Effect of Column Temperature and Eluent Flow Rate on the High Performance Liquid Chromatographic Analysis of Cyclosporin A and D

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Abstract: On a reversed-phase C_{18} analytical column using an eluent of 70 : 30 acetonitrile and water, the following effects were observed with increasing column temperature (from 25 to 75°C) for cyclosporin A (CSA) and cyclosporin D (CSD). The peak heights and number of theoretical plates (N) increased. The height equivalent to a theoretical plate (HETP) decreased. The areas under the peaks, retention times and capacity factors (k') for both compounds did not vary with temperature. With increasing eluent flow rate (from 0.5 to 2.5 ml/min), the peak heights, peak areas, retention times and N all decreased for both compounds. A slight decrease in k' for CSA and CSD was also observed. HETP increased with increasing flow. The separation factor, α, remained relatively constant for the ranges of temperatures and flow rates investigated.

Cyclosporin A (CSA) is a potent immunosuppressive agent that has been shown to prolong allograft survival of various organs and tissues (1, 2). CSA, cyclosporin D (CSD) and other cyclosporins, are derived from the fungi *Trichoderma polysporum Rifia* and *Cylindrocarpum Lucidum* (3). CSA and CSD are neutral, hydrophobic cyclic peptides consisting of 11 amino acids with molecular weights of approximately 1200 daltons (Fig. 1).

The development of a high performance liquid chromatographic assay method for a drug requires knowledge of the variables that affect its retention and resolution to obtain optimum separation conditions. The following factors are known to influence the retention and resolution characteristics of a chromatographed substance: column composition, column temperature, eluent composition and eluent flow rate (4). The purpose of this study was to investigate the effects of column temperature and eluent flow rate on the chromatographic behavior of CSA and CSD on a reversed-phase C_{18} column. These two factors were felt to be particularly important in the liquid chromatographic analysis of CSA and CSD owing to their unique molecular structures and physicochemical properties. Previous investigators (5–7) have shown that the retention time of small peptides (less than 20 amino acid residues) in reversed-phase analysis is influenced by the number and type of amino acid residues as well as by the molecular structure and conformation.

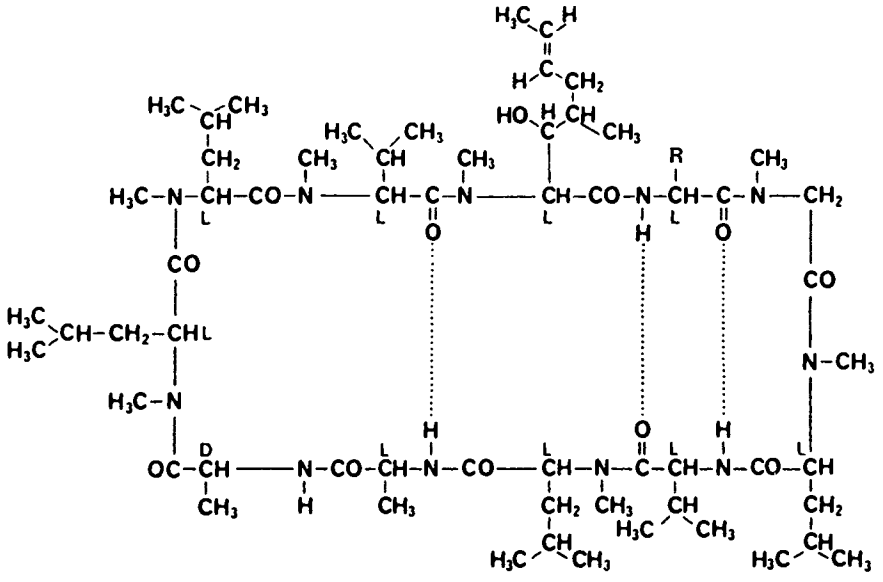

Fig. 1 Structural formula of cyclosporin A [R = CH_2 – CH_3] and cyclosporin D [R = CH – $(CH_3)_2$].

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Materials and Methods

Drugs and Standard Solutions

CSA and CSD were obtained from Sandoz Laboratories in Basel, Switzerland. Standard solutions containing 20 mg of CSA or CSD were prepared in 10 ml of the mobile phase. From these stock solutions, working drug solutions with a concentration of 5 μg of CSA (or CSD) per ml of solution were prepared by dilution with additional mobile phase. Twenty microliter samples of these working solutions were injected onto the column per injection.

