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LOW TEMPERATURE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF *cis–trans* PROLINE DIPEPTIDES

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SUMMARY

Isomeric dipeptides which contain proline at the C-terminus and undergo on-column *cis–trans* isomerization were separated by reversed-phase chromatography at subambient column temperature. Chromatography at low temperatures offers a convenient method for measuring the relative concentrations of the isomers and, thus, the calculation of the equilibrium constants for such *cis–trans* isomerization at various conditions of solvent, pH, and temperature. Pure fractions of the *cis* and *trans* isomers were collected and used in kinetic studies of the isomerization. High-performance liquid chromatography at temperatures near the freezing point of the eluent is a potentially useful tool for the study of a wide range of biochemical molecules.

INTRODUCTION

The application of low-temperature high-performance liquid chromatography (HPLC) for the separation of thermally labile and unstable molecules has been reviewed recently[1]. The technique has mostly been used for inorganic separations of metal coordination complexes[2,3] and organometallic compounds[4]. The only biochemical application known to us of low-temperature HPLC has been the reversed-phase chromatography of native papain at subambient temperature by Benedek *et al.*[5] In each case the column temperature was lowered to slow down on-column reactions of the eluites such as decomposition and denaturation that preclude their separation and quantitation at room temperature.

Recent work by Melander *et al.*[6] has provided a theoretical framework for the treatment of on-column reactions in order to predict the expected chromatographic behavior in the case of on-column conformational changes and to determine the pertinent kinetic and equilibrium parameters from the observed chromatographic profiles. In a companion paper[7] their approach was demonstrated by a study of the effect of *cis–trans* isomerization of proline dipeptides in reversed-phase chromatography. The results of this work shed light on the effect of temperature when on-

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column chemical reactions occur and facilitates the selection of optimal conditions for a given separation under such conditions.

Proline is different from the other proteogenic amino acids because its side chain forms a covalent bond with the nitrogen. Due to the formation of the pyrrolidine ring and the concomitant loss of the amide hydrogen the conformational space of the proline residue and the hydrogen bonding ability of the peptide bond in peptides and proteins is unique[8]. As a result, peptide bonds involving proline nitrogen are relatively rigid and the rate of *cis–trans* isomerization is relatively slow. For small proline peptides such as those used in this study the equilibrium constant for *cis–trans* isomerization is such that in most cases both isomers are present at room temperature. The *cis–trans* isomerization is believed to play a key role in protein denaturation and the work of Brandts and co-workers[9,10] has shown the relationship between the presence of proline residues and the rate of denaturation. Previous studies of this isomerization have employed calorimetry[9] and NMR methods[11]. Chromatographic methods for such investigations offer certain advantages, most notably the isolation of the pure isomers for further study.

Reversed-phase chromatography of *cis-* and *trans*-proline dipeptides was examined in great detail by Melander *et al.*[12] They found that the separation of *cis-* and *trans*-L-alanyl-L-proline (Ala-Pro) under usual conditions was impeded by the on-column interconversion of the isomers. Chromatography of other proline dipeptides suffered from the same problem. Only at elevated column temperatures was the rate of interconversion sufficiently high for the peptide to be eluted as a single peak. Thus by varying the column temperature, the appropriate rate constants for the isomerization reaction under conditions present in the HPLC column could be estimated from chromatographic data. However, their approach can be applied only when the on-column reaction is reversible under the conditions investigated and does not provide a general method for kinetic measurements outside the range of the chromatographic conditions.

In this study low-temperature HPLC was used to bring about the separation of the *cis–trans* isomers of proline dipeptides. In this way each isomer was isolated in pure form and used for extra-chromatographic measurements of the equilibrium and kinetic constants of the isomerization reaction under various conditions. In contrast with the previous use of elevated temperature for the analytical determination of total peptide content[7], the present approach employs low column temperature so that the isomers present in the sample can be determined individually.

