cis/trans Isomerization Rate of Oxazolam in Organic Solvents Measured by High-Performance Liquid Chromatography[1)]

Yukihisa KURONO,*,[a] Yasuhiro JINNO,[a] Tomonari KUWAYAMA,[b] Naomi SATO,[a] Tamotsu YASHIRO,[a] and Ken IKEDA[a]

Faculty of Pharmaceutical Sciences, Nagoya City University,[a] 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan and Pharmacy, Tokai Teishin Hospital,[b] 2-17-5 Matsubara, Naka-ku, Nagoya 460, Japan. *Received August 26, 1988*

Diastereoisomers of oxazolam (2*R-cis*, 2*R-trans*, 2*S-cis*, and 2*S-trans* isomers) were resolved and assigned by high-performance liquid chromatography (HPLC). By means of HPLC the *cis/trans* isomerization rates of oxazolam in some organic solvents could be measured, although such rate measurements had previously been attempted only by an nuclear magnetic resonance (NMR) method and it was reported that in most organic solvents the isomerization was too fast to allow the rates to be measured. In some cases (depending on the lot number of the solvent), rapid isomerization was observed in chloroform-*d* (the ratio of *cis* isomer to *trans* isomer (2 : 3) did not change with time, that is, the isomerization was at equilibrium immediately after dissolution of oxazolam in the $CDCl_3$). This fast isomerization was suggested to be catalyzed by an acid contaminant, since the iminium intermediate of oxazolam was detected in the reaction solution by fluorescence spectroscopy.

Keywords oxazolam; diastereoisomer; HPLC; resolution; *cis/trans* isomerization rate; kinetics; iminium intermediate; organic solvent; fluorescence; ^{1}H-NMR

From the viewpoint of drug behavior after oral administration, we previously studied[2-5)] the kinetics and mechanism of the oxazolidine ring-opening and ring-closing reactions of benzodiazepinooxazoles[2,4)] and the subsequent hydrolysis of the diazepine ring[3,5)] in aqueous solution. Recently diastereoisomers of oxazolam (2*R-cis*, 2*R-trans*, 2*S-cis*, and 2*S-trans* isomers, see Chart 1 for the chemical structures) were resolved by high-performance liquid chromatography (HPLC).[6,7)] The peaks, however, were not assigned. Furthermore, the kinetics and mechanism of the *cis/trans* isomerization of oxazolam in organic solvents have not been fully elucidated.[8)]

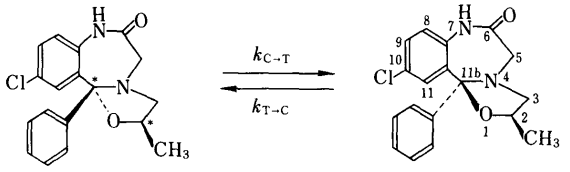

Chart 1

To assign the resolved peaks of racemic oxazolam we synthesized 2*R*- and 2*S*-oxazolam, and employed them as standards for the HPLC analysis. As an application of this HPLC analysis, *cis/trans* isomerization rates of oxazolam were measured. The rates had previously been measured only by the nuclear magnetic resonance (NMR) method. In this paper we describe the results of the assignment of the diastereoisomers and of the rate measurements by HPLC.

Experimental

Materials Oxazolam (lot No. 8) was supplied by Sankyo Co., Ltd. It was recrystallized from dichloromethane and the ratio of *cis* isomer to *trans* isomer was about 1 : 4, as estimated from the ^{1}H-NMR measurement immediately after dissolving the solid in dimethylformamide-d_7 (DMF-d_7). When oxazolam was recrystallized slowly from a relatively large volume of ethanol, the ratio was about 11 : 9. At present it is unknown what factor determines the *cis/trans* ratio. Using (*R*)-(−)-1-amino-2-propanol and (*S*)-(+)-1-amino-2-propanol as starting materials, 2*R*-oxazolam and 2*S*-oxazolam were synthesized, respectively, by procedures similar to those reported by Deriege *et al.*,[9)] Miyadera *et al.*,[10)] and Lemke and Hanze.[11)]

Deuterated solvents ($CDCl_3$, CD_2Cl_2, and DMF-d_7) were purchased commercially. The company and the lot number are cited in the text as necessary. *n*-Hexane, dichloromethane, and dioxane of HPLC grade were bought commercially. The other organic solvents were obtained commercially and were distilled before use.

