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## The Behavior of 1,4-Benzodiazepine Drugs in Acidic Media. VI.<sup>1)</sup> Hydrogen Exchange Reaction and Proton and Carbon-13 Nuclear Magnetic Resonance Spectra of Estazolam

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Estazolam deuterated in the triazole ring was isolated and its carbon-13 nuclear magnetic resonance signals were assigned in relation to those of estazolam. The coupling constant ratio,  $^1J_{^{13}\text{C}^1\text{H}}/^1J_{^{13}\text{C}^2\text{H}}$ , for the triazole C-1 atom was 6.66. Rates of hydrogen exchange of estazolam with deuterium were measured at 23°C in acidic aqueous solution (0.5N DCl) and in methanol-*d*<sub>4</sub> containing trifluoroacetic acid-*d* and triethylamine. The exchange reaction was accelerated in the presence of acid or base, indicating that the exchange occurs in a base-catalyzed reaction and that the triazole C-1 atom of estazolam behaves as an acid.

**Keywords**—estazolam; alprazolam; benzodiazepine; hydrogen exchange reaction; <sup>1</sup>H-NMR; <sup>13</sup>C-NMR; isotope effect; carbon acid; triazole ring; reaction rate

Previously we reported the structural changes in acidic aqueous solution of 1,4-benzodiazepines (including benzodiazepinooxazoles), based mainly on the proton and carbon-13 nuclear magnetic resonance (<sup>1</sup>H- and <sup>13</sup>C-NMR) spectra.<sup>2-4)</sup> As regards triazolobenzodiazepines (I) with a 1,2,4-triazole ring condensed at the 1 and 2 positions of the 1,4-benzodiazepine ring, the reversible reaction of estazolam (IE-H, R=H in Chart 1) in acidic aqueous solution (0.1N HCl) has been investigated kinetically by Inotsume and Nakano.<sup>5)</sup> The kinetic analysis was made with reference to ultraviolet (UV) spectral changes so that the nature of the chemical species involved in the ring-cleaving reaction at the azomethine bond has not been clarified completely. Although the <sup>1</sup>H-NMR signals of IE-H in chloroform-*d* (CDCl<sub>3</sub>) have been assigned,<sup>6)</sup> the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra in acidic aqueous solution and also the <sup>13</sup>C-NMR spectrum in organic solvents have not been analyzed.

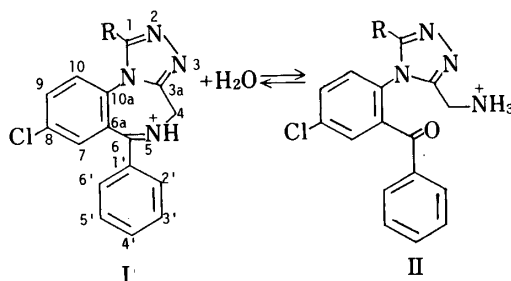


Chart 1

When the structural changes of IE-H were examined by <sup>1</sup>H- and <sup>13</sup>C-NMR spectroscopy, as a part of our continuing studies on the behavior of 1,4-benzodiazepine drugs in acidic media, an exchange reaction of hydrogen attached to the triazole ring with deuterium was

found. In this paper we describe the isolation of deuterated estazolam (IE-D), assignments of the  $^{13}\text{C}$ -NMR signals of IE-D in  $\text{CDCl}_3$  in relation to those of IE-H, and the exchange reaction rates in 0.5 N DCl and methanol- $d_4$ . For comparison with the spectra of IE-D and/or IE-H, the spectrum of alprazolam (IA,  $\text{R} = \text{CH}_3$ ) in acidic aqueous solution is also discussed.

### Experimental

**Materials and Instruments**—Estazolam (IE-H, Lot No. OB 033) and alprazolam (IA, Lot No. OB 002) were supplied by Takeda Pharmaceutical Co. and were used without further purification. All other chemicals were purchased and were of reagent grade. The  $^1\text{H}$ -NMR spectra were obtained with a JEOL JNM-MH 100 or JNM-FX 100 spectrometer at 100 MHz. The  $^{13}\text{C}$ -NMR spectra were recorded on a JEOL JNM-FX 100 or JNM-GX 400 spectrometer at 25 or 100 MHz. The mass spectra (MS) were obtained with a JEOL DX-300 mass spectrometer.

