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Study on protolytic equilibria of lorazepam and oxazepam by UV and NMR spectroscopy

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Abstract

Protolytic equilibria in homogeneous and heterogeneous systems of lorazepam and oxazepam, which are sparingly soluble ampholytes from the class of 1,4-benzodiazepines, were studied at 25 °C and ionic strength of 0.1 M. Acidity constants and equilibrium constants in a heterogeneous system were determined. On the basis of the analysis of the corresponding ^{13}C- and ^{1}H-NMR spectra, deprotonation site in the molecules of the investigated compounds was predicted. Finally, the correlation between chemical shifts in the ^{1}H-NMR spectra and the acidity of the amide proton of 1,4-benzodiazepines was established.

Keywords: Oxazepam; Lorazepam; Acidity constants; Solubility

1. Introduction

Benzodiazepines belong to a class of compounds interesting not only for their pharmacological (tranquilizer) activity but also because their numerous derivatives can serve as model substances for the study of structure–activity relationships. Their complex chemical behavior is evident from the fact that, in addition to acid–base reactions to which benzodiazepines as protolytes are subjected in aqueous solutions, benzodiazepines undergo hydrolysis with changes in molecular structure [1–9].

Investigations presented here are a continuation of the study on acid–base equilibria in homogeneous and heterogeneous systems and hydrolysis of compounds from the class of benzodiazepines [9–13] and a consideration of the influence of structural changes on acid–base equlibria. Investigated benzodiazepines are lorazepam and oxazepam. The pK_a values of these two benzodiazepines were reported (oxazepam, p$K_{a1} = 1.7$, p$K_{a2} = 11.6$; lorazepam, p$K_{a1} = 1.3$, p$K_{a2} = 11.5$) [14] previously. The first acidity constant corresponds to the protonation of the nitrogen in the azomethine group. However, both >N–H group [14] and –O–H group [2] were suggested to be potential deprotonation sites. Due to that, one of the aims of the present study was to locate deprotonation site in the molecules of oxazepam and lorazepam

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applying NMR spectroscopy. In addition, correlation of chemical shifts in the ^{1}H-NMR spectra and acidity of the amide proton in 1,4-benzodiazepines was investigated.

The studies of solubility and protolytic equilibria of pharmacologically active substances are of special importance in biochemical pharmacology, because absorption of drugs in the gastrointestinal tract and transport through the blood–brain barrier and cell membranes are affected by the properties of the chemical species involved. As lorazepam and oxazepam, like most of the benzodiazepine derivatives, undergo hydrolysis, these investigations are necessary for kinetic analysis and elucidation of the reaction mechanisms. Besides that, knowledge of solubility and distribution of species as a function of pH are important for choice of optimal conditions for drug analysis.

2. Experimental

2.1. Apparatus and reagents

For spectrophotometric measurements a GBC 911A spectrophotometer (GBC Scientific Equipment Pty Ltd., Dandenong, Australia) with 1 cm silica cells was used. A PHM240 pH-meter (Radiometer) with a combined GK2401B electrode (Radiometer) served to determine pH values. Conversion of the measured pH values [$t = 25$ °C, $I = 0.1$ (NaCl)] into pc_H was done according to the relation [10]: $pc_H = -\log[H_3O^+] = pH - A$. Value of the correction factor A (0.04) was obtained by titrating standard HCl solution with standard NaOH solution at $t = 25$ °C and ionic strength of $I = 0.1$ (NaCl).

NMR spectra were recorded on a Varian/Gemini 200 instrument (^{1}H at 200 MHz, ^{13}C at 50 MHz) in d^6-DMSO using TMS as an internal standard; δ in ppm.

Lorazepam (Lz) and oxazepam (Ox) were products of Hoffmann La Roche. Stock solutions of the drugs were prepared by weighing accurately the dry substance and dissolving it in methanol. Other reagents: hydrochloric acid, sodium hydroxide, sodium chloride, sodium acetate, methanol, dimethylsulphoxide-d_6, acetanilide and potassium *tert*-butoxide (Merck) were of analytical grade of purity.

