Report

Epimerization Kinetics of Moxalactam in Frozen Urine and Plasma Samples

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The epimerization of moxalactam (LMOX) in frozen urine and plasma samples was studied during long-term storage. The *R/S* ratio at equilibrium [(*R/S*)eq] at −10°C was similar in urine and in rat and human plasma ultrafiltrate but differed from that in water. The (*R/S*)eq values in human plasma and its ultrafiltrate differed slightly, while they were the same in rat plasma and in its ultrafiltrate. The difference for the human plasma and ultrafiltrate may result from differences in plasma protein binding between *R*- and *S*-epimers in the liquid region of the frozen plasma. The change of *R/S* ratio in frozen human plasma continued below the collapse temperature of LMOX aqueous solution, where the liquid region appeared still to exist as determined by NMR measurement. Consequently, the biological LMOX samples should be preserved at or below −70°C to prevent changes in the *R/S* ratio.

KEY WORDS: moxalactam; epimerization; frozen plasma; frozen urine; electrolyte; protein binding.

INTRODUCTION

Moxalactam (LMOX) 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 our previous study (4), the *R/S* ratio of LMOX was found to change gradually, and the *R/S* ratio at equilibrium depended on the storage temperature of frozen LMOX aqueous solutions. The change in the *R/S* ratio ceased below the collapse temperature of the LMOX aqueous solution. The study suggested that the drug was unstable even if frozen. In the case of the LMOX aqueous solution, it was recommended to store it below the collapse temperature (−19°C) (4). Aqueous solutions, in general, should be stored below the eutectic or collapse temperature.

Biological samples of LMOX (plasma or urine) were stored in a refrigerator (about −20°C). However, the *R/S* ratio changed to a value below 1.1 of the authentic sample of LMOX. This study addresses the factors regulating the change of the *R/S* ratio in frozen biological samples to optimize storage conditions.

MATERIALS AND METHODS

Materials

Moxalactam disodium (LMOX), a mixure of *R*- and *S*-epimers at a ratio of about 1.1, was used as obtained from our laboratories. The other mixtures of *R*- and *S*-epimers at various ratios were obtained by mixing LMOX and an *R*- or *S*-epimer-rich compound synthesized at our laboratories (5). Human serum albumin (HSA; Fraction V) was obtained from Sigma Chemical Co., St. Louis, MO. Ovalbumin (2 × Cryst.) was obtained from Seikagaku Kogyo Co., Ltd, Tokyo. All other chemicals were of reagent grade.

The rat plasma was collected from male Jcl-SD rats (250–300 g) fasted overnight. The human plasma and urine were collected from three healthy male volunteers fasted overnight. The ultrafiltrate of human or rat plasma was obtained by an ultrafiltration method (Amicon Centriflo Ultrafiltration Membrane Filter Cone, Type MPS 1, Membrane Type YMT 14 mm, Lexington, MA). A 5% HSA solution was made by dissolving HSA with the ultrafiltrate of human plasma.

Analytical Procedures

The *R*- and *S*-epimers were quantified separately by high-performance liquid chromatography (HPLC). The samples pretreated by the method described below were injected onto a column (4.6 × 150 mm) packed with Nucleosil 5C18 (Macherey-Nagel Co., Düren). The mobile phase was 0.1 *M* ammonium acetate. Samples were analyzed by UV detection at 254 nm and peak areas were calculated with a Model 5000A, System Instruments Intelligent Integrator (Tokyo).

In the case of plasma or 5% HSA, 100-μl samples were added to 200 μl methanol for deproteinization. After immediate mixing and centrifugation for 3 min at 10,000*g* and approximately 0°C by a refrigerated centrifugator (MR-15A, Tomy Seiko Co., Ltd., Tokyo), an aliquot of the clear supernatant was applied to HPLC. The change in the *R/S* ratio was not observed during these procedures.

Samples of plasma ultrafiltrate and urine were directly applied to HPLC without any pretreatment.