**Isolation of Deuterated Estazolam**—A solution of 150 mg of IE-H in 2.5 ml of 0.5 N DCl was stirred at room temperature for 4 d. The reaction mixture was neutralized with 2 N NaOD and extracted with chloroform. The organic layer was washed with  $\text{D}_2\text{O}$ , and dried with  $\text{MgSO}_4$ . Removal of the solvent gave 120 mg of colorless prisms (from methanol- $d_4$ ), mp 232.5–234 °C (uncorrected).

**Measurement of NMR Spectra**—A solution was prepared by dissolving 20 mg of the sample in 0.4 ml of 0.5 N deuterium chloride (DCl). The conditions of measurement for the  $^{13}\text{C}$ -NMR spectra of 25 MHz were as follows: spectral width, 6000 Hz with 8K memory points; repetition time, 2.0 s; pulse width, 5.0  $\mu\text{s}$ ; number of scans accumulated, 2000 to 8000. When the isotope effect in deuterated estazolam was examined, the resolution employed for the  $^{13}\text{C}$ -NMR measurement was 0.73 Hz/point at 100 MHz, and the concentration was 45 mg of sample in 0.4 ml of  $\text{CDCl}_3$ . The chemical shift values ( $\delta$ ) are expressed in ppm relative to tetramethylsilane used as an internal or external standard, and the coupling constants ( $J$ ) are expressed in hertz (Hz). Two equilibrium species derived from estazolam in 0.5 N DCl solution were assigned by using relative signal intensities.

**Kinetic Measurement of the Hydrogen Exchange Reaction of Estazolam**—Solutions studied were prepared by dissolving about 20 mg of IE-H in 0.4 ml of 0.5 N DCl or methanol- $d_4$ . The solutions were placed in a constant-temperature bath (23 °C), and the integrated areas of the 1-hydrogen signal were compared with the aromatic hydrogen signal area (as a nonexchanging standard) as a function of time. The pseudo first-order rate constant ( $k$ ) was calculated from the slope of a linear plot based on the following equation:  $\log[8(1\text{-H signal})/(\text{aromatic H signal})] = -k \cdot t/2.303 + \text{constant}$ , where 8(1-H signal) is eight times the integrated area of the 1-proton signal and (aromatic H signal) is the integrated area of the aromatic proton signal.

### Results and Discussion

#### MS and $^{13}\text{C}$ -NMR Spectra

To confirm the structure of deuterated estazolam (IE-D), the mass spectra of IE-D and IE-H were measured and the data are summarized in Table I. IE-H exhibited a molecular ion peak at  $m/e$  294 and IE-D at  $m/e$  295. The mass numbers of fragment peaks of IE-D were greater by one atomic mass unit than those of IE-H. These results confirm that only one IE-H hydrogen is replaced by deuterium in IE-D. The chlorine atom was removed in similar fragmentations in both IE-H and IE-D. The IE-D fragmentation occurs without removal of deuterium.

When the  $^1\text{H}$ -NMR spectra of IE-D and IE-H in  $\text{CDCl}_3$  were compared, a sharp singlet at  $\delta$  8.68 due to the triazole ring proton (1-position) of IE-H<sup>6)</sup> was absent in the case of IE-D, while the other signals of IE-H were nearly identical with those of IE-D. These Mass and  $^1\text{H}$ -NMR results clearly show that IE-D has the structure in which the hydrogen alone attached to the triazole ring of IE-H is exchanged with deuterium.