Instrumentation

A modular liquid chromatographic system consisting of a Constametric III pump (Laboratory Data Control, Riviera Beach, FL.), SpectroMonitor III variable wavelength ultraviolet detector (Laboratory Data Control), 20 μl loop injector (Model 2175, Rheodyne Inc., Cotati, CA.) and chart recorder (Coleman Instruments, Oak Brook, IL.) was used.

Chromatographic Conditions

The samples were analyzed with a reversed-phase, C_{18} μ-Bondapak, 30 cm × 3.9 mm I. D. column (Waters Associates, Inc., Milford, MA.) using an eluent consisting of a 70:30 mixture of acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegon, MI.) and water. Distilled and deionized water was used. Prior to use, the mobile phase was filtered through a Fluoropore filter (Millipore Corp., Bedford, MA.) and then degassed, first under vacuum and then with sonication. At the end of each analysis, the column was rinsed thoroughly with methanol (Burdick and Jackson Laboratories, Inc.). Between studies, it was stored containing methanol.

To investigate the effects of eluent flow rate on the chromatographic behavior of CSA and CSD, the following flow rates were studied: 0.5, 1.0, 1.5, 2.0, and 2.5 ml/min. The eluent was monitored at 205 nm with an attenuation of 0.01 a.u.f.s. and a chart speed of 0.25 cm/min.

The effects of column temperature were studied over the range of 25–75°C in a water-jacketed column. The column was maintained at the desired temperature by circulating heated water from a temperature-controlled circulating water bath (Beckman Instrument Co., Fullerton, CA.). Furthermore, the mobile phase and pre-column tubing were equilibrated at the desired column temperature prior to each temperature study.

Data Analysis

The chromatographic parameters, N, number of theoretical plates, HETP, height equivalent to a theoretical plate, and k′, the capacity factor, were determined for CSA and CSD using standard methods (4). The separation factor, α, was assessed by determining the ratio of the capacity factors for CSD and CSA, i.e., $\alpha = k'_{CSD}/k'_{CSA}$. Peak areas were determined by the tangent method.

For each parameter and drug, the mean value for 3 determinations was obtained for a given condition and plotted using a three-dimensional plotting program and digital computer.

Results and Discussion

During the development of a high performance liquid chromatographic assay method for CSA, the selection of a suitable mobile phase was approached with the aim of achieving a value for the capacity factors for CSA and CSD (k'_{CSA} and k'_{CSD}, respectively) in the range of 1–5 at room temperature and under isocratic elution. Using these criteria, a mobile phase of acetonitrile-water (70:30) was chosen since an optimum value for k′ for the band-pair of interest was obtained. With this mixture, the retention times decreased from 27.0 and 36.8 min at a flow rate of 0.5 ml/min to 6.0 and 8.4 min at a flow of 2.5 ml/min for CSA and CSD, respectively, at ambient column temperature. These observations showed that the relationship between the retention time and flow rate for CSA and CSD was nonlinear (Fig. 2).

As shown in Figures 3 and 4, respectively, the peak heights and areas under the peaks for CSA and CSD both decreased as the eluent flow rate was increased. Of the two parameters, the changes observed in the resultant areas were more pronounced. As a result, the net effect of the increase in eluent flow was a reduction in the sensitivity of the method for both compounds.

While the peak heights of CSA and CSD increased with increasing column temperature, no changes in the peak areas

