Pharmaceutical Research, Vol. 5, No. 5, 1988

Research Article

Epimerization Kinetics of Moxalactam in Frozen Solution

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Received July 17, 1987; accepted December 16, 1987

The epimerization rate constants of R- and S-epimers of moxalactam (LMOX) in a frozen aqueous solution decreased as the temperature decreased. The reaction proceeded in the unfrozen region remaining in the frozen solution, without being affected by the ice. The reaction stopped completely below the collapse temperature of the LMOX aqueous solution. The ratio of R- and S-epimers at equilibrium, which was equal to the ratio of the epimerization rate constant, increased as the temperature decreased. This change in the ratio at equilibrium could be ascribed to the difference in the activation energy between the two epimers.

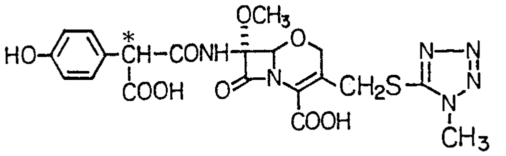
KEY WORDS: moxalactam; epimerization; frozen solution; ice; activation energy.

INTRODUCTION

Moxalactam (LMOX), a new semisynthetic antibiotic with a 1-oxacephem nucleus (Scheme I), exists as a mixture of R- and S-epimers at the C-7 side chain at an R/S ratio (R- to S-epimer) of about 1.1. The R-epimer is antimicrobially more active *in vitro* than the S-epimer (1–3).

In the course of our studies with LMOX, we found that when the aqueous solution of LMOX was frozen and stored at −17°C, the R/S ratio increased after 20 days. On the other hand, the R/S ratio hardly changed when the solution was kept for the same period at 3°C. Thus, the epimerization reaction had proceeded even in the frozen state with a resulting change in the R/S ratio at equilibrium.

Several studies (4–7) have presented evidence that reactions proceed in a liquid region containing high concentrations of reactants and that the increase in their concentrations resulting from the freezing out of the water in the liquid region accelerates the reaction rates in the case of second-order reactions. However, some experimental features of reactions in frozen solutions have seemed inconsistent with the expectations from the concentration effect. Other suggested factors (8–13) are that ice itself participates in the reactions by causing a favorable positional orientation between reactants, the ice crystal surface serves as a replacement for a catalyst molecule, and proton transfers are facilitated to or from the reactants. However, the factors in the frozen solution have not been clearly identified and seem different for each reaction. This study was done to clarify the epimerization kinetics of LMOX in a frozen solution.



Scheme I

MATERIALS AND METHODS

Materials

Moxalactam disodium (LMOX), a mixture of R- and S-epimers at a ratio of about 1.1, was used as obtained from our laboratories. The other mixture of R- and S-epimers at a ratio of about 2.5 was obtained by mixing LMOX and an R-epimer-rich compound (14) synthesized at our laboratories. All other chemicals were of reagent grade.

Analytical Procedures

The R- and S-epimers were quantified by high-performance liquid chromatography (HPLC), based on peak areas calculated with a Model 5000A, System Instruments Intelligent Integrator (Tokyo). LMOX was eluted on a stainless-steel column (4.6 × 150 mm) packed with Nucleosil 5C$_8$ (Macherey-Nagel Co., Düren), at a flow rate of 2.0 ml/min. The eluant employed to separate the R- and S-epimers was 0.1 *M* ammonium acetate.

Microscopic Photography

A drop of water or 0.1% aqueous solution of LMOX was placed on a slide glass and covered with a microscope cover glass (25 mm square). The solution was spread out between the two glass plates by gentle pressure of a cover glass. The preparations were frozen and maintained at −25.0°C in a cell by circulating methanol from a controlled low-temperature bath. Photographs of the preparations in the frozen solution were taken at various temperatures, with the temperature being raised at 1°C/min; nitrogen gas was blown into the cell to prevent frost from forming on the

