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EFFECT OF MOLECULAR STRUCTURE AND CONFORMATIONAL CHANGE OF PROLINE-CONTAINING DIPEPTIDES IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

Peptides that contain proline residues may yield multiple peaks in high-performance liquid chromatography if the proline is not at the N-terminus. The phenomenon is caused by slow kinetics of isomerization that are on the same time-scale as the chromatographic separation with such peptides. In this study, the shape and number of peaks are examined qualitatively in view of the influence of isomerization kinetics on the bandspreading, and as functions of pH, temperature and flow velocity. These effects in the chromatography of alanylproline are shown in detail. Single peaks are obtained for each peptide investigated by proper adjustment of these variables in light of their effect on the pertinent rate and equilibrium constants.

INTRODUCTION

The appearance of more peaks in a chromatogram than expected from the number of sample components is usually attributed to impurities. It is known, however, that a given substance can yield multiple peaks for a variety of physical reasons, including poor sample introduction, maldistribution of eluent flow[1] and non-uniform radial temperature profile in the column[2]. In the present work we examine peak splitting that occurs as a consequence of a conformation change of an eluite at a rate that is commensurable with that of the chromatographic distribution process between the mobile and stationary phases. Dipeptides that contain L-proline have been chosen as model substances because their pertinent properties are well documented in the literature. Thus, we can relate the effect of operational conditions on the observed unusual behavior to the molecular structure of the sample components and the underlying secondary equilibria. Similar phenomena have been observed in thin-layer chromatography (TLC)[3] and in high-performance liquid chromatography (HPLC) with other substances[4,5]. As early as 1960, band-spreading, due to concomitant migration and reaction, was treated quantitatively by Giddings[6,7]. Recently the significance of slow kinetics in determining column efficiency in HPLC has been pointed out in a general fashion by Horváth and Lin[8]. Results presented here are expected to facilitate

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recognition of such secondary equilibria in practice. A qualitative understanding of the relationship between operating conditions and separation efficiency can lead to optimization of separation, particularly in the case of biological substances.

All peptides can be in either the *cis* or *trans* conformation with respect to the amide bond, although most of them exist exclusively in *trans* form[9,10]. However, certain peptides linked via the imino nitrogen of proline are peculiar because they can be present both in *cis* and *trans* forms under usual conditions[10–21]. Furthermore, the rate at which a new equilibrium composition of their mixture is attained upon slight change in conditions is relatively slow[10,13,14,17,18]. In some cases relaxation times are of the order of minutes, *i.e.*, on the time-scale of chromatographic runs in HPLC. If the retention factors of the *cis* and *trans* form are not identical, peak splitting, or at least excessive band-spreading, can be expected as a result of the relatively slow kinetics of the conformation change.

Examination of appropriate molecular models of dipeptides containing proline reveals that the hydrophobic surface area that affects the strength of eluite binding to the stationary phase[22,23] is different for the two conformers. This can be seen from the space-filling models for *cis*- and *trans*-alanylproline shown in Fig. 1. In the *cis* conformer a plane through the two α-carbons separates non-polar residues from the polar amino and carboxyl groups. No such plane that separates the polar from the non-polar residues can be found for the *trans* form of alanylproline, however. Consequently the solvophobic theory[22–24] predicts that in reversed-phase chromatography the retention of the *cis* conformer, which can have a larger contact area upon binding to the hydrocarbonaceous ligates of the stationary phase, will be greater than that of the *trans* form, which has a smaller hydrophobic surface area.

