8-Ethyl-5,8-dihydro-2-(2-hydroxy-1-pyrrolidinyl)-5-oxopyrido[2,3-*d*]pyrimidine-6-carboxylic Acid (8)——a) Aqueous 10% H_2SO_4 (0.2 ml) was added with stirring to a suspension of **7** (3.0 g) in a mixture of dioxane (20 ml) and water (10 ml). The reaction mixture was heated on a steam bath, becoming clear and immediately precipitating a yellowish solid. Heating was continued for several minutes and then the reaction mixture was cooled. The solid formed was collected by filtration, and washed with hot dioxane. Recrystallization of the crude product gave **8** (1.0 g) as yellowish fine needles (see Table I). The spectral data are given in Table II.

b) Water (10 ml) was added to a hot solution of **9a** (2.82 g) in dioxane (20 ml) with stirring. The mixture was heated on a steam bath for 5 min, during which period yellowish crystals separated out. The crystals collected by filtration and recrystallized, giving **8** (1.91 g).

8-Ethyl-2-(2-ethoxy- and -methoxy-1-pyrrolidinyl)-5,8-dihydro-5-oxopyrido[2,3-*d*]pyrimidine-6-carboxylic Acids (9a and 9b)——Aqueous 10% H_2SO_4 (15 ml) was added to a suspension of **7** (4.0 g) in EtOH or MeOH (200 ml). The mixture was heated on a steam bath for 5 min. The resulting precipitate was collected by filtration and the filtrate was concentrated to about 70 ml under reduced pressure, giving an additional crop of the precipitate. Recrystallization of the combined precipitate gave the corresponding 2-alkoxy derivatives **9a** (3.02 g) and **9b** (2.73 g) as yellowish fine needles (see Table I). The spectral data are given in Table II.

8-Ethyl-5,8-dihydro-2-(2-pyrrolin-1-yl)-5-oxopyrido[2,3-*d*]pyrimidine-6-carboxylic Acid (10)——Compound **9a** (500 mg) was heated to *ca.* 200° under reduced pressure (30 mmHg), melting into a yellow liquid which then solidified. The solid was taken up in $CHCl_3$ and chromatographed on silica gel (10 g) with $CHCl_3$ as an eluent. Recrystallization of the crude product gave **10** (106 mg) as yellow needles (see Table I). Compound **9b** was treated in a similar manner to give **10**. The spectral data are given in Table II.

2-(3-Ethoxycarbonylpropylamino)-8-ethyl-5,8-dihydro-5-oxopyrido[2,3-*d*]pyrimidine-6-carboxylic Acid (12)——2-Chloro-8-ethyl-5,8-dihydro-5-oxopyrido[2,3-*d*]pyrimidine-6-carboxylic acid (**11**)[3b] (4.0 g) was added to a solution of ethyl 4-aminobutyrate hydrochloride (3.02 g) and triethyl amine (3.8 g) in EtOH (200 ml). The mixture was heated under reflux for 2 hr. After removal of the EtOH by distillation, the residue was taken up in $CHCl_3$. The $CHCl_3$ solution was washed with water, dried, and the solvent was distilled off to leave the crude product, which was recrystallized, giving **12** (3.4 g) (see Table I). The spectral data are given in Table II.

2-(3-Carboxypropylamino)-8-ethyl-5,8-dihydro-5-oxopyrido[2,3-*d*]pyrimidine-6-carboxylic Acid (13)——A solution of **12** (1.74 g) in aqueous 5% NaOH (20 ml) was heated on a steam bath for 10 min. The mixture was adjusted to pH 4 with AcOH under cooling, giving precipitates which were collected, washed with water, and recrystallized to afford **13** (0.98 g) as colorless fine needles (see Table I). The spectral data are given in Table II.

Acknowledgement The authors are grateful to Drs. H. Takamatsu, M. Shimizu, and H. Nishimura of these laboratories for their encouragement throughout this work. Thanks are also due to members of the analytical division of the laboratories for elemental analysis and spectral measurements.

