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REVERSIBLE RING-OPENING REACTION OF DIAZEPAM IN ACID MEDIA AROUND BODY TEMPERATURE

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SUMMARY

The hydrolytic reaction of diazepam in acid solution at body temperature was studied spectrophotometrically. The reversible azomethine bond cleavage reaction of diazepam took place and an open-ring compound was in equilibrium with a closed-ring compound (protonated diazepam) The rate of ring closure was found to be greater than that of ring-opening, indicating that the concentration at equilibrium of the closed-ring compound is larger than the open-ring species. The activation energies of the forward and reverse reactions were obtained from Arrhenius type plots. In addition, the effect of pH on these reactions was examined.

INTRODUCTION

Although the degradation kinetics of benzodiazepines at elevated temperatures have been studied and the degradation products such as benzophenones (2-amino-5-chlorobenzophenone, 2-methylamino-5-chlorobenzophenone, or 2-amino-5-nitrobenzophenone) and quinolones (3-amino-6-chloro-1-methyl-4-phenyl-2(1H)-quinolone and 3-amino-6-nitro-4-phenyl-2-(1H)-quinolone) have been identified (Carstensen et al., 1971; Mayer et al., 1972, 1974; Maulding et al., 1975; Han et al., 1976, 1977a, b), no detailed study on the reaction of benzodiazepines at body temperature has been published. The authors (Kohri et al., 1978) as well as others (Yoneda et al., 1977; Umezawa et al., 1977) recently observed spectral changes of benzodiazepines in acid solution at 37°C. Since the rate of the reaction at body temperature has been found to be fast enough to warrant further study in view of possible effects on the bioavailability of these drugs following oral administration of dosage forms containing such drugs, the authors have further investigated the reaction of diazepam in acid solution. The results indicate that a ring-opening reaction takes place at an appreciable rate even at body temperature, and that the open-

ring compound is in equilibrium with a closed-ring compound (protonated diazepam) and reverts back to diazepam when the pH value of the medium is increased.

MATERIALS AND METHODS

Materials

Diazepam and 2-glycyl(methyl)amino-5-chlorobenzophenone hydrochloride were generously supplied by Yamanouchi Pharmaceutical Co., Tokyo and Pharmaceutical Division, Sumitomo Chemical Co., Osaka, respectively. 2-Methylamino-5-chlorobenzophenone was synthesized following a standard procedure (Sternbach et al., 1962). Chloroform and ethanol were distilled prior to use. Hydrochloric acid, phosphate salts, and citric acid were of reagent grade and were purchased from Wako Pure Chemical Industries, Osaka.

Kinetic studies

All spectra were recorded on a ACTA MVI spectrophotometer (Beckman Instruments, Fullerton, Calif.) Concentrated solutions of diazepam (6.56×10^{-4}–8.30×10^{-3} M) in ethanol were employed as stock solutions. The stock solution was diluted with preincubated 0.1 N HCl (resultant solvent: water : ethanol = 19 : 1) to prepare the test solution of diazepam. In order to prepare the solution of the chloroform-unextractable species in situ, unreacted diazepam was selectively extracted into chloroform from the mixture which was obtained by incubating the diazepam test solution in 0.1 N HCl at 37°C.

In this study, no neutral salt was added to the test solution to raise its ionic strength since little effect of ionic strengths on the rate of reaction of diazepam is expected from kinetic considerations (Han et al., 1977a), although this should be verified experimentally. The pH value of the test solution in 0.1 N HCl was 1.1 and did not change significantly during experiments. Experiments were carried out in duplicate and the mean values for the rate constants were obtained. Deviations from the mean in each set of experiments were less than 3%. For experiments at pH 2.2 and 7.4, the buffer prepared from 0.1 M citric acid and 0.2 M Na_2HPO_4 (19.6 : 0.4) and 0.1 M phosphate buffer were used, respectively.