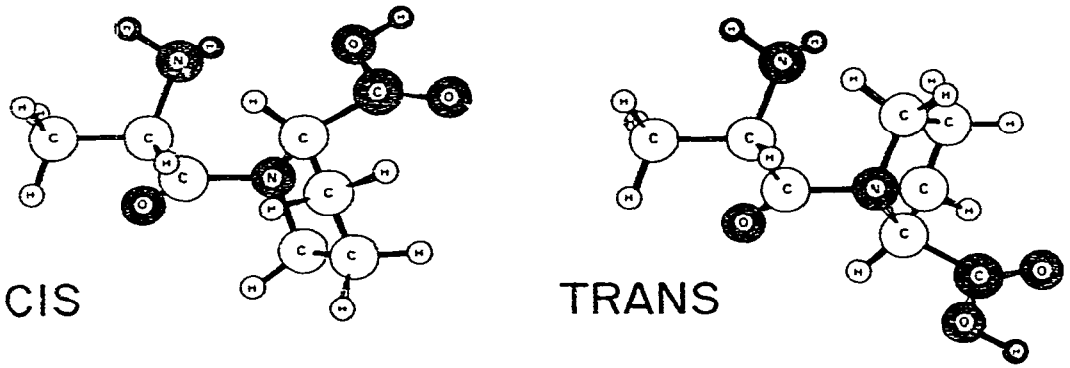

Fig. 1. Perspective molecular structures of *cis*- and *trans*-L-alanyl-L-proline with the polar functions shaded. Whereas polar and non-polar residues in the *cis* conformation can be placed on opposite sites of a hypothetical plane, no such plane exists for *trans* conformation. Consequently, retention of the *cis* conformer in reversed-phase chromatography is expected to be greater than that of the *trans* conformer.

EXPERIMENTAL

The apparatus consisted of a Model 100A pump (Altex, Berkeley, CA, U.S.A.), a Model 70-10 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) a Model LC-55 variable-wavelength spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Model 123 recorder (Perkin-Elmer). The precolumn heat exchanger and the

column were thermostatted by using a Model K-2/R circulating water bath (Messgeraetewerke, Lauda, G.F.R.). The column effluent was monitored at 210 nm detector setting.

The 10-μm LiChrosorb RP-18 column (250 × 4.6 mm I.D.) was obtained from Knauer (Berlin, G.F.R.). Reagent phosphoric acid, NaH_2PO_4 and Na_2HPO_4 were obtained from Fisher (Pittsburgh, PA, U.S.A.). The peptides were purchased from Sigma (St. Louis, MO, U.S.A.).

Perspective drawings of L-alanyl-L-proline and L-prolyl-L-alanine molecules were prepared by using the PROPHET system.

RESULTS AND DISCUSSION

Chromatograms of L-prolyl-L-alanine and L-alanyl-L-proline obtained on octadecyl-silica with plain aqueous buffer, pH 6, as the eluent at 25 C are depicted in Fig. 2. The second peptide, in which proline is the C-terminal residue, yielded a broad "peak" with fairly sharp spikes on the leading and trailing edges, whereas more normal peak shape has been observed for the first peptide. Similar results were obtained with the dipeptide pairs L-valyl-L-proline and L-prolyl-L-valine, as well as L-prolyl-L-phenylalanine and L-phenylalanyl-L-proline. In each case, the recorder response of the dipeptide with a C-terminal proline residue, which we will call a *proline peptide*, yielded "peaks" that consisted of two spikes and an intermediary plateau region. In contradistinction the tracing of the corresponding peptide isomer with proline at the N-terminus, which we will call *prolyl peptide*, showed only one sharp

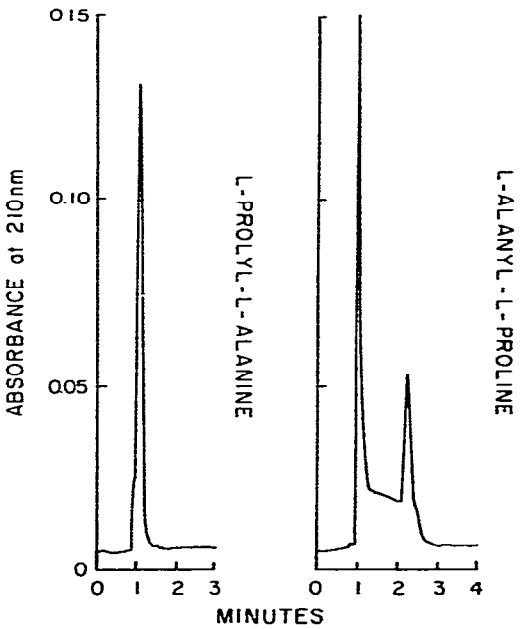

Fig. 2. Chromatograms of L-prolyl-L-alanine and L-alanyl-L-proline. The data were obtained with neat aqueous 0.05 *M* phosphate buffer, pH 6.0, at 3 ml/min flow-rate he column (250 × 4.6 mm I.D.) was packed with 10-μm LiChrosorb RP-18. The temperature was 25°C.