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Reversible Ring-opening Reactions of Triazolobenzo- and Triazolothienodiazepines in Acidic Media at around Body Temperature

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Hydrolytic reactions of triazolobenzo- and triazolothienodiazepines (estazolam and etizolam) as well as a thienodiazepine (clotiazepam) were studied spectrophotometrically. Cleavage reactions of the azomethine bonds of estazolam and etizolam were reversible and the open-ring compounds were in equilibrium with the closed-ring compounds (protonated forms of the parent drugs). However, little spectral change was observed in

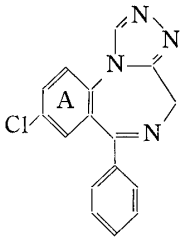
1) Location: *Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan.*

clotiazepam. The rate of ring-closure reaction was greater than that of ring-opening reaction in estazolam whereas the opposite was the case in etizolam. The activation energies of the forward and reverse reactions were obtained from Arrhenius-type plots. In addition, the pK_a value of etizolam was determined.

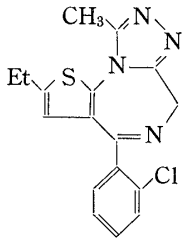
Keywords——reversible reaction; acid hydrolysis; estazolam; etizolam; clotiazepam; triazolobenzodiazepine; triazolothienodiazepine; thienodiazepine; pK_a determination; kinetics

Although the hydrolytic cleavage of benzodiazepine antianxiety agents was considered to take place only at elevated temperature,[2,3] pyrazolodiazepinones have been shown to be easily hydrolyzed at ambient temperature.[4] However, even in the case of benzodiazepines, reversible ring-opening of diazepam at its azomethine bond in acidic media at 37° has been demonstrated recently.[5] In a study of the stability of triazolobenzodiazepine and thienodiazepines as a part of an examination of the physicochemical properties of these drugs, only a small degree of degradation was observed in estazolam at 50° in 5.6 N HCl,[6] whereas marked degradation was observed in etizolam at 60° and pH 2.1.[7] Clotiazepam was apparently more stable than etizolam, since a smaller degree of degradation was observed at 60° and pH 1.2.[8]

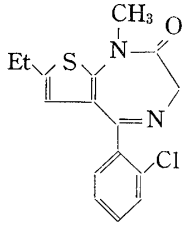
Other than the above reports, no detailed report on the reactions of these drugs at body temperature has been published. Therefore, the authors have investigated spectrophotometrically the reactions of these drugs in acidic solutions. It was found that estazolam and etizolam are susceptible to hydrolysis in mildly acidic solutions, whereas clotiazepam, which is structurally related to etizolam, was stable under the same conditions.



estazolam



etizolam



clotiazepam

Experimental

Materials——Estazolam was generously supplied by Takeda Chemical Industries, Osaka, and etizolam and clotiazepam by Yoshitomi Pharmaceutical Industries, Osaka. Chloroform and ethanol were distilled prior to use. Other chemicals were of reagent grade and were purchased from Wako Pure Chemical Industries, Osaka.

Kinetic Studies and pK_a Determination——The kinetic studies and pK_a determination were carried out spectrophotometrically. The procedures have been described in detail previously.[5,9] Reaction rate constants were calculated from changes in absorbance at fixed wavelengths at λ_{max}.

Since estazolam was not quantitatively extracted into chloroform after acidification of the aqueous estazolam solution, the molar absorptivity of the chloroform-unextractable species was calculated from the absorbance measured by the methods shown in Chart 1 and described below. After estazolam solutions in 0.1 N HCl at time zero and at equilibrium had been shaken for 5 min at 10° with the same volume of chloro-

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4) W. Hong, C. Johnston, and D. Szulcozewski, *J. Pharm. Sci.*, **66**, 1703 (1977).
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7) A. Kudo, T. Tawara, M. Nobutoki, and Y. Ota, *Clinical Report*, **6**, 1879 (1972).
8) J. Sakai, M. Nobutoki, Y. Ota, and H. Matsuki, *Iyakuhin Kenkyu*, **9**, 1012 (1978).
9) A. Albert and E. Serjeant, "Ionization Constants of Acids and Bases," 2nd ed., Chapman and Hall, London, 1971, pp. 44—64.

