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ANALYSIS OF THE *cis–trans* ISOMERIZATION KINETICS OF L-ALANYL-L-PROLINE BY THE ELUTION-BAND RELAXATION METHOD

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SUMMARY

The "elution-band relaxation method" has been applied to the analysis of the *cis–trans* kinetics isomerization of the proline of L-Ala-L-Pro using reversed-phase liquid chromatography. A procedure suitable for cases where neither isomer can be injected separately is described.

INTRODUCTION

High-performance liquid chromatography (HPLC) is an indispensable tool in biochemistry and biophysics because of its high speed and resolution which cannot be attained by conventional chromatography[1]. Though it is usually used as a separation tool, we have developed methods to use it for conformational and kinetic analyses of molecules and have demonstrated clearly that HPLC has high potential for such applications[2–4]. We also affirmed that this potential can be fully exploited through the use of a micro-computer and mathematics.

We recently described the "elution-band relaxation method" for the analysis of reversible isomerization kinetics where the time constant is comparable to the time of elution[4]. The concept is as follows. Isomerizing molecules give different chromatograms for different flow-rates. When the flow-rate is high, the molecules have little time to isomerize and give a chromatogram consisting almost entirely of peaks due to the injected molecules. In contrast, when the flow-rate is low, the molecules have time to isomerize and give a broad band; this band relaxes and the first moment of the chromatogram shifts depending on the flow-rate. In our elution-band relaxation method the rate constants are obtained by analyzing this shift of the first moment. In the previous study[4] we applied the method to the denaturation–renaturation of protein using the size of the molecule as a probe.

Another approach to the study of isomerization using HPLC was developed by Horváth's group[5,6]. The mathematical basis of their method is quite similar to ours, however, the methods derived are different. They used two procedures to analyze isomerization kinetics: one is to evaluate the plate height contribution due to isomerization, and the other to simulate chromatograms by solving differential equations numerically.

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Separations of isomers of several compounds by reversed-phase liquid chromatography have been reported[7–9]; L-Ala-L-Pro is one such compound. The separation of its *cis* and *trans* isomers was reported by Horváth and co-workers[7], and the kinetics of isomerization was analyzed using the above methods[5,6]. In this study we analyzed this reaction by our elution-band relaxation method with a new procedure appropriate for the present case.

THEORETICAL

The procedures described in the previous study[4] assumed implicitly that each isomer can be injected separately, or that one of the isomers is stabilized under a certain solvent condition. In the present case of the *cis–trans* isomerization, however, this assumption is not valid. Therefore a new procedure must be used as follows.

The reaction under consideration is:

$$A \underset{k'}{\overset{k}{\rightleftarrows}} B$$

The conclusions of our previous study were: (1) in HPLC the diffusion terms have a negligible contribution to the first moment, and (2) the first moment of the chromatogram is expressed in terms of the scaling time, t_S, given by $t_S = l/v$, where l is the column length and v is a velocity or an analogous characteristic of operation, *e.g.*, the flow-rate. The first moment of a chromatogram obtained by injecting isomer A, given as the scaled moment [equal to $M_{1A}(t_S)/t_S$ in ref. 4], applies directly to the first moment of the elution volume when t_S is changed by changing v for a single column

$$M_{1A}(t_S) = \alpha_A[1-\exp(-\beta t_S)]/t_S+\gamma \tag{1}$$

where α_A, β and γ are functions of the velocities of the isomers and the rate constants (see eqns. 18–20 in ref. 4). The same quantity for B is:

$$M_{1B}(t_S) = \alpha_B[1-\exp(-\beta t_S)]/t_S+\gamma \tag{2}$$

When a mixture of the isomers is injected, the first moment is the sum of their contributions

$$M_1(t_S) = \alpha^*[1-\exp(-\beta t_S)]/t_S+\gamma \tag{3}$$

where $\alpha^* = f_A\alpha_A+f_B\alpha_B$ and f_A and f_B are the fractions of A and B injected. By measuring $M_1(t_S)$ for various values of t_S (usually by changing the flow-rate), α^*, β and γ can be determined. The rate constants are determined by β and γ in combination with the peak elution times of the isomers (see eqns. 19 and 20 in ref. 4)