peak in all cases. In some other cases the effect was less dramatic; *e.g.*, glycylproline did not yield split peaks but only a trailing shoulder under similar chromatographic conditions. Results shown in Fig. 2 can be interpreted on the basis of the above-mentioned facts that (i) the prolyl peptide exists only in *trans* form, (ii) the proline peptide can be present both in *cis* and *trans* forms, and (iii) owing to its smaller hydrophobic surface area, the *trans* conformer elutes faster than the corresponding *cis* conformer. Indeed the first spike of the reaction "peak" of a proline peptide has the same retention as the single peak obtained when the corresponding prolyl peptide is chromatographed. Whereas with the proline peptides the concentrations of the *cis* and *trans* conformers are commensurate, according to the literature[13,14,16,20,25] the prolyl peptides exist only in *trans* form under the conditions investigated here.

The observation that chromatographic peak splitting does not occur with peptides having N-terminal proline supports the proposition that the behavior described here is not due to the presence of proline *per se* in the peptide but only occurs when proline is not at the N-terminus of the peptide. Peak splitting can be readily explained by the slow kinetics of isomerization that are expected on the basis of literature data when proline is not at the N-terminus of the peptides. In some experiments we collected fractions of the column effluent corresponding to various portions of the "reaction peak" obtained with proline peptides. In each case the original chromatogram was reproduced upon rechromatographing these fractions, and this finding suggests that the effects reported here were not caused by sample impurities.

The effect of flow-rate on the shape of alanylproline "peak" is shown in Fig. 3. The results were obtained at two flow-rates, 1.0 and 9.0 ml/min, under conditions otherwise identical with those stated in Fig. 2. The "reaction peak" obtained at the higher flow-rate of 9.0 ml/min is more disperse and bimodal than that obtained at 1.0 ml/min, or even than that recorded at a flow-rate of 3.0 ml/min, and is shown in Fig. 2. It is seen that with decreasing flow-rate the proline peptide elutes as an increasingly monodisperse broad peak. This is consistent with the hypothesis that peak splitting is the consequence of slow kinetics of isomerization. Deviations from equilibrium concentrations develop in the mobile phase as the *cis* conformer of the proline peptide is preferentially bound by the stationary phase. The respective concentrations of the two eluite forms in the mobile phase will relax toward the equilibrium concentrations. When the isomerization rate is much less than the rate for the equilibration of the less retained component between the mobile and stationary phase, the two conformational isomers migrate down the column nearly independently and consequently two recorder peaks or, to be more precise, a bimodal reaction peak is observed. As the rate of isomerization becomes greater, however, the peptide appears as a single peak and, in the limit of infinitely rapid isomerization, only a monodisperse peak with no excessive broadening due to kinetic effects will be observed.

The effect of temperature on the retention and the peak shape of alanylproline is shown in Fig. 4. The chromatograms were run under the conditions given in Fig. 2 except that the column temperature was kept at 25, 40 or 55 C. It is seen that with increase of temperature the tracing becomes increasingly monodisperse until, in this case at 55 C, only somewhat excessive peak broadening hints at the peak splitting found at lower temperatures. This result is consistent with the dominant role of kinetic effects in the determination of peak shape. As the temperature increases the rate of isomerization will increase, because the activation energy for the isomerization