EXPERIMENTAL

The liquid chromatograph used for measurements above 0°C consisted of a Model 870 pump, Model 8800 gradient controller, and a Model 52001-901 UV spectrophotometer (DuPont, Wilmington, DE, U.S.A.). Chromatograms were recorded and integrated using a Model Cl-10 computing integrator (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Columns were immersed in a Lauda WB-20/DR constant temperature bath (Brinkman, Westbury, NY, U.S.A.). Before entering the injection valve the eluent was passed through a 2.7-m long heat exchanger made of No. 316 stainless-steel tubing having 0.75 mm I.D. and 1.6 mm O.D. placed prior to a Model 7010 injection valve equipped with a 10-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). The column effluent was monitored at 210 nm.

The 250 × 4.6 mm I.D. column was packed with 10 μm LiChrosorb RP-18 (E. M. Science, CherryHill, NJ, U.S.A.).

Aqueous 0.050 *M* phosphate was prepared by dilution from a 0.50 *M* stock solution of monobasic sodium phosphate, which had been adjusted to pH 6.00 with sodium hydroxide, and the pH of the diluted solution was further adjusted if necessary with 0.10 *M* sodium hydroxide. Mobile phases were filtered and degassed with helium before use. Flow-rates were 2.00 ml/min unless otherwise noted.

All pH measurement were made using an Accumet 420 pH meter (Fisher Scientific, Pittsburgh, PA, U.S.A.) calibrated at pH 4.00 and 10.00.

Data manipulations for the calculation of the absorbance ratio for the two isomers and iterative solution of eqn. 3 was conducted using TK! Solver (Software Arts, Wellesley, MA, U.S.A.) on a Rainbow 100 PC (Digital Equipment, Maynard, MA, U.S.A.).

Reagent grade orthophosphoric acid, monobasic and dibasic sodium phosphate, and sodium hydroxide and HPLC grade methanol and acetonitrile were obtained from Fisher (Pittsburgh, PA, U.S.A.). The dipeptides were obtained from Sigma (St. Louis, MO, U.S.A.).

Collection of fractions for kinetic measurements

The following procedure was used to collect fractions containing pure *cis* or *trans* isomers for the kinetic study of isomerization, and to maintain them at 0°C throughout the study. An amount of 2 mg of L-valyl-L-proline (Val-Pro) was dissolved in 1 ml of mobile phase. First a 10-μl sample of the Val-Pro solution was injected and the retention times of the *cis* and *trans* forms were measured. Thereafter, samples of 10 μl were injected and the peaks of the pure isomers were collected and kept at low temperature to prevent re-isomerization. This was accomplished by connecting a 1/16-in. needle with luer fitting to the column outlet fitting with a compression fitting. A 1.0-ml serological syringe (Becton, Dickinson and Co., Rutherford, NJ, U.S.A.) was sealed with a crimped needle to prevent contamination and cooled in the bath. At times corresponding to half heights of the peaks to be trapped, the cooled syringe first was connected to the luer fitting of the needle, and the fraction was collected with the syringe residing in the bath with the column. The syringe was then removed and transferred to a similar needle attached with a compression fitting to the injection valve. The syringe and valve were kept immersed in the bath throughout. The valve was immediately flushed with 0.2 ml of the eluate to remove any traces of the bath solvent which were present in the needle. Injections of the trapped fractions were made periodically to follow the course of the reaction.

Preparation of peptide solutions with various pH

To 5.00 ml of 0.10 *M* orthophosphoric acid in water 0.500 ml of a solution containing 5.27 mg/ml Val-Pro was added and the mixture diluted to 10.00 ml with water. The pH was found to be 1.80. To 5.00 ml of 0.10 *M* orthophosphoric acid solid sodium hydroxide pellets and 0.500 ml of the Val-Pro solution were added and the mixture diluted to 10.00 ml with water. The pH was found to be 12.90. The concentrations of phosphate (0.0500 *M*) and of Val-Pro (0.264 mg/ml) were the same in both solutions. By mixing various quantities of the two solutions samples having intermediate pH values were prepared.

