A Spectrophotometric Method for the Determination of Equilibrium Constants of the Reversible 1,4-Benzodiazepine Ring-opening Reaction

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A general spectrophotometric method is proposed for the quantitative investigation of the hydrolysis of 1,4-benzodiazepines which, under the effect of nucleophilic hydration, undergo reversible cleavage of the 4,5-azomethine bond, followed by simultaneous simple acid–base reactions. The method is based on establishing the pH region of the solution in which, at the state of complete equilibration, three species dominate, as well as on using suitable isosbestic points and the differences in rates of simple protolysis and hydrolysis. The method is presented using flurazepam as an example, but it also can be applied to other compounds which hydrolyse by a similar mechanism.

It is well known that in acidic media most 1,4-benzodiazepines hydrolyse to a certain extent. Investigations of the hydrolytic mechanism have shown that in molecules of these compounds there are two sites potentially susceptible to nucleophilic hydration: the 1,2-amide linkage and the 4,5-azomethine bond. Both these bonds undergo a two-step sequential reaction: reversible ring-opening and degradation of the molecule to the corresponding benzophenone and glycine derivative.[1] 1,4-Benzodiazepines in which the 1,2-bond is fused to a heterocyclic ring[2–5] or in which the resident positive charge at C-5 is increased due to the resonance effect of the relevant substituents,[6,7] undergo preferential cleavage of the 4,5-azomethine bond. At room temperature, in the presence of not too high an acid concentration, the initial reversible hydrolytic step in the case of these benzodiazepines is stabilized. As this process is simultaneously accompanied by simple acid–base reactions, an equilibrium between a mixture of the ring-opened and ring-closed forms is established in the solution and is pH dependent. Although the kinetic and hydrolytic mechanisms in such benzodiazepines and related compounds have been extensively