (FK506) ON THE DESIGN FOR INJECTABLE FORMULATION

Y. Namiki, A. Fujiwara, N. Kihara, S. Koda, K. Hane, and T. Yasuda

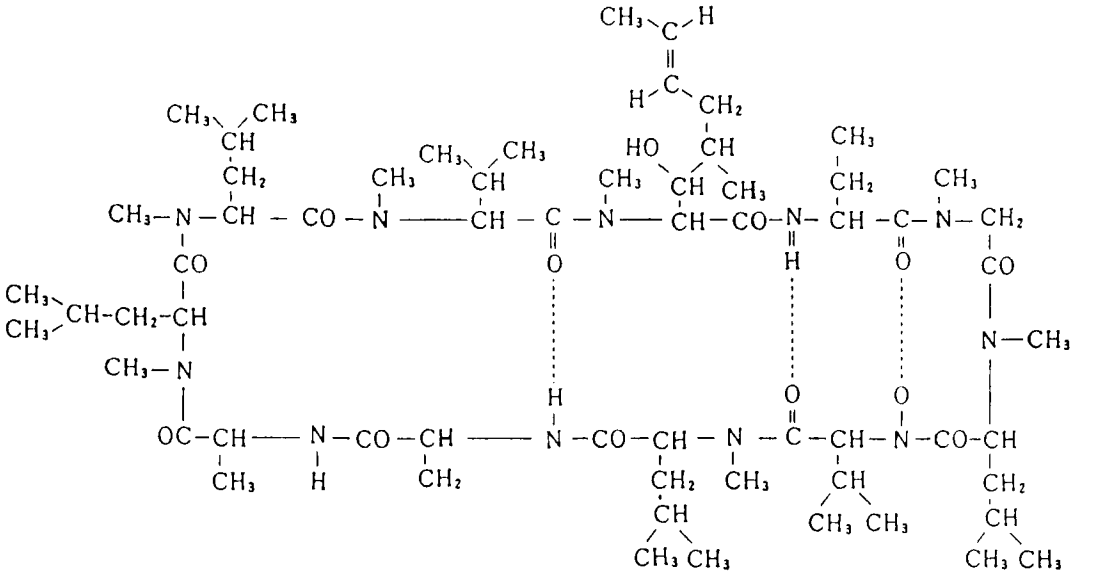
Analytical Research Laboratories,
Fujisawa Pharmaceutical Co., Ltd.,
Kashima, Yodogawa-ku, Osaka 532, Japan

ABSTRACT

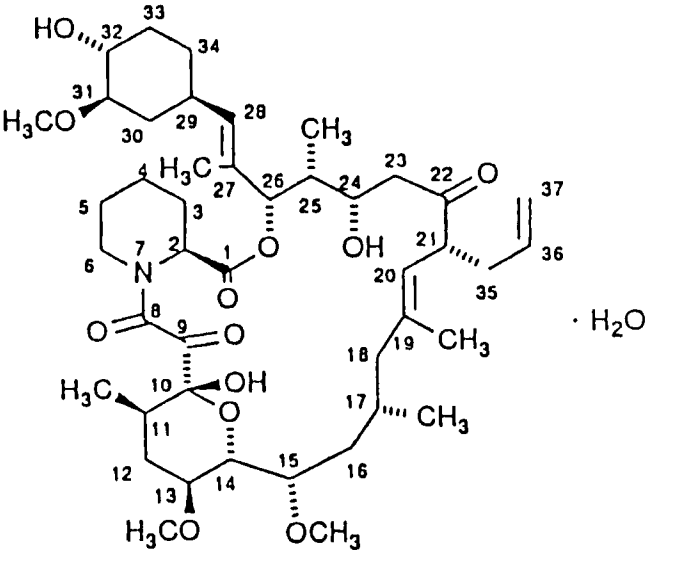
Due to its poor solubility in water, immunosuppressive drug substance FK506 was aimed to use nonaqueous solvent such as dehydrated ethanol for designing the injectable formulation. Ethanol, however, has been known to generate the tautomeric compounds from FK506. Herein, factors affecting the tautomeric phenomenon have been studied. Polyoxyethylene hydrogenated castor oil 60 (HCO-60, nonionic surfactant), employed as a solubilizer to avoid producing precipitates in FK506 admixtures, restrained the formation of tautomeric compounds. High temperature accelerated the equilibrium among FK506 and tautomeric compounds, whereas pH and concentration of FK506 solution had no effect on the tautomeric phenomenon.