Solutions of HCl and NaOH were standardized potentiometrically. Acetate buffer (0.25 M, pH 5.0) was prepared by dissolving sodium acetate in double distilled water and adding HCl to pH 5.0.

2.2. Determination of the acidity constants

The acidity constants of the examined benzodiazepines were determined spectrophotometrically, at 25 °C and ionic strength of 0.1 M, using a fast working procedure.

For each benzodiazepine two series of solutions in pH range 0–6.5 (for K_{a1}) and 6.5–14 (for K_{a2}) were prepared. Working solutions of lorazepam (1×10^{-4} M) and oxazepam (5×10^{-5} M) were prepared by diluting the appropriate volume of stock benzodiazepine solution. Acidity of solutions was adjusted with standard solutions of HCl or NaOH. pc_H values of the solutions were determined on the basis of the pH values measured in the pH range 2–12, whereas outside this region pc_H values were calculated from the concentration of HCl or NaOH. The spectra were recorded within the wavelength range from 220 to 500 nm at the scanning speed of 500 nm/min. Maximum content of methanol in working solutions was 1% (v/v).

2.3. Determination of equilibrium constants in heterogeneous systems

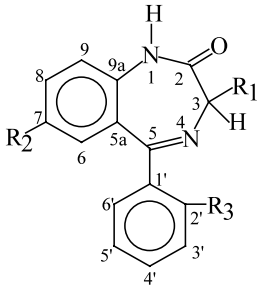
Saturated aqueous solutions used for determinations of the constant K_{s0} were prepared by treating an excess of solid benzodiazepine with 0.1 M NaCl solution. The samples were thermostated at 25 °C with occasional stirring until complete equilibration (4 h). The pH value of such heterogeneous systems was approximately 6.5 for both investigated benzodiazepines. After equilibration, the solutions were separated from the insoluble part by filtration. Aliquots of the filtrates were diluted with acetate buffer (0.025 M, pH 5.0). Actual concentration of molecular form (HA) of the benzodiazepines was determined spectrophotometrically at the wavelength of the absorption maxima, i.e. at 230 and 229 nm for lorazepam and

oxazepam, respectively, conformity with Beer's law having been previously verified.

For determination of the equilibrium constant K_{s2} saturated aqueous solutions of the benzodiazepines were prepared in the pH range from 11 to 12. The suspensions were obtained by a partial dissolution of solid diazepines in NaOH solution of known concentration with the addition of NaCl up to the ionic strength of 0.1 M. The samples were thermostated at 25 °C with occasional stirring until complete equilibration (4 h). After the measurement of the pH values, the solutions were separated from the insoluble part by filtration and filtrates were further processed as described for determination of the constant K_{s0}.

3. Results and discussion

In order to determine deprotonation site in the molecules of oxazepam and lorazepam their ^{1}H- and ^{13}C-NMR spectra obtained in d^6-DMSO and in the same solvent with addition of a base, potassium *tert*-butoxide (p$K_a = 18$ [15]) were compared. Benzodiazepines of an analogous structure without the hydroxyl group at the position 3 (nitrazepam and clonazepam) served as references:



lorazepam: $R_1 = OH$, $R_2 = Cl$, $R_3 = Cl$
oxazepam: $R_1 = OH$, $R_2 = Cl$, $R_3 = H$
nitrazepam: $R_1 = H$, $R_2 = NO_2$, $R_3 = H$
clonazepam: $R_1 = H$, $R_2 = NO_2$, $R_3 = Cl$

Upon addition of an equimolar amount of the base, the signal at $\delta \approx 11$ (δ 11.33 clonazepam; δ 11.19 nitrazepam; δ 10.97 lorazepam; δ 10.86 oxazepam) corresponding to the resonance of the proton bound to the amide nitrogen N-1 [16] completely disappeared in the ^{1}H-NMR spectra of all four benzodiazepines. On the other hand, the signal of the hydroxyl proton of oxazepam and lorazepam was present upon addition of the base and its chemical shift changed (from δ 4.81 to 4.47 for oxazepam and from δ 4.86 to 4.45 for lorazepam). This result might indicate deprotonation at N-1. However, as amide proton signals might be extensively broadened or even disappear in presence of a base, because of the increased exchange kinetics, unambiguous evidence for deprotonation at N-1 was obtained by analysis of ^{13}C-NMR spectra.