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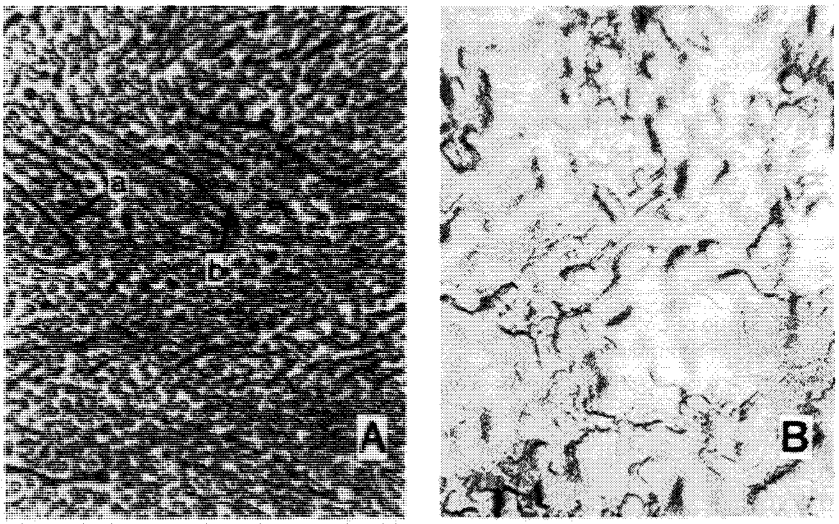


Fig. 1. Microscopic photographs of a LMOX aqueous solution (A) and water (B) in the frozen state at −10°C. The channels (a), between ice (b), contain the unfrozen, concentrated solution.

cover glass. The preparations were observed with a microscope (Nikon, Tokyo) equipped with a 100× objective and a 147-nm polarized filter.

Phase Diagram

The experiment was followed by the method reported by Inoue *et al.* (15). A 1-ml portion of LMOX aqueous solution was pipetted into a test tube equipped with platinum electrodes and a thermistor terminal. The sample portion was frozen in a dry ice–acetone bath, then heated at the rate of 0.5°C/min by introducing a flow of nitrogen gas into the test tube. The electric conductivity was measured using a conductometer (E 518, Metrohm, Herisau), and the temperature was measured with a thermometer (Takara SP-01-5A thermistor, Tokyo). The changes in conductivity and temperature were recorded with a Hitachi 056 recorder (Tokyo).

Kinetic Procedures

LMOX was dissolved at various concentrations with deionized distilled water. The pH of the LMOX solution was in the range of 5.2 (0.1%, w/w) and 5.4 (35.0%, w/w) at room temperature (25°C). The solution, 100 or 200 μl, was pipetted into 1-ml ampoules which were then sealed and kept at a given temperature.

To follow the reaction in the frozen solution, the LMOX solutions in the ampoules were first frozen rapidly in a dry ice–acetone bath and then placed in a controlled low-temperature bath (TRL-N135, Thomas Scientific Co., Ltd., Tokyo). The ampoules were removed from the bath at appropriate intervals and thawed within a minute by immersion in a water bath (25°C) to determine the R- and S-epimers by HPLC.

To follow the reaction in the unfrozen solution, the LMOX solution in ampoules was cooled carefully to the reaction temperature in a controlled low-temperature bath to prevent freezing of the sample. The posttreatment was similar to that described above.

RESULTS

Microscopic Photography of the LMOX Solution in the Frozen State

Microscopic photographs were taken to observe the difference between a 0.1% (w/w) aqueous solution of LMOX and water in the frozen state. Figure 1 shows representative photographs at −10°C. The channels (a) in which LMOX was dissolved exist in ice when the LMOX aqueous solution was frozen. On the other hand, the channels were not found when water was frozen and a layer of ice was present. It was confirmed by this experiment that the liquid region existed as a channel at the temperature between the freezing point and the collapse point even when the LMOX solution was frozen. A similar existence of the liquid region has been reported in several studies (16–18).

Phase Diagram

Figure 2 presents a phase diagram of LMOX aqueous solution. When a quantity of LMOX is added to water, for

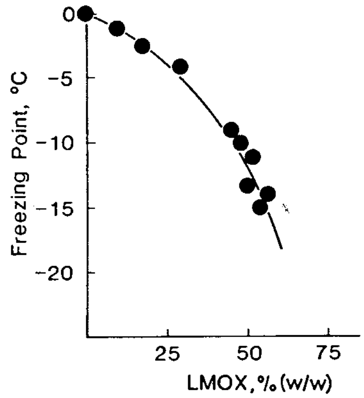


Fig. 2. Freezing-point phase diagram of a LMOX aqueous solution.

