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Note

Investigation of the mechanism of peak broadening observed in the high-performance liquid chromatographic analysis of cyclosporine

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Cyclosporine (Fig. 1) is a cyclic undecapeptide with potent immunosuppressive activity in animals and in man. A product of the fungus *Trichoderma polysporum*, the peptide contains one unique unsaturated amino acid and seven methylated amide groups. Cyclosporine is a hydrophobic peptide, having a partition ratio between octanol and water of 120:1.

It has been shown that therapeutic monitoring of cyclosporine is important because of its potential to cause renal complications. One significant feature of all of the high-performance liquid chromatographic (HPLC) methods developed for its

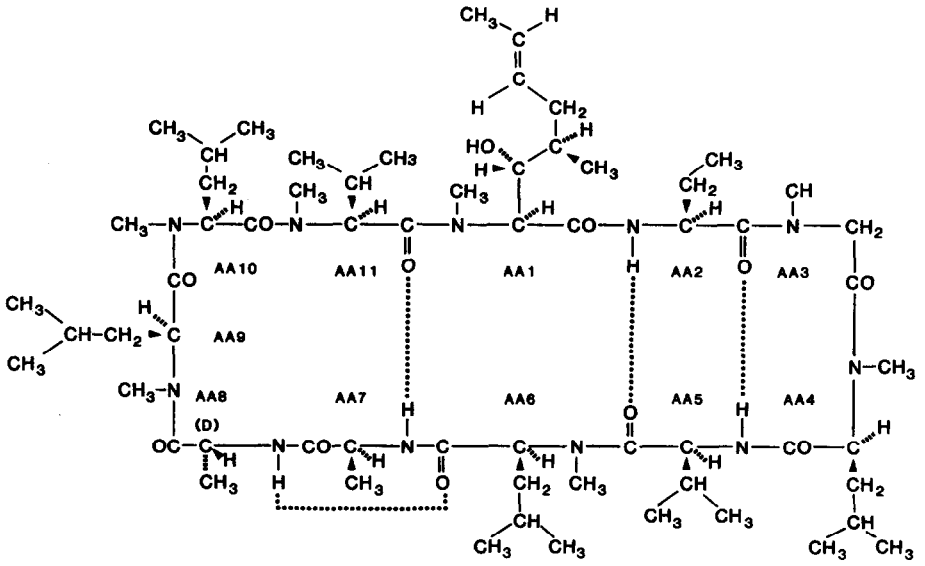

Fig. 1. Structure of cyclosporine as determined by X-ray crystallographic and C^2HCl_3 NMR data.

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measurement[1–4] is the necessity to use elevated temperatures to achieve reasonable peak shapes. In order to select rationally conditions to simplify the analysis of cyclosporine and to learn more about the molecule itself, we have undertaken a study to elucidate the mechanism(s) responsible for the peak broadening.

EXPERIMENTAL

Cyclosporine (cyclosporine A) and cyclosporine D were provided by Sandoz. All solvents were glass-distilled HPLC grade materials (Omnisolve).

The solvent delivery system was a Varian 5060 (Walnut Creek, CA, U.S.A.). Detection was achieved with a Laboratory Data Control 1203 detector modified with a Max N cell and a zinc hollow-cathode lamp (214 nm). Columns of various dimensions (150 × 4.6, 50 × 4.6 and 20 × 4.6 mm I.D.) and particle sizes (3 and 5 μm) were purchased from Supelco (Bellefonte, PA, U.S.A.). Temperature control was maintained with a glass jacket through which liquid was circulated from a Haake refrigerated circulator. When peak moments were calculated, the data were collected using an ADALAB system (IMI, State College, PA, U.S.A.) and a simple baseline-corrected summation method was used with operator selection of the integration region. Diffusion coefficient measurements were made with a peristaltic pump (Rainin) and 0.15 mm I.D. PTFE tubing attached to the LDC detector(s).

Circular dichroism studies were performed on a Jasco J-14C computer-assisted circular dichroic spectrometer. Fourier-transform proton NMR studies were run on a Nicolet NT-300-WB 300 MHz system using normal and pulse (180–τ–90) techniques.

RESULTS AND DISCUSSION

We considered three possible causes of a peak such as that observed for cyclosporine (Fig. 2): (a) slow diffusion of cyclosporine; (b) slow kinetic interactions between the cyclosporine and the stationary phase; (c) inability to resolve conformational isomers of cyclosporine. The effect of an increase in temperature, as shown in Fig. 2, was slightly greater for the peptide than for phenol (ΔN = 1400 *vs.* 1000, where N is the number of plates), although this was of limited value in establishing a mechanism. The increase in the observed column efficiency for cyclosporine was logrithmically related to temperature over the range 10–80°C, doubling every 7°C.

