[Chem. Pharm. Bull. 33(12)5503—5510(1985)]

The Behavior of 1,4-Benzodiazepine Drugs in Acidic Media. IV.[1] Proton and Carbon-13 Nuclear Magnetic Resonance Spectra of Diazepam and Fludiazepam in Acidic Aqueous Solution[2]

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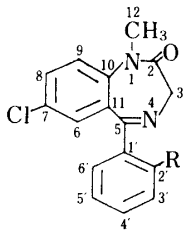
(Received May 1, 1985)

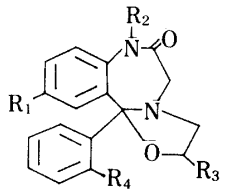
Chemical species of diazepam and fludiazepam in an acidic aqueous solution (0.5 N DCl) were studied by means of proton and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy. These compounds were present in the form of a protonated iminium structure immediately after preparation of the solution and subsequently underwent slow hydrolysis of the iminium moiety to give a ring-opened benzophenone structure. The structures produced were in equilibrium with the corresponding ring-closed iminium structures. Furthermore, the benzophenone structures were present as an equilibrium mixture of rotating isomers around an amide (*cis* and *trans* isomers). The proportion of the benzophenone structure of fludiazepam in the equilibrium solution was greater than that of diazepam. The ratio of rotating isomers of the benzophenone structure that arose from fludiazepam was almost identical to that from diazepam. Assignments of the characteristic proton and carbon-13 resonances of these species and of the aromatic carbon-13 resonances of the iminium structure were carried out.

Keywords——diazepam; fludiazepam; benzodiazepine; acidic media; ^{1}H-NMR; ^{13}C-NMR

We previously reported structural changes in acidic aqueous solution among benzodiazepinooxazoles having oxazolidine rings at positions 4 and 5 of the benzodiazepine ring (such as oxazolam, cloxazolam, haloxazolam and flutazolam, belonging to the category of 1,4-benzodiazepine drugs), on the basis of proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectral analyses.[1,3] We here discuss 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one (diazepam) (Id) and 7-chloro-1,3-dihydro-5-(2-fluorophenyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one (fludiazepam) (If), which are representative 1,4-benzodiazepine drugs lacking an oxazolidine ring, in comparison with the above compounds.



Id: R=H diazepam
If: R=F fludiazepam



benzodiazepinooxazoles

	R_1	R_2	R_3	R_4
oxazolam	Cl	H	CH_3	H
cloxazolam	Cl	H	H	Cl
haloxazolam	Br	H	H	F
flutazolam	Cl	$(CH_2)_2OH$	H	F

Chart 1

The structures of Id and If in acidic aqueous solution (0.1 N HCl) have been reported by Nakano and Inotsume,[4] who carried out a kinetic investigation based on ultraviolet (UV) spectral changes. They proposed that these compounds undergo a reversible ring-cleaving equilibrium at the azomethine bond of the benzodiazepine ring. However, the nature of the molecular species involved in the ring-cleaving reaction at the azomethine bond was not clarified.

Structural studies of Id on the basis of NMR spectral analysis have been carried out in organic solvents[5-7] but there is no report on the assignment of the molecular species in acidic aqueous solution.

In order to study the behavior of the molecular species of Id and If, these compounds were dissolved in 0.5 N DCl to immediately form iminium structures (IId and IIf) by the addition of deuterium to the nitrogen atom at position 4. They gave equilibrium mixtures with benzophenone structures (IIId and IIIf) formed by slow hydrolysis at the iminium moiety. Each of the compounds (IIId) and (IIIf) is also present as an equilibrium mixture of rotational isomers around the amide group (amide-rotamers). We here report these chemical changes and assignments of the ^{1}H- and ^{13}C-NMR spectra of the various molecular species.

Experimental

Materials——Diazepam (Lot No. OB 171) and fludiazepam (Lot No. K 32) were kindly supplied by Takeda Pharmaceutical Co., Ltd., and Sumitomo Pharmaceutical Co., Ltd., respectively, and were used without further purification.

Instruments——^{1}H-NMR spectra were obtained with a JEOL JNM-MH 100 or JNM-FX 100 spectrometer at 100 MHz. ^{13}C-NMR spectra were obtained with a JEOL JNM-FX 100 spectrometer at 25 MHz.

