Kinetic Analysis of Molecular Interconversion of Immunosuppressant FK506 by High-Performance Liquid Chromatography

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High-performance liquid chromatography of FK506, a macrolide immunosuppressant, was performed on a reversed-phase column. The peak was broad with the column kept at room temperature, which was accounted for by slow interconversion between the two forms of FK506. With the use of a heated column, a sharp peak was observed because of the rapid interconversion at high temperature. When the column was cooled to 0°C, two sharp peaks were observed because essentially no interconversion occurred at 0°C during elution. Analysis of the chromatograms obtained at various eluant flow rates indicated that the conversion of the two forms follows first-order kinetics, and the apparent activation energies for the conversions were calculated. The interconvertibility between two molecular forms may be related to the immunosuppressive activity.

KEY WORDS: FK506; cyclosporins; immunosuppressant; conformational isomerization; HPLC.

INTRODUCTION

FK506 is a macrolide immunosuppressant (Fig. 1) used for preventing the rejection of organ transplants. Though this compound is chemically distinct from the clinically used immunosuppressant cyclosporin A (a cyclic 11-amino acid peptide), the two drugs inhibit the same step in the activation of helper T cells (1–4).

The three immunosuppressive cyclosporins (cyclosporins A, C, and D) gave characteristic temperature-dependent profiles in high-performance liquid chromatography (HPLC), and we found that these profiles resulted from interconversion between two isomers (5). Since the interconvertibility may be involved in the biological effect of cyclosporins, we tested whether FK506 also undergoes interconversion between isomers.

MATERIALS AND METHODS

FK506 was a gift from Fujisawa Pharmaceutical Co. Ltd., Osaka. A Hitachi 655A HPLC system was used. A reversed-phase Inertsil ODS-2 column (GL Sciences, Tokyo) was placed in a temperature-controlled incubator bath, and the mobile phase of an acetonitrile–water mixture was eluted. The injection volume was 25 μL, unless otherwise indicated. The peak area was measured by a Hitachi D-2500 integrator, but the area of the peak isolated from a fused or complexed peak was measured using Macintosh FlexiTrace software.

RESULTS

Effect of Column Temperature on HPLC Profile

FK506 was chromatographed at a 1.0 mL/min flow rate of the mobile phase and detected by absorption at 214 nm. The difference in the HPLC profiles obtained at various column temperatures was remarkable (Fig. 2): a sharp and symmetrical peak was observed at 60°C; the symmetrical peak became low and broad at a temperature between 60 and 23°C; the peak was asymmetrical, with a gradually ascending and steeply descending slope at a temperature between 23 and 20°C; two sharp peaks appeared at a temperature between 15 and 4°C, one peak on the leading edge and the other on the trailing edge (peak P and peak Q in Fig. 2) of the broad peak; and they were the main peaks at 0°C. The integrated area was constant with a CV of 3.4% at the various temperatures (60, 50, 40, 30, 23, 20, 15, 10, 8, 4, and 0°C) despite the remarkable profile difference, indicating that the injected solute was always completely recovered. Neither the peak broadening nor the appearance of the two peaks was due to column deterioration because one sharp peak was always observed for the control substance pregnenolone at all temperatures tested.

All HPLC profiles can be accounted for by the reversible conversion of FK506 molecular forms (form P $\rightleftarrows$ form Q) which occurred as a random event with a certain probability at a certain temperature. Since the conversion rate was rapid at 60°C, the solute migrated at an apparently constant velocity, which gave the fused sharp peak. The broad peak at a temperature between 60 and 20°C should result from slow interconversion. The peaks P and Q obtained at low temperatures could be ascribed to the two individual nonconverting forms, and the interposed broad peak represents the solute that had changed its form once or more during the elution.

Theoretical Plate Number

The theoretical plate number, representing peak sharpness, was plotted against the column temperature (Fig. 3). The plate number of the FK506 peak at 60°C was as large as 2100 though smaller than that of pregnenolone (about 5000), and that at 23°C was as small as 200. The large and constant plate numbers (2500 to 3000) of peaks P and Q at the low temperatures supported that either peak was ascribed to the nonconverted form.