Kinetic studies in the spectrophotometer cell

In order to calculate rate constants without sampling procedures, reactions were carried out in the cell. The molar absorptivity of the chloroform-unextractable species, which is required for calculation of the concentration of the species, was calculated based on the following assumptions: (1) protonated diazepam is quantitatively extractable with chloroform, (2) the chloroform-unextractable species is not extracted with chloroform at all, (3) no compound other than this species is formed from protonated diazepam under the experimental conditions, and (4) it is cyclized quantitatively to form diazepam at pH 7.4 so that its concentration can be calculated from the amount of diazepam formed.

One-centimeter cells equipped with tight caps were placed in a thermoregulated rectangular cell holder, the temperature of which was maintained constant by means of a

portable circulator (model FJ, Haake, Karlsruhe, G.F.R). In one set of experiments, the stock solution was diluted directly in the cell placed in the thermoregulated cell holder. In another set of experiments, a 3-ml portion of the solution of the chloroform-unextractable species, prepared in situ, was quickly placed in the spectrophotometer cell. In both sets of experiments, a repeat scan time accessory for ACTA M-series spectrophotometer was employed to obtain a series of spectra of the solution in the cell at predetermined time intervals.

Employing the molar absorptivity of protonated diazepam and the chloroform-unextractable species, the concentrations of both species were computed from the observed absorbance in the UV spectra. The forward and reverse reaction rate constants for the reversible first-order reaction were obtained using the following equations (Espenson, 1974):

$$\log \frac{P_t - P_\infty}{P_0 - P_\infty} = -\frac{k_f + k_r}{2.303} t \quad (1)$$

$$K_{eq} = \frac{k_f}{k_r} = \frac{P_0 - P_\infty}{P_\infty} \quad (2)$$

where P_0, P_t and P_∞ are concentrations or fractions of unreacted diazepam at times zero, t, and infinity, respectively; k_f and k_r are rate constants for the forward and reverse reactions, respectively, and K_{eq} is an equilibrium constant.

Kinetic studies in the constant temperature bath

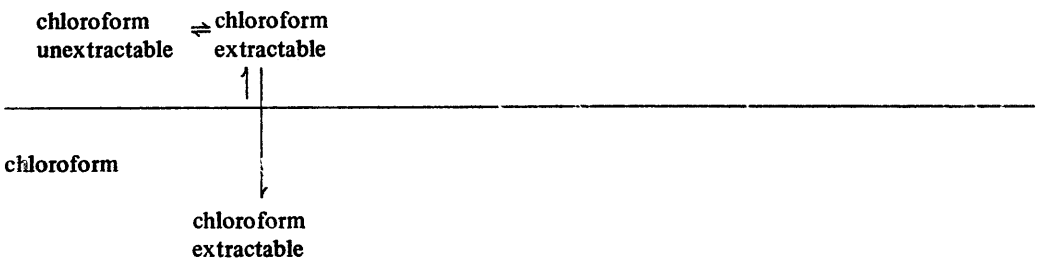
In order to determine the concentrations of both protonated diazepam and the chloroform-unextractable species separately at each sampling time, the sampled solution was subjected to extraction. In this experiment, it is assumed that protonated diazepam is quantitatively extracted into chloroform whereas the chloroform-unextractable species remains totally in the aqueous layer.

The diazepam stock solution was diluted with preincubated hydrochloric acid in a 100-ml volumetric flask placed in a thermostated waterbath (Thermounit C-600 with Taitec bath, Taiyo Kagaku Kogyo Co., Tokyo). At predetermined time intervals, a 5-ml aliquot of the solution was pippeted into a test tube with glass stopper and immediately shaken with a 5-ml portion of chloroform. Spectra of both the chloroform layer (corresponding to protonated diazepam) and the aqueous layer (corresponding to the chloroform-unextractable species) were immediately taken. Equations 1 and 2 were employed to calculate rate constants.