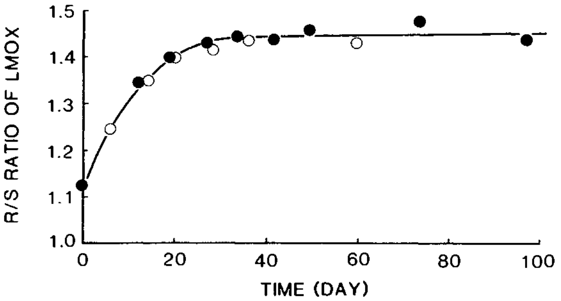


Fig. 3. Time courses of the R/S ratio of LMOX at −10°C in the frozen solution. The initial concentrations were 0.1% (w/w) (●) and 9.1% (w/w) (○).

example, 16.7% (w/w), the freezing point of water is depressed to about −2.3°C. If this frozen solution is cooled further, ice continues to crystallize out, and the solution in the liquid region will become concentrated according to the phase diagram. Both ice and LMOX solidify in a mixture and the liquid region disappears when the temperature falls below the collapse temperature (−19°C) (15).

Influence of the Initial Concentration on the Epimerization Reaction in the Frozen Solution

Although the initial concentration affected the epimerization rates of R- and S-epimers in the unfrozen solution as discussed later (Fig. 9), almost no effect was found in the frozen solution. Changes in the time course of the R/S ratio were compared in the frozen state at −10.0°C between 0.1% (w/w) and 9.1% (w/w) aqueous solutions. In spite of the difference in initial concentrations, the R/S ratios at equilibrium and the time courses to reach equilibrium were almost the same, as shown in Fig. 3. This result implies that a difference in initial concentrations does not affect the R/S ratio at equilibrium and the time course in the frozen solution.

Influence of Ice on the Epimerization Reaction

The time courses of the R/S ratio were compared at −6.5°C between the frozen 0.1% (w/w) LMOX aqueous solution and the unfrozen 35.0% (w/w) LMOX aqueous solution, which depresses the freezing point to −6.5°C according to the phase diagram (Fig. 2). As shown in Fig. 4, the time courses were not different from each other and the R/S ratios attained were almost equal. This means that ice does not influence the epimerization reaction as discussed later in detail.

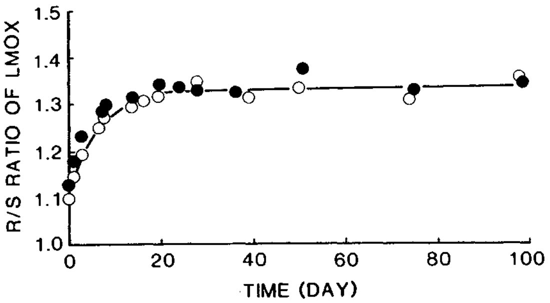


Fig. 4. Time courses of the R/S ratio of LMOX at −6.5°C in the frozen solution (●) and the unfrozen solution (○). The initial concentrations were 0.1% (w/w) in the frozen solution and 35% (w/w) in the unfrozen solution.

In addition to the above results, no crystallization was observed in the unfrozen solution of 35.0% (w/w) LMOX for the experimental period. Thus, the change in the R/S ratio was not thought to be due to the selective crystallization of the R- or S-epimer.

Influence of Temperature on the Epimerization Reaction in the Frozen Solution

The changes of the R/S ratio in the frozen 0.1% (w/w) LMOX aqueous solution were followed over the temperature range of −4.0 to −17.0°C. The R/S ratio at equilibrium and the time required to attain the equilibrium increased as the reaction temperature decreased, as shown in Fig. 5. However, the changes in the R/S ratio stopped completely at −20°C within the experimental period of 60 days [−19.0°C is the collapse temperature of LMOX (15)].