RESULTS AND DISCUSSION

Effect of temperature on isomer resolution

The effect of temperature on the chromatography of species which undergo chemical reaction or conformational change is often dramatic. We believe a large number of species of biological interest exhibit such behavior for which the chromatography of the proline dipeptides can serve as a simple model. One diagnostic tool for conformational changes occurring in the column is the effect of changing flow-rate. If the characteristic time of the reaction is commensurate with that of the chromatographic retention process in the column, instead of the usual peaks, unchromatographic concentration profiles are observed. In the case of irreversible reactions a fraction or the whole of the injected substance is transformed in the column. The results of a reversible on-column reaction at room temperature are exemplified by the unchromatographic elution profile depicted in Fig. 1 that show the effect of flow-rate on the separation of L-leucyl-L-proline (Leu-Pro). At low flow-rates only a very broad peak is recognizable containing both the *cis* and *trans* isomers, and its shape reflects that they underwent isomerization in the column. At higher flow-rate, peaks of both the *cis* and *trans* forms are recognizable but, the major fraction of the peptide is present in the reaction zone between the two peaks. The chromatograms in Fig. 1 are similar to those reported earlier[12] and demonstrate the value of changing the flow-rate in the diagnosis of the interplay of chemical reactions and chromatographic retention.

However, the effect of lowering the column temperature can be even more dramatic and informative. The chromatogram of Leu-Pro shown in Fig. 2 was obtained at 1°C at the lower flow-rate, that at room temperature resulted in the very broad peak as seen in Fig. 1. At reduced temperature the two isomers were resolved almost completely because the rate of reaction on the column was sufficiently atten-

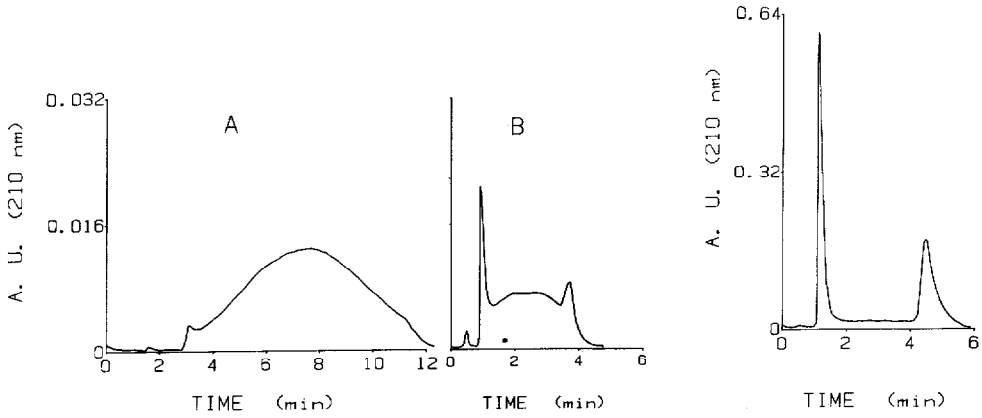

Fig. 1. Effect of flow-rate on the chromatography of Leu-Pro. Column 250 × 4.6 mm I.D. packed with 10 μm LiChrosorb RP-18; mobile phase, methanol–0.050 *M* phosphate, pH 6.0 (10:90, v/v); temperature, 23°C; UV detection at 210 nm; sample, 10 μl of a solution of 2.7 mg/ml Leu-Pro in phosphate buffer pH 6.0 (A) Flow-rate 2.0 ml/min; (B) flow-rate 5.0 ml/min.

Fig. 2. Chromatogram of Leu-Pro by low-temperature HPLC at 1°C and flow-rate 2.0 ml/min. Other conditions as in Fig.1.