Partition kinetic experiments in the constant temperature bath

In order to determine the rate constant of the reverse reaction, a sink condition as shown in Scheme 1 was employed. Thus, protonated diazepam formed from the chloroform-unextractable species in 0.1 N HCl solution was quickly extracted into chloroform. In this experiment, it is assumed that the rate of transfer of protonated diazepam from the aqueous to the organic layer is much faster than the role of formation of protonated diazepam from the chloroform-unextractable species.

A stoppered Erlenmeyer flask containing 100 ml of the diazepam test solution was



Scheme 1. Partition behaviors of two species.

placed with water in a jacketed beaker connected to the thermostated waterbath (Thermounit C-600 with Taitec bath) maintained at 37°C and the solution was equilibrated for 15 h. Then 100 ml of prewarmed chloroform was added and the lower chloroform layer was agitated by means of a magnetic bar. The aqueous layer was sampled at predetermined time intervals and the concentration of the chloroform-unextractable species was immediately measured spectrophotometrically. The following equation was employed to calculate the rate constant, k_r:

$$\log \frac{A_t}{A_0} = -\frac{k_r}{2.303} t \tag{3}$$

where A_t and A_0 are absorbance values due to or concentrations of the chloroform-unextractable species at times t and zero, respectively.

RESULTS AND DISCUSSION

Nature of a reaction and properties of a product

The spectral change of diazepam in acid solution (0.1 N HCl) at 37°C is shown in Fig. 1. The following observations indicate that the fact that no further spectral change occurred after 11 h was due to attainment of the equilibrium state for a reversible reaction rather than completion of the reaction. The spectrum of the aqueous layer, following extraction with chloroform of the acid solution equilibrated for more than 11 h, was quite different from that of the equilibrated solution (before extraction). This observation indicates the presence of at least two species which markedly differ in their partition coefficients in the equilibrated solution.

Since diazepam was quantitatively extracted into chloroform immediately after acidification of aqueous diazepam solution, protonated diazepam was shown to be chloroform-extractable. The species which remained in aqueous solution after chloroform extraction was shown to be essentially unextractable by chloroform, since the second extraction with the same solvent did not affect the spectrum of the aqueous layer to a significant extent.

The reversible nature of the reaction was demonstrated by the formation of protonated diazepam from the chloroform-unextractable species. A spectrum of the

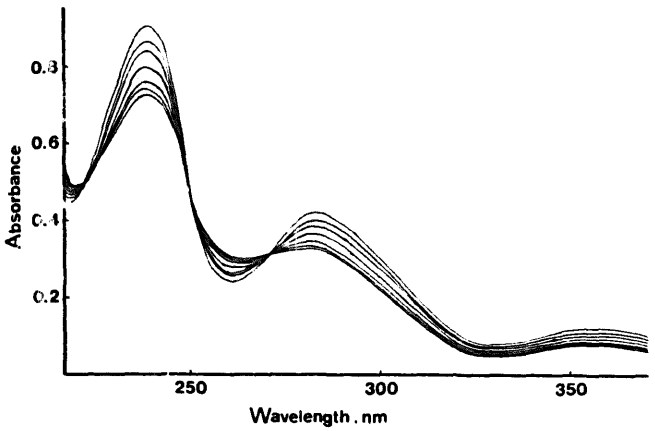


Fig. 1. Typical spectral changes for the hydrolysis of 3.28×10^{-5} M diazepam in 0.1 N HCl at 37°C. Absorbance at 238 nm decreased with time (0, 0.5, 1, 2, 4, 6, and 11 (∞) h).

chloroform-unextractable species with a λ_{max} of 255 nm, which was taken immediately following the extraction (Fig. 2, 0 h), hanced with time to give a spectrum (Fig. 2, 15 h) which is quite similar to that of the equilibrium mixture starting from protonated diazepam (Fig. 1, 11 h). In addition, the species extracted by chloroform from the equilibrated mixture starting from the chloroform-unextractable species was identified as protonated diazepam.

Fig. 3 depicts changes in concentrations of both species as obtained by separate determinations of each species. As approach to and an eventual attainment of the equilibrium is apparent.