To ascertain whether diffusion could account for the very broad peaks at room temeprature, the diffusion coefficient of cyclosporine in the HPLC mobile phase was measured by an open-tube dynamic flow technique[5]. The diffusion coefficient was then used to calculate the expected plate number using the Knox equation[6]. As shown in Table I, there is a major discrepancy between the calculated and observed values for cyclosporine, whereas the agreement for toluene and albumin is reasonable.

The possible role of a kinetically slow interaction with the stationary phase was more difficult to exclude. One might envisage a slow conformational change at the interface between the mobile and stationary phases, for example, or dissociation and association of an adduct of several of the hydrophobic peptide molecules. According to the Giddings random walk model[8], chemical reactions that are slow rel-

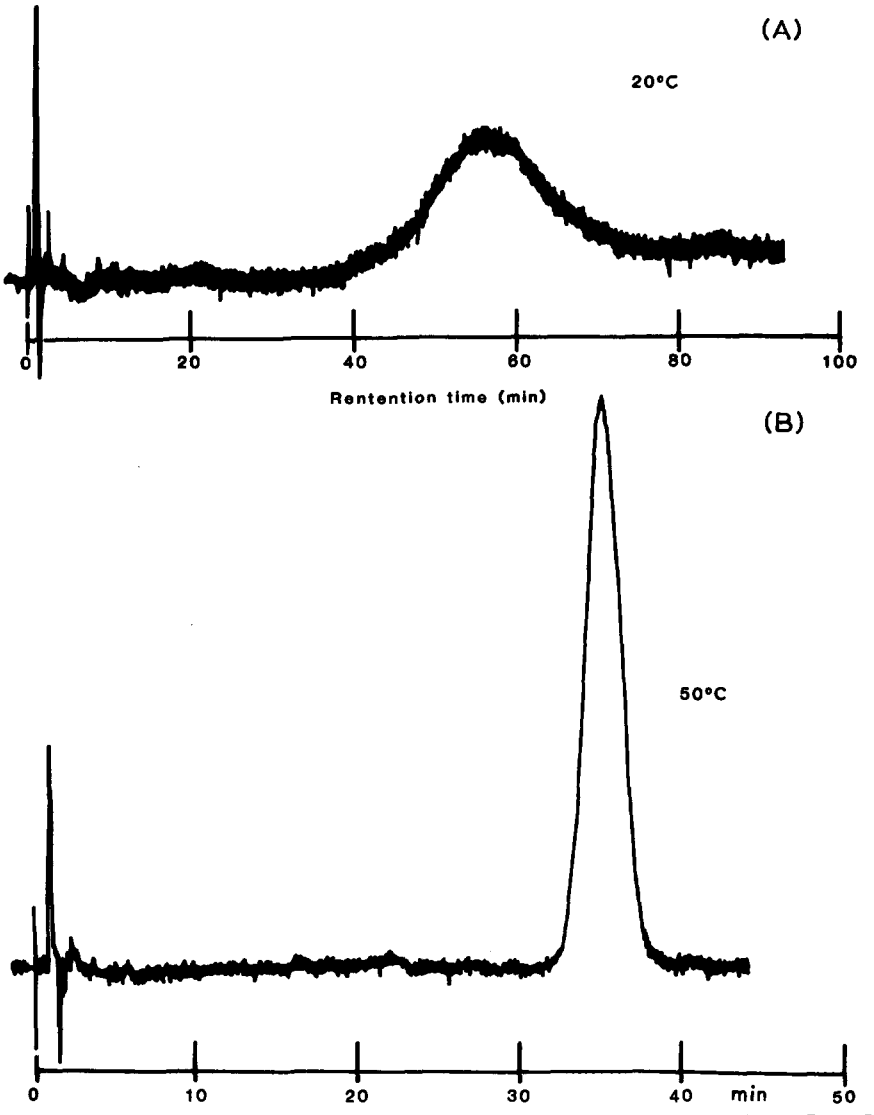

Fig. 2. Chromatograms of cyclosporine at (A) 20°C and (B) 50°C using reversed-phase HPLC. Conditions: mobile phase, acetonitrile–methanol–water (58:10:32); column, Supelco LC-18, 150 × 4.6 mm I.D.; flow-rate, 1 ml/min.

ative to the chromatographic process result in asymmetric peak shapes. Clearly this is not the case for cyclosporine (Fig. 2). Further consideration of this observation, however, convinced us that for a strongly retained compound (capacity factor, $k' > 10$) a significant amount of kinetic broadening could occur before any tailing would be observed[9]. Therefore, to rule out kinetics as a broadening mechanism, we performed a flow-switching experiment and measured peak broadening as a function of flow-rate on two packing materials with different particle diameters.