Rechromatography

The three fractions (peak P, peak Q, and the broad peak or bridge R) eluted from the column cooled to 0°C were

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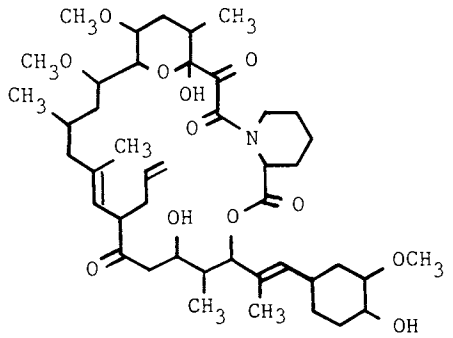

Fig. 1. Structure of FK506.

reinjected, and each fraction reproduced the original profile (Fig. 4). When the three fractions were injected into the column kept at 10°C, they all gave a profile of two sharp peaks and an interposed bridge, whereas all gave a profile of a single sharp peak when injected into the column heated to 60°C. These results demonstrated that the solute in the P and Q fractions was interconvertible and that the bridge R fraction consisted of the two forms. The rechromatography under the different conditions (first HPLC at 15 or 40°C, second HPLC at 0, 15, or 40°C) supported the interconversion.

Multiple UV Absorption Monitoring

The HPLC profiles obtained by monitoring absorption at various wavelengths were compared. The profiles were the same, and the ratio of the absorbances at 254, 234, and 214 nm was 1:4:8 at 0, 10, and 40°C, respectively. Hence the UV spectrum of form P was similar to that of form Q.

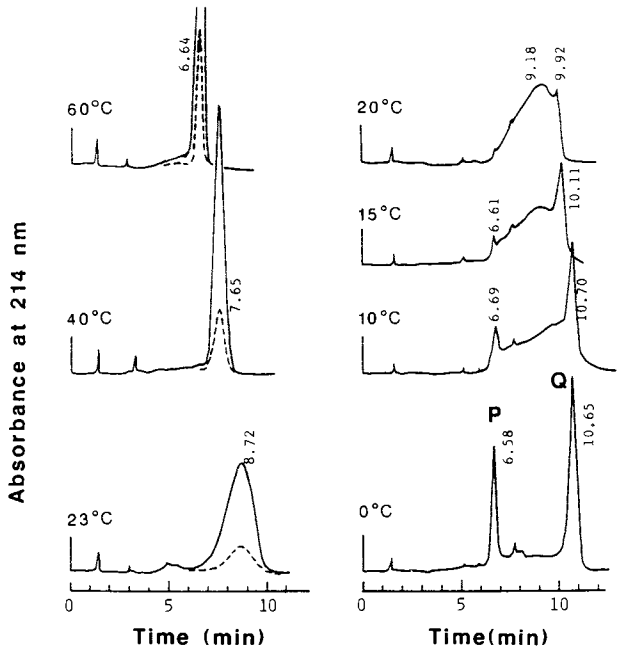

Fig. 2. Chromatograms of FK506 at various column temperatures. The FK506 solution (200 μg/mL) was injected into the 25-cm-long column and eluted with a mixture of CH_3CN/H_2O (75/25) at a 1.0 mL/min flow rate. Absorbance recorded at attenuations 5 and 7 is represented by the solid line and the dashed line, respectively. The number at the top of each peak represents the retention time.

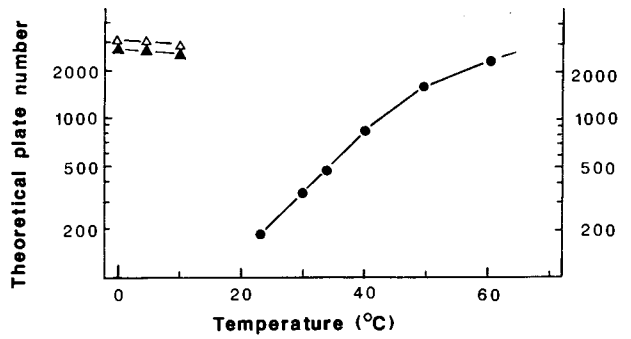

Fig. 3. Theoretical plate numbers at various column temperatures. Theoretical plate numbers (N) were calculated as $N = 16 \times (t/W)^2$, where t is the retention time and W is the width of the peak bottom in time units. (●) The plate number of the peak at 60 to 23°C; (▲) that of peak P at 10 to 0°C; (△) that of peak Q at 10 to 0°C.