Results of partition kinetic experiments are shown in Fig. 4. An essentially first-order decrease in the amount of the chloroform-unextractable species in the aqueous layer with time was observed. Thus, when one species is removed from the system as soon as it is formed, the reaction can go into completion. This process may be used to synthesize diazepam from the chloroform-unextractable species in aqueous solutions.

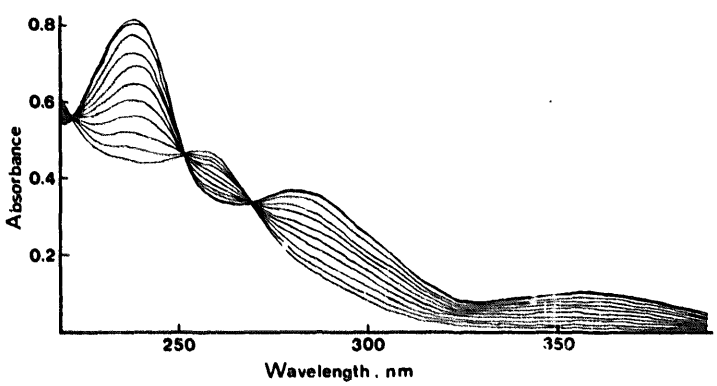


Fig. 2. Typical spectral changes for the ring closure reaction of 3.73×10^{-5} M of the chloroform-unextractable species in 0.1 N HCl saturated with chloroform at 37°C. Absorbance at 238 nm increased with time (0, 0.25, 0.58, 1, 1.5, 2.17, 3, 4, 6, 11, and 15 (∞) h).

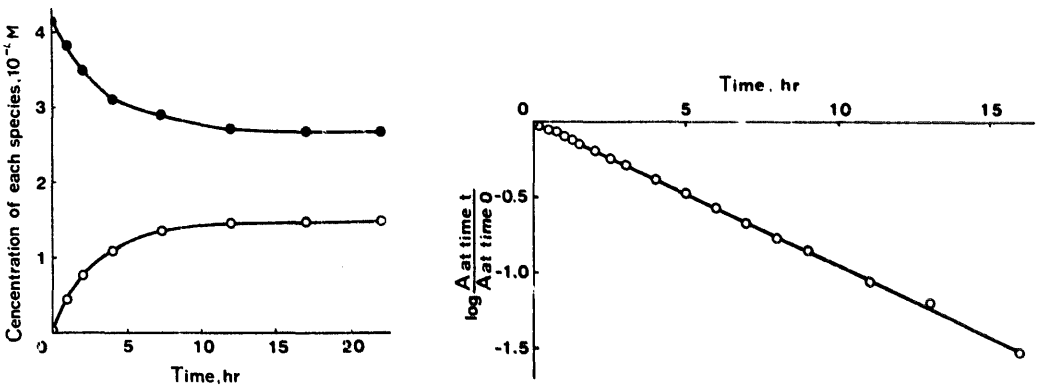


Fig. 3. Separately determined concentrations of protonated diazepam (•) and the chloroform-unextractable species (○) for the hydrolysis of 4.15×10^{-4} M diazepam in 0.1 N HCl at 37°C.

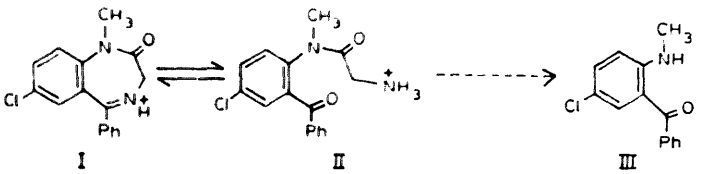
Fig. 4. Change in absorbance (A) with time of the chloroform-unextractable species in the aqueous layer (0.1 N HCl) in the partition kinetic experiment using chloroform at 37°C. Diazepam solution (1.64×10^{-4} M) was incubated in 0.1 N HCl at 37°C for 15 h prior to the partition experiment.