Assignations of the signals in the ^{13}C-NMR spectra (Table 1) were established on the basis of the DEPT spectra and the spectra of model compounds or taken from the literature [17,18]. Addition of the base did not result in significant changes of chemical shifts at C-2 and C-3. However, prominent changes of the chemical shifts were observed in the benzene ring fused to the diazepine ring of all four benzodiazepines. These changes occur at C-7 (a decrease for δ 3.5–5.7), C-9 (an increase for δ 3.0–3.5) and C-9a (an increase for δ 7.1–10). Such changes would be absolutely impossible to occur if –OH group of lorazepam and oxazepam would be the deprotonation site, because the resulting charge at oxygen would be localized and would not influence the above mentioned chemical shifts. In addition, there is a satisfactory agreement between the changes of the chemical shifts in all four benzodiazepines indicating deprotonation of the amide group, because in nitrazepam and clonazepam deprotonation can occur only at the N-1. Finally, the direction of the chemical shift change upon addition of the base (downfield at C-9a and C-9 atoms and upfield at C-7 atoms) is in accordance with behavior of analogous systems. The model compound was acetanilide. The spectra of acetanilide were recorded under the same conditions as the spectra of the benzodiazepines. Upon deprotonation of acetanilide by addition of the equimolar amount of the base the chemical shift changes were +1.8 ppm for the methyl carbon, +1.8 ppm for the carbonyl carbon, +11.0 ppm for the *ipso*-carbon, +3.4 ppm for the *ortho*-carbons, −1.0 ppm for the *meta*-carbons and −4.0 ppm for the *para*-carbon.

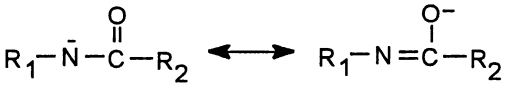
Table 1
Chemical shifts (ppm) in ^{13}C-NMR spectra of 1,4-benzodiazepines in d^6-DMSO in the absence and in the presence of potassium *tert*-butoxide (a base)

	Oxazepam		Lorazepam		Nitrazepam		Clonazepam	
	Ox	Ox+base	Lz	Lz+base	Nz	Nz+base	Cz	Cz+base
C-2	170.0	168.7	169.4	169.6	170.2	168.6	169.6	168.3
C-3	83.1	83.3	83.1	83.3	57.9	59.9	57.3	59.2
C-5	162.6	161.5	162.3	160.9	168.7	168.1	168.2	167.7
C-5a	126.9	127.5	127.1	128.2	126.6	125.0	127.2	126.1
C-6	129.5	128.9	130.0	127.5	126.3	124.9[a]	125.2	124.7[a]
C-7	128.1	122.4	127.9	122.4	141.7	136.9	141.9	138.4
C-8	132.1	130.0	132.1[a]	130.6[a]	126.8	127.7	126.7	125.4[a]
C-9	123.4	126.6	123.4	126.8	122.5	125.5[a]	122.5	126.0[a]
C-9a	138.3	147.0	138.0[b]	146.5	145.3	155.3	144.6	151.7
C-1′	138.0	139.3	137.4[b]	139.1	138.6	139.7	138.3	139.1
C-2′	129.5	129.6	132.1	132.4	129.9	129.7	132.1	132.3
C-3′	128.7	128.4	129.0	129.8	128.7	128.5	130.0	129.9
C-4′	130.7	130.8	131.7[a]	131.7[a]	131.0	130.1	131.8	131.2
C-5′	128.7	128.4	127.7	127.4	128.7	128.5	127.8	127.6
C-6′	129.5	129.6	131.6	130.8[a]	129.7	129.7	131.8	131.8

[a] Assignations can be interchanged.
[b] Assignations can be interchanged.

These changes are in full accordance with changes recorded with the investigated benzodiazepines. Good agreement with the direction of the chemical shift changes obtained in this study was also obtained by comparison of spectra of aniline and of deprotonated aniline [19].