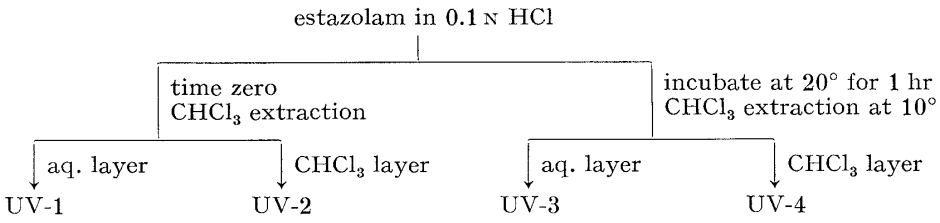

Chart 1

form to extract unreacted estazolam, the spectra of the aqueous layer (UV-1 for time zero and UV-3 for equilibrium) and the chloroform layer (UV-2 for time zero and UV-4 for equilibrium) were obtained. The chloroform/0.1 N HCl partition coefficient and molar absorptivity in chloroform saturated with 0.1 N HCl were calculated from the absorbances in UV-1 and UV-2. If reaction products are assumed to be unextractable by chloroform, UV-4 represents estazolam, the concentration of which could be calculated by using the molar absorptivity of estazolam. Since the absorbance of estazolam in UV-3 could be calculated from the partition coefficient and the concentration of estazolam in the chloroform extract at equilibrium, the concentration and the molar absorptivity of the reaction products could be calculated from the concentration of estazolam at time zero minus those of estazolam in chloroform (UV-4) and in the aqueous layer (UV-3) at equilibrium.

Results and Discussion

The Nature of the Reactions

The spectral changes of estazolam and etizolam in 0.1 N HCl are shown in Figs. 1 and 2, respectively. Because estazolam reacted vary rapidly at 37°, its spectral change was recorded at 20°, whereas the spectral scan for etizolam was made at 37°. The reactions reached equilibria in 5 min for estazolam and in 290 min for etizolam, whereas little spectral change was observed for clotiazepam in 100 min at 37°. Following chloroform extractions of the acidic solutions of estazolam and etizolam at equilibrium, the spectra of the aqueous layers (Fig. 3, time zero) were quite different from those of the equilibrated solutions (Figs. 1 and 2, time ∞). These observations indicate the presence of at least two species, which differed markedly in their partition coefficients, in equilibrated solutions of both drugs. The spectrum of the chloroform-unextractable species from etizolam, taken immediately following the extraction (Fig. 3, time zero), changed with time to give a spectrum (Fig. 3, time ∞) which was quite

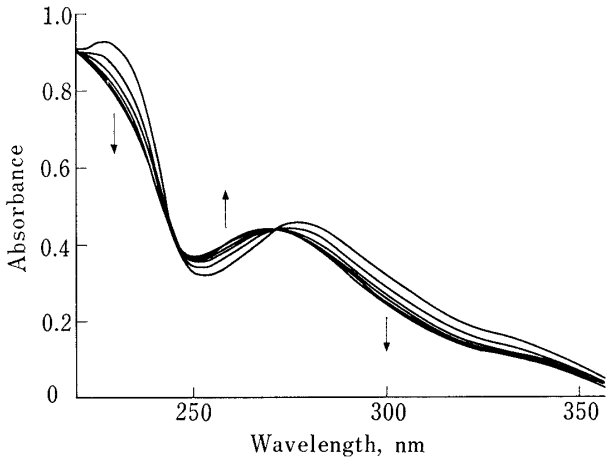

Fig. 1. Typical Spectral Changes During the Hydrolysis of Estazolam (4.02×10^{-5} M) in 0.1 M in HCl at 20°

The absorbance at 228 nm decreased with time (0, 3, 6, 12, 18, and 40 (∞) min, from the top).