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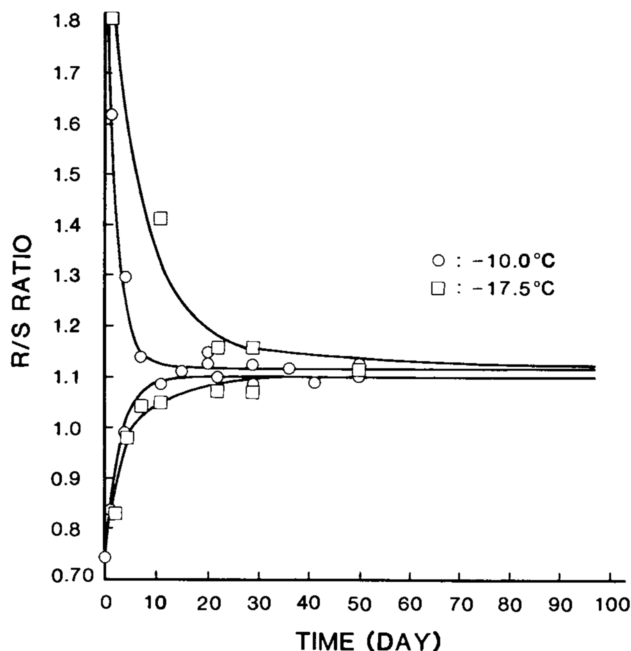


Fig. 1. Time courses of the R/S ratio at -10.0 and -17.5°C in frozen human urine. The initial concentration of a R/S mixture was 0.1% (w/w).

Kinetic Procedures

LMOX, R-epimer-rich samples, or S-epimer-rich samples were dissolved to 0.1% (w/w) in electrolyte solutions (prepared with deionized distilled water), rat plasma, its ultrafiltrate, 5% HSA (dissolved in the ultrafiltrate of human plasma), human urine, human plasma, and its ultrafiltrate unless otherwise mentioned. The resultant solutions, 100 or 200 μl, were pipetted into 1-ml ampoules, which were then sealed and kept at a given temperature.

To follow the epimerization reaction in frozen solution, the LMOX solutions in the ampoules were first frozen rapidly in a dry ice–acetone bath, 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.

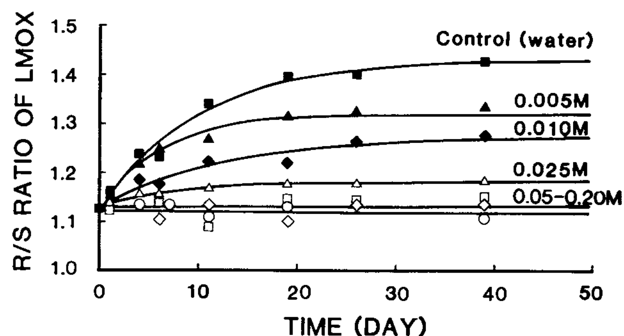


Fig. 3. Effect of sodium chloride on the R/S ratio in the frozen state. The initial concentration of LMOX was 0.1% (w/w).

Protein Binding

Binding of LOMX to HSA was measured at various HSA concentrations at -5.0°C by an ultrafiltration method using an Amicon Centriflo Ultrafiltration Membrane Filter Cone, Type MPS 1, Membrane Type YMT 14 mm (Lexington, MA). Five hundred microliters of HSA solutions and 20 μl of LMOX aqueous solutions of various concentrations were mixed and applied to the membrane cone. After incubation at -5°C for 20 min, approximately 10% of the initial volume was ultrafiltrated at -5°C using a refrigerated centrifugator (KUBOTA KR/702, Kubota Seisakusho, Tokyo). The centrifugation was done for about 10 min at 1000g in the case of a diluted HSA solution but at 3500g for a concentrated HSA solution because the ultrafiltration became difficult by an increase in the viscosity. The concentration of LMOX in the ultrafiltrate was determined by HPLC. The adsorption of LMOX to the membrane was negligible. The concentration ratio of LMOX to HSA was always kept constant (LMOX:HSA = 1:50, w/w) because LMOX and albumin were concentrated in the liquid water regions in frozen samples at the same concentration ratio as the initial concentration ratio before freezing. The protein binding ratio was calculated from the unbound and total concentrations of the R- and S-epimer.