The results obtained from ^{1}H- and ^{13}C-NMR spectra undoubtedly demonstrate that deprotonation in the molecules of oxazepam and lorazepam occurs at the nitrogen N-1. A higher acidity of the >N–H group compared with –OH group can be explained by resonance stabilization of the resulting anion:



A great difference in the acidity of the azomethine and the amide nitrogens makes it possible to determine K_{a1} and K_{a2} independently of each other. In order to avoid the consequences of hydrolysis, determinations were performed applying a rapid working procedure. Electron withdrawing HO– group decreases the electron density at the azomethine nitrogen, i.e. decreases pK_{a1} value which is about 3 in 1,4-benzodiazepines that do not contain this group [10,13]. An additional decrease of the pK_{a1} value of lorazepam is due to the presence of the chlorine atom in the *ortho*-position of the phenyl group. Therefore, pK_{a1} values of lorazepam and oxazepam could be expected to be below 2. Thus, for the determination of this constant, the basic spectrophotometric equation [20] was transformed into the following linear dependence:

$$A = A_{H_2A^+} - K_{a1}\frac{A - A_{HA}}{[H_3O^+]} \quad (1)$$

where $A_{H_2A^+}$, A_{HA} and A represent absorbances of protonated (H_2A^+) and molecular (HA) benzodiazepine forms, and their mixtures at a given wavelength, respectively. The constants K_{a1} were calculated from the slope of the corresponding lines at two wavelengths for oxazepam (285 and 361 nm) and three wavelengths for lorazepam (280, 287 and 370 nm) (Fig. 1). The acidity constant K_{a2} of both benzodiazepines was determined according to the classical spectrophotometric equation [20] at two wavelengths: 270 and 343 nm (Ox) and 275 and 350 nm (Lz). The values

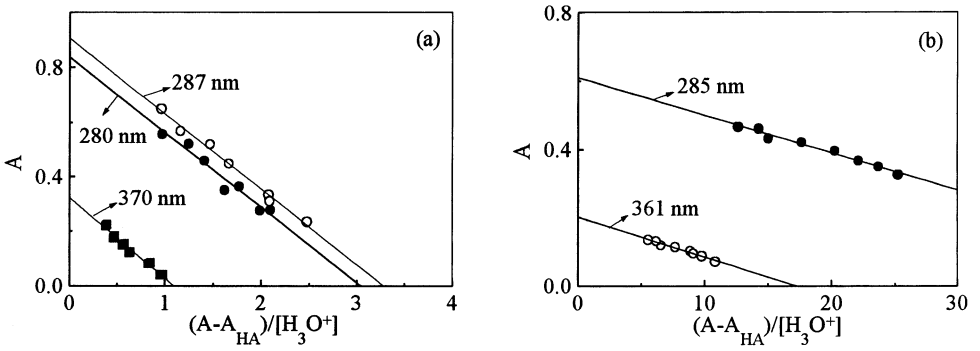


Fig. 1. Spectrophotometric determination of K_{a1} according to equation Eq. (1): (a) lorazepam, $c = 1 \times 10^{-4}$ M; (b) oxazepam, $c = 5 \times 10^{-5}$ M.

Table 2
Concentration equilibrium constants ($p\bar{K}_a \pm t_{0.95}s/\sqrt{n}$) in homogeneous and heterogeneous systems of lorazepam and oxazepam ($I = 0.1$ M; $t = 25$ °C)

Constant	Equation	Lorazepam	Oxazepam
pK_{a1}	Classical spectrophotometric	0.56±0.05	1.90±0.04
pK_{a2}	Eq. (1)	10.96±0.01	11.19±0.01
	Eq. (6)	10.96±0.02	11.05±0.02
pK_{s0}	Eq. (7a)	3.54±0.01	4.16±0.01
	Eq. (7b)	3.58±0.09	4.08±0.05
pK_{s1}	Eq. (5)	2.98±0.05	2.26±0.04
pK_{s2}	Eq. (7a)	14.50±0.02	15.21±0.02

of determined acidity constants of oxazepam and lorazepam are listed in Table 2.