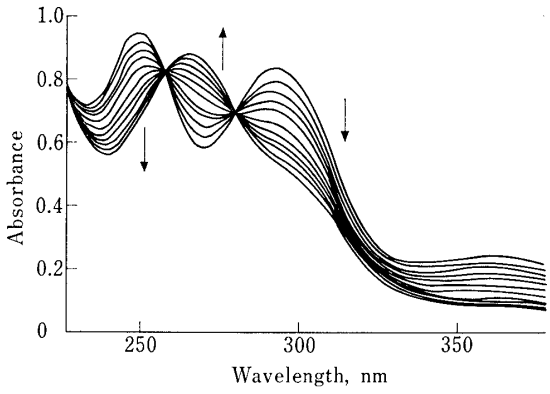

Fig. 2. Typical Spectral Changes During the Hydrolysis of Etizoalm (7.8×10^{-5} M) in 0.1 N HCl at 37°

The absorbance at 293 nm decreased with time (0, 10, 20, 30, 50, 70, 90, 110, 150, and 230 (∞) min, from the top).

similar to that of the equilibrium mixture from the parent drug (Fig. 2, time ∞). The spectrum of the aqueous layer of the equilibrated solution of estazolam extracted with chloroform changed to a lesser extent with time (not shown), since protonated estazolam was not quantitatively extracted into chloroform, but the spectrum following chloroform extraction was quite similar to that of the equilibrium mixture starting from estazolam (Fig. 1, time ∞). In addition, the cyclization reaction at neutral pH was so fast at 37° that the spectra of solutions immediately following addition of pH 7.4 phosphate buffer corresponded to those of the parent drugs.

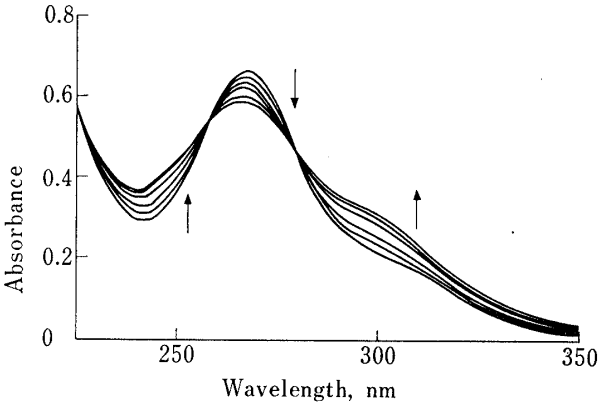

Fig. 3. Typical Spectral Changes due to the Ring-closure Reaction of the Chloroform-unextractable Species of Etizolam (4.54×10^{-5} M) in 0.1 N HCl at 37°

The absorbance at 267 nm decreased with time (0, 10, 20, 40, 90, and 210 (∞) min, from the top).

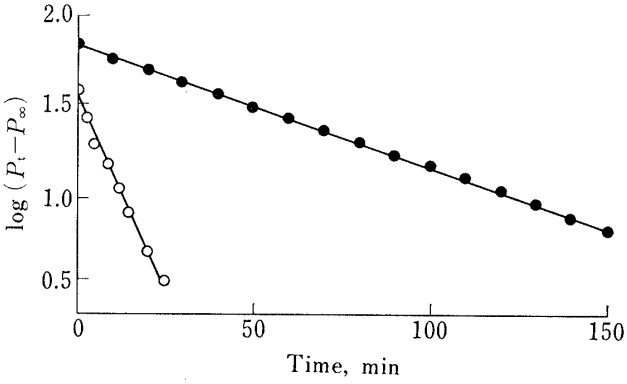

Fig. 4. Plots according to the Equation for a Reversible First Order Reaction for 3.76×10^{-5} M Estazolam (○) at 15° and 7.27×10^{-5} M Etizolam (●) at 37°, both in 0.1N HCl

P_t and P_∞ are the percentages of the drug remaining unreacted at time t and infinity, respectively.

Structural Assignments of the Chloroform-unextractable Species

Although the chloroform-unextractable species from estazolam and etizolam are yet to be definitely identified, the observations rule out hydrolysis products produced by hydrolyses at 1,2- and 4,5- bonds, which are not expected to be cyclized. Since open-ring structures at the azomethine bond for the chloroform-unextractable species do not conflict with any spectrophotometric or partition characteristics of the species, these structures are tentatively assigned to the chloroform-unextractable species.