Influence of the Initial R/S Ratio on the Equilibrium R/S Ratio

The variations of the R/S ratio in a 0.1% (w/w) LMOX aqueous solution were compared in the frozen state at −10.0°C between different initial R/S ratios, 1.13 and 2.49. As shown in Fig. 6, both curves approached almost the same R/S ratio at equilibrium. This result indicates that the R/S ratio at equilibrium is not dependent on the initial R/S ratio and that the epimerization reaction is a reversible process.

DISCUSSION

The epimerization reaction kinetics of LMOX in the frozen state were elucidated in this study. The experimental results showed that the change in the R/S ratio with temperature was not caused by the crystallization of the R- or S-epimer and that it was a reversible process. The microscopic photograph (Fig. 1) of the LMOX aqueous solution in the frozen state showed that there was a liquid region in which LMOX was concentrated by crystallization of water in the frozen solution. The possibility of the epimerization reaction occurring in the unfrozen liquid region was suggested by the fact that the changes in the R/S ratio ceased below the collapse temperature. Further support for this came from the following two findings.

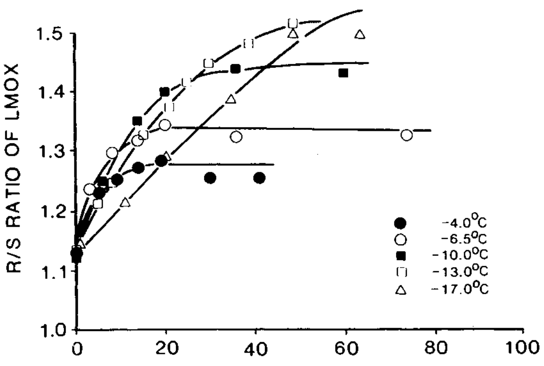


Fig. 5. Time courses of the R/S ratio of LMOX at various temperatures in the frozen solution. The initial concentration was 0.1% (w/w).

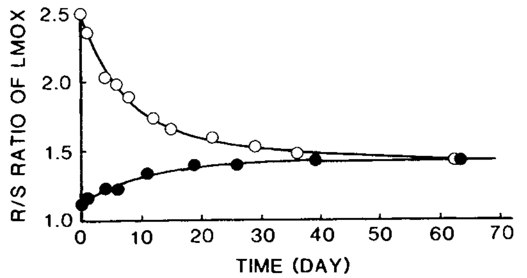


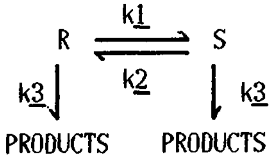
Fig. 6. Time courses of the R/S ratio of LMOX in the frozen solution at − 10.0°C between different initial R/S ratios, 1.13 (●) and 2.49 (○). LMOX concentration was 0.1% (w/w).

Study of the influence of the initial concentration on the epimerization reaction in the frozen solution showed that it did not affect the R/S ratio at equilibrium and the time course at the same temperature (Fig. 3). The LMOX concentration in the liquid region of the frozen solution at − 10°C will become approximately 45% (w/w) irrespective of the initial concentration before freezing according to the phase diagram in Fig. 2. Therefore, even if LMOX aqueous solutions of different concentrations are stored in the frozen state at the same temperature, the epimerization reaction should proceed at the same concentration regardless of the initial concentration because the concentration in the liquid region of the frozen solution is governed by the temperature.

We next compared the epimerization reaction between the frozen and the unfrozen states. The R/S ratio at equilibrium and the time courses did not differ, as shown in Fig. 4. An aqueous solution of 0.1% (w/w) LMOX is concentrated to about 35% (w/w) at −6.5°C by crystallization of water according to the phase diagram in Fig. 2. Therefore, the LMOX concentration in the liquid region remaining in the frozen solution is equal to that of the unfrozen solution. The difference between their conditions is only the existence of ice. While some compounds seem to be subject to catalysis of the ice surface (11,13), the epimerization reaction of LMOX was not affected by the presence of ice. The good superposition of the two time courses strongly supports the idea that the epimerization reaction of LMOX occurs in the liquid region remaining in the frozen solution without being affected by the ice.