Structural assignment of a chloroform-unextractable species

Although definite identification of the chloroform-unextractable species is yet to be achieved, a tentative assignment of a possible structure of the species may be made on the basis of spectrophotometric studies. The initial assignment of 2-glycyl(methyl)amino-5-chlorobenzophenone (II) for the structure of the chloroform-unextractable species was based on the observation that the UV spectrum of the chloroform-unextractable species in 0.1 N HCl solution was quite similar to (but not identical with) that of an authenic sample of 2-methylamino-5-chlorobenzophenone (III). Thus the chloroform-unextractable species is likely to have a structure similar to that of III. The chloroform-unextractable nature of the species suggests the presence of an ionic group in the molecule. III itself cannot be assigned to the structure of the chloroform-unextractable species because III is chloroform-extractable.

Recent procurement of an authentic sample of II hydrochloride (Inaba et al., 1975) and its kinetic and spectrophotometric identity with the chloroform-unextractable species made the assignment of II for the chloroform-unextractable species more certain. Specifically, the authentic sample of II, which gave a spectrum identical to that of the chloroform-unextractable species at time zero, reacted with the same rate constant ($k_r = 0.25\ h^{-1}$) as the chloroform-unextractable species ($k_r = 0.23\ h^{-1}$, see Table 1) to give a spectrum which was identical to the spectrum of Fig. 2 at time infinity. Therefore, the spectrum of the acidic solution immediately following chloroform extraction of the equilibrated solution (Fig. 2 at time zero) corresponds to II unless any other chloroform-unextractable products are present.

Thus, the reaction of diazepam in acid solution (protonated diazepam I) may be represented tentatively as follows.



I II III

A definite identification of the chloroform-unextractable species could not be made since it could not be isolated from aqueous solutions as such because of the reversible nature of the reaction in 0.1 N HCl and because it is rapidly cyclized to form diazepam at pH values above the pKa value of diazepam (3.3, Barrett et al., 1973). The cyclization reaction at neutral pH is so fast at 37°C that a spectrum of the solution immediately following neutralization of the solution of the chloroform-unextractable species by addition of pH 7.4 phosphate buffer corresponded to that of diazepam. Thus, any attempt to extract the unionized form of the chloroform-unextractable species into organic solvent after neutralization failed.

Quantitative aspects

The molar absorptivity of an authentic sample of II was found to be only 3% greater than that of the chloroform-unextractable species calculated under assumptions cited in the *Kinetic Studies* section. The decrease in percenf of protonated diazepam with time calculated from data such as shown in Fig. 1, is plotted in Fig. 5 (solid circles), while the increase in percent of protonated diazepam with time, calculated from data such as shown in Fig. 2 (100 minus the percent of the chloroform-unextractable species), is plotted in the same figure (open circles). Slight disagreement in the equilibrium values is observed in the figure, possibly because of experimental errors involved in separate experiments and errors in computation of concentrations of the chloroform-unextractable species.

Reaction rate constants calculated according to Eqns. 1 and 2 and 3 are recorded in Table 1. Although the general magnitude of rate constants agreed reasonably, they differed

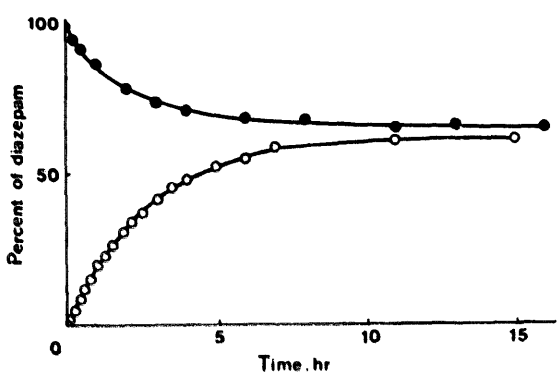


Fig. 5. Decrease in protonated diazepam with time starting with diazepam (•) and increase in protonated diazepam with time starting with the chloroform-unextractable species (○) in 0.1 N HCl solution at 37°C.

