REVERSIBLE ISOMERIZATION OF RAPAMYCIN DEMONSTRATED BY LIQUID CHROMATOGRAPHY

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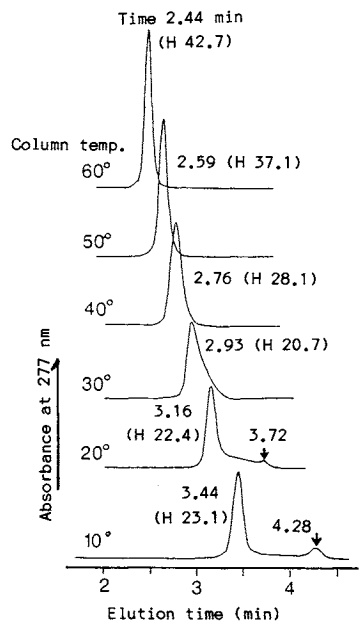
Rapamycin, an immunosuppressive macrolide initially evaluated as an antifungal agent, prevents the rejection of transplanted organ by blocking the activation of helper T cells in analogy with cyclosporin A (a cyclic peptide) and FK506 (a macrolide). These three drugs constitute a group of immunosuppressants that have a property of binding to an enzyme, peptidyl prolyl *cis-trans* isomerase[1~4]. As we had reported, cyclosporin A and FK506 gave broadened, tailed, or splitted peaks on high-performance liquid chromatography (HPLC), which can be explained by reversible conversion between two isomers[5,6]. Therefore, the chromatogram of the other drug, rapamycin, was investigated.

Rapamycin was separated on a reversed-phase HPLC column and monitored by UV absorption. As Fig. 1 shows, the elution patterns were enormously different depending on column temperature: a sharp and symmetric peak at 60° and 50°C; a broader and asymmetric peak at 40°C; a peak with a tailing at 30°C; two peaks (P for the first, Q for the second appearing peak) and a bridge interposed by the two peaks at 20° and 10°C. As Fig. 2 shows, the patterns were also enormously different depending on flow-rate. When the column was kept at 40°C, the slower flow-rate produced the sharper and higher peak. With the column kept at 20° or 15°C, a fast 2.0 ml/minute flow-rate produced two peaks, and the slower flow-rate produced smaller two peaks.

The total area was constant at the various temperatures and various flow-rates. Re-chromatography of the separated fractions P and Q produced the original pattern. The HPLC with multiple-wavelength detection at 214, 234, 254 and 277 nm gave the similar pattern, indicating that UV spectra of substances P and Q were similar. The chromatographic system seemed to work normally without trouble during the study because pregnenolone, a reference compound, always gave one sharp peak.

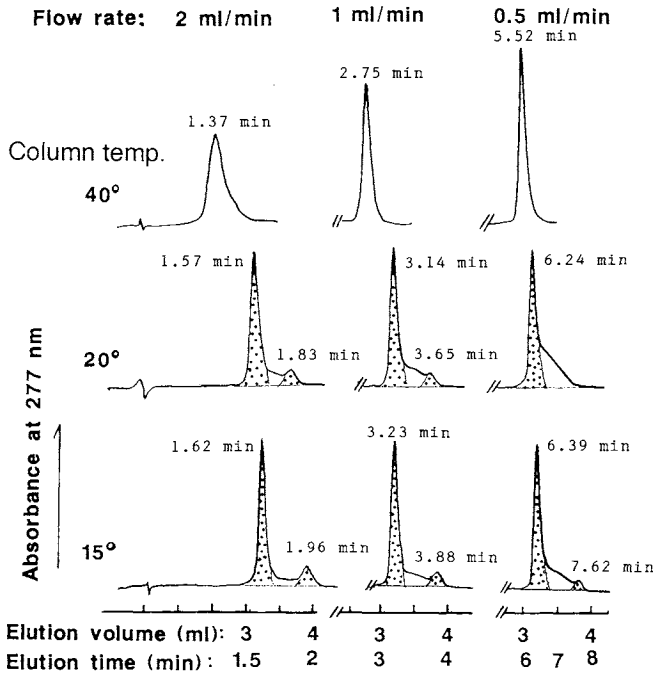
These HPLC patterns could be accounted for only by on-column conversion between two forms of rapamycin, though some other mechanisms were suggested such as precipitation of the drug, existence of a number of isomers and high stability of the conformation at high temperature[7]. At high temperature of 60°C, the molecules should undergo interconversion so frequently that they move at an apparently constant velocity in the column to give one sharp peak. The conversion should occur less frequently at 50° and 40°C, and the deviation of the conversion frequency should be wider as seen in the Poisson distribution mode, giving a broader peak. At 10°C, the conversion should occur so rarely that most molecules move at the velocity of each form, giving one peak for each form. The tailing or bridge fraction should contain the molecules that undergo conversion once or only a few times in the column. When flow-rate is slow, the molecules take a long time to come out of the column having more chances to undergo conversion in the column, which accounts for the effect of flow-rate on the pattern.

Fig. 1. Chromatograms at various column temperatures.



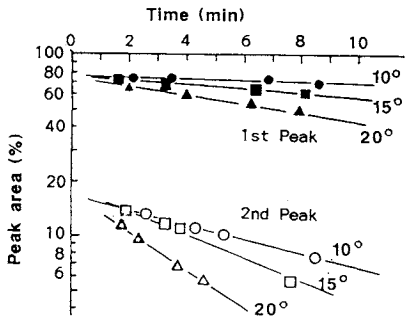
The 25 μl solution containing 1.25 μg rapamycin was injected. Column: Inertsil ODS-2 (4.5 mm i.d. × 150 mm). Eluant: $CH_3CN/H_2O = 90/10$. Flow-rate: 1.0 ml/minute. Detection: absorbance at 277 nm at attenuation 10. The peak height (H) is shown in mm on a chart of the chromatography integrator.

Fig. 2. Chromatograms at various flow-rates at column temperature 15°, 20° and 40°C.



The 25 μl solution containing 0.15 μg rapamycin was injected. Recorded at attenuation 7. The other conditions were same as those in Fig. 1. The dotted peaks could be assigned to the nonconverted isomers.

Fig. 3. Relationship between retention time and peak area ratio (%) to the total area for the first and second peaks.



Half-life for the 1st and 2nd peak was 45 minutes and 7.6 minutes at 10°C, 23 minutes and 4.6 minutes at 15°C, 12 minutes and 2.9 minutes at 20°C, respectively.

Areas of P and Q peaks decreased in a logarithmic manner with their half-lives dependent on temperature, indicating that the reaction followed first-order kinetics (Fig. 3). The existence ratio was 78% and 18% for P and Q at time zero, respectively, indicating that no other major components existed. The previous NMR study showed that rapamycin exists as a mixture of two isomers in a ratio 80:20 in chloroform, a non-aqueous solvent[8], whereas our study demonstrated not only the existence of isomers but also their interconversion.

Cyclosporins, FK506 and rapamycin bind to the enzyme that catalyzes *cis-trans* isomerization of proline peptide at peptide bond as described before, and thermodynamic property of proline peptide isomerization is quite similar to the property of these immunosuppressants[5,6,9]. Both rapamycin and FK506 have a homoproline moiety, and cyclosporins are peptides. The UV spectra of their isomers are similar for the three drugs. These facts suggest that the conversions of these three drugs are analogous to the *cis-trans* conformational isomerization of proline peptide, though steric structures of the two separated forms are not yet determined. These facts suggest also that such reversibility is a prerequisite for binding to the enzyme and for immunosuppressive activity.

Acknowledgments

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