Since both investigated benzodiazepines in molecular form are sparingly soluble in water, the following equilibria between the solid phase (HA_s) and solution are possible in the heterogeneous system:

$$HA_s \rightleftharpoons HA \quad K_{s0} = [HA] \tag{2}$$

$$HA_s + H_3O^+ \rightleftharpoons H_2A^+ + H_2O \quad K_{s1} = \frac{[H_2A^+]}{[H_3O^+]} \tag{3}$$

$$HA_s + H_2O \rightleftharpoons A^- + H_3O^+ \quad K_{s2} = [A^-][H_3O^+] \tag{4}$$

Between acidity constants and equilibrium constants in the heterogeneous system the following relationships exist:

$$K_{a1} = \frac{K_{s0}}{K_{s1}} \tag{5}$$

$$K_{a2} = \frac{K_{s2}}{K_{s0}} \tag{6}$$

Equilibrium constants of lorazepam and oxazepam in heterogeneous systems were determined by the solubility method. Solubility (S) of the benzodiazepines in aqueous solution is given by the following expression:

$$S = [H_2A^+] + [HA] + [A^-] \tag{7}$$

The concentrations of H_2A^+ and A^- forms could be neglected within the range of $pK_{a1}+2 < pH < pK_{a2}-2$ and the Eq. (7) gets transformed into the following dependence:

$$S = [HA] = K_{s0} \tag{7a}$$

which represents the solubility of the molecular form (intrinsic solubility). On the basis of spectrophotometrically determined solubility of the benzodiazepines in 0.1 M NaCl solution (pH 6.5) and the Eq. (7a), the constants K_{s0} of both benzodiazepines were calculated.

In solution at $pH > pK_{a2}-2$, there are practically only HA and A^- particles and the Eq. (7) becomes:

$$S = [HA] + [A^-] = K_{s0} + \frac{K_{s2}}{[H_3O^+]} \tag{7b}$$

On the basis of experimentally determined solubility at various pH values (pH 11–12) and dependence given by Eq. (7b) the constants K_{s0} and K_{s2}

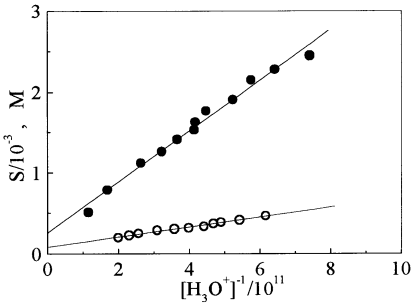


Fig. 2. Determination of K_{s0} and K_{s2} according to the equation Eq. (7b): (●) lorazepam, (○) oxazepam.

of both benzodiazepines were calculated by linear regression analysis (Fig. 2).

Study on heterogeneous equilibria of the examined benzodiazepines in acidic media requires low pH values of the solutions (pH < 2). Due to a slow equilibration in the heterogeneous system and the fact that in an acid medium both benzodiazepines undergo a relatively rapid hydrolysis, the constants K_{s1} were calculated on the basis of the constants K_{a1} and K_{s0} according to the Eq. (5).

The equilibrium constants determined and calculated for lorazepam and oxazepam are listed in Table 2. It can be seen that the constants K_{s0} determined employing the two approaches are in accordance. The same holds true for the K_{a2} values determined directly and indirectly (on the basis of the equilibrium constants in the heterogeneous system). A comparison of the pK_a values of lorazepam and oxazepam provide an indication of the electronic effect of the *ortho* chlorine atom in the phenyl group. The presence of this substituent decreases pK_{a1} and pK_{a2} for 1.34 and 0.23 U, respectively. This is in accordance with the data obtained for clonazepam (pK_{a1} = 1.70, pK_{a2} = 10.29) and its non-chlorinated analogue nitrazepam (pK_{a1} = 3.05, pK_{a2} = 10.51) where a decrease of 1.35 and 0.22 U was observed for pK_{a1} and pK_{a2}, respectively [13].