Effects of Additives in Mobile Phase

When using the mobile phase containing the acid (0.2% trifluoroacetic acid, v/v), the alkali (25 mM K_2HPO_4), or the detergents (0.1% sodium dodecyl sulfate, w/v; 0.1% Tween 20, v/v), the HPLC profiles showed no change at 0, 10, 20, and 40°C, indicating that these reagents had no significant effect on the conversion rate or the retention time of each molecular form.

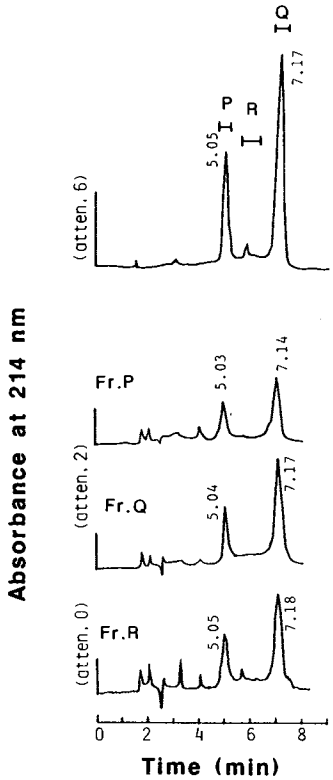

Fig. 4. Rechromatograms of the fractions eluted from the column cooled to 0°C. Fifty microliters of the solution (200 μg/mL) was injected into the 25-cm column, and fractions P, Q, and R were collected as shown at the top. Twenty-five-microliter portions of the fractions were injected, and the rechromatograms obtained are shown below. The mobile phase was a mixture of CH_3CN/H_2O (90/10), and the other HPLC conditions were the same as for Fig. 2.

Effect of Flow Rate and Kinetic Analysis

The effect of the flow rate is shown in Figs. 5–8. The integrated, adjusted peak area was constant for a given amount of the injected solute, indicating that the recovery was complete. The retention volume of each corresponding peak was constant as observed with pregnenolone, which gave a sharp peak with a constant theoretical plate number with a CV of 7.5% (n = 21, at the three flow rates at the seven temperatures).

With the cooled column, the ratio (%) of the peak P and Q area to the integrated area decreased at the slower flow rate, with a complementary gain in the bridge area (Fig. 5). The plot of this ratio against the retention time was linear on a semilogarithm graph for the two peaks (Fig. 6), indicating that the conversion in either direction followed first-order kinetics. The half-lives of the two forms P and Q at each temperature were determined from the plot, for example, 0.5 and 3.0 min, respectively, at 24.5°C. The rate constants (k = 0.693/half-life) were calculated, and the apparent activation energy was determined to be 17.5 ± 0.5 kcal/mol for form P and 18.0 ± 0.5 kcal/mol for form Q by the Arrhenius plot method. The ratio was estimated to be 40% (P) and 60% (Q) at time 0 by extrapolation, indicating that no other forms existed.

The peak was always single and symmetrical with the heated column (40, 50, and 60°C) at the various flow rates and slightly asymmetrical at 30°C (Fig. 7). The peak height and sharpness (theoretical plate number) increased at the slower flow rates, which was not seen for the conventional compounds (e.g., pregnenolone). Elution at a reduced flow rate leads to an increase in the retention time and an increase in the number of conversions, which results in a smaller deviation of the number distribution, to give a higher and

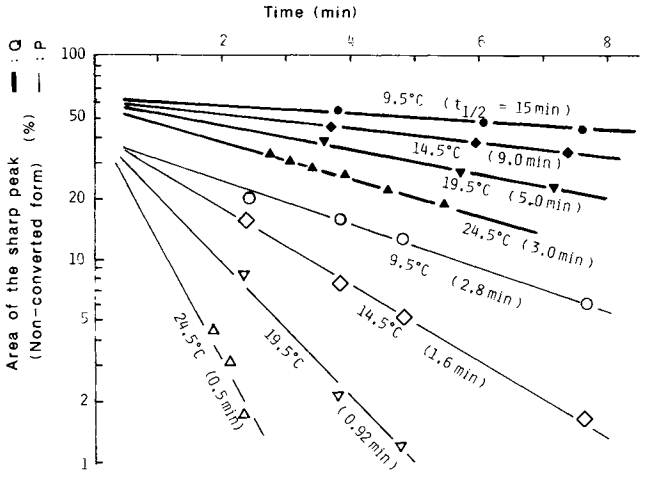

Fig. 6. Time course of percentage of the nonconverted form area to the integrated area.