Measurement of NMR Spectra——A solution was prepared by dissolving 30 mg of the sample in 0.4 ml of chloroform-*d* ($CDCl_3$) or 20 mg of the sample in 0.4 ml of 0.5 N deuterium chloride (DCl). The conditions of measurement for the ^{13}C-NMR spectra were as follows: spectral width, 6000 Hz with 8 K memory points; repetition time, 2.0 s; pulse width, 6 μs; number of scans accumulated, 2000 to 10000. The chemical shift values (δ) are expressed in ppm relative to tetramethylsilane (TMS) used as an internal or external standard, and the coupling constans (J) are expressed in hertz (Hz). The various carbon resonances were assigned on the basis of chemical shift theory, multiplicities in single-frequency off-resonance decoupled spectra, and coupling constant values with the fluorine atom. The various temperatures shown in Fig. 2 are only approximate, having been read from the variable temperature control device of the JNM-FX 100.

Results and Discussion

^{1}H-NMR Spectra of Id and If in 0.5 N DCl

Though both Id and If showed time-dependent spectra in 0.5 N DCl, we will discuss the spectrum of If, which gave clearer spectral changes.

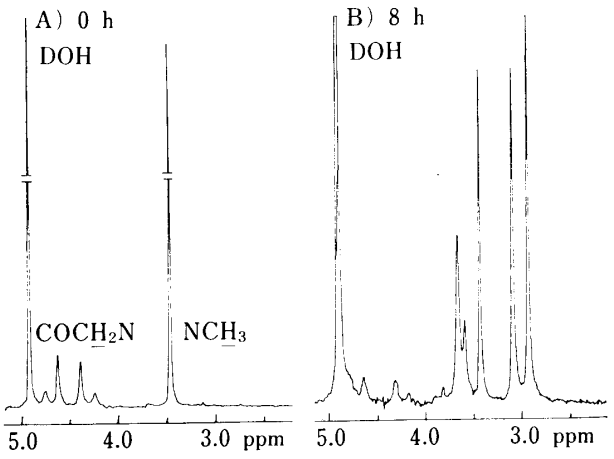


Fig. 1. The Upfield Region of the ^{1}H-NMR Spectrum of Fludiazepam in 0.5 N DCl

A) Immediately after dissolution. B) After attainment of equilibrium.

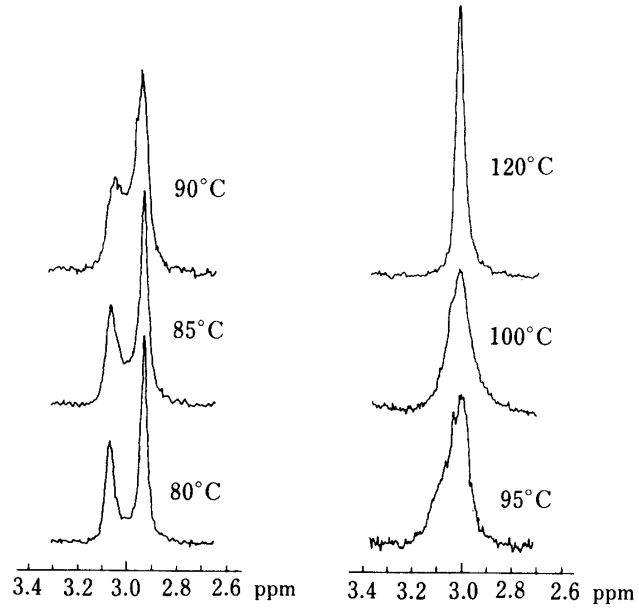


Fig. 2. The Upfield Region of the ^{1}H-NMR Spectrum of *N*-Methyl Proton Signals of IIIf at Various Temperatures in 0.5 N DCl

TABLE I. ^{1}H-NMR Spectral Data for Diazepam and Fludiazepam in 0.5 N DCl at Equilibrium[a]

	Iminium form (II)		Benzophenone form (III)			Ratio[b]	
	NCH_3	$COCH_2N$	NCH_3 *cis*	NCH_3 *trans*	$COCH_2N$	II/III	III_{cis}/III_{trans}
Diazepam	3.51 (s)	4.36 (d, J=14.0) 4.68 (d, J=14.0)	2.93 (s)	3.14 (s)	3.60—3.82 (m)	4.0	1.1
Fludiazepam	3.46 (s)	4.34 (d, J=13.0) 4.68 (d, J=13.0)	2.92 (s)	3.08 (s)	3.52—3.76 (m)	0.33	1.2