TABLE 1

RATE CONSTANTS OF RING-OPENING AND RING-CLOSURE REACTIONS ESTIMATED BY FIVE DIFFERENT PROCEDURES

Estimation procedure	Rate constant at 37°C (h^{-1})	
	Ring-opening	Ring-closure
Spectral change starting from protonated diazepam, Figs. 1 and 5	0.15	0.30
Spectral change starting from the chloroform-unextractable species, Figs. 2 and 5	0.14	0.23
Assay of diazepam extracted into chloroform layer, Fig. 3	0.099	0.18
Assay of the chloroform-unextractable species remaining in aqueous layer, Fig. 3	0.13	0.24
Decrease in absorbance in aqueous layer during partition kinetic experiments, Fig. 4	–	0.22

somewhat from each other depending on the experimental method employed. In any case, the rate constant of ring-closure was greater than that of ring-opening. This observation indicates a greater concentration of the closed-ring compound (protonated diazepam) than the chloroform-unextractable species in equilibrium.

Effect of temperature

The rate constant at 25 and 50°C determined from spectral changes similar to that shown in Fig. 1, are presented in Table 2 along with the calculated percentages of protonated diazepam in the equilibrated mixtures. The energies of activation of the forward and reverse reactions, calculated from the Arrhenius equation, were 18.3 ± 1.2 (S.E.) kcal/mol for the ring-opening reaction and 15.7 ± 0.9 (S.E.) kcal/mol for the ring-closure reaction.

TABLE 2

RATE CONSTANTS OF RING-OPENING AND RING-CLOSURE REACTIONS AT THREE TEMPERATURES

Temperature (°C)	Rate constant (h^{-1})		Protonated diazepam at equilibrium (%)
	Ring-opening	Ring-closure	
25	0.033	0.086	72
37	0.15	0.30	67
50	0.49	0.79	62

Effect of pH

The preliminary studies indicated that spectral change similar to that shown in Fig. 1 took place in the buffer solution at pH 2.2. However the change in absorbance was smaller than that in 0.1 N HCl solution, indicating that the reaction proceeded to a lesser extent. At pH values higher than the pKa of diazepam, little change in absorbance with time was observed. As indicated earlier, a possible open-ring compound, once formed in acid solutions, reverts back to diazepam when the pH of the medium is raised.

GENERAL DISCUSSION

Near body temperature, the reversible reaction involving hydrolysis of the 4,5-azomethine bond of diazepam is expected to be a major reaction. At higher temperatures, on the other hand, the open-ring compound further degrades to form 2-methylamino-5-chlorobenzophenone as reported by several investigators (de Silva et al., 1964; Carstensen et al., 1971; Mayer et al., 1972; Han et al., 1977a). For low water concentrations or at extreme temperature, formation of a quinolone can become appreciable (Carstensen et al., 1971; Mayer et al., 1972).

The experimental data obtained in the present in vitro study suggests that after administration of a dosage form of diazepam, somce diazepam dissolved in the stomach is hydrolyzed to form the open-ring compound because of the acidic pH of stomach contents. When the open-ring compound enters the intestine, it will revert back to diazepam as a result of the increase in pH. Therefore, there will be little loss in drug bioavailability.

Although it has been predicted that recyclization of the open-ring species of diazepam would not occur at pH value below its pKa (Han et al., 1977a), the present studies suggest that such a recyclization does take place even if the rate of recyclization is much smaller than that at pH values above pKa. Mechanistic aspects of the reaction will be discussed later when more information is obtained from further studies on hydrolysis of other benzodiazepines, now in progress. The reversible ring-opening hydrolysis at the 4,5-azomethine bond may not be general among all benzodiazepines since a preliminary study on hydrolysis of oxazepam showed that oxazepam behaved differently from diazepam in 0.1 N HCl solution at 37°C.

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