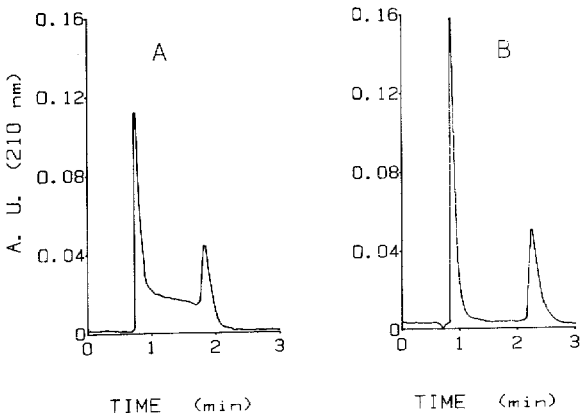

Fig. 3. Effect of temperature on the chromatography of Ala-Pro. (A) 23°C; (B) 1°C. Mobile phase 0.050 *M* phosphate, pH 6.0; flow-rate 2.0 ml/min. Other conditions as in Fig. 1.

uated to avert the reaction and the formation of a broad reaction peak as seen in Fig. 2. Thus low-temperature HPLC offers the means to separate the two isomers which undergo isomerization at room temperature. Similar results were obtained for the separation of other proline dipeptides at low temperature as seen from the comparison of the chromatograms of Ala-Pro in Fig. 3 that were obtained at 25°C and 1°C under otherwise identical conditions.

Determination of the equilibrium distribution of the isomers

The possibility of separating and isolating the individual isomers by low temperature HPLC allows us to study the isomerization equilibrium. The equilibrium constant for the isomerization of Val-Pro was determined in the pH range of 1.8 to 10.95 at 23°C and 0°C. All solutions were prepared to contain identical quantities of Val-Pro and identical total phosphate concentrations within 2 ppt* in order to facilitate accurate determination of the absorbance ratio for the two isomers. Chromatograms obtained at three different pH values are shown in Fig. 4. Samples were equilibrated at 23°C and the column was operated at 0°C. The distinct shift from predominantly *trans* isomer at low pH to predominantly *cis* isomer at high pH is clearly seen in these chromatograms. The *cis* form was the major component of the solid dipeptide as purchased. Dissolution of the solid peptide Val-Pro in neat methanol and acetonitrile yields solutions containing predominantly the *cis* isomer and the *trans* isomer, respectively. Similar behavior was noted for Ala-Pro. Upon dissolving Val-Pro in aqueous buffer of pH 6.0 the *cis* isomer predominated initially, but after five minutes at room temperature, the concentrations of the two isomers were about the same.

The peak areas were determined for the *cis* and *trans* peaks at pH values from 1.80 to 10.95. The total amount of Val-Pro for a single solution (*M*), is related to the total area of the two isomer peaks represented by A_c and A_t weighted by the absorptivities of the two isomers, ε_c and ε_t, and a conversion factor k.

* ppt = Parts per thousand.

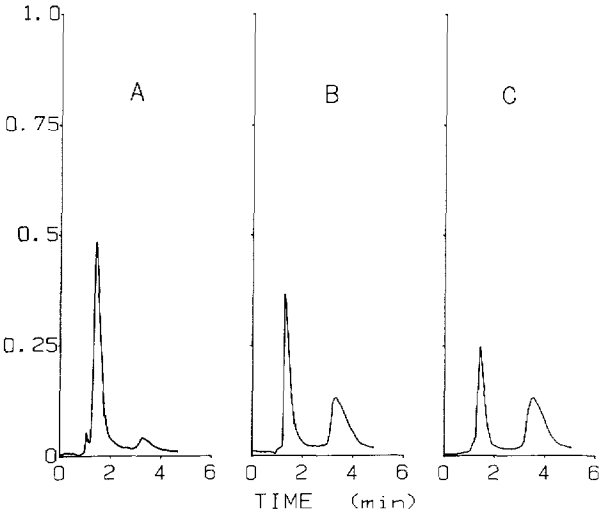

Fig. 4. Chromatograms obtained at 0°C of aqueous Val-Pro solutions having different pH. (A) pH 3.1; (B) pH 6.0; (C) pH 10.0. Mobile phase: methanol–0.050 *M* phosphate, pH 6.0 (20:80, v/v); flow-rate 2.0 ml/min. Other conditions as in Fig. 1.