Quantitative Aspects

Linear relations were obtained in plots based on the equation for reversible first order reactions[10] (Fig. 4). Reaction rate constants at 37° obtained for etizolam are presented in Table I. Those for estazolam were calculated from rate constants obtained at lower temperatures

The finding that forward and reverse reaction rate constants of estazolam are much greater than those of diazepam[5] indicates increased reactivity of the triazolo derivatives, possibly because of a contribution of the triazolo ring to the electron-deficient state of the 5-carbon. This was also seen in the increased reactivity of etizolam over clotiazepam. Since the forward reaction rate constant was smaller than the reverse reaction rate constant, the closed-ring compound (protonated estazolam) is expected to predominate in an equilibrated mixture.

The observation that etizolam showed lower reactivity than estazolam indicates that a chlorobenzene ring contributes more to the electron deficiency of the 5-carbon than a thiophene

10) J.H. Espenson, Homogenous inorganic reactions. In E.S. Lewis (ed.), "Investigation of Rates and Mechanisms of Reactions," 3rd ed., Part I, Wiley-Interscience, New York, N.Y., 1974, p. 558.

ring. The similar trend with diazepam and clotiazepam may be explained in the same way.

The forward reaction rate constant of etizolam was greater than the reverse reaction rate constant. This may be explained by interference with the cyclization by steric hindrance due to the chloro substituent in the 5-phenyl group.

Although the reaction rate constants of pyrazolodiazepinones[4] were not reported, their reactivity appears to be much greater than that of clotiazepam and greater than that of diazepam.[5] These results indicate that the reactivity is dependent on the structure of the A-ring (see formula), but it is difficult to reach a definite conclusion because of differences in the substituents in the A-ring and the 5-phenyl ring.

Effect of Temperature

Arrhenius-type rate *vs.* temperature plots for data obtained at three temperatures for estazolam (10°, 15°, and 20°) and etizolam (25°, 37°, and 50°) were linear (not shown) and the calculated activation energies are tabulated in Table I.

Table I. Rate Constants[a] and Activation Energies of the Forward and Reverse Reactions in 0.1N HCl at 37°

Drug	Forward reaction		Reverse reaction		Percentage at equilibrium %
	Rate const. hr^{-1}	Ea kcal/mol	Rate const. hr^{-1}	Ea kcal/mol	
Estazolam	25.5[b]	18.0	32.4[b]	18.1	44[b]
Etizolam	0.671	17.3	0.283	15.0	30

a) Average of two determinations.
b) Calculated by means of the Arrhenius equation from data obtained at lower temperatures.

pK_a of Etizolam

The pK_a value of etizolam was calculated to be 2.76±0.03 (SE). Since the reported pK_a value of clotiazepam is 4.11±0.05,[8] the lower value in etizolam may be explained by reduced electron density on the nitrogen at the 4-position in the presence of the triazolo ring.

The pK_a value of estazolam could not be calculated because of its extremely fast reaction over the pH region around its pK_a. No means of obtaining pK_a values is available for drugs which react rapidly under the conditions of pK_a measurements.

General Discussion

Near body temperature, reversible reactions involving hydrolysis of the 4,5-azomethine bonds of estazolam and etizolam are expected to take place in the same way as with diazepam.[5] Since the magnitude of the reaction rate constants differs greatly among drugs, reactivity in ring-opening reactions is vary dependent on the A-ring (see formula) and the triazolo ring of diazepines.

The experimental data obtained in the present *in vitro* study suggest that after administration of these drugs in dosage forms, a fraction of the drug dissolved in the stomach is quickly hydrolyzed to form the open-ring compound because of the acidic pH of the stomach contents. Because of the large k_f values, *i.e.* short $t_{1/2}$ values, the hydrolytic cleavage is expected to reach equilibrium in a short time. When the open-ring compounds pass from the stomach to the intestine, they are expected to revert to the parent drugs upon increase in the environmental pH. Therefore, there will be little loss of drug bioavailability. However, measurements of dissolution rates of estazolam and etizolam in simulated gastric fluids should be made in the light of the knowledge that a fraction of the dissolved drug is hydrolyzed to form the open-ring compounds which exhibits a different absorption spectrum.

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