Instruments The HPLC apparatus (TRI ROTOR®, JASCO) was equipped with a chiral stationary phase column, SUMIPAX OA-2000 (Sumitomo Kagaku Co., Ltd., 5 μm, 4 mm i.d. × 250 mm), and a variable-wavelength spectrophotometric detector (UVIDEC 100-II, JASCO) operated at 265 nm. Peak area analyses were carried out with a Shimadzu C-R1B data processor.

^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-FX 100 spectrometer at 100 and 25 MHz, respectively. A Shimadzu RF-520 spectrofluorophotometer was used for the measurement of the fluorescence spectra.

Kinetic Runs Ten microliters of oxazolam dissolved in an organic solvent (about 2×10^{-3} M) was injected into the HPLC apparatus at appropriate time intervals. The mobile phases were as follows: *n*-hexane: dichloromethane: dioxane = 10 : 1 : 1 and 7 : 4 : 2 (v/v) for the resolutions of racemic oxazolam (3 peaks) and of *cis/trans* oxazolam (2 peaks), respectively. The flow rate was 1.0 ml/min and the temperature was 25 °C. The change in peak areas due to the *cis* or *trans* isomer as a function of time were subjected to pseudo first-order analysis according to Eq. 1.

$$\log|CP-CP_\infty| = -(k_{obs}/2.303)\cdot t + \text{const} \quad (1)$$

where CP and CP_∞ are the percentages of *cis* isomer at time t and at infinity, respectively, and k_{obs} is the pseudo first-order rate constant for the isomerization.

For the NMR spectral measurements about 10 mg of oxazolam was dissolved in 0.4 ml of deuterated organic solvent. The solution was placed in a constant temperature bath (25 °C), and at appropriate time intervals the NMR spectrum was measured. The integrated area of 2-CH_3 signals due to the *cis* or *trans* isomer was compared with the aromatic hydrogen signal area as a function of time. The pseudo first-order rate constant (k_{obs}) was calculated by using Eq. 1.

Fluorescence Measurements Fluorescence spectra of oxazolam in organic solvents were measured in the presence and absence of trifluoroacetic acid (TFA). An excitation wavelength of 350 nm was used for the measurements.

Results and Discussion

Assignment of Diastereoisomers of Oxazolam by HPLC Since the carbons at the 2- and 11b-positions of oxazolam are asymmetric, oxazolam is a mixture of four diastereoisomers, that is, 2*R-cis*, 2*R-trans*, 2*S-cis*, and 2*S-trans* isomers.[6,7)] Figure 1a illustrates the chromatogram of racemic oxazolam on SUMIPAX OA-2000. The racemate was

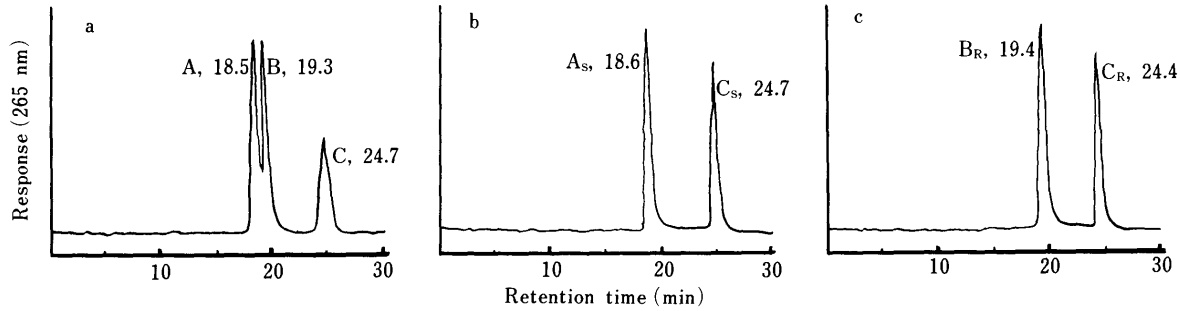

Fig. 1. HPLC Chromatograms of Oxazolam Dissolved in the Eluent

a. Racemic oxazolam (eluent, *n*-hexane : dichloromethane : dioxane = 10 : 1 : 1). b. 2*S*-Oxazolam (eluent, *n*-hexane : dichloromethane : dioxane = 10 : 1 : 1). c. 2*R*-Oxazolam (eluent, *n*-hexane : dichloromethane : dioxane = 10 : 1 : 1).