As to the degradation of LMOX, the decomposition of the initial total of R- and S-epimers was less than 8% at the end of the experimental period in the frozen state at −4°C, where LMOX was most unstable among the conditions tested in this report. The total of R- and S-epimers decreased linearly in the semilogarithmic plot. We have stated (19) for the epimerization of LMOX in aqueous solution that the degradation rate constants of the R- and S-epimers can be regarded as equal when the total of the R- and S-epimers decreases linearly in the semilogarithmic plot. Therefore, one can assume that the degradation rate constants of the R- and S-epimers are also equal in the frozen state. The decomposition rate decreased as the temperature was lowered, and degradation of the R- and S-epimers was no longer observed below − 10.0°C over the experimental period.

Based on these findings, we postulate that the epimerization reaction proceeds in the liquid region remaining in the frozen solution according to Scheme II, which was shown in the previous report (19).



Scheme II

$$(R) = \frac{(R_0)k_2 + (S_0)k_2}{k_1 + k_2}e^{-k_3t} + \frac{(R_0)k_1 - (S_0)k_2}{k_1 + k_2}e^{-(k_1 + k_2 + k_3)t} \tag{1}$$

$$(S) = \frac{(R_0)k_1 + (S_0)k_1}{k_1 + k_2}e^{-k_3t} + \frac{-(R_0)k_1 + (S_0)k_2}{k_1 + k_2}e^{-(k_1 + k_2 + k_3)t} \tag{2}$$

The constants k_1 and k_2 are the epimerization rate constants from R-epimer to S-epimer and vice versa, respectively. The concentrations of the R-epimer (R) and S-epimer (S) at time t can be expressed by the factors of the initial concentrations of the R-epimer ($R_{\text{lf030°lr030}}$) and S-epimer ($S_{\text{lf030°lr030}}$), k_1, k_2, k_3, and t in Eqs. (1) and (2), where k_3 is the degradation rate constant of LMOX.

$$(P) = \frac{(P_0 + 1)k_2 + (P_0k_1 - k_2)e^{-(k_1 + k_2)t}}{(P_0 + 1)k_1 - (P_0k_1 - k_2)e^{-(k_1 + k_2)t}} \tag{3}$$

The R/S ratio (P) can be expressed as Eq. (3) by dividing Eq. (1) by Eq. (2) and removing k_3, where P_0 is the R/S ratio at $t = 0$.

$$\frac{(P_0 + 1)(P - P_\infty)}{(P + 1)(P_0 - P_\infty)} = e^{-(k_1 + k_2)t} \tag{4}$$

Equation (4) is obtained by substituting the R/S ratio at equilibrium (P_∞), which is equal to k_2/k_1, into Eq. (3) and rearranging it. Equation (3) was used for curve fitting by

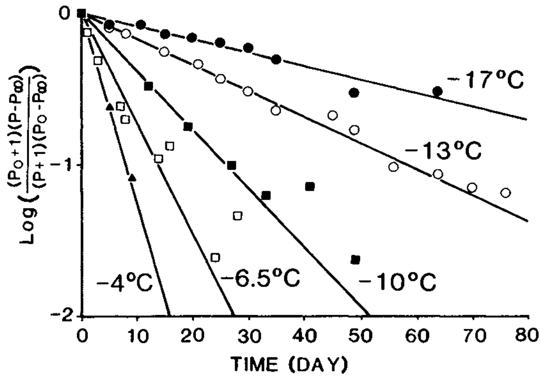


Fig. 7. Semilogarithmic plot for $(P_0 + 1)(P - P_\infty)/(P + 1)(P_0 - P_\infty)$ versus time. The slope of each solid line shows the value of $-(k_1 + k_2)$ at the respective temperature ($k_1 + k_2$) were obtained by NONLIN).

Table I. Effect of Temperature on the Epimerization Reaction in a Frozen Solution[a]

Temperature (°C)	k_1 (days^{-1})[b]	k_2 (days^{-1})[b]	k_2/k_1
−4.0	0.1306	0.1650	1.26
−6.5	0.0715	0.0961	1.34
−10.0	0.0386	0.0566	1.47
−13.0	0.0178	0.0280	1.57
−17.0	0.0076	0.0129	1.69
−20.0	—[c]	—[c]	—[c]

[a] The initial concentration was 0.1% (w/w).