$$k = \frac{\alpha t_{SO}(\gamma t_{SO}-t_A)}{t_A(t_B-t_A)} \tag{4}$$

$$k' = \frac{\alpha t_{SO}(\gamma t_{SO}-t_B)}{t_B(t_A-t_B)} \tag{5}$$

where t_A and t_B are the respective peak times of A and B in the chromatogram the scaling time of which is t_{SO}; t_A and t_B are used as an approximation in order to obtain the unknown velocities of A and B. For this approximation to be valid, t_A and t_B must be obtained from a chromatogram where the reaction extent is so small that two separate peaks are observed.

We can determine also the isomer fraction of the sample by use of the determined parameters:

$$f_A = \frac{(\alpha^*\beta+\gamma)t_{SO}-t_A}{t_B-t_A} \tag{6}$$

EXPERIMENTAL

L-Ala-L-Pro was obtained from Sigma (St. Louis, MO, U.S.A.). A few hours before the experiment it was dissolved in buffer to give a 0.1% solution. An ODS-120T (10 μm) column (7.5 cm × 4.6 mm I.D.) was kindly provided by Toyo Soda (Tokyo, Japan). An HLC-803D pump (Toyo Soda) with a 2.7-μl sample loop was employed. The absorbance at 210 nm was monitored with a detector UV-8 (Toyo Soda), A/D converted by a micro-computer and stored on a flopy disk. Flow-rates were determined from the positions of ghost peaks by taking the value of 0.5 ml/min as standard. Least squares analysis was done by use of the program SALS of the Computer Center of the University of Tokyo[10].

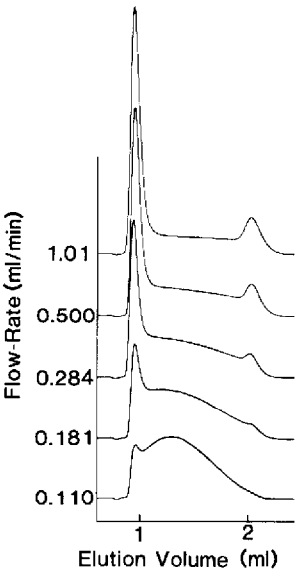
RESULTS AND DISCUSSION

The reaction was analyzed at pH 6.0 and 20°C. The pH was that of the eluent, however, the compound was dissolved in 50 m*M* phosphate, pH 2.4, equilibrated and injected. To obtain a precise value of β, α^* should be as large as possible (see eqn. 3). When the injection mixture is equilibrated in the eluent, α^* is equal to zero; it is necessary to shift the equilibrium towards one of the isomers. Therefore the pH of the sample was made low, because the *trans* isomer of L-Ala-L-Pro was reported to predominate at low pH[7,11].

Fig. 1 shows some of the chromatograms obtained for various flow-rates. Contributions from volumes other than that of the column itself, *e.g.*, connection tubes, detector cell, were subtracted.

These volumes make no contribution to the separation and only increase the first moment by a constant value (25 μl here). Fig. 1 confirms the observations of Melander and co-workers[6,7]: two peaks occur for a high flow-rate, and the lower the flow-rate the greater the chromatogram relaxes. According to their assignment the left peak is due to the *trans* and the right to the *cis* isomer.

The first moment of the elution volume is plotted *versus* the inverse of the flow-rate in Fig. 2, *i.e.*, M_1 (t_S) *vs.* t_S. From the results of the least squares analysis and by use of t_A, t_B and t_{SO} obtained from the chromatogram at the flow-rate of 1.01 ml/min, eqns. 4 and 5 give the rate constants of 0.445 ± 0.049 min^{-1} for the *trans* to *cis*, and 0.290 ± 0.032 min^{-1} for the *cis* to *trans* isomerization. We can also evaluate the isomer fraction of the injected sample by use of eqn. 6; for the *trans* isomer at