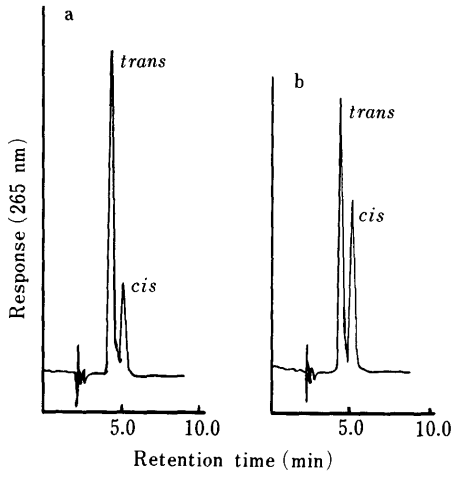

Fig. 2. HPLC Chromatograms of Racemic Oxazolam Dissolved in $CHCl_3$

a. Immediately after dissolution in $CHCl_3$ (eluent, *n*-hexane : dichloromethane : dioxane = 7 : 4 : 2). b. At 1500 min after dissolution in $CHCl_3$ (eluent, *n*-hexane : dichloromethane : dioxane = 7 : 4 : 2).

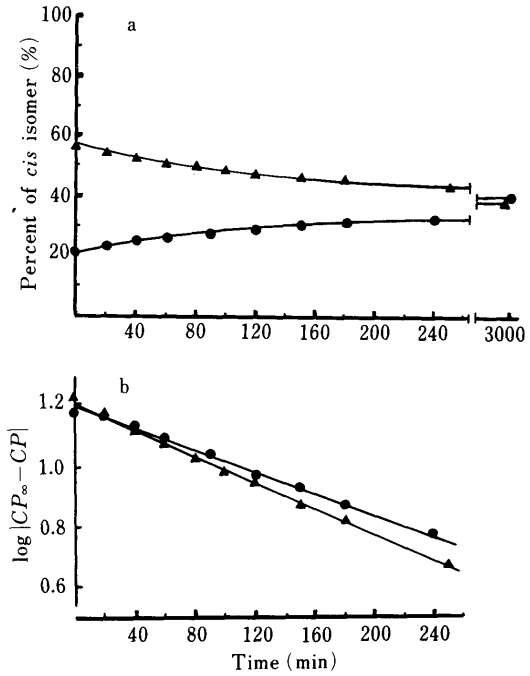

Fig. 3. Time Courses and First-Order Plots of *cis*/*trans* Isomerization of Oxazolam in CCl_4 at 25 °C

a. Time courses. ●, started from the sample of *cis* : *trans* = 1 : 4; ▲, started from the sample of *cis* : *trans* = 11 : 9. b. First-order plots. ●, started from the sample of *cis* : *trans* = 1 : 4; ▲, started from the sample of *cis* : *trans* = 11 : 9.

dissolved in mobile phase solvents (eluent) before application to HPLC. Three peaks were found. We could not obtain four peaks by using this column and various mobile phase solvents. In order to assign the peaks in Fig. 1a to the respective isomers, we synthesized 2*R*- and 2*S*-oxazolam and subjected them to the chromatography (Figs. 1b and 1c). Each oxazolam shown in Figs. 1b and 1c was equilibrated in the eluent before the application to HPLC.[12] The retention times (t_R) of the peaks in Figs. 1b and 1c imply that peaks A and C and peaks B and C in Fig. 1a are due to 2*S*-oxazolam and 2*R*-oxazolam, respectively.

In most organic solvents the ratio of *trans* isomer to *cis* isomer at equilibrium is suggested to be about 3 : 2 (the *trans* isomer amounts to about 60%).[4,8] The ratio of the peak area with the smaller t_R (A_S or B_R) to that with the larger t_R (C_S or C_R) in Figs. 1b or 1c is about 3 : 2. The peaks A_S (or B_R) and C_S (or C_R) are, therefore, due to *trans* isomer and *cis* isomer, respectively.

Figures 2a and 2b show the chromatograms of the racemic oxazolam, with different eluents from that in Fig. 1a. In this eluent system, only *cis*/*trans* isomers are resolved and not 2*S*/2*R* isomers. The rates of the *cis*/*trans* isomerization could, however, be followed under these conditions, because the isomerization rates were found to be identical for the 2*R* and 2*S* isomers (k_{obs} in $CHCl_3 \fallingdotseq 2.1 \times 10^{-2}$ min^{-1}).