Table II shows the assignments of carbon signals of IE-D and its parent compound (IE-H) in  $\text{CDCl}_3$ . Fourteen signals were observed in the  $^{13}\text{C}$ -NMR spectrum of IE-H, which were carefully assigned with reference to the spectra of alprazolam,<sup>7)</sup> triazolam,<sup>8)</sup> diazepam,<sup>4)</sup> and fludiazepam<sup>4)</sup> reported already. Among these signals, the signal at  $\delta$  141.05 of IE-H ( $^1J_{^{13}\text{C}^1\text{H}} = 213$  Hz) is attributable to the C-1 atom. In the  $^{13}\text{C}$ -NMR spectrum of IE-D, the signal at  $\delta$  141.05 was absent and a new triplet appeared at  $\delta$  140.89. The signal ( $^1J_{^{13}\text{C}^2\text{H}} = 32$  Hz) presumably resulted from an isotope-induced upfield shift of 0.16 ppm, because the exchange

TABLE I. Mass Spectral Data for Estazolam (IE-H) and Deuterated Estazolam (IE-D)

Compound	M <sup>+</sup>	M <sup>+</sup> - 35			
IE-H	294 (82.9%)	259 (100%)	239 (55.7%)	231 (25.6%)	205 (88.2%)
IE-D	295 (100%)	260 (92.3%)	240 (26.0%)	232 (19.6%)	206 (35.3%)

Relative intensities (%) are given in parentheses.

TABLE II. <sup>13</sup>C-NMR Chemical Shifts of Estazolam (IE-H) and Deuterated Estazolam (IE-D) in CDCl<sub>3</sub> at 100 MHz

Carbons	IE-H	IE-D	Carbons	IE-H	IE-D
C-1	141.05 <sup>a)</sup>	140.89 <sup>b)</sup>	C-9	132.48	132.48
C-3a	153.94	153.92	C-10	123.73	123.73
C-4	45.85	45.84	C-10a	132.34	132.32
C-6	168.17	168.17	C-1'	139.04	139.04
C-6a	129.15	129.14	C-2',6'	129.40	129.40
C-7	132.07	132.07	C-3',5'	128.43	128.43
C-8	133.15	133.15	C-4'	130.85	130.85

a) <sup>1</sup>J<sub>13C1H</sub> = 213 Hz. b) 1:1:1 triplet, <sup>1</sup>J<sub>13C2H</sub> = 32 Hz.