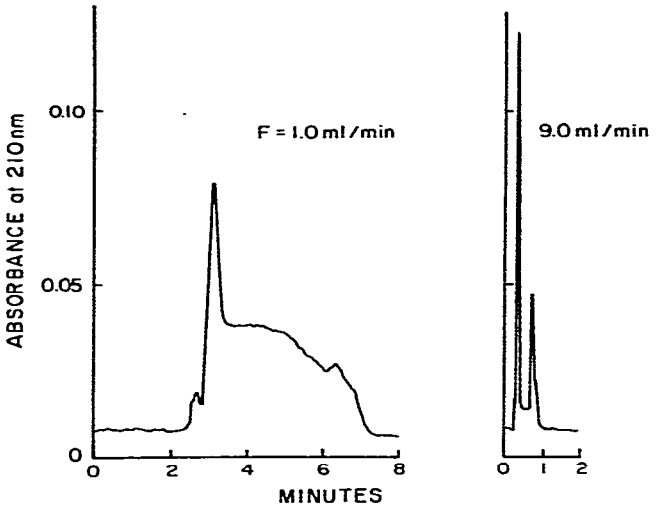

Fig. 3. Effect of flow-rate on the shape of the L-alanyl-L-proline peaks. Chromatographic conditions were those stated in Fig. 2 except the flow-rate, *F*, which was 1.0 or 9.0 ml/min.

is typically *ca.* 20 kcal/mol[10,14,17,20,26,27]. On the other hand the effect of temperature on retention is less dramatic. The enthalpy of the *cis–trans* equilibrium is nearly zero in aqueous solutions[17,18]. As a result the equilibrium composition will not change palpably with temperature and therefore no change in retention factor is anticipated

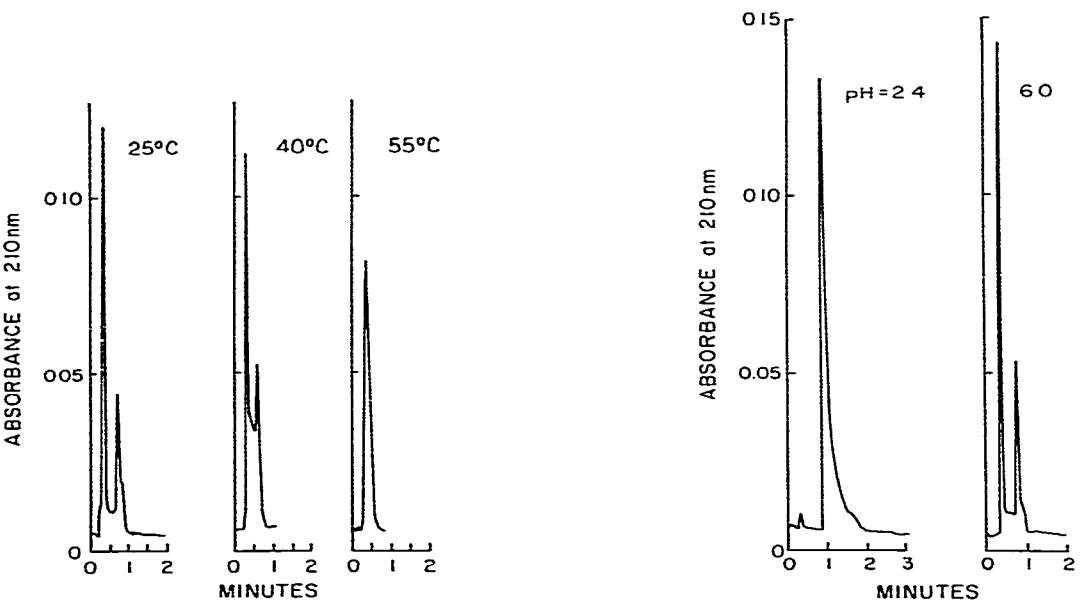

Fig. 4. Effect of column temperature on the elution of L-alanyl-L-proline. The temperature was 25, 40, or 55°C, and the flow-rate was 9.0 ml/min. Other conditions as in Fig. 2.

Fig. 5. Effect of pH on the elution of L-alanyl-L-proline. The mobile phase was 0.05 *M* phosphate buffer, pH 2.4 or 6.0. The flow-rate was 9.0 ml/min. Other conditions as in Fig. 2.

from that source, although some decrease in retention is expected because the retention enthalpies in reversed-phase chromatography are usually negative[24].