***cis*/*trans* Isomerization Rate of Oxazolam in Organic Solvents** Figure 3a shows the time courses of *cis*/*trans* isomerization of oxazolam (racemates) in carbon tetrachloride (CCl_4). At equilibrium the ratio of *cis* isomer to *trans* isomer is about 2 : 3. The k_{obs} values obtained from the increase in *cis* isomer and from the decrease in *cis* isomer (Fig. 3b) were 4.25×10^{-3} and 5.17×10^{-3} min^{-1}, respectively, and agreed approximately with each other. This agreement is consistent with the reaction scheme shown in Chart 1. According to Chart 1, k_{obs} is represented by Eq. 2.

$$k_{obs} = k_{C \to T} + k_{T \to C} \quad (2)$$

The equilibrium constant K is expressed by Eq. 3.

$$K = [BF_{cis}]_{eq}/[BF_{trans}]_{eq} = k_{T \to C}/k_{C \to T} \quad (3)$$

From Eqs. 2 and 3 the individual rate constants can be calculated and are listed in Table I.

Table I summarizes the rate constants obtained in several organic solvents. The isomerization in chloroform-*d* was

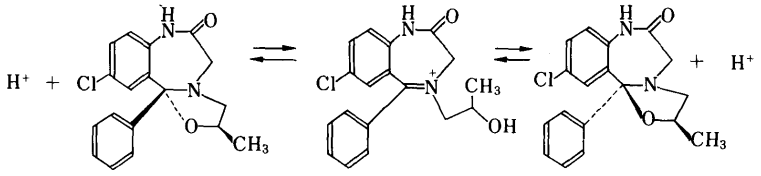

Chart 2

TABLE I. The First-Order Rate Constants for the *cis/trans* Isomerization of Oxazolam in Various Organic Solvents at 25 °C

Solvent	$k_{obs} \times 10^3$ (min^{-1})	$k_{C \to T} \times 10^3$ (min^{-1})	$k_{T \to C} \times 10^3$ (min^{-1})
CCl_4	4.25[a]	2.55	1.70
CCl_4	5.17[b]	3.10	2.07
$CHCl_3$	20.9	12.5	8.36
$CDCl_3$	24.0	14.4	9.60
(Merck lot No. 446W366520)			
$CDCl_3$	20.6 (17.5[c])	12.4	8.24
(Aldrich lot No. 03315JT)			
CH_2Cl_2	5.61	3.37	2.24
CD_2Cl_2	8.69	5.21	3.48
CH_2ClCH_2Cl	72.7	43.6	29.1

a) Started from a sample of *cis*: *trans* = 1 : 4. *b*) Started from a sample of *cis*: trans = 11 : 9. *c*) Determined by the NMR method.

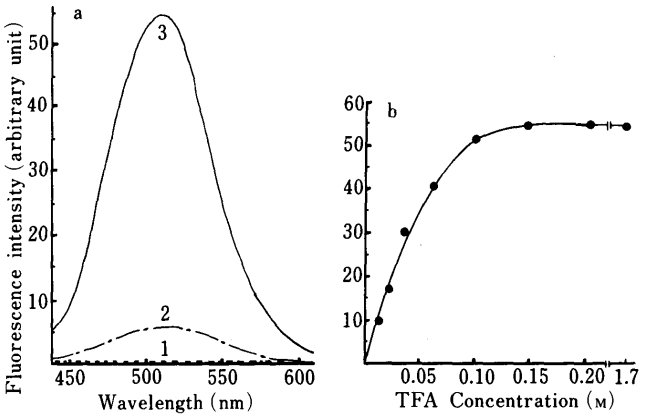

Fig. 4. Fluorescence Emission Spectra of Oxazolam Excited at 350 nm and Effect of TFA

a. Fluorescence emission spectra. 1 (----), 1.06×10^{-4} M oxazolam in $CHCl_3$; 2 (—·—), 1.06×10^{-4} M oxazolam in $CDCl_3$ (Aldrich lot No. 00301TM); 3 (——), 1.06×10^{-4} M oxazolam in $CHCl_3$ containing 1.50×10^{-1} M TFA. b. Effect of TFA concentration on the fluorescence intensity. Concentration of oxazolam was 1.06×10^{-4} M.

reported to be rapid at room temperature.[8] Indeed we also sometimes observed rapid isomerization, *i.e.*, 2 : 3 ratio of *cis* isomer to *trans* isomer immediately after dissolving the solid in $CDCl_3$ (depending on the lot number of $CDCl_3$, *e.g.*, Aldrich lot No. 00301TM, 00126CT, *etc.*). For the *cis/trans* isomerization of oxazolam, an iminium intermediate (Chart 2) had been considered to be necessary[2,4,8] and the intermediate exhibits fluorescence which is not observed in the original oxazolam.[13] To elucidate the reason for the difference in the rates, we measured the fluorescence spectra of chloroform-*d* solutions of oxazolam.