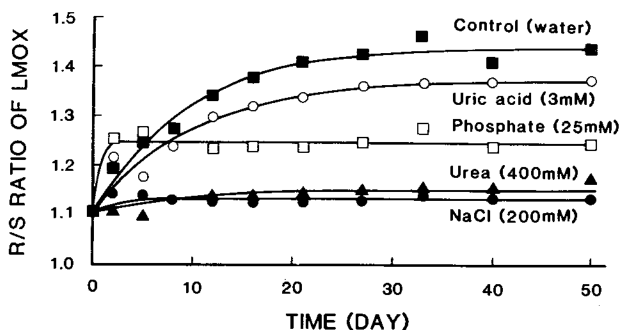


Fig. 2. Effect of typical urine components on the R/S ratio at -10.0°C in the frozen state. The initial concentration of LMOX was 0.1% (w/w). Uric acid, phosphoric acid, urea, and sodium chloride were dissolved in deionized distilled water and adjusted to pH 6.5 with sodium hydroxide and hydrogen chloride.

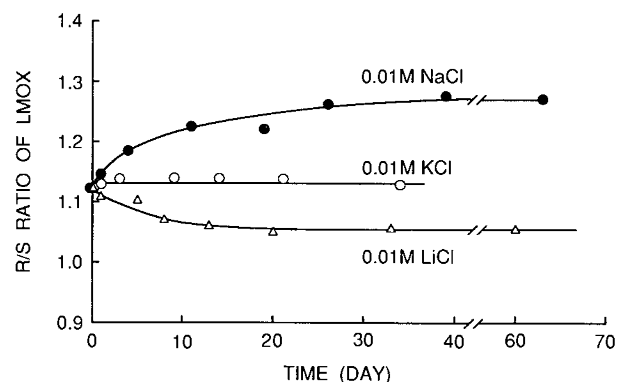


Fig. 4. Effect of cations on the R/S ratio at -10.0°C in the frozen state. The initial concentration of a R/S mixture was 0.1% (w/w).

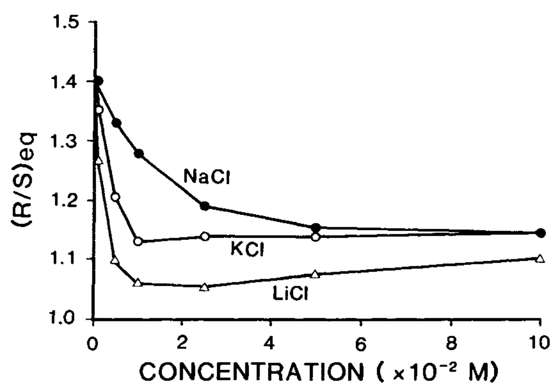


Fig. 5. Effect of cations on the (R/S)eq at -10.0°C in the frozen state. The initial concentration of a R/S mixture was 0.1% (w/w).

NMR Measurements

The proton NMR spectra of liquid water in the frozen state were measured on a Varian XL-200 NMR spectrometer at 200.057 MHz. The Fourier transform measurement conditions were as follows: spectral width, 10,000 Hz; acquisition time, 0.3 sec; pulse flipping angle, 45°; pulse delay time, 0.7 sec; and number of transients, 32 or 128. The sample solution (0.5 ml) was poured into a 4-mm Pyrex tube. The tube was immersed in liquid nitrogen. The relative amount of unfrozen water of each sample was determined by comparison with the peak area of the NMR signal produced by a 0.5% ovalbumin solution, in which the water content was reported to be 0.32 g H_2O/g protein between -20 and -40°C by Hanafusa (6).