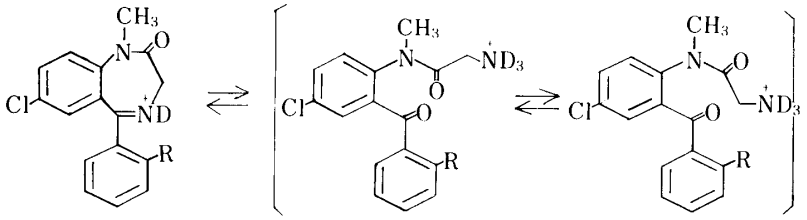
a) Coupling constants (Hz) and splitting patterns (s=singlet, d=doublet, m=multiplet) are given in parentheses. *b*) Ratios were calculated from the integrated curve of *N*-methyl signals in the equilibrium solution at 23 °C.

Figure 1 shows the ^{1}H-NMR spectra of If dissolved in 0.5 N DCl at room temperature. The spectrum of If measured immediately after dissolution (Fig. 1A) and that at equilibrium 8 h after dissolution (Fig. 1B) both gave characteristic signals. The signals of the partial spectrum shown in Fig. 1A were assignable to five aliphatic protons of IIf. The doublets at δ 4.68 and 4.34 (each corresponding to 1H) are derived from a geminal-coupled AB-type quartet (J=13.0 Hz) arising from methylene protons at position 3 of the benzodiazepine ring. The singlet at δ 3.46 (corresponding to 3H) was assignable to the methyl protons on the nitrogen atom at position 1. Other aliphatic proton signals were absent. Accordingly, the only molecular species present in the solution at time zero was IIf, formed by addition of deuterium to the nitrogen atom at position 4.

The spectrum in Fig. 1B showed, in addition to the signals of IIf found in Fig. 1A, a multiplet at δ 3.52—3.76, presumably derived from the signals of a AB-type quartet and a singlet, and two singlets at δ 3.08 and 2.92. The ratio of signal intensity was δ 3.52—3.76: δ 3.08 and 2.92=2:3 on the basis of the integrated curve. For this reason, these signals were considered to be derived from the methylene and methyl protons of molecular species newly produced in the solution. Since the equilibrium solution was neutralized to form only If, the parent compound, the two newly observed methyl proton signals suggest the presence of two structures that regenerate If in addition to IIf. These structures were produced from IIIf hydrolyzed at positions 4 and 5 of the benzodiazepine ring, as shown by the ^{13}C-NMR spectrum. It was also found that IIIf was present in the form of two kinds of molecular species

distinguishable in terms of the NMR spectrum.

Changes in the proton signals of the two methyl groups at δ 3.08 and 2.92 were observed at various temperatures to examine the two isomers of IIIf. As shown in Fig. 2, the spectra varied with temperature, and coalescence was observed at about 100 °C. This suggests that there are two forms in equilibrium that are distinguishable on the NMR time scale at room temperature. These two forms are provably rotational isomers around an amide bond (amide-rotamers)[8] (IIIf *cis* and IIIf *trans*), as shown in Chart 2.



trans *cis*

IId: R=H IIIf: R=F

IIId IIIf

Chart 2

Similar spectral changes in 0.5 N DCl were also observed for Id. The shift values are summarized in Table I. Assignments of the methyl signals of the III_{cis} and III_{trans} conformations of the amide-rotamer were based on the assumption that the methyl signal of the III_{cis} isomer, in which the carbonyl group is in a *cis* relation with regard to the methyl group, is shifted upfield[8] as compared with the methyl signal of the III_{trans} isomer, in which these two groups are in a *trans* relationship.

The percentages of IIId and IIIf (ring-opened molecular species) in the equilibrium solution were 20% and 75%, respectively, on the basis of the integrated curve of the methyl proton signals. The results are in good agreement with the equilibrium kinetic results of Nakano and Inotsume.[4] Of the two amide-rotamers of III, the *cis* isomer was predominant in both IIId and IIIf.

^{13}C-NMR Spectra of Id and If in 0.5 N DCl

Figure 3 shows the aliphatic and carbonyl carbon region of the ^{13}C-NMR spectra of If before (A) and after (B) attainment of equilibrium in 0.5 N DCl solution. Four signals can be seen in the spectrum in Fig. 3A. The signal at δ 174.3 was assignable to the iminium carbon atom characteristic of IIf, and the signal at δ 169.1 to the amide carbonyl carbon in the downfield region. The two signals at δ 51.5 and 37.4 were assignable to methylene and methyl carbon atoms, respectively, in the upfield region based on multiplicities in the off-resonance spectrum. Therefore, compound If was present only in the form of IIf in 0.5 N DCl solution at time zero, as also concluded from the ^{1}H-NMR spectrum.