Figure 4a shows the fluorescence spectra of oxazolam in $CDCl_3$ (Aldrich lot No. 00301TM) and also in $CHCl_3$ containing trifluoroacetic acid (TFA). These spectra indicate the presence of the iminium form in the $CDCl_3$ solution of oxazolam. Figure 4b shows the dependence of the fluorescence intensity on the TFA concentration. Above 1.50×10^{-1} M TFA, the fluorescence intensity of 1.06×10^{-4} M oxazolam is constant and all of the oxazolam is, thus, considered to exist as the iminium form. The intensity of the $CDCl_3$ solution in Fig. 4a implies that 10.4% ((5.7/55) × 100) of oxazolam exists as the iminium form. In the examples of rapid isomerization of oxazolam in $CDCl_3$ so far examined, the range of concentration of the iminium form was from 5% to 20%, depending on the lot number. These samples of deuterated chloroform may be contaminated with impurities (*e.g.*, phosgene, water, *etc.*). These fast isomerizations were consequently considered to be probably catalyzed by an acid contaminant in the solvents.

The data for the other solvents examined are also listed in Table I. The order of the magnitude of the k_{obs} values for all solvents seems to be the same as the order of the instability[14] (*e.g.*, contents of impurities) of the chlorinated hydrocarbons ($ClCH_2CH_2Cl > CHCl_3 > CH_2Cl_2 = CCl_4$). Further work on the quantitative interpretation of the data in Table I is required.

Acknowledgments We wish to thank Mr. M. Kurita, Director of Tokai Teishin Hospital Pharmacy, and Miss. S. Kato, Faculty of Pharmaceutical Sciences, Nagoya City University, for encouragement and NMR spectral measurements, respectively. We are also grateful to Sankyo Co., Ltd. for the gift of oxazolam.

References and Notes

1) This reports constitutes Part XIII of the series entitled "The Behavior of 1,4-Benzodiazepine Drugs in Acidic Media," Part XII: T. Kuwayama, S. Kato, Y. Kurono, T. Yashiro, and K. Ikeda, *Yakugaku Zasshi*, **108**, 641 (1988).
2) Y. Kurono, T. Kuwayama, K. Kamiya, T. Yashiro, and K. Ikeda, *Chem. Pharm. Bull.*, **33**, 1633 (1985).
2) T. Kuwayama, Y. Kurono, T. Muramatsu, T. Yashiro, and K. Ikeda, *Chem. Pharm. Bull.*, **34**, 320 (1986).
4) Y. Kurono, T. Kuwayama, Y. Jinno, K. Kamiya, E. Yamada, T. Yashiro, and K. Ikeda, *Chem. Pharm. Bull.*, **36**, 732 (1988).
5) Y. Kurono, Y. Jinno, T. Kuwayama, T. Yashiro, and K. Ikeda, *Chem. Pharm. Bull.*, **36**, 2582 (1988).
6) G. Blaschke, *J. Liq. Chromatogr.*, **9**, 341 (1986).
7) *Chem. Abstr. Formula Index*, **105**, 1877*F* (1986).
8) T. Miyadera, A. Terada, C. Tamura, M. Yoshimoto, and R. Tachikawa, *Ann. Rep. Sankyo Res. Lab.*, **28**, 1 (1976).
9) M. E. Deriege, J. V. Earley, R. I. Fryer, R. J. Lopresti, R. M. Schweiniger, L. H. Sternach, and H. Wharton, *Tetrahedron*, **25**, 2591 (1971).
10) T. Miyadera, A. Terada, M. Fukunaga, Y. Kawano, T. Kamioka, C. Tamura, H. Takagi, and R. Tachikawa, *J. Med. Chem.*, **14**, 520 (1971).
11) T. L. Lemke and A. R. Hanze, *J. Heterocycl. Chem.*, **8**, 125 (1973).
12) The *cis/trans* isomerization of oxazolam in the eluent was slow and it took about 10 d to reach equilibrium.
13) M. Ikeda and T. Nagai, *Chem. Pharm. Bull.*, **30**, 3810 (1982).
14) J. A. Riddick, W. B. Bunger, and T. K. Sakano, "Organic Solvents," 4th ed., John Wiley and Sons, Inc., New York, 1986, pp. 1009—1017.