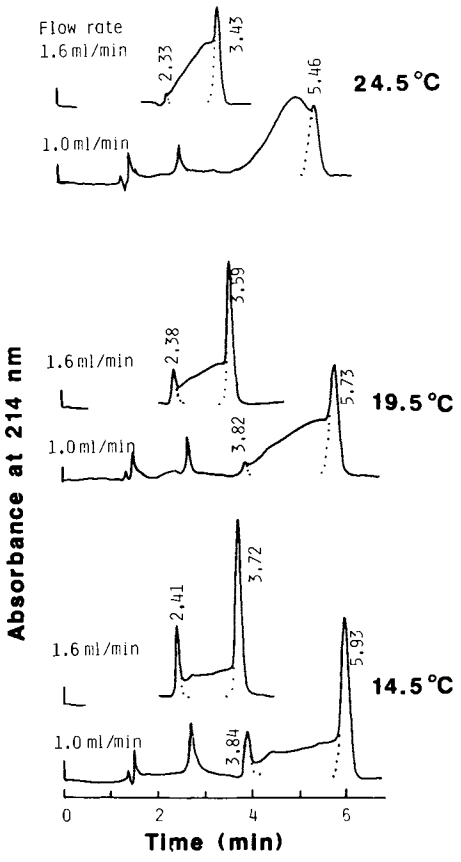

Fig. 5. Chromatograms of FK506 at various flow rates with a 15-cm-long, cooled column. The mobile phase was a mixture of CH_3CN/H_2O (80/20). Absorbance was recorded at attenuation 2. The dotted line shows how the nonconverted form peak was isolated from the fused or complexed profile.

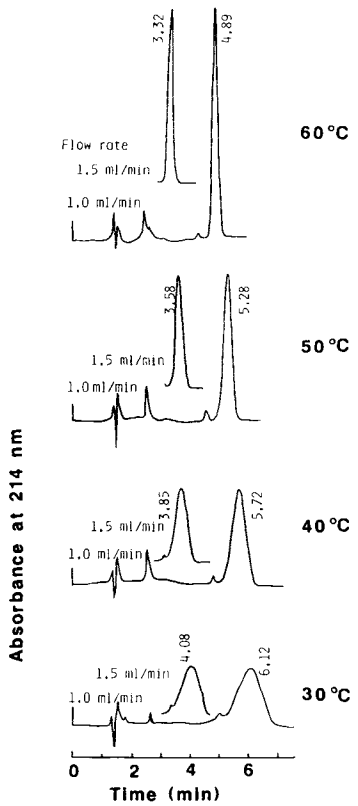

Fig. 7. Chromatograms of FK506 at various flow rates with a 15-cm-long heated column. The mobile phase was a mixture of CH_3CN/H_2O (75/25). The other HPLC conditions were the same as for Fig. 5.

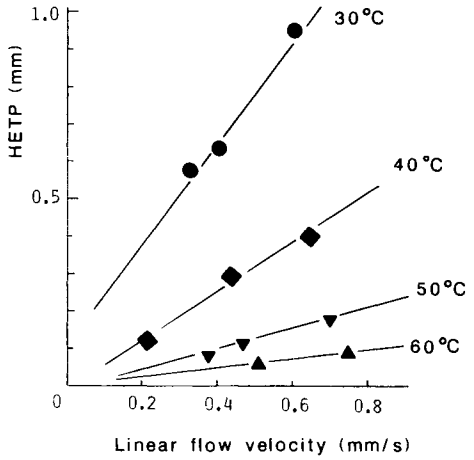

Fig. 8. Effect of temperature on the plot of HETP (height equivalent to a theoretical plate) against apparent linear flow velocity (v) of the solute in the column.

sharper peak. The height equivalent to a theoretical plate (HETP = column length/theoretical plate number) was proportional to the observed linear flow velocity (v = column length/retention time) (Fig. 8). According to a report on the peak broadening of ethylenediaminetetraacetate-chromium(III), the activation energy (E_a) can be calculated by the following equation: $E_a = -R[(1/T_1) - (1/T_2)]^{-1} \ln(H_1/H_2)$, where R is the gas constant, and H_1 and H_2 are the HETP at temperature T_1 and T_2, respectively (6). From this equation (the data at 30°C were deleted from the calculation because of its peak asymmetry), the activation energy was calculated to be 17.5 ± 1.0 kcal/mol, which agreed well with the values determined before by the Arrhenius plot method.