INTRODUCTION

New immunosuppressive drug, FK506 (1) has been found to possess biological properties, effectiveness in organ transplantation by inhibiting the transcription of early T-cell activation genes (2), similar to cyclosporin A (3) in spite of considerable difference in their structures (Figure 1). Both drugs are designed for injectable dosage form with which administered intravenously during the immediate postoperative period (4, 5).



CsA



FK506

FIGURE 1
Structures of CsA and FK506

FK506, however, showed the poor solubility of 2-5 μg/ml in water being independent of pH value (6), thereby considering the employment of nonaqueous solvents such as dehydrated ethanol, propylenegycol or polyethyleneglycol 400 (PEG 400) for parenteral use (7). Appropriate solubilizer, e.g. polyoxyethylene hydrogenated castor oil 60 (HCO-60), should also be added to prevent a formation of FK506 precipitates in admixture obtained after dilution with 0.9% NaCl injection or 5% dextrose injection essential to alleviation of hemolysis and local irritation at injection site (5, 8).

Under such conditions, FK506 particularly has the possibility to generate the tautomeric compounds (**1** and **2**, Figure 2) resulting from the hemiketal moiety at C-10 (6, 9).

The objective of this study is to determine the influence of pH, temperature, concentration of FK506, and HCO-60 on the tautomeric phenomenon for designing preferable FK506 injectable formulation.

EXPERIMENTAL

Materials

FK506 ($C_{44}H_{69}NO_{12}\cdot H_2O$) and FK506 ampoule for injection (10-mg/ml) were obtained from Fujisawa Pharmaceutical Company. Tautomeric compounds **1** and **2** generated in water:dehydrated ethanol (1:1) were obtained by the method described in our previous report (9).

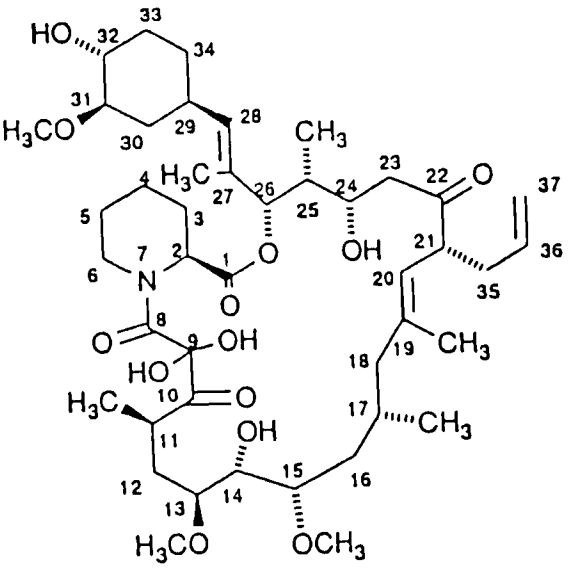
Tetrahydrofuran (THF) and isopropyl alcohol (IPA) were of HPLC-grade from Wako Pure Chemical Industries and Hayashi Pure Chemical Industries, respectively. HCO-60 was purchased from Nihon Surfactant Kogyo. Water was deionized and purified with Milli-Q SP reagent water system manufactured by Millipore before use. All other reagents used were of reagent grade.

Hydrochloric acid-NaCl (pH 1, 2), phosphate buffer (pH 3-8), and carbonate buffer (pH 9) were used for kinetic study. All hydrochloric acid, phosphate, and carbonate solutions were 0.1M, adjusted to an ionic strength of 0.3 with NaCl as described in our report (10).

Containers made of polyethylene filled with 0.9% NaCl injection or 5% dextrose injection were purchased from KENDALL McGAW Laboratories.

Instrumentation

The HPLC system consisted of a Shimadzu LC-6A pump, a SCL-6A system controller, a SPD-6A variable-wavelength UV detector, a CTO-6A column oven, and a C-R4AX Chromatopac integrator. In order to keep solutions at approximately 4, 14, 25 and 35°C, cooling system WIG-7000 manufactured by Ishido Group Company was used. The pH was measured by a TOA model HM-30S.