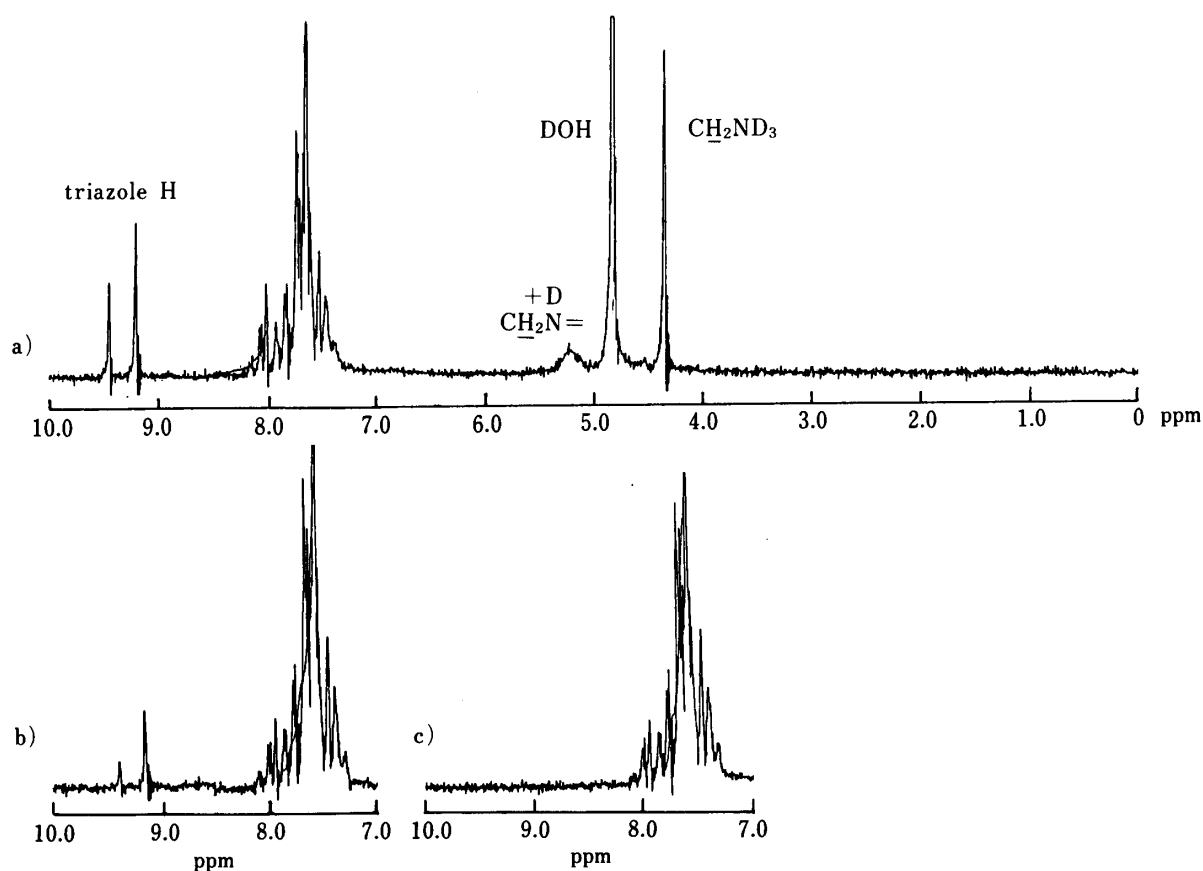


Fig. 1. <sup>1</sup>H-NMR Spectral Changes of Estazolam in 0.5 N DCl with Time at 23°C  
a, 0.5 h; b, 23 h; c, 100 h.

of a hydrogen attached to a carbon atom with deuterium causes splitting to a characteristic 1:1:1 triplet due to  $^{13}\text{C}$ - $^2\text{H}$  coupling.<sup>9)</sup> The  $^1J_{^{13}\text{C}^1\text{H}}/^1J_{^{13}\text{C}^2\text{H}}$  value is 6.66, which is close to the value of 6.55<sup>9)</sup> observed generally for the coupling constant ratio. The other carbon signals of IE-D were in close agreement with those of IE-H. An upfield shift of 0.02 ppm was observed for C-3a and C-10a. These carbons should show an isotope-induced shift because they are relatively close to the C-1 atom.

### $^1\text{H}$ -NMR Spectra of Estazolam in 0.5 N DCl

Prior to the measurement of the hydrogen exchange rate in acidic aqueous solution, assignment of  $^1\text{H}$ -NMR signals of IE-H in solution is necessary because of the existence of the two equilibrium species<sup>5)</sup> as shown in Chart 1. Figure 1a shows the  $^1\text{H}$ -NMR spectrum in 0.5 N DCl solution, in which IE-H attained equilibrium.<sup>5)</sup> Both a broad singlet at  $\delta$  5.22 and a sharp singlet at  $\delta$  4.36 observed in the upfield region were due to the aliphatic protons. The sum of the signal intensities of the two signals was equivalent to two protons, when calculation was made based on the signal intensity of aromatic protons at  $\delta$  7.30–8.20. Accordingly, these signals were assigned to methylene protons derived from the two chemical species present in the solution. Of these two methylene proton signals, the broad singlet at  $\delta$  5.22 was considered to be due to IE-H with deuterium on the nitrogen atom at position 5, on the basis of the assumption that the methylene protons of IE-H in chloroform-*d* give a very broad singlet.<sup>6)</sup> The sharp singlet at  $\delta$  4.36 was, thus, assignable to the methylene protons of IIE-H having the benzodiazepine ring-cleaved structure. The ratio of the chemical species present in the equilibrium solution (IE-H/IIE-H) was about 3/7 as estimated from the signal intensities.