RESULTS AND DISCUSSION

The Epimerization Reaction in Frozen Urine

Mixtures of R- and S-epimers at different R/S ratios

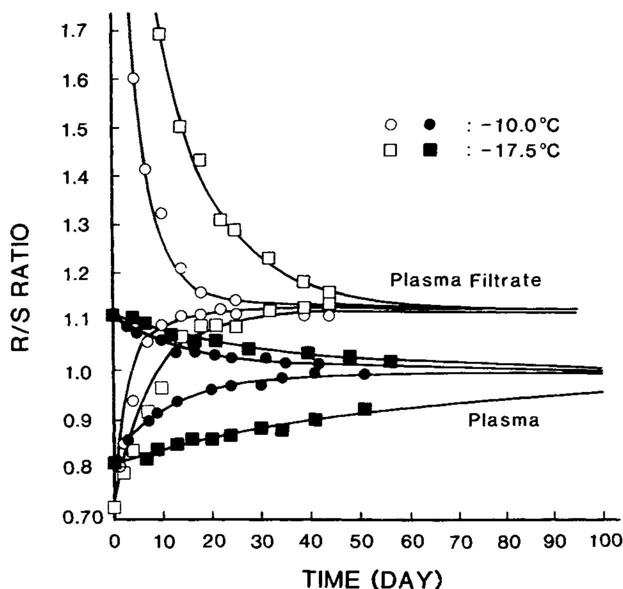


Fig. 6. Time courses of the R/S ratio at -10.0°C (circle) and -17.5°C (square) in frozen human plasma (filled) and its ultrafiltrate (open). The initial concentration of the R/S mixture was 0.1% (w/w).

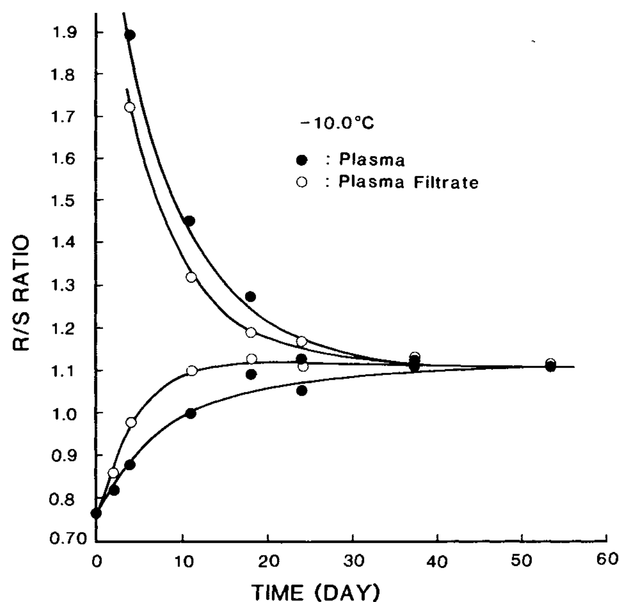
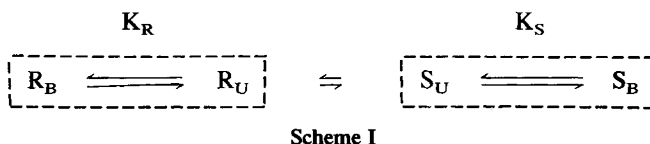


Fig. 7. Time courses of the R/S ratio at -10.0°C in frozen rat plasma and its ultrafiltrate. The initial concentration of the R/S mixture was 0.1% (w/w).

were dissolved in human urine (0.1%, w/w), and the changes in the R/S ratios were followed in the frozen states at -10.0 and -17.5°C, as shown in Fig. 1. The R/S ratios at equilibrium [(R/S)eq] were almost the same (about 1.12) at both temperatures; and the time required to attain equilibrium took longer at -17.5°C than at -10.0°C. We reported previously (4) that the (R/S)eq values were about 1.47 at -10.0°C and 1.70 at -17.5°C in frozen LMOX aqueous solutions (4). The (R/S)eq in frozen urine was different from that in the frozen aqueous solution. Therefore, some components in urine seem to affect the (R/S)eq.