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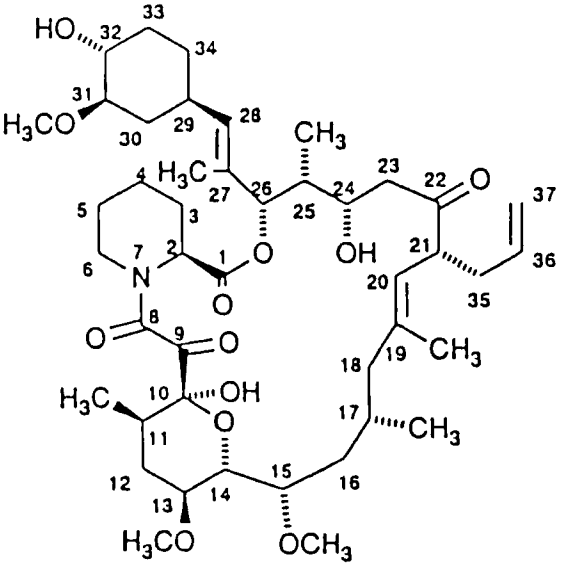

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FIGURE 2
Structures of 1 and 2

Kinetic measurements

The decomposition of FK506 was studied in mixtures of buffer solutions and dehydrated ethanol (1:1) at 40°C. Accurately weighed FK506 (0.02 g) was dissolved in 10.0 ml of dehydrated ethanol, and allowed to stand at room temperature (about 25°C) for 6 hours in order to keep in equilibrium (6, 9). Then, various buffer solutions were added to obtain the resulting concentration of 1 mg/ml. All these solutions were stored in a 40°C oven. At appropriate intervals, sample solutions were taken to measure pH and the remaining FK506 by HPLC Method I. The observed rate constants (k_{obs}) were determined from the slopes of linear plots of the logarithm of FK506 remaining percentage versus time.

Examination of factors affecting tautomeric phenomenon

Dehydrated ethanol was selected as a vehicle, in which FK506 was most soluble (560 mg/ml) at 25°C and most stable compared with propylenegycol and PEG 400. However, the mixture of the FK506 alcoholic solution with 0.9% NaCl injection and 5% dextrose injection became turbid due to crystallization of FK506 immediately after dilution, so that solubilizers were examined to prevent this unfavorable precipitation. As a result, satisfactory solubility and stability were accomplished only by solution containing surfactant, HCO-60, forty times (400 mg) as much weight as FK506 (10 mg) in one ml of dehydrated ethanol, in which FK506 is expected to show the tautomeric phenomenon (6, 9). In addition, this phenomenon can also be observed in the diluted solution with appropriate vehicles before administration. Therefore, factors affecting the tautomeric phenomenon were investigated: pH, temperature, concentration of FK506, and HCO-60. (a) pH; FK506 was dissolved in dehydrated ethanol at the concentration of 4 mg/ml, and then various buffer solutions of which pH ranging from 1 to 9 were added to make 2 mg/ml at 25°C, respectively. (b) temperature; dehydrated ethanol solution containing about 4 mg/ml of FK506 was prepared, and each of pre-cooled and pre-heated (4-35°C) water was added to make 2 mg/ml. (c) concentration; FK506 solutions in water:dehydrated ethanol (1:1) were prepared at three concentrations of 0.1, 0.5 and 2 mg/ml. (d) HCO-60; FK506 ampoule was diluted with water:dehydrated ethanol (1:1) to make 0.5 mg FK506/ml. The composition ratios among FK506, **1** and **2** in solutions stored under the above various conditions were measured by peak area percentage method at regular time intervals using HPLC Method I. Additionally, the remaining percentage of FK506 in the admixtures was determined by the same method.

Determination of **1** and **2** in designed injectable dosage form

The amounts of **1** and **2** in five lots of FK506 ampoule for injection (10-mg/ml) were determined by HPLC Method II after dilution with n-hexane:n-butyl chloride (2:1) to make 1.5 mg/ml.