$$M = k\,(A_c/\varepsilon_c + A_t/\varepsilon_t) \tag{1}$$

Since all of the Val-Pro solutions injected into the column had identical volumes and total concentrations and since the actual peak areas are measured at the constant pH of the mobile phase (6.00), the quantity A_cM/k must be constant and given by

$$\varepsilon_cM/k = A_c + (\varepsilon_c/\varepsilon_t)A_t \tag{2}$$

The ratio $\varepsilon_c/\varepsilon_t$ was calculated using data for seven sample solutions having different pH values in the range 1.80 to 10.04 and the standard deviation of ε_cM/k was calculated. The standard deviation showed a minimum value of 2.2% at which point the ratio $\varepsilon_c/\varepsilon_t$ had a value of 0.68. The ratio thus obtained is different from the value of 1.47 reported by Jacobson *et al.*[7] who used NMR data obtained under different conditions for concentration ratios of Val-Pro isomers in order to evaluate the absorbance ratio from absorptivities measured in dilute phosphate buffers. In contradistinction, in this study the concentrations of Val-Pro conformers in the mobile phase were measured directly.

The overall *cis–trans* isomerization can be described by the equation

$$trans \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} cis$$

where k_1 and k_{-1} are the appropriate rate constants and the equilibrium constant K_{eq} is given by

$$K_{eq} = k_1/k_{-1} = [cis]/[trans]$$

As previously noted, K_{eq} is dependent on the state of ionization of the peptide[6,13,14] the temperature, and the solvent. The complete description of all equilibria for this dipeptide is shown below.

$$\begin{array}{ccccc} \textit{trans}\text{-}^{+}\text{HVal-Pro} & \overset{K_{1t}}{\rightleftarrows} & \textit{trans}\text{-}^{+}\text{Val-Pro}^{-} & \overset{K_{2t}}{\rightleftarrows} & \textit{trans}\text{-Val-Pro}^{-} \\ K_{eqA}\uparrow\downarrow & & K_{eqZ}\uparrow\downarrow & & K_{eqB}\uparrow\downarrow \\ \textit{cis}\text{-}^{+}\text{HVal-Pro} & \underset{K_{1c}}{\rightleftarrows} & \textit{cis}\text{-}^{+}\text{Val-Pro}^{-} & \underset{K_{2c}}{\rightleftarrows} & \textit{cis}\text{-Val-Pro}^{-} \end{array}$$

The equilibrium constants for the *cis–trans* isomerization of each form of the dipeptide are indicated by K_{eqA}, K_{eqZ}, and K_{eqB} for the acidic, zwitterionic, and basic forms respectively. The acid dissociation constants for the *cis* and *trans* forms are denoted as K_{1c}, K_{2c}, K_{1t}, and K_{2t}.

The variation of the equilibrium constant with pH can be described exactly by eqn. 3.

$$K = \frac{K_{eqA}K_{eqZ}K_{eqB}([H^+]^2 + [H^+]K_{1t} + K_{2t}K_{1t})}{K_{eqZ}K_{eqB}[H^+]^2 + K_{eqA}K_{eqB}[H^+]K_{1t} + K_{eqA}K_{eqZ}K_{1t}K_{2t}} \quad (3)$$

The *cis* isomer content of Val-Pro solutions of various pH is listed in Table I. The variation of K_{eq} for the *cis–trans* isomerization of Val-Pro with pH is illustrated in Fig. 5. The shift toward *cis* isomer, *i.e.* increasing K_{eq}, at pH 3 is indicative of the pK_1 of Val-Pro and the fact that the negative charge on the proline moiety increases the stability of the *cis* form[13]. The second sigmoidal break is due to the loss of the second proton to form the anion with another increase in the stability of the *cis* form. NMR studies of similar dipeptides show the same trend.