If a kinetically controlled process were occurring in a normal elution exper-

TABLE I

EXPECTED AND OBSERVED COLUMN EFFICIENCIES FOR VARIOUS MOLECULES

Compound	*M.W.*	*N*	
		*Calculated**	*Observed*
Toluene	92	3205**	3574
Albumin	66 000	31***	53
Cyclosporine	1202	1195	61

* Calculated for a well packed 5 cm column, 5 μm particles, 0.5 ml/min mobile phase flow-rate.
** Diffusion constant calculated from the Wilke–Chang equation.
*** From ref. 7.

iment, numerous repetitions of that process would occur in the time required for elution. By loading the cyclosporine on to the head of a column, then reversing the direction of flow, one could achieve, in theory, a single interaction. Variation of the contact time between the solute and the stationary phase should then show the effect of a kinetically slow process by changes in the peak profile[10]. Execution of this experiment for cyclosporine showed no peak profile changes over a wide range of contact times.

Kinetic control of peak broadening processes has also been shown to be manifest in the flow-dependent term of the plate height–flow-rate relationship (such as the Knox equation). When kinetic broadening is absent, there should be differences in the value of this flow-dependent coefficient for different particle sizes[10]. A constant value for different sized particles, in this instance 3 and 5 μm, would be consistent with a kinetically controlled process. Using peak moments, a significant difference between C terms of the Knox equation of the two particle sizes could not be proved, probably as a result of the imprecision in the second peak moment due to the very broad peak and the associated problem of precisely defining the initial and terminal points for the peak.

As a result of the inconclusive results with regard to the kinetic limitations, the chromatographic conditions were altered in an attempt to resolve the possible conformational isomers. By decreasing the temperature, the interconversion of the conformers was expected to be decreased. It was also found empirically that the removal of methanol from the mobile phase resulted in substantial peak broadening and changes in peak shape (Fig. 3A). The pattern observed at 4°C was reminiscent of the chromatographic pattern observed for the conformational isomers of the dipeptide L-alanyl-L-proline[11]. One possible explanation for such a trace is that the first peak retention time (15 min) and the last peak (retention time 35 min) are pure conformers and the area connecting the two peaks was the result of on-column re-equilibration of the two forms during elution. In an effort to verify that the peak at 15 min was a pure component, the eluent was collected and stored at 0°C until re-injection. Injection of this band resulted in the chromatogram shown in Fig. 3B. Clearly, the compound in the collected effluent has re-equilibrated into the previously observed conformers. Similar results were observed for the last peak in the original chromatogram (Fig. 3C). These results were clearly compatible with the presence of conformers, although the mechanism of the rearrangement is not apparent. Hence, the role

of the column in the conformer interconversion *versus* solution phase equilibrium cannot be ascertained from these experiments.

In an effort to confirm the existence of conformers, circular dichroism spectra of cyclosporine in a variety of solvents including water, methanol, octanol and chloroform were measured. A classical pattern attributable to a β-pleated sheet was present in all solvents. It was impossible to detect any significant differences in the spectra. This was not surprising, as the optical activity of cyclosporine can only originate from the fixed ring orientation of the peptide. It does demonstrate, however, that the basic ring structure was left intact. Using 300 MHz Fourier transform nuclear magnetic resonance, a relatively complex pattern was obtained using C^2HCl_3 as a solvent. The pattern for the four amide protons, however, was relatively simple (Fig. 4A), indicating equivalent areas for each of the four protons. Temperature studies indicated that three of the amide groups were internally hydrogen bonded. The spectrum obtained using H_2O–^2H_2O–C^2H_3CN (20:20:60) or $C^2H_3O^2H$–CH_3OH (50:50) was considerably more complex (Fig. 4B). Using the fact that the size of each of the four amide groups on each conformer must be the same, the presence of at least four conformers can be supported.