TABLE 1
Stability of FK506 in Mixtures of Various Buffer Solutions and Dehydrated Ethanol at 40°C

pH value of buffer solution used	Apparent pH value*	Observed rate constant (hr^{-1})
1	1.17	8.0×10^{-4}
2	2.26	2.1×10^{-4}
3	3.91	1.2×10^{-4}
4	4.80	1.5×10^{-4}
5	5.63	1.8×10^{-4}
6	6.76	5.8×10^{-4}
7	8.04	5.7×10^{-3}
8	9.06	6.2×10^{-2}
9	10.39	8.5×10^{-1}

*: The initial pH value for each of the sample solutions was higher than that of added buffer solutions due to the influence of ethanol. During this test, no significant change of pH values was observed.

Observations of tautomeric phenomenon in admixtures

In order to ensure the tautomeric phenomenon in admixtures for clinical studies, FK506 ampoule for injection was diluted with 0.9% NaCl injection and 5% dextrose injection in polyethylene container to prepare admixtures at a concentration of 40 μg/ml of FK506. The admixtures thus obtained were stored at room temperature (about 25°C) under fluorescent light for 24 hours. At the appropriate intervals, exactly 5 ml each of the admixture was diluted with dehydrated ethanol to make exactly 10 ml for determining the remaining FK506 by HPLC Method I. The amounts of **1** and **2** in the solution obtained from the following procedures were measured by HPLC Method II. Each 10 ml of the admixture was mixed with 10 ml of ethyl acetate, and the resulting mixture was shaken vigorously for a few minutes, and was centrifuged at 1660G (3000 rpm, arm length: 16.5 cm) for 10 min. The solution thus obtained was filtered through ADVANTEC TOYO No. 2S. The supernatant liquid (5 ml) was evaporated under reduced pressure, and the residue was reconstituted in 1.0 ml of n-hexane:n-butyl chloride:acetonitrile (7:2:1). Approximately 10 μl of this solution was injected into the normal phase HPLC at scheduled time.

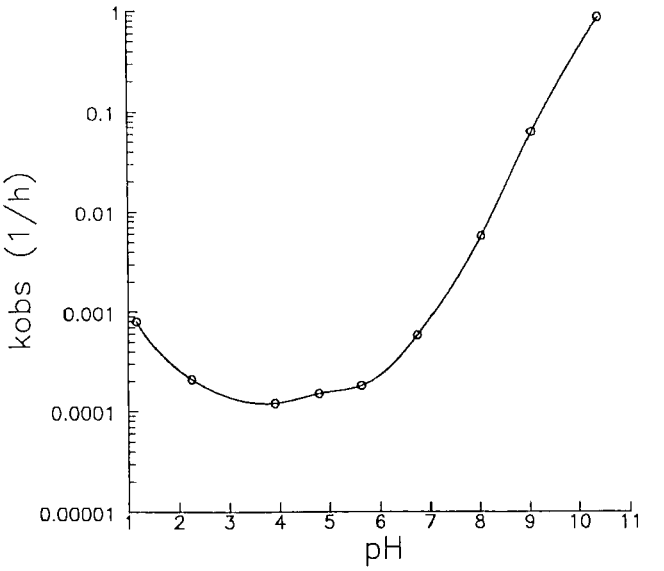

FIGURE 3
pH-rate profile of FK506 at 40°C

HPLC methods

Two different HPLC methods were used. Method I is a reverse phase method which was performed on a TOSOH TSKgel ODS-80T_M (5 μm, 4.6 x 150-mm) at 50°C, using water:THF:IPA (5:2:2) as a mobile phase. The pump delivered the mobile phase at a flow rate of 0.8 ml/min, in which the retention time of FK506 detected at 220 nm was around 10 minutes. Method II employed two columns each packed with 5 μm Supelcosil LC-FUJI-Diol from Supelco Inc., 4.6 x 250-mm, in tandem at ambient temperature and detection at 225 nm with peak area percentage method. The mobile phase consisted of n-hexane:n-butyl chloride:acetonitrile (7:2:1) at 1.5 ml/min.