Eqn. 3 was solved by iteration to determine the theoretical relationship between the overall equilibrium constant and the other five equilibrium constants by using initial values for the equilibrium constants of Ala-Pro determined by

TABLE I

FRACTION OF *cis* ISOMER AT TWO TEMPERATURES IN AQUEOUS SOLUTIONS OF L-VALINE-L-PROLINE HAVING DIFFERENT pH VALUES

pH	*cis (%)*	
	23°C	*0°C*
1.80	9.3	19.6
3.07	24.2	–
4.94	52.9	42.9
6.00	51.8	–
7.14	50.4	50.8
8.00	54.2	50.7
8.88	59.3	–
10.00	65.6	–
10.96	65.6	–

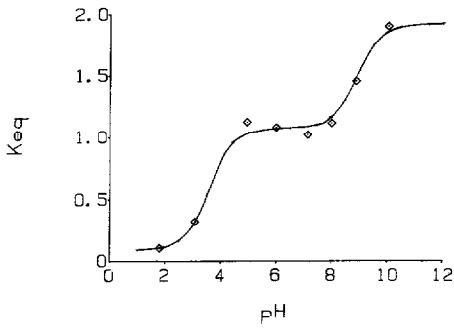

Fig. 5. Equilibrium constant for *cis–trans* isomerization of Val-Pro as a function of pH at 23°C. Conditions as in Fig. 4.

NMR[13,14]. The best values for Val-Pro were 11.46, 0.930, 0.520 for K_{eqA}, K_{eqZ}, K_{eqB}, and 3.57 and 8.91 for pK_{1t} and pK_{2t} respectively. The data were used to evaluate K_{eq} values for the plot in Fig. 5. The maximum error between theoretical and experimental points is 8% and the average deviation is 2.5%.

Table II lists the pK values for isomers of the Val-Pro as determined as well as data reported previously for Gly-Pro and Ala-Pro[13]. The date for pK_1 suggests that the stability of the zwitterionic form of the *cis* isomer increases with the size of the non-polar hydrocarbonaceous moiety in the amino acid at the N-terminus of such dipeptides. This is further supported by the observation that Phe-Pro[14,15] is predominantly in the *cis* form. In this study, Phe-Pro gave only a single chromatographic peak with a retention similar to that of the *cis* isomer of Leu-Pro as shown in Table III.

The data for the percent *cis* isomer of Val-Pro at 0°C shown in Table I show a definite shift to more *trans* isomer at lower temperature. A similar shift was observed by Cheng and Bovey[16] for Gly-Pro in NMR studies[16].

Isomerization kinetics in free solution

The technique of low-temperature HPLC was also employed to isolate *cis* and *trans* forms of Val-Pro for subsequent determination of the rate of the isomerization in free solution. Effluent fractions containing the *trans* and *cis* isomers were collected at 0°C. Chromatograms of the fractions immediately reinjected showed only a single peak of the particular isomer. After 5 min at 0°C, however, the samples already

TABLE II

SUMMARY OF pK VALUES FOR *cis* AND *trans* ISOMERS OF PROLYL DIPEPTIDES

Dipeptide	pK_1		pK_2	
	trans	*cis*	*trans*	*cis*
Gly-Pro[13]	3.03	2.56	8.55	8.49
Ala-Pro[13]	3.22	–	8.50	8.24
Val-Pro	3.57	2.48	8.91	8.66

TABLE III

RETENTION FACTORS OF PROLINE DIPEPTIDES AT 0°C

Mobile phase methanol–0.05 *M* phosphate, pH 6.0 (80:20, v/v); column 250 × 4.6 mm I.D. packed with 10 μm LiChrosorb RP-18.

Dipeptide	*Retention factor, k'*	
	trans	*cis*
Ala-Pro	0.08	0.19
Val-Pro	0.31	1.58
Leu-Pro	0.63	2.63
Phe-Pro	*	2.00

* No *trans* isomer present under these conditions.

showed the presence of a noticeable amount of the other isomer upon rechromatography. Storage at subzero temperatures is required to arrest isomerization.