When the solution attained equilibrium, as shown in Fig. 3B, four paired signals were observed in addition to the signals found in Fig. 3A. The two adjoining signals at δ 194.2 and 193.8 in the most downfield region with nearly the same intensity were clearly assignable to the benzophenone carbonyl carbon atoms. The signals suggest the presence of IIIf formed by hydrolysis of IIf at the iminium site. Signals at δ 168.6 and 168.2 were due to the amide carbonyl carbon atoms. Upfield signals at δ 41.9 and 41.4 were assignable to the methylene carbon atoms and the remaining signals at δ 38.6 and 38.2 to the methyl carbon atoms on the basis of the off-resonance spectrum. The ^{13}C-NMR data are summarized in Table II.

The methylene carbon resonance of IIIf was shifted to higher field by about 10 ppm compared to that of IIf, and this is consistent with the structural change from IIf to IIIf.

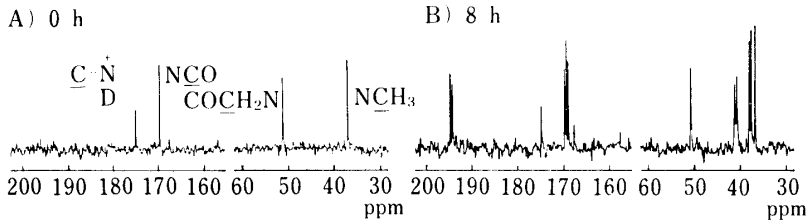


Fig. 3. The Aliphatic and Carbonyl Carbon Regions of the ^{13}C-NMR Spectrum of Fludiazepam in 0.5 N DCl

A) Immediately after dissolution. B) After attainment of equilibrium.

TABLE II. ^{13}C-NMR Spectral Data for Diazepam and Fludiazepam in 0.5 N DCl at Equilibrium

	Iminium form (II)					Benzophenone form (III)			
	NCH_3	$COCH_2N$	$C=\overset{+}{N}D$	NCO		NCH_3	$COCH_2N$	Ar_2CO	NCO
Diazepam	37.4	51.2	178.7	169.3	*cis*	38.3	42.0	197.6	168.3
					trans	38.9	41.4	198.0	168.4
Fludiazepam	37.4	51.5	174.3	169.1	*cis*	38.2	41.9	194.2	168.2
					trans	38.6	41.4	193.8	168.6

Paired signals observed in Fig. 3B can be explained in terms of the presence of the amide-rotamers.

Data on Id, which exhibited a similar spectrum, are also summarized in Table II. The isomers of IIId and IIIf (III_{cis} and III_{trans}) were assigned on the basis of their signal intensity, taking account of the ratio of isomers deduced from the ^{1}H-NMR spectra and literature data on amide-rotamers.[9] The iminium carbon resonance of IIf was shifted to higher field by about 4 ppm compared to that of IId. A similar result was also found for the benzophenone carbonyl carbon atoms of III. This may be explained in terms of the effect of fluorine substituted at position 2′.[6] Because other carbon atoms of the benzodiazepine ring are distant from the fluorine atom, the carbon resonances of IIf were the same as those of IId, and those of IIIf were the same as those of IIId.

Assignments of Aromatic Carbon Resonances of IId and IIf in 0.5 N DCl

Figures 4A and 4B show the complete decoupled and off-resonance decoupled spectra in the aromatic carbon region of IIf in 0.5 N DCl solution. Table III lists the chemical shifts and $^nJ_{CF}$ values of various carbon resonances. Eleven separate signals were observed in the spectrum shown in Fig. 4A. Because a fluorine atom causes a remarkable downfield shift in the signal of the *ipso* carbon in the benzene nucleus,[10] the one remaining aromatic carbon atom (C-2′) gave a signal at δ 161.8 ($^1J_{CF}=257.4$ Hz), which dose not appear in Fig. 4A. Of the eleven carbon signals in Fig. 4A, four signals were observed as doublets, due to coupling with the fluorine atom. The signals at δ 139.9, 126.9, δ 119.7 and 118.5 had $^nJ_{CF}$ values of 8.8, 4.4, 10.3 and 20.5 Hz respectively. Because the spectrum in Fig. 4B shows that the signal at δ 119.7 corresponds to a quaternary carbon atom, the signal is assignable to C-1′. Based on the fluorine atom substituent shift and $^nJ_{CF}$ values,[10] the carbon signals at δ 139.9, 126.9 and 118.5 were assigned to C-4′, C-5′ and C-3′, respectively.