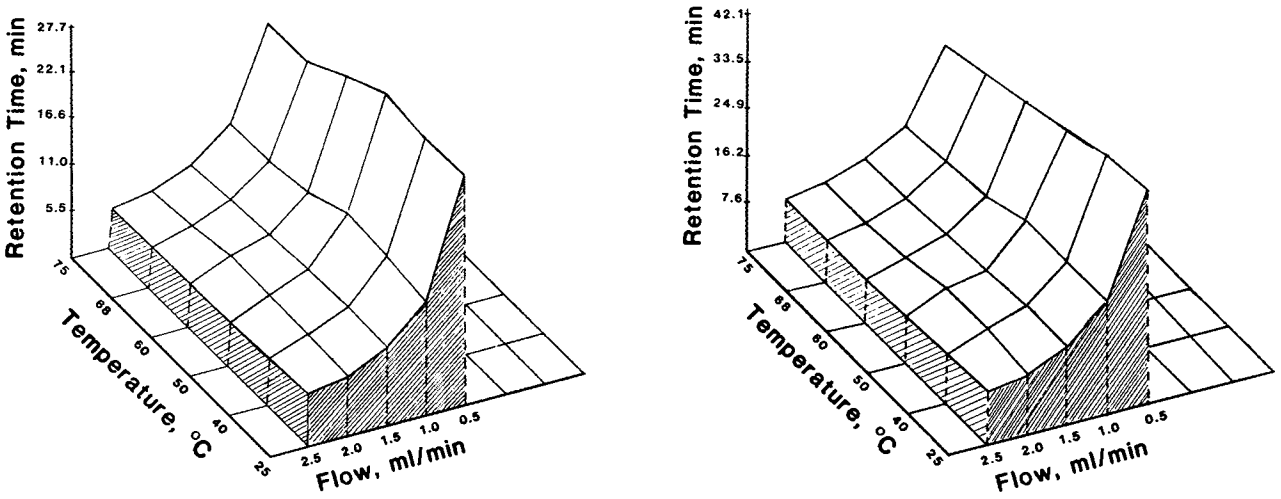

Fig. 2 The effects of column temperature and eluent flow rate on the retention time of CSA (left) and CSD (right).

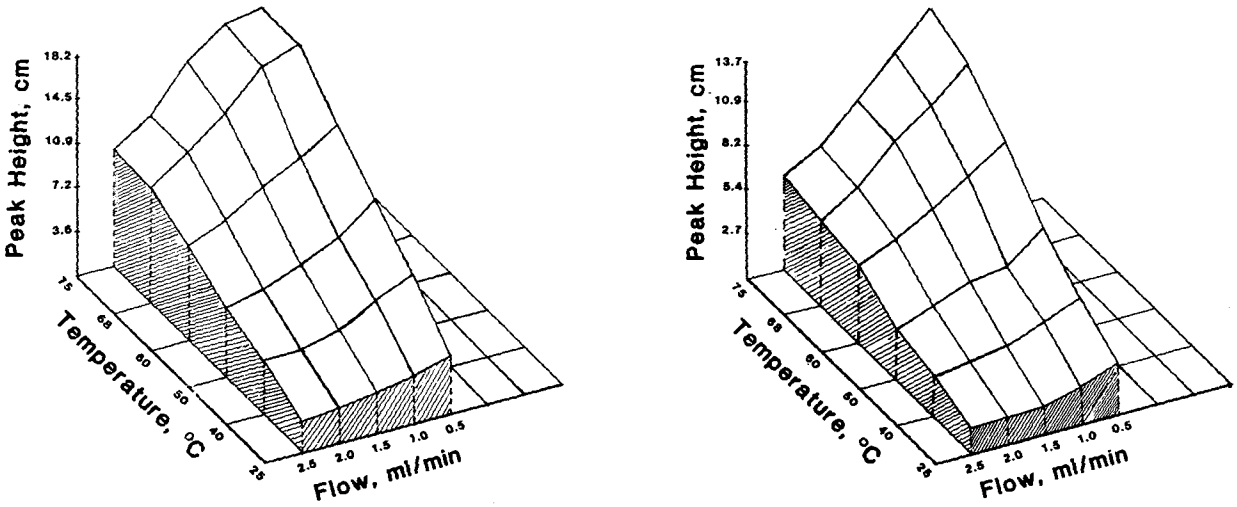

Fig. 3 The effects of column temperature and eluent flow rate on the peak height of CSA (left) and CSD (right).