The singlets at  $\delta$  9.44 and 9.19 in the downfield region were both due to triazole ring protons, because the signals diminished gradually as shown in Figs. 1b and 1c and no such signal was seen in the case of alprazolam (IA, R =  $\text{CH}_3$ ). The spectrum of IA (not shown here), instead of the signals in the downfield region, gave a singlet at  $\delta$  2.47 corresponding to 3H, due to methyl protons on the triazole ring. The sum of the triazole proton signals in Fig. 1a does not afford a signal intensity corresponding to one proton because of the slow exchange reaction with deuterium in the solution. The signals at  $\delta$  9.44 and 9.19 are, however, assignable to IE-H and IIE-H, respectively, based on the ratio of the signal intensity at  $\delta$  9.44 to that at  $\delta$  9.19 (3:7).

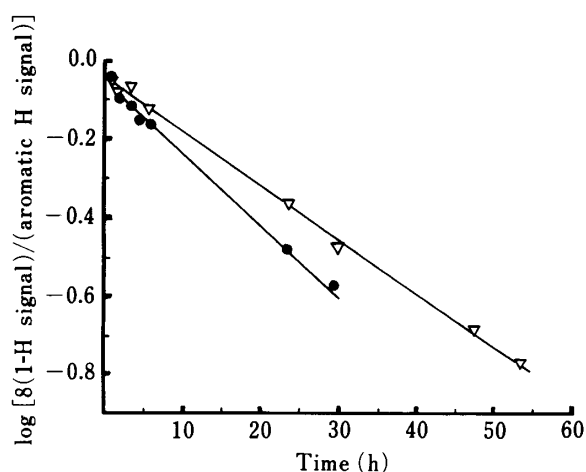


Fig. 2. First-Order Plots for the Hydrogen Exchange of Estazolam at 23 °C

●, 0.5 N DCl; ▽, methanol- $d_4$ .

TABLE III. Hydrogen Exchange Rate Constants of Estazolam at 23 °C

Solvent system	$k \times 10^2$ ( $\text{h}^{-1}$ )	Correlation coefficient	$t_{1/2}$ (h)
0.5 N DCl	4.02 (4.02 <sup>a</sup> )	0.9978 (0.9978 <sup>a</sup> )	17.2 (17.2 <sup>a</sup> )
Methanol- $d_4$	3.16	0.9982	21.9
Methanol- $d_4$ + TEA <sup>b</sup>	3.66	0.9976	18.9
Methanol- $d_4$ + TFA <sup>b</sup>	4.02	0.9978	17.2

<sup>a</sup>) Values calculated from the signal at  $\delta$  9.19 due to IIE-H. <sup>b</sup>) The amount of triethylamine (TEA) or trifluoroacetic acid-*d* (TFA) added was 2.5 eq with respect to estazolam.

### Exchange Rate Constant of the Triazole Ring Hydrogen of Estazolam

The exchange reaction of hydrogen attached to the triazole ring of IE-H was observed not only in 0.5 N DCl but also in methanol- $d_4$ , having exchangeable deuterium. The exchange reaction of hydrogen with deuterium was an apparent first-order reaction with respect to IE-H as shown in Fig. 2, and the rate constants were obtained from the analysis described in the experimental section. The accuracy of rate constants determined by the NMR method is generally  $\pm 5\%$ .<sup>10)</sup> The rate constants along with the respective correlation coefficients are summarized in Table III. The constant for IIE-H in 0.5 N DCl was identical with that for IE-H. The rate of IE-H in methanol- $d_4$  was promoted in both acidic and basic systems, containing trifluoroacetic acid- $d$  (TFA) and triethylamine (TEA), respectively.<sup>11)</sup> The exchange rate constant in the presence of TFA was  $4.0 \times 10^{-2} \text{ h}^{-1}$  and was the same as that in 0.5 N DCl. The exchange reaction, therefore, is probably a base-catalyzed reaction, as is the reaction observed for heterocyclic compounds,<sup>13)</sup> and the C-1 atom of IE-H behaves as a carbon acid.

IE-D isolated from IE-H was dissolved in methanol or 0.5 N HCl for hydrogen exchange, regenerating IE-H and thus demonstrating that the hydrogen exchange reaction is reversible. The rate constant for the reverse exchange reaction with hydrogen, however, could not be determined accurately because of large  $^1\text{H}$ -NMR signals due to methanol and water.

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