The kinetics of *trans* Val-Pro isomerization was investigated at 0°C. The kinetics of the reversible reaction is assumed to be first order, and give rise to the following relationship:

$$\ln\left(\frac{C - C_{eq}}{C_o - C_{eq}}\right) = \frac{K_{eq} + 1}{K_{eq}} k_1 t \tag{4}$$

where C is the concentration of *trans* isomer at time t, C_o is the initial concentration of *trans* isomer, C_{eq} is the equilibrium concentration of *trans* isomer. First the equilibrium distribution for the isomers of Val-Pro in the mobile phase consisting of methanol–0.05 *M* phosphate, pH 6.0 (20:80, v/v) was determined in order to evaluate C_{eq}. The dipeptide was dissolved in the mobile phase and the solution was stored at 0° for seven days. The solution was then analyzed by HPLC at 0°C and found that 51.0% of the Val-Pro was present in the *trans* form for a value of K_{eq} of 0.96. The

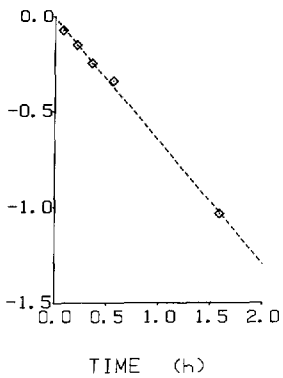

Fig. 6. Kinetic plot of data determined by low-temperature HPLC for the *trans–cis* isomerization of Val-Pro at 0°C. Conditions as in Fig. 4.

procedure described above was then employed to collect a pure fraction of the *trans* isomer which was rechromatographed over a period of 24 h to observe the return to equilibrium. The concentrations of *cis* and *trans* isomers at each time were determined from peak areas.

The plot of data according to eqn. 4 is shown in Fig. 6. The slope is $-1.78 \cdot 10^{-4}$ s^{-1} with a relative standard deviation of 2.7%. From eqn. 4, the rate constant for the *trans* to *cis* conversion, k_1, is $-8.7 \cdot 10^{-5}$ s^{-1} and the reversed-rate constant k_{-1} is $9.1 \cdot 10^{-5}$ s^{-1} at 0°C. These values of k_1 and k_{-1} are about an order of magnitude lower than the respective values of $1.0 \cdot 10^{-3}$ s^{-1} and $1.5 \cdot 10^{-3}$ s^{-1} obtained by Jacobson *et al.*[7] at room temperature in neat aqueous phosphate at pH 7.07. The more than ten-fold reduction in the isomerization rates by lowering the temperature accounts for the complete separation of the two isomers by low-temperature HPLC. This can be substantiated by evaluating the Damkohler number, D_a, for the isomerization reaction of Val-Pro. This dimensionless parameter which is used for the characterization of the interplay of reaction and chromatographic transport in reactions and has been applied to reactive chromatographic systems[6] is given in eqn. 5

$$D_a = Lk_1(1 + 1/K_{eq})/u_o \tag{5}$$

where L is the column length and u_o is the mobile phase velocity as measured with an unretained tracer. For the *trans* to *cis* conversion of 0°C we obtain a Damkohler number $1.3 \cdot 10^{-3}$. As noted previously the interplay of chemical reaction and chromatographic retention gives observable results on the chromatogram only when $10^{-3} < D_a < 10^3$. In our system the value of D_a is sufficiently low for the on-column reaction to be negligible so that the individual isomers traverse the column without change and can be readily quantified from the chromatogram.

CONCLUSIONS

Low-temperature HPLC is a powerful technique for the analysis and study of labile biochemical molecules. It can facilitate the measurement of physicochemical quantities such as kinetic and equilibrium constants by HPLC and the investigation of the behavior of biochemical substances. Furthermore, it is a useful tool to prepare conformational isomers in pure form for subsequent study by other techniques. In this regard, displacement chromatography[17] at subambient temperatures may offer particular advantages. When the rate of reaction at column temperatures above the freezing point of aqueous eluents is not sufficiently low, the employment of non-aqueous mobile phases can be considered. It is believed that for a significant number of biochemical molecules low-temperature HPLC may represent the method of choice to study conformation and changes and/or isolate pure conformers.

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