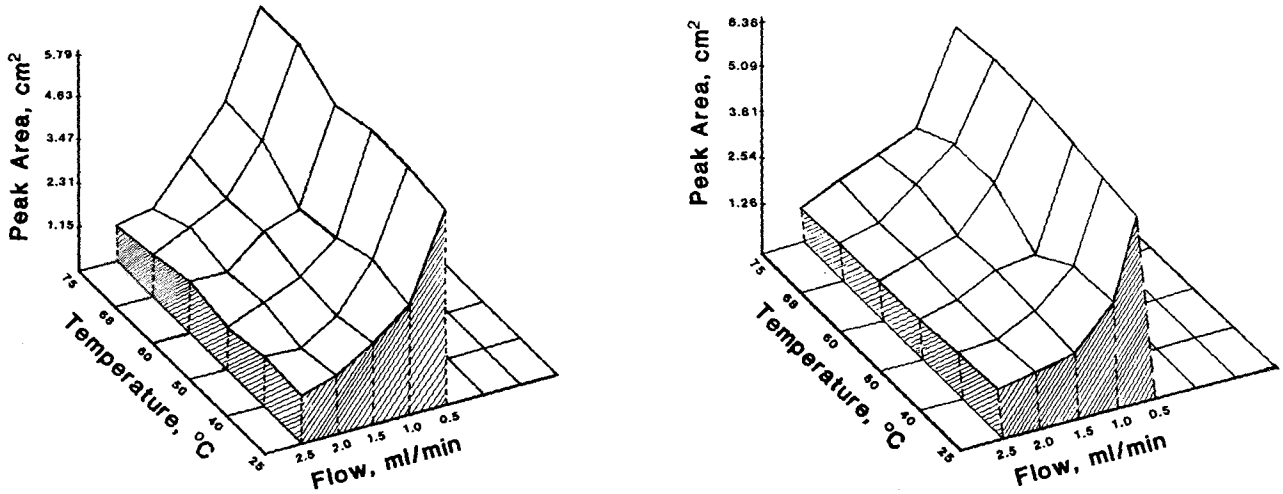

Fig. 4 The effects of column temperature and eluent flow rate on the area under the peak for CSA (left) and CSD (right).

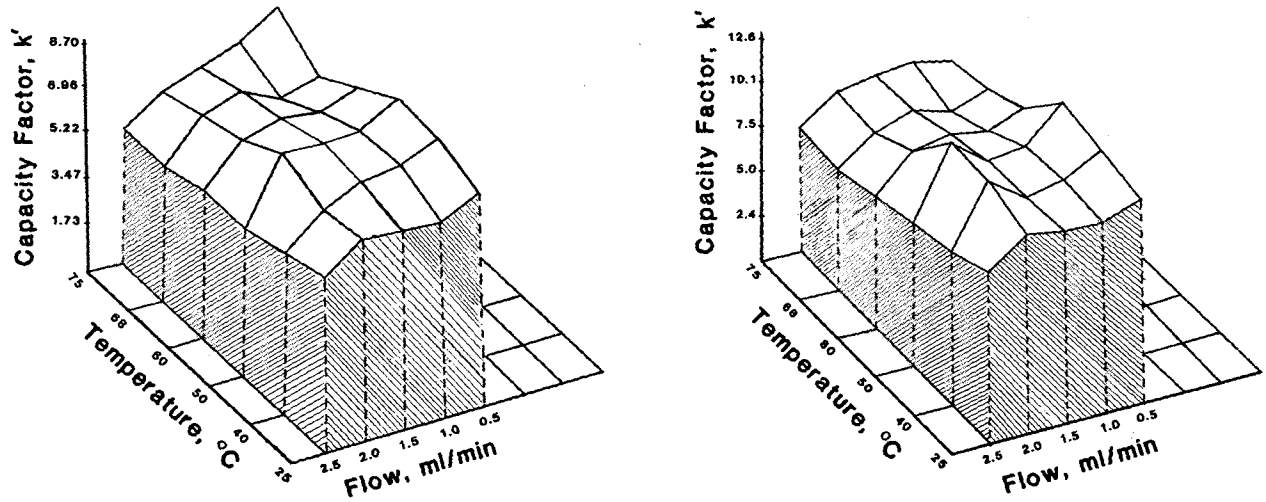

Fig. 5 The effects of column temperature and eluent flow rate on the capacity factor for CSA (left) and CSD (right).

were observed for the range of temperatures investigated (Figs. 3 and 4). These findings were in agreement with the general observation that increasing the column temperature markedly sharpens the shape of a peak (4).