Results and Discussion

Stability of FK506 in various pH buffer solutions

The semilogarithmic plots of the remaining percentage of FK506 versus time were reasonably linear in all pH values tested, and showed that the decomposition of FK506 followed apparent first-order kinetics. The pH dependence of the overall first-order profile is presented in Table 1. FK506 takes a J-shaped stability profile (see Figure 3); FK506 is fairly stable in the pH range between 2 and 6, but is significantly unstable at higher pH values. These data indicate that FK506 designed to be administered intravenously after dilution with 0.9% NaCl injection or 5% dextrose injection is stable as

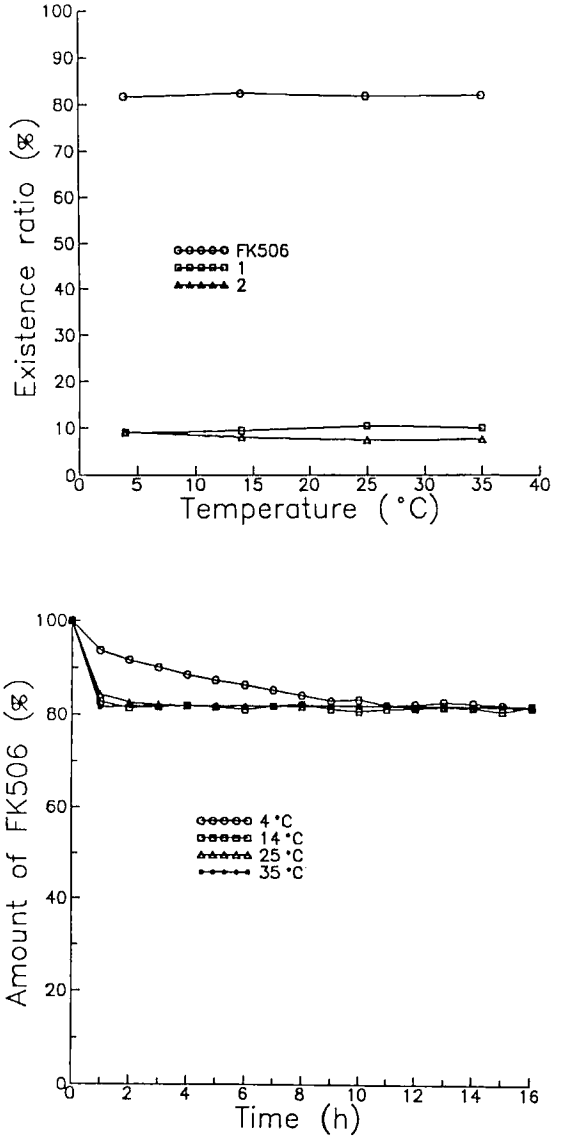

FIGURE 4
Influence of temperature from 4°C to 35°C in water:dehydrated ethanol (1:1) [top: existence ratio among FK506 and tautomeric compounds in the equilibrated state (16 h), bottom: equilibration for FK506]