Influence of Typical Urine Components

The R/S ratio was followed at -10.0°C in the aqueous solutions of various urine components to know what component in urine affects the (R/S)eq. The concentration of the urine component was selected based on the reported daily amount of each component excreted in urine (7), and the pH was adjusted to 6.5 by the addition of sodium hydroxide or hydrogen chloride. As shown in Fig. 2, every main component in urine lowered the (R/S)eq, and especially, the (R/S)eq in sodium chloride solution was quite similar to that in urine. In addition, other components present at high concentrations changed the (R/S)eq to a similar value as in frozen urine. Hence, the main components in urine caused an (R/S)eq value close to 1.12, and the effect depended on the dissolved species and the concentration.



Scheme I

Table I. Protein Binding of LMOX at $-5°C$[a]

LMOX concn. (w/v, %)	HSA concn. (w/v, %)	Unbound R-epimer (%)	Unbound S-epimer (%)	$(1 + KR)/(1 + KS) \cdot 1.1$[b]
2.6	5.0	46.0 ± 2.7	26.3 ± 2.8	0.63
8.1	15.7	51.7 ± 9.9	32.1 ± 7.5	0.68
21.1	40.9	52.4 ± 6.1	32.8 ± 5.3	0.69
23.5	45.0	49.2 ± 1.3	30.5 ± 1.9	0.68
26.1	50.0	41.2 ± 0.6	26.8 ± 1.0	0.72
31.4	60.2	34.9 ± 1.7	23.2 ± 1.1	0.73

[a] The LMOX aqueous solution, 20 μl, was added to HSA aqueous solution, 500 μl, with a constant LMOX-to-HSA concentration ratio (LMOX:HSA = 1:50, w/w) (N = 3).
[b] 1.1 was the (R/S)eq in plasma ultrafiltrate at $-10.0°C$.

Effect of Sodium Chloride on the (R/S)eq

The (R/S)eq at different concentrations of sodium chloride, the main urinary ion, was followed at $-10.0°C$ in the frozen state. As shown in Fig. 3, the value of the (R/S)eq approached 1.12 at higher concentrations of sodium chloride.

The solutes, LMOX and sodium chloride, are concentrated in the liquid region. The concentration of sodium chloride in the liquid region reached similar values at the fixed temperature regardless of the concentration before being frozen, because the molar ratio of sodium chloride to LMOX was so high that the contribution of LMOX to the freezing point depression was negligible. Further, the concentration ratio of LMOX and sodium chloride in the liquid region remained unchanged during freezing. The (R/S)eq was approximately 1.43 in the absence of sodium chloride. When sodium chloride was added, LMOX and sodium chloride were concentrated in the liquid region in the frozen state. As a result, the favorable environment for the R-epimer in the liquid region was destroyed by excess electrolytes at the higher concentration ratio of sodium chloride to LMOX, and the (R/S)eq approached 1.12.

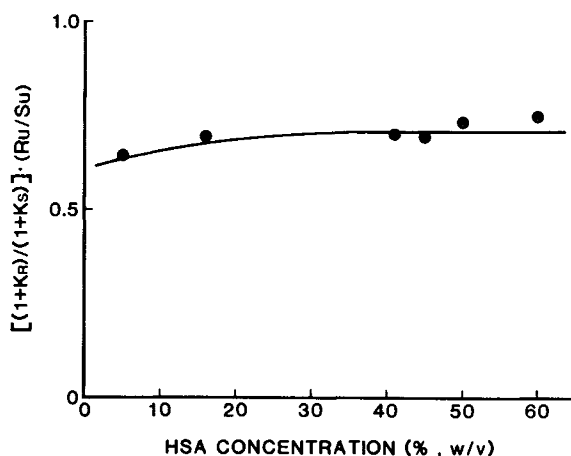


Fig. 8. Effect of HSA protein binding on the R/S ratio of LMOX at $-5.0°C$ in the unfrozen state. The (R/S)eq in frozen plasma was the product of $(1 + K_R)/(1 + K_S)$ and the (R/S)eq value (1.1) in the plasma ultrafiltrate.