The effects of column temperature and eluent flow rate on CSA and CSD retention time are shown in Figure 2. The retention times of both compounds were found to be independent of the column temperature but dependent on the flow rate of the mobile phase, the retention time decreasing with increasing flow. The former observation is unusual in that previous workers (8–11) have noted that the retention times of proteins as well as nonproteinaceous substances decreased with an elevation in column temperature. Bovine serum albumin is the only other protein reported where the retention time did not change as a function of column temperature (8).

Since the capacity factor is dependent on the retention time, k'_{CSA} and k'_{CSD} were unaffected by a change in column temperature (Fig. 5). On the other hand, k'_{CSA} and k'_{CSD} decreased slightly as the eluent flow rate was increased (Fig. 5). Thus, although the eluent flow rate had a pronounced effect on the retention times of CSA and CSD, the net effect on k'_{CSA} and k'_{CSD} was not as dramatic, thereby showing the importance of the composition of the mobile phase on the capacity factor for a given substance.

Several studies (11–13) suggest that temperature can significantly affect α and k'. In the present investigation, the values for the separation factor, α, varied slightly (range: 1.29–1.47) for the ranges of temperatures and flow rates investigated as shown in Figure 6.

The improvement in column efficiency for CSA and CSD (i.e., increase in N and decrease in HETP) in this study was due to the increase in column temperature (Figs. 7 and 8). This effect could be explained by the influence of temperature on the viscosity of the mobile phase, where a decrease in the viscosity would result in a reduction in column pressure. Alternatively, the improvement in efficiency with increasing temperature could also have been due, in part, to the fact that CSA and CSD are both relatively high molecular weight polypeptides of unique cyclic form. As a result, they might have low diffusion or penetration at low temperatures. An elevation in column temperature reduces the viscosity of the mobile phase. Furthermore, it produces an increase in the mass transfer or diffusion of the solute to the stationary phase (14). Giddings (14) reported that both of these effects decrease HETP.

Several high performance liquid chromatographic methods for the analysis of CSA using elevated column temperatures of 70 to 75°C have been reported (15–18). The results of this study show that the use of increased temperature for the determination of CSA enhances the column efficiency rather than decreasing the analysis time (i.e., retention time).

In the present investigation, the retention order of CSA and CSD as well as the α values were not altered by a change in column temperature. These observations follow the behavior that would be expected of a "regular" temperature or enthalpy-entropy compensated effect (19, 20) and might be due to the similarities of CSA and CSD in their structure and degree of compactness.

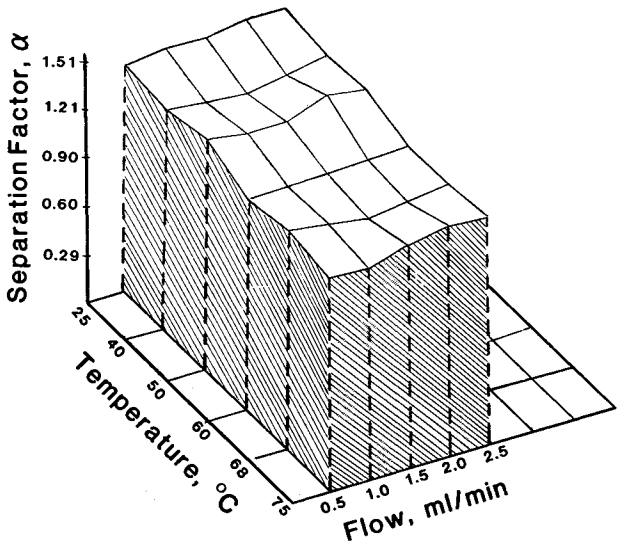

Fig. 6 The effects of column temperature and eluent flow rate on the separation factor for CSA and CSD.