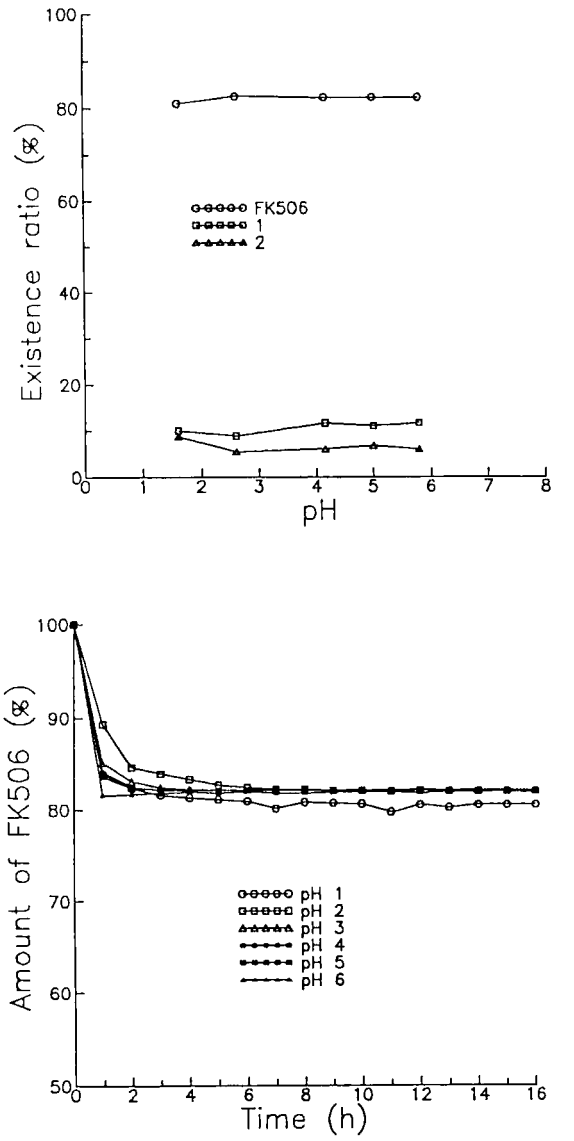

FIGURE 5
Influence of pH from 1 to 6 at 25°C
[top: existence ratio among FK506 and tautomeric compounds in the equilibrated state (16 h), bottom: equilibration for FK506]

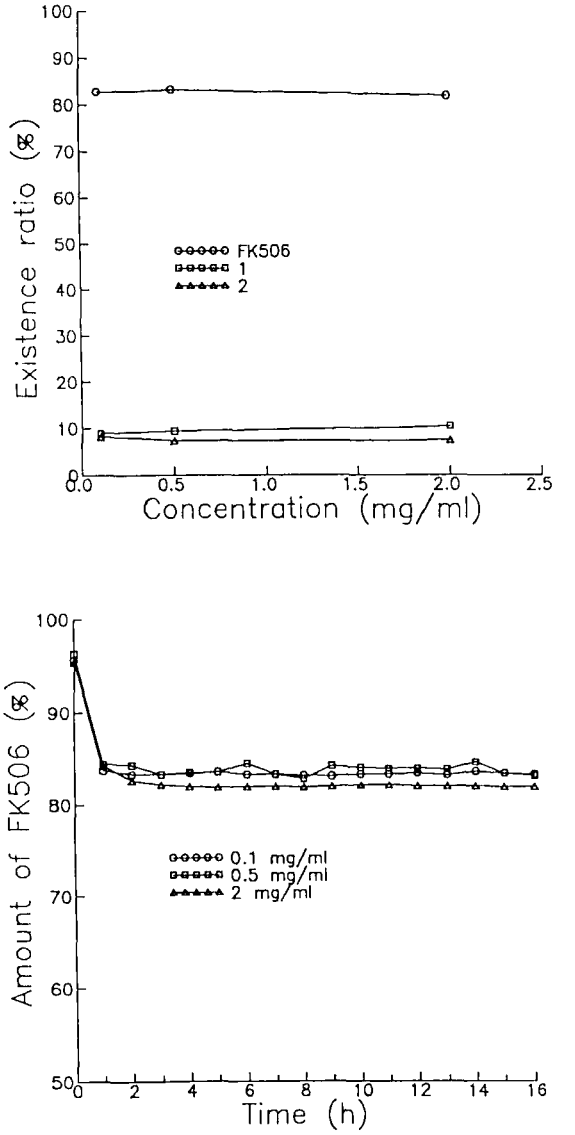

FIGURE 6
Influence of concentration from 0.1 mg/ml to 2 mg/ml at 25°C in water:dehydrated ethanol (1:1)
[top: existence ratio among FK506 and tautomeric compounds in the equilibrated state (16 h), bottom: equilibration for FK506]

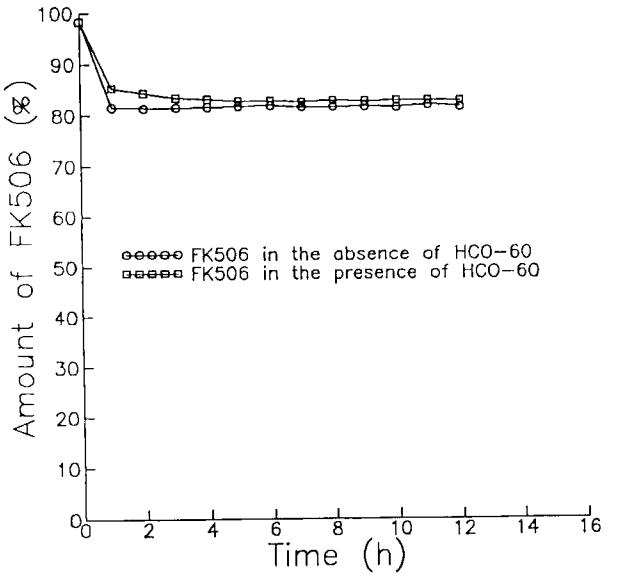

FIGURE 7
Equilibration of FK506 in the presence and absence of HCO-60 in water:dehydrated ethanol (1:1)

Taormina et al. described (5), because pH values of these admixtures are within the relatively stable pH range (see Table 3).