The effect of pH on peak shape of alanylproline is illustrated in Fig. 5. The single peak obtained at the lower pH is somewhat better retained than the split reaction peak of the peptide obtained at higher pH. Such an effect is to be expected if the peak splitting is a consequence of (i) the presence of the *cis* and *trans* forms in approximately equal amounts and (ii) the relatively slow kinetics of the interconversion.

The equilibrium composition contains *ca.* 50% of the *cis* conformer under zwitterionic conditions and decreases to *ca.* 10% under cationic conditions[13–15]. Furthermore, at pH 2.4, the relaxation rate for isomerization is much larger than that found at pH 6.0[14,15]. These effects both reduce the axial dispersion of the peptide peak at low pH values. The observation that the retention appears to increase with decrease of pH conforms with the general experience that the magnitude of the retention factors for the zwitterionic form of amino acids or peptides is lower than that of the corresponding cationic form[29] under otherwise identical chromatographic conditions.

Indeed, decreasing pH and increasing temperature can be employed simultaneously to improve peak shapes so that the chromatogram is suitable for extraction of quantitative data without elaborate computer analysis. The combined effect of relatively low eluent pH and high column temperature is seen on the chromatogram

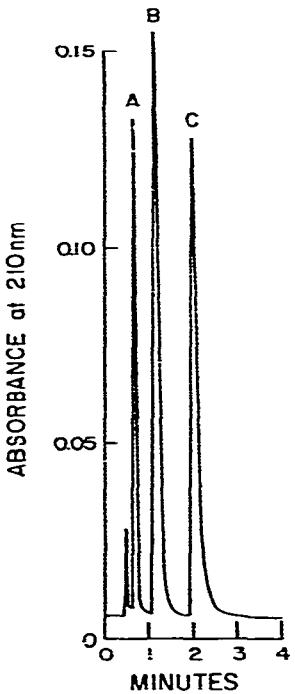

Fig. 6. Chromatogram of L-prolyl-L-glycine (A), L-alanyl-L-proline (B) and L-prolyl-L-valine (C) obtained at low eluent pH and elevated temperature in order to reduce excessive bandspreading of alanylproline. Conditions as in Fig. 2, except the eluent pH, column temperature and flow-rate were 2.4, 55° and 5 ml/min, respectively.

in Fig. 6, where the peak exhibits neither bimodal character nor excesssive band-spreading.

CONCLUSIONS

The data presented here indicate that molecules that exist in two or more distinguishable conformations can display anomalously broad and/or split chromatographic peaks. Knowledge of the effects of operating conditions, such as temperature, pH, mobile phase composition, and salts, on the equilibria and kinetics, however, can be used to reduce the influence of such phenomena on separation efficiency. In the cases examined here, decrease of eluent pH and increase of column temperature both decrease the relaxation time for isomerization with concomitant improvement in the peak shape, as illustrated in Figs. 4–6.

The results are consistent with the interpretation that multiple peaks observed in the chromatography of proline-containing peptides arise from the presence of the peptide in two slowly interconvertible *cis* and *trans* conformers. By extension, multiple peaks should be anticipated in the chromatography of other molecules when the rates of conformational change are slow on the time-scale of the separation by HPLC. Such a situation is likely to occur when free rotation about a bond in the eluite molecule is hindered. Indeed, multiple spots in TLC of cyclic peptides, such as D-methylalanine[1]-tentoxin[2], and multiple peaks in HPLC of the peptides gramicidin and 2-melanotropin[22] were explained by assuming the existence of different conformers of the peptides.

The phenomena investigated here are the consequence of relatively slow kinetics of conformational change in the mobile phase. An understanding of the underlying chemistry facilitates optimization of the chromatographic system as far as the separation efficiency is concerned. In turn, it also seems likely that study of the chromatographic system may facilitate understanding of the physical chemistry of the system insofar as the peak shape contains information on the pertinent equilibrium and rate constants. Extraction of these physico-chemical parameters from chromatographic data appears to be an intriguing and challenging task and is currently under investigation in our laboratory.

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