Comparison of Effect on the (R/S)eq Among Various Cations

When an ion dissolves in water, it produces a hydration structure around itself. An ion is classified as a structure-maker ion or structure-breaker ion according to the hydration number per ion. Lithium, sodium, and potassium cations are called structure-maker ions, and the magnitude of structure-making properties decreases in this order (8). Therefore, the (R/S)eq was measured at $-10.0°C$ at various concentrations of sodium chloride, potassium chloride, or lithium chloride to investigate how these electrolytes influence the (R/S)eq. Representative time courses of LMOX in 0.01 M aqueous solutions are shown in Fig. 4. Figure 5 shows that the (R/S)eq approached similar values when the electrolyte concentration exceeded 0.1 M. In the diluted concentration range, the (R/S)eq showed different profiles depending on the cationic species. In all the cases tested, LMOX and the electrolyte were still dissolved in the liquid region at $-10.0°C$.

The environment around LMOX molecule was influenced by the concentration and the species of electrolyte, and the stability of each epimer seemed to be affected differently. At higher concentrations of electrolytes, different effects seen with each ion disappeared by the mutual interaction of the same ions (9).

The Epimerization Reaction in Frozen Plasma and Its Ultrafiltrate

The changes in the R/S ratio in frozen human plasma

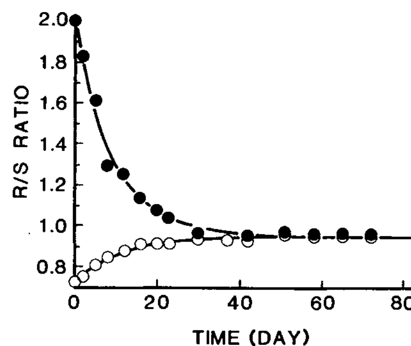


Fig. 9. Time courses of the R/S ratio at $-10.0°C$ in frozen 5% HSA. The initial concentration of the R/S mixture was 0.1% (w/w).

Table II. Amount of Unfrozen Water in Frozen Samples (g H_2O/g Protein)[a]

Sample	Temperature (°C)							
	−10.6	−20.7	−30.4	−40.7	−50.5	−60.5	−70.5	−80.4
0.5% ovalbumin	0.46	0.34	0.32	0.32	0.27	0.22	0.20	0.10
Human urine	93.44	52.00	16.00	5.87	5.08	4.96	0.11	0.11
Human plasma	50.40	26.88	20.48	16.64	14.08	8.96	4.28	1.21
5% HSA	9.08	7.60	7.20	6.88	6.32	5.44	2.37	0.67
LMOX aqueous solution (0.1%)	0.89[b]	0.30	0.21	0.18	0.00	0.00	0.00	0.00

[a] The amount of the unfrozen water was represented as the relative peak area with respect to the peak area of 0.5% ovalbumin measured between −20 and −40°C (0.32 g H_2O/g protein).
[b] Measured at −9.5°C.

were followed at −10.0 and −17.5°C (Fig. 6). The (*R*/*S*)eq values were 1.0 for the two temperatures tested, but the time required to attain equilibrium increased at the lower temperature. In another experiment with frozen rat plasma, the (*R*/*S*)eq was 1.1 at −10.0°C (Fig. 7). This value was equal to the *R*/*S* ratio (1.1) of LMOX. There appeared to be a small but significant difference between the (R/S)eq values in human and rat plasma frozen at −10.0°C. The marked differences in (*R*/*S*)eq in water (1.43 at −10°C) and in rat or human plasma may be caused by plasma components. Therefore, the *R*/*S* ratio was followed in frozen human or rat plasma ultrafiltrates (MW cutoff >10,000). As seen from the comparison of Figs. 6 and 7, the (*R*/*S*)eq values for both human and rat plasma ultrafiltrates approached 1.1, as found in human urine. Hence, the (*R*/*S*)eq values in plasma ultrafiltrate are likely to be governed by sodium chloride as the major plasma component (7).