Influence of factors on tautomeric phenomenon

As shown in Figure 4, the time to reach equilibrium was obviously dependent on the temperature, approximately 16 hours at 4°C and within one hour between 14°C and 35°C, although the existence ratio among three components was similar at any temperature.

On the other hand, pH and concentration of FK506 were proved to have no effect on tautomeric phenomenon. No significant difference between pH 1 and pH 6, and no dependence on the concentration from 0.1 to 2 mg/ml were observed with respect to the equilibration rate and the existence ratio among FK506, **1** and **2** as shown in Figures 5 and 6, respectively. However, peak shapes of **1** and **2**, dissolved in the mixture of pH 6 buffer solution and dehydrated ethanol (1:1) showing pH 6.76 apparently, were too broad to measure their peak areas exactly.

In addition, Figure 7 demonstrates that the sample solution in the presence of HCO-60 takes 6 hours at 25°C to reach equilibrium remarkably slower than that in the absence of HCO-60. This delay will be explained by the following reason relevant to the micelle formation between FK506 and HCO-

TABLE 2
Amounts of **1** and **2** in FK506 Ampoule for Injection (10-mg/ml)

Lot No.	I10-1	I10-2	I10-3	I10-4	I10-5
Amount (%) of **1**	2.55	2.64	2.42	2.26	2.45
Amount (%) of **2**	5.42	5.36	5.28	5.28	5.49
Total amount (%)	7.97	8.00	7.70	7.54	7.94

TABLE 3
Stability Test Results of FK506 Admixtures with 0.9% NaCl Injection and 5% Dextrose Injection

Test items[a] Vehicles		Initial	Minute 30	Hour 1	Hour 3	Hour 6	Hour 24
0.9% NaCl	Assay (% to the initial)	100	---[b]	---	---	99	98
	1 and **2** (%)	9.9	12.0	11.4	10.8	10.3	12.6
5% dextrose injection	Assay (% to the initial)	100	---	---	---	97	97
	1 and **2** (%)	8.2	10.0	10.5	10.3	10.2	12.4

[a]: Throughout the test period, the appearance of these solutions was colorless clear as initial solution. With respect to pH values, no significant change was observed. Initial pH: 5.60 (0.9% NaCl), 4.82 (5% dextrose injection).
[b]: Not done

60; relatively hydrophilic part in surfactant oriented to C-10 hemiketal moiety interferes the activity of crystalline water as a driving force to cause the tautomeric phenomenon (6, 9).

The results obtained above suggest that relatively long storage is indispensable in order to achieve a complete equilibrium to provide constant amounts of **1** and **2** if HCO-60 is formulated. Accordingly, the mixed dehydrated ethanol solution of FK506 and HCO-60 has been determined to be

stored for one night in practical manufacturing process for FK506 ampoules. As shown in Table 2, the amounts of **1** and **2** in five lots of FK506 injectable ampoules were verified to be almost constant.

Formation of **1** and **2** in admixtures

Injectable dosage forms have to be designed principally based on solubility and stability of the active ingredient. Particularly FK506 is insoluble in water, thereby being developed as nonaqueous formulation for injections and intended to administer after dilution with NaCl or dextrose solution as mentioned before. Thus, the stability and the tautomeric phenomenon of FK506 in admixtures with 0.9% NaCl injection or 5% dextrose injection were examined at around 25°C (concentration of FK506: 40 μg/ml). The results obtained from both admixtures were summarized in Table 3. Besides being stable of FK506, no precipitates were observed in both admixtures for 24 hours, and the equilibrium was completed within almost one hour to generate **1** and **2** in total at the constant level of 10-13%. Consequently, FK506 injectable dosage form was found to be administered at a constant quality after dilution with each of 0.9% NaCl injection and 5% dextrose injection, even if HCO-60 and low temperature around 4°C could restrict the tautomeric phenomenon. Moreover, this evidence suggests that discerning the biological action of **1** and **2** would be of importance, about which will be presented elsewhere.

Acknowledgement

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