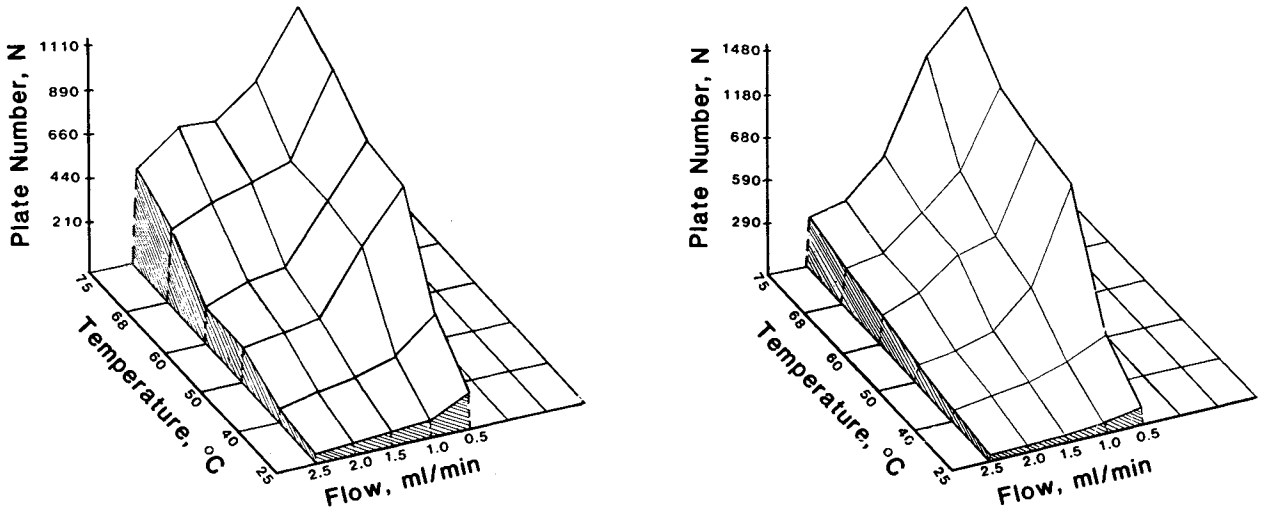

Fig. 7 The effects of column temperature and eluent flow rate on the number of theoretical plates for CSA (left) and CSD (right).

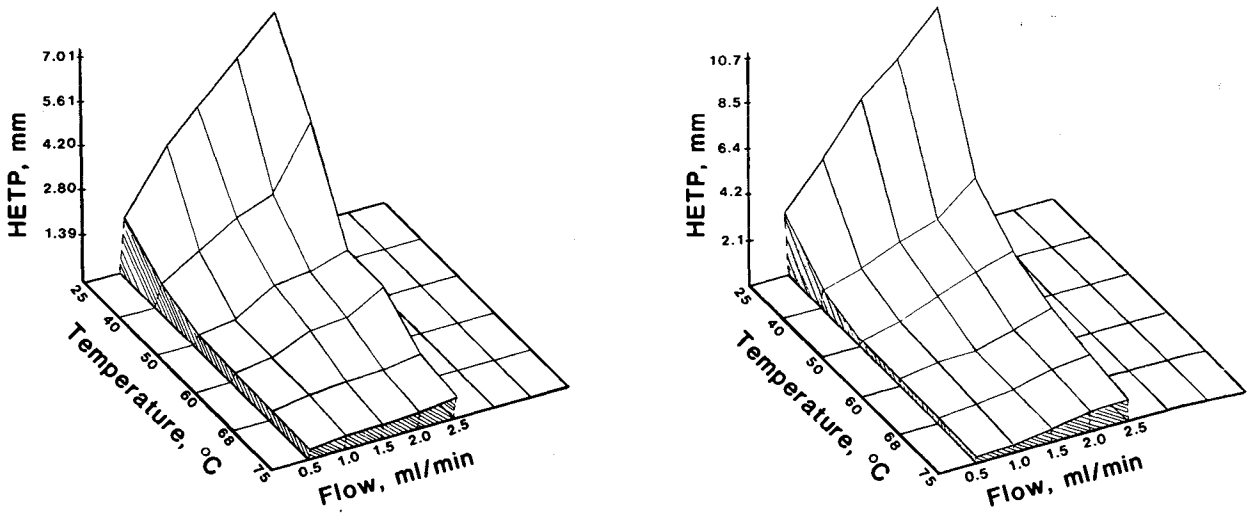

Fig. 8 The effects of column temperature and eluent flow rate on height equivalent to a theoretical plate (HETP) for CSA (left) and CSD (right).

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