As to human plasma, the (*R*/*S*)eq difference in plasma and ultrafiltrate seemed due to a plasma component impermeable to the ultrafiltration membrane used (Fig. 6), while the (*R*/*S*)eq in rat plasma was identical to that in rat plasma ultrafiltrate (Fig. 7). In parallel, human plasma protein binding of the *S*-epimer was previously shown to be higher than that of *R*-epimer (10), and the *R*- and *S*-epimers were found to bind to albumin in plasma (11). On the other hand, no difference was observed in rat plasma protein binding between both epimers (12). The *R*- and *S*-epimers could thus equilibrate in plasma as shown in Scheme I. The R_B and S_B show the bound concentrations for *R*- and *S*-epimers, and

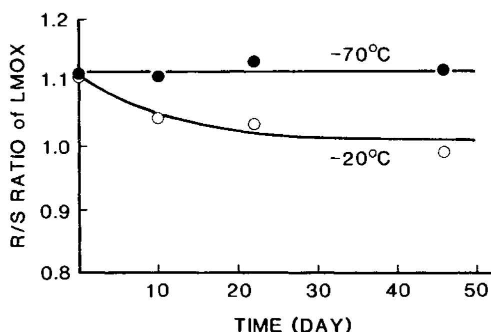


Fig. 10. Time courses of the *R*/*S* ratio of LMOX in frozen human plasma at −20 and −70°C. The initial concentration of LMOX was 0.1% (w/w).

the R_U and S_U the unbound concentrations. The (*R*/*S*)eq in plasma is given as the ratio of (R_U + R_B) to (S_U + S_B) in Eq. 1.

$$(R/S)\text{eq} = \frac{(R_U + R_B)}{(S_U + S_B)} = \frac{(1 + K_R) \cdot R_U}{(1 + K_S) \cdot S_U} \quad (1)$$

By dividing numerator and denominator by R_U and S_U, respectively, the (*R*/*S*)eq is represented as the product of $(1 + K_R)/(1 + K_S)$ and R_U/S_U, which is the (*R*/*S*)eq (1.1) in the plasma ultrafiltrate. The K_R and K_S are the binding constants of each epimer. When the two binding constants are equal as in the case of rat (12), $(1 + K_R)/(1 + K_S)$ become unity and the (*R*/*S*)eq in plasma is exactly equal to that in plasma ultrafiltrate. Therefore, if the stereospecific protein binding ($K_R < K_S$) still exists between *R*- and *S*-epimers in the frozen plasma, it causes the different (*R*/*S*)eq values between human plasma and its ultrafiltrate. To test this hypothesis, the protein binding of LMOX at a high HSA concentration was measured at −5°C in the unfrozen state by an ultrafiltration method. When human plasma was frozen, water was solidified and LMOX and albumin were concentrated in the liquid water regions at the same ratio as the LMOX-to-albumin ratio before freezing. The concentration ratio of LMOX to HSA was kept constant (LMOX:HSA = 1:50, (w/w). The unbound fractions of *R*- and *S*-epimers are listed in Table I. As shown in Fig. 8, the stereospecific binding of HSA to *R*- and *S*-epimers still existed even at high HSA concentrations at low temperatures and can account for the difference in the (*R*/*S*)eq values between human plasma and its ultrafiltrate. In agreement with this prediction, the *R*/*S* ration of *R*-epimer-rich samples or *S*-epimer-rich samples approached 1.0 in the frozen 5% HSA solution at −10.0°C (Fig. 9), as found in human plasma.

Hanafusa reported that the amount of unfrozen water of a 0.5% ovalbumin solution was 0.32 g H_2O/g protein between −20 and −40°C (6). We measured the unfrozen water of human plasma, 5% HSA, and human urine at various temperatures by NMR. The results are listed in Table II. The amount of unfrozen water is shown by the relative peak area ratio with respect to the average peak area of 0.5% ovalbumin between −20 and −40°C. Considerable unfrozen water still existed below the collapse temperature of an LMOX aqueous solution. It is thus possible that the *R*/*S* ratio changes even below this collapse temperature of the aqueous

solution when storing urine and plasma samples. As shown in Fig. 10, the *R/S* change occurred at −20°C but not at −70°C in human plasma for the period examined.

In conclusion, the *R/S* ratio of LMOX in urine and plasma was influenced by the electrolytes and plasma protein even in the frozen state. Accordingly, special care must be taken when clinical and biological samples of drugs such as LMOX are frozen for storage, and the recommended preservation temperature is below −70°C where liquid water regions are negligible.

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