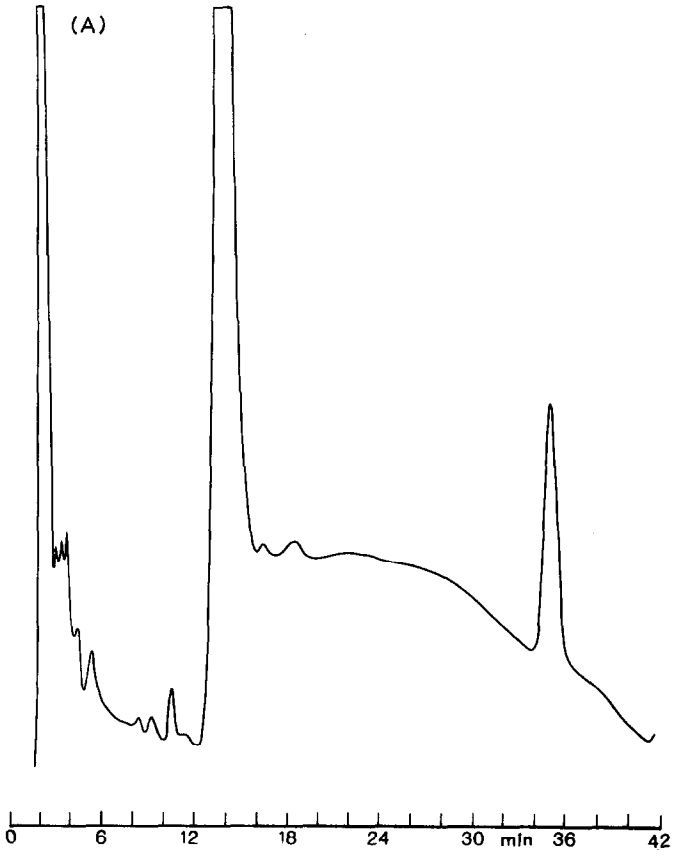

Fig. 3.

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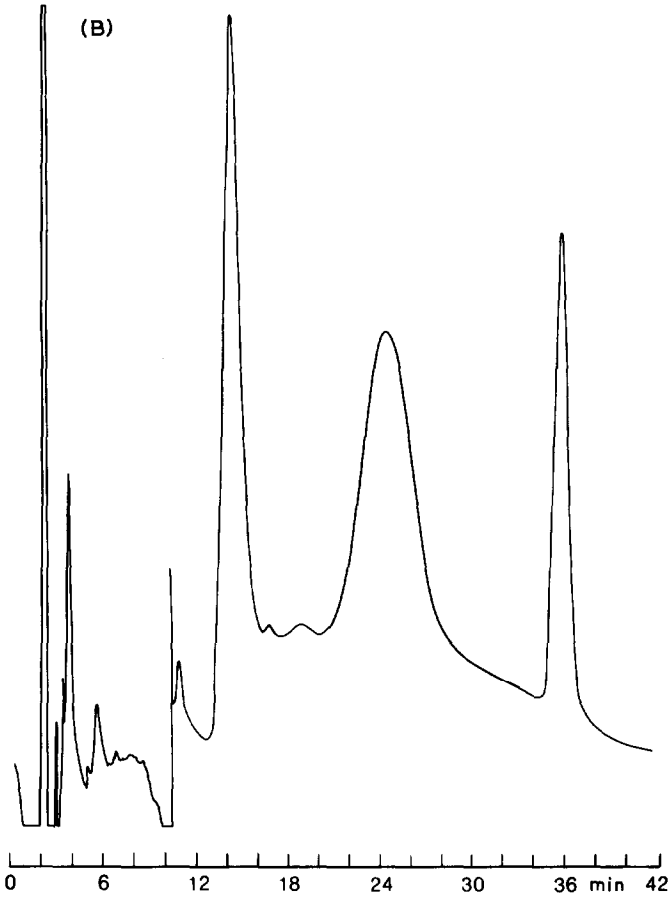

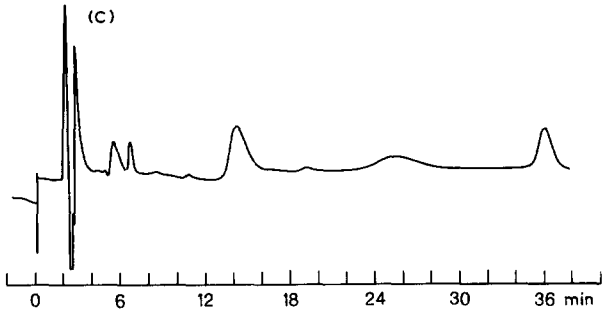

Fig. 3. Chromatograms of cyclosporine and re-chromatography of eluted fractions at 2°C. (A) Original chromatogram [conditions: mobile phase, acetonitrile–water (68:32); column, Supelco LC-18, 50 × 4.6 mm I.D.; flow-rate, 1 ml/min]; (B) re-injection of fraction collected from 13 to 15 min; (C) re-injection of fraction collected from 33 to 37 min.