Correlation of the acidity of amide proton (pK_{a2}) with its chemical shifts in ^{1}H-NMR spectra of five 1,4-benzodiazepines (Table 3) revealed an expected increase of the chemical shifts parallel to the increasing acidity constants. It was found that there is a linear dependence between the chemical shifts and the pK_{a2} values that can be expressed by

Table 3
Chemical shifts (δ) of the amide proton in ^{1}H-NMR spectra obtained in d^6-DMSO and pK_{a2} values of 1,4-benzodiazepines

Benzodiazepine	δ (ppm)	pK_{a2}[a]
Clonazepam	11.33	10.29 [13]
Nitrazepam	11.19	10.51 [13]
Lorazepam	10.97	10.96 (this work)
Oxazepam	10.86	11.19 (this work)
Bromazepam[b]	10.70	11.60 [21]

[a] t = 25 °C; I = 0.1 M (NaCl).
[b] 7-Bromo-1,3-dihydro-5-(2-pyridinyl)-2H-1,4-benzodiazepin-2-one.

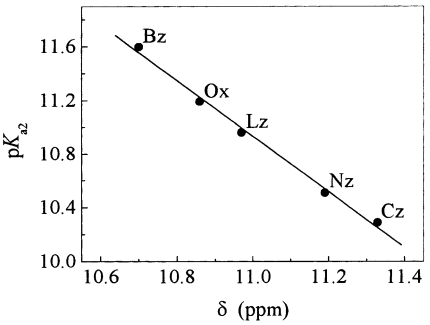


Fig. 3. Plot of pK_{a2} values vs. chemical shift in ^{1}H-NMR spectra of the amide proton in 1,4-benzodiazepines in d^6-DMSO: Bz, bromazepam; Ox, oxazepam; Lz, lorazepam; Nz, nitrazepam; Cz, clonazepam.

the equation:

$$pK_{a2} = 33.72 - 2.07\delta \quad (r = 0.997;\ s = 0.05) \qquad (8)$$

The obtained linear dependence (Fig. 3) can be explained by the fact that both values, the chemical shift and the acidity, depend on the electron density. Numerical values in Eq. (8) represent empirical parameters characteristic for the examined class of compounds.

On the basis of the measured chemical shifts, the derived equation enables the prediction of pK_a values of the amide proton in benzodiazepines of analogous structure. This is especially significant for those benzodiazepines in which due to hydrolytic degradation and/or very weak acidity, it is impossible to apply classical methods for the determination of the pK_a.

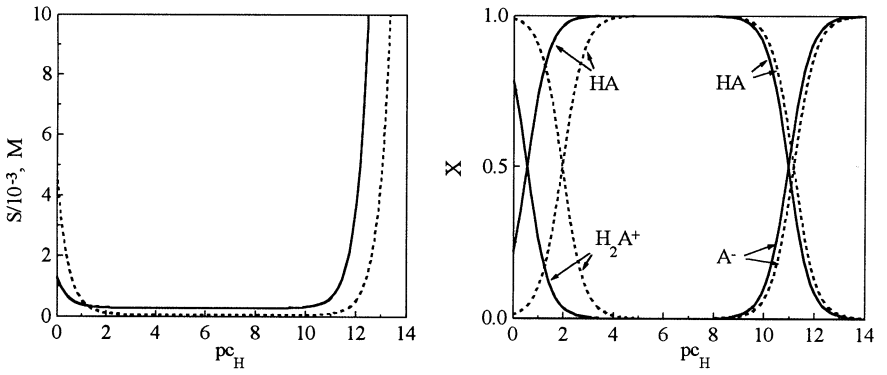


Fig. 4. Solubility curve (left) and distribution diagram (right) of lorazepam (——) and oxazepam (------) as a function of pc_H. t = 25 °C; $pc_H = pH - 0.04$.

On the basis of the values obtained for the equilibrium constants, the solubility and the distribution of equilibrium species (X) of lorazepam and oxazepam were calculated as a function of pc_H (Fig. 4). Minimal solubility of the examined benzodiazepines ($dS/dpc_H = 0$) is achieved when:

$$pc_H = \frac{pK_{s2} - pK_{s1}}{2} = \frac{pK_{a1} + pK_{a2}}{2} \quad (9)$$

Minimum solubilities of lorazepam and oxazepam are 2.88×10^{-4} M (pc_H 5.76) and 7.25×10^{-5} M (pc_H 6.47), respectively.

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