Of the carbon signals with no J_{CF} values, those at δ 144.7, 132.5 and 125.9 were found to be attributable to quaternary carbons on the basis of the multiplicity observed in Fig. 4B. The most downfield signal at δ 144.7 was assignable to amide nitrogen-substituted C-10. As will be described later, the C-1′ atom was affected by the iminium carbon atom (C-5) because of the structural change from If to IIf. This afforded an upfield shift of about 7 ppm. If the C-11

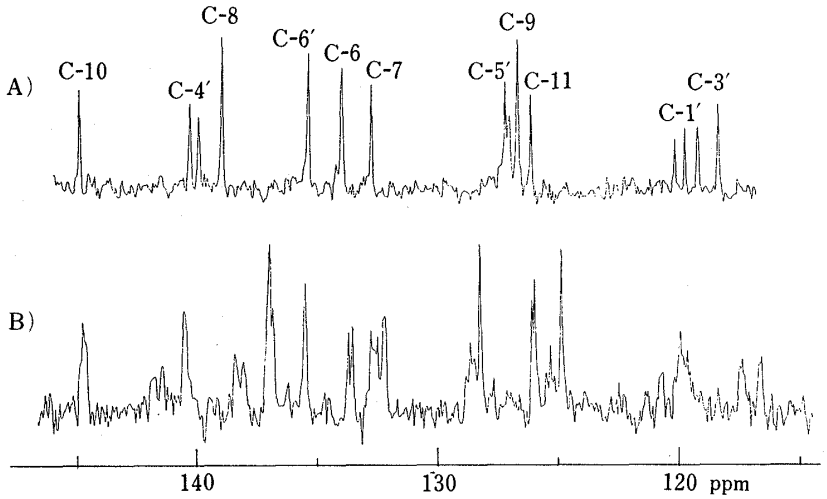


Fig. 4. The Aromatic Carbon Region of the ^{13}C-NMR Spectrum of IIf in 0.5 N DCl

A) Decoupled spectrum. B) Off-resonance spectrum.
C-1′, C-3′, C-4′ and C-5′ atoms were observed with $^{n}J_{CF}$ coupling.

TABLE III. ^{13}C-NMR Spectral Data for Diazepam and Fludiazepam[a)]

Carbon	Id[b,c)]	IId[d)]	If[c)]	IIf[d)]
C-2	170.0	169.3	169.3	169.1
C-3	56.9	51.2	56.9	51.5
C-5	168.7	178.7	165.8	174.3
C-6	129.7	134.7	128.3	133.7[e)]
C-7	129.9	132.2[e)]	129.6	132.5
C-8	131.3	138.5[f)]	131.5	138.7
C-9	122.5	126.3	122.7	126.4
C-10	142.5	145.1	141.3	144.7
C-11	129.1	125.5	131.2	125.9
C-12	34.7	37.4	34.9	37.4
C-1′	138.1	131.5[e)]	126.7 (11.7)	119.7 (10.3)
C-2′	129.3	133.4	160.4 (251.3)	161.8 (257.4)
C-3′	128.3	130.9	116.2 (22.0)	118.5 (20.5)
C-4′	130.5	137.5[f)]	132.3 (8.8)	139.9 (8.8)
C-5′	128.3	130.9	124.4 (2.9)	126.9 (4.4)
C-6′	129.3	133.4	131.3 (2.2)	135.1[e)]

a) The $^{13}C^{19}F$ coupling constants, in Hz, are given in parentheses. *b*) Assignments made by Haran and Tuchagues.[6)] *c*) In $CDCl_3$. *d*) In 0.5 N DCl. *e,f*) Assignments may be reversed in each column.

atom is assumed to be similarly affected, the signal at δ 125.9 can be assigned to C-11 and the other signal at δ 132.5 to C-7.