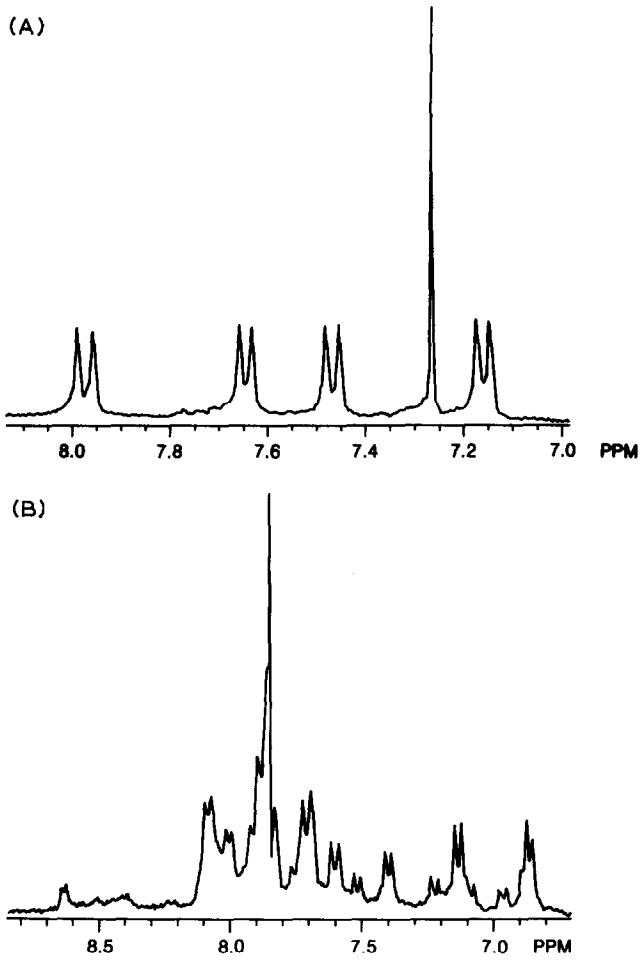

Fig. 4. NMR pattern from the amide region of cyclosporine in (A) C^2HCl_3 and (B) in C^2H_3CN–^2H_2O–H_2O (60:20:20) using a 180–τ–90 pulse technique.

CONCLUSIONS

From the above results, the extreme peak broadening observed for cyclosporine seems to be the result of the inability of an octadecyl-bonded phase to resolve completely several conformers. There are a number of implications of this finding for the HPLC analysis of cyclosporine. It is clear that there is no alternative to elevating the column temperature. For octadecyl-bonded columns, a temperature of 70–80°C is required. For hexyl- or cyano-bonded phases, acceptable column efficiency has been reported at 50–60°C[12]. The present results support the concept that the lower temperature is useful for these columns because they are less able to resolve the various conformers. Hence rapid interconversion of the conformers is not as important for narrowing the peak. The contention that the latter columns at 50°C are more

stable than an octadecyl-bonded column at 70°C is not supported by our studies. An average of 1200 injections per column has been achieved on an octadecyl-bonded column at 75°C over the past 2 years (11 000 analyses) in our laboratory.

A second consideration is the thermal equilibration of the mobile phase. Several groups have reported a strong dependence of column efficiency on flow-rate[1,13]. Although a chromatographic mechanism was implied, the strong effect of temperature on the separation can overshadow any other potential mechanism. Specifically, as the flow-rate is increased the mobile phase does not achieve thermal equilibrium with the temperature-controlling device and thus the peak is inordinately broadened. Several coils of 0.005 in. I.D. tubing are required either before the injection valve or between the injection valve and the column to pre-equilibrate the mobile phase. When this is done, the relationship between column efficiency and flow-rate is similar to that observed for more conventional solutes.

Finally, let us consider the role of the silica base material in the generation of conformers or in their partial separation. Clearly, the conformers exist in solution, as demonstrated by NMR spectroscopy. The fact that relatively rapid (*ca.* 30 min) re-equilibration occurs at 0°C, as illustrated in Fig. 3, would support a relatively small energy barrier between the conformers. The energy barrier for the cyclic tetrapeptide tentoxin has been reported as 13 kcal/mol[14], a value that is certainly consistent with the observations reported here.

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