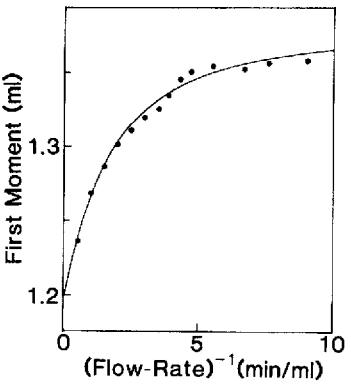


Fig. 1. Examples of chromatograms of L-Ala-L-Pro. Column: ODS-120T (10 μm, 7.5 cm × 4.6 mm I.D.). Eluent: 50 mM phosphate, pH 6.0, 20°C. Detection: UV, at 210 nm.

Fig. 2. Plot of first moment *vs.* inverse flow-rate. The curve is the best fit line (see eqn. 3 of text).

pH 2.4 this fraction is calculated as 0.76, *cf.*, 0.89 for the cationic form of L-Ala-L-Pro obtained by NMR spectroscopy[11].

Now we examine the results according to the scheme presented by Melander *et al.*[5]. They considered isomerization in both the mobile and stationary phases. Although our model equations (eqn. 1, 2 in ref. 4) involve only two rate constants, they can be shown to be identical to the "governing differential equations" (eqn. 10 in ref. 5) of Melander *et al.*[5]:

$$c_1 + c_2 = (1 + k_A)c_2 = \rho_A$$

$$c_3 + c_4 = (1 + k_B)c_3 = \rho_B$$

$$u_0/(1 + k_A) = v_A,\ u_0/(1 + k_B) = v_B$$

$$k_2/(1 + k_A) = k,\ k_3/(1 + k_B) = k'$$

The quantities on the left-hand sides are expressed in the notation of Melander *et al.*[5]; k_A and k_B are the capacity factors of molecules A and B. The equilibrium constant in the mobile phase is given by $K_m = k_2/k_3$ (eqn. 12 in ref. 5); in our notation it is:

$$K_m = t_A k/t_B k'$$

With our rate constants, k and k', this relationship yields the fraction 0.41 for the *cis* isomer *cf.*, 0.35 by NMR spectroscopy[11]. With the knowledge of k_A and k_B, and of the rate constants in solution[12], one may discuss the rate constants in the stationary phase just as Jacobson *et al.*[6] have done.

Next, we consider the advantages and disadvantages of our method in comparison with those of other researchers[5,6]. The major advantage of our method is its simplicity. This is attained by focusing on the first moment and by manipulating the equilibrium of the mixture injected. The first moment is easier to measure precisely than the moments of higher orders, and a method using a higher order moment must deal with intrinsic band spreading; the procedure is fairly complex[6]. Without the manipulation of the equilibrium, the first moment would never relax and one would have to resort to another means. Conversely, this is a drawback of our method. There are two other disadvantages. One is the use of t_A and t_B. Because our method employs only the first moment, the velocities of the isomers, or the capacity factors, must be obtained independently. This leads to a restrictive condition mentioned in the Theoretical section: a chromatogram of small reaction extent is indispensable. The other disadvantage is concerned with the different extinction coefficients of the isomers. When the isomers have different extinction coefficients, eqn. 3 is invalid and the expression of the first moment is a little bit complex. The simulation method[6] is free from this problem though it has a trial-and-error character. The important point is that our method can extract as much information as other methods, while at the same time being very simple.

Another method described by Lebl and Gut[8] has one weak point. According to their discussion, the peaks observed correspond to the remainder of the starting compound, and the plateau between them corresponds to the mixture of isomers generated during the time of separation. However, the peaks do contain isomers generated during that time. Under their peak B (see Fig. 1 in ref. 8), for example, not only is there the remainder of species B, but also A generated just before elution, B generated from A just after injection and A and B interconverted several times.

In conclusion, we have demonstrated that the elution-band relaxation method is applicable to reactions whose time constants are approximately 1 min. For the present, the procedure described here together with the two presented previously completes our methodology. Further development of the method may include its application to isomerizations comprising more species, and more rapid separation of isomers with sufficient resolution will enlarge the application time domain of the method.

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