[b] Values were estimated by NONLIN using Eq. (3) and the data in Fig. 5.

[c] Epimerization rates were unable to be estimated because the change of R/S ratio was almost not found.

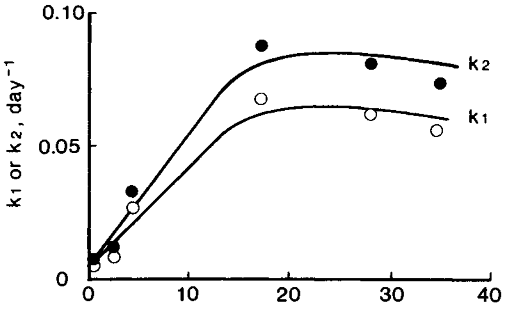


Fig. 9. LMOX concentration dependency of the epimerization rate constants, k_1 (○) and k_2 (●), at −7.5°C in the unfrozen solution.

NONLIN (20) to estimate the values of k_1 and k_2, which allowed us to know the accurate P_∞ in Eq. (4).

If Scheme II can be used for the epimerization reaction in the frozen solution, a linear relationship must be obtained when the logarithmic values on the left side of Eq. (4) are plotted against time. In Fig. 7, the lines indicate the relationship calculated from k_1 and k_2 obtained by NONLIN, and the points show experimental values calculated from P and P_∞. Figure 7 shows that they lie almost on straight lines at various temperatures. The good linearities of the plots demonstrate the validity of Scheme II for analyzing the epimerization of LMOX in frozen solutions.

The epimerization rate constants and the ratios (k_2/k_1) at various temperatures are listed in Table I. As the temperature decreased from −4.0 to −17.0°C, the epimerization rate constants decreased and the ratios increased. Below the collapse temperature of the LMOX aqueous solution, the epimerization reaction stopped completely because the liquid region solidified. When k_1 and k_2 were plotted against the reciprocal of the temperature, good linear relationships were observed in the temperature range studied regardless of the change in LMOX concentration in the liquid region of the frozen solution (Fig. 8). In the aqueous solution of the unfrozen state at −7.5°C, for example, k_1 and k_2 increased linearly with an increase in the concentration of LMOX up to near 17% (w/w), however, they approached plateau values, then showed descending curves with moderate slopes beyond this concentration as shown in Fig. 9. The good linear relationships in the Arrhenius plot in Fig. 8 were owing to this slight concentration dependency in a concentrated LMOX solution. The apparent activation energies obtained from each slope were 29.7 kcal/mol for k_1 and 26.6 kcal/mol for k_2; the difference was statistically significant ($P < 0.05$). The difference in the apparent activation energies between the two epimers can be seen more clearly from the k_2/k_1 versus reciprocal of temperature plot, which shows a positive slope (see inset in Fig. 8).

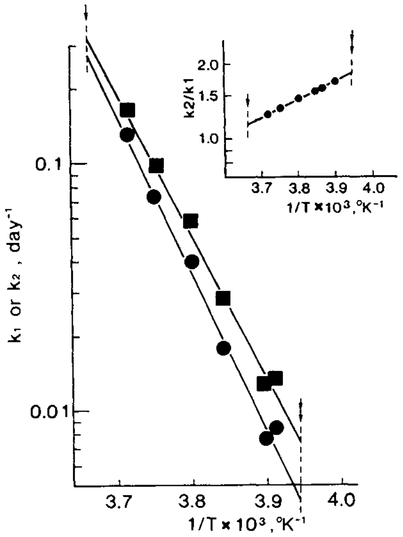


Fig. 8. Arrhenius plots of the epimerization rate constants, k_1 (●) and k_2 (■), in the frozen solution. The inset shows Arrhenius plots of (k_2/k_1). The arrows (↓ and ↓) are the freezing temperature of water and the collapse temperature of the LMOX aqueous solution, respectively.

The conclusion from this study is that the epimerization reaction of LMOX proceeds in the liquid region in a frozen solution, without any influence of ice, and that the change in the R/S ratio at equilibrium with temperature can be ascribed to the differences in apparent activation energies between the two epimers. Further studies will be needed to know what contributes to the difference in the activation energies.

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