DISCUSSION

The characteristic HPLC behavior of FK506 can be accounted for by interconversion between the two forms. Though the actual steric structure is still uncertain, the conversion is considered to be a conformational isomerization, as indicated by ^{13}C NMR studies that revealed the existence of conformational isomers with major (*cis*) and minor (*trans*) amide bonds in $CDCl_3$ (3,7). It is further possible that additional rapid conformational changes occur that require less activation energy, which might explain the smaller plate numbers of peaks P and Q than those of a conventional compound such as pregnenolone.

The equilibrium constant (K = Q/P) seemed to be variable depending on the environment or solvent. The Q/R abundance ratio at time 0 was 1.5 (60%:40%) (Fig. 6), however, the average ratio of the half-life (or reciprocal of the rate constant) was 5.6. This discrepancy was probably caused by the different conditions before and during elution, during which interaction of the solute with the column affected the conversion rate. The energy difference between the two forms was calculated to be 0.22 to 1.0 kcal/mol ($\Delta E = -RT\ln K$), when K ranged from 1.5 to 5.6 and T ranged 273 to 297 K), indicating that the activation energy for the conversion from P to Q was only marginally lower than that for the reverse conversion (approximately 17.5 and 18.0 kcal/mol, respectively). Because of the dependence on the environment, the abundance ratios in biological samples cannot be predicted.

Let us assume that one molecule migrates in a column undergoing interconversion between form P and form Q at a time ratio of r and $(1 - r)$; the column length (L) is

$$\begin{aligned} L &= v_P \times t_P = v_Q \times t_Q = v \times t \\ &= [v_P \times t \times r] + [v_Q \times t \times (1 - r)] \\ &= L \times t \times [r/t_P + (1 - r)/t_Q] \end{aligned}$$

where v_P, v_Q, and v are the linear flow velocities and t_P, t_Q, and t are the retention times of the nonconverted P, the nonconverted Q, and the converted molecule, respectively. Then the following equation is obtained:

$$1/t = [(1/t_P) \times r] + [(1/t_Q) \times (1 - r)].$$

This equation shows that t is variable only in the range between t_P and t_Q under the condition of $0 \leqq r \leqq 1$, giving the mathematical bases for the profile of the bridge interposed by the two peaks and for the determination of the existence ratio of r = P/(P + Q).

As will be reported separately (5), the immunosuppressive drugs, cyclosporins A, C, and D, showed similar temperature-dependent HPLC profiles because of interconversion between two forms, and the activation energy for the conversion of the cyclosporins was 15 to 18 kcal/mol, which agrees well with that for the FK506 conversion. In summary, these drugs were found to share the following properties: (i) they are interconvertible between two major forms in an acetonitrile–water solvent; (ii) they require nearly equal activation energy for the conversion, which occurs on a time order of minutes at room temperature; and (iii) they are highly lipophilic but with substantial differences in lipophilicity between the two forms, as indicated by the different HPLC retentions of the two forms. These unusual properties may be relevant to their immunosuppressive activity.

The macrolide FK506 and the peptide cyclosporins share two specific properties in addition. First, both are taken up by erythrocytes at low temperatures (8,9), and, second, both bind to peptidyl prolyl *cis–trans* isomerase, although the target enzyme proteins are not identical for the two drugs (3,4,10–12). The pipecolinic acid (homoproline) moiety in the FK506 molecule is considered as a binding site because of its similarity to proline (Fig. 1) and because of the existence of FK506 *cis–trans* isomers as described before. Hence, both drugs bound to this enzyme may be in a transition or intermediate form between their two isoforms. Since the complexes of both drugs with this *cis–trans* conversion-catalyzing enzyme are reported to mediate the immunosuppressive activity (13), the kinetic analysis of the interconversion may help clarify the relationship between the structure and the immunosuppressive activity.

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