Four carbon signals at δ 138.7, 135.1, 133.7 and 126.4 were all attributable to tertiary carbon atoms with $^{1}J_{CH}$ coupling. By comparison of the assignment of If (described later) with that of IIf, the iminium carbon atom (C-5) of IIf causes an upfield shift of the C-1′ and C-11 signals, and a downfield shift of the C-4′ signal (C-4′ is *para* to C-1′).[11)] If a similar effect is assumed at the C-8 atom (*para* to C-11), the signal at δ 138.7 can be assigned to C-8. The shift effect on the *para*-carbon atom derived from the iminium carbon is in agreement with assignments for medazepam under acidic conditions reported by Kovar and Linden.[12)] Of the other three carbon signals at δ 135.1, 133.7 and 126.4, the most upfield signal at δ 126.4 was assignable to the C-9 atom adjoining C-10, which is directly attached to a nitrogen atom. This

assignment is supported by the fact that the signal at δ 126.4 is a sharp doublet without a $^3J_{CH}$ value in the off-resonance spectrum. The remaining two carbon signals at δ 135.1 and 133.7 were attributable to either C-6 or C-6′, and these assignments could be interchanged. Accordingly, $^3J_{CF}$ of C-6′ would not be observed due to the intermediacy of C-1′, which is a quaternary carbon atom.

The ^{13}C-resonances of If were assigned with reference to the results of Haran and Tuchagues.[6] The aromatic carbon signals of IId in 0.5 N DCl were similarly assigned using the ^{13}C-chemical shifts of If and IIf. The results are listed in Table III.

When the carbon resonances of I were compared with those of II, the resonances of C-3, C-11 and C-1′ were shifted more upfield in 0.5 N DCl than in $CDCl_3$. In this case, C-11 and C-1′ appeared to be especially affected by the iminium carbon atom (C-5). The resonance of the C-3 atom showed a similar upfield shift due to addition of deuterium to the nitrogen atom at position 4. Variation in the shift value of the amide carbonyl carbon atom (C-2) was negligible in these solvents. Other carbon signals appeared at more downfield regions in 0.5 N DCl than in $CDCl_3$. The signals of carbons at the *para* positions to C-11 and C-1′, both attached to C-5 (C-8 and C-4′), showed large downfield shifts (about 7 ppm). Because of the addition of deuterium to the nitrogen atom at position 4, the resonance of C-5 in II showed a characteristic downfield shift of 10.0 ppm for IId and 8.6 ppm for IIf.

In summary, compounds Id and If were initially present in the form of an iminium structure II in 0.5 N DCl at room temperature and then underwent slow hydrolysis at the iminium moiety, attaining equilibrium with the benzophenone structure III. The equilibration of two isomers of III was also observed in the solution. ^{1}H-NMR spectra at various temperatures revealed that the signals were attributable to the equilibrium between the amide-rotamers (III_{cis} and III_{trans}). With regard to the slower equilibrium between II and III, fludiazepam favored the benzophenone structure III as compared with diazepam. Accordingly, the equilibrium between II and III was clearly affected by the fluorine substituent at position 2′. On the other hand, in the equilibrium of the amide-rotamers observed in IIId and IIIf (III_{cis} and III_{trans} isomers) the proportions were nearly the same, although the III_{cis} isomer was slightly predominant. Thus, the 2′-F substituent exerted no effect on the equilibrium between the two amide-rotamers.

Interestingly, acidic aqueous solutions of flutazolam[1] contain only the benzophenone component. Accordingly, in the case of flutazolam, it appears that both the 2′-F substituent and the 2-hydroxyethyl group attached to the iminium nitrogen atom promoted hydrolysis at the iminium moiety, suggesting an apparent uni directional hydrolysis. Equilibrium between the amide-rotamers of flutazolam favored the *cis*-conformation. Because the 2′-F substituent did not affect the equilibrium, however, the substituent attached to the amide nitrogen is presumably responsible for this effect. In view of the fact that the *cis*-conformation of flutazolam, having a 2-hydroxyethyl group (bulkier than the methyl group), was predominant, the rotamer ratio seems to be affected not only by the steric size of the substituents but also by the presence of the hydroxyl group.

Molecular species produced by the two types of equilibria of diazepam and fludiazepam resulted in regeneration of the parent compounds due to an increase in pH value. It is suggested that 1) these drugs are probably present in the form of a benzophenone structure partially hydrolyzed at the iminium moiety in the acid environment of the stomach after internal administration, and 2) then reproduce the parent compounds because of the elevated pH values in the intestine. These reactions probably do not affect drug bioavailability.

Acknowledgement The authors are grateful to Professor K. Ikeda and Dr. Y. Kurono of the Nagoya City University for valuable guidance throughout the course of this work. We also thank Miss S. Kato, Faculty of Pharmaceutical Sciences, Nagoya City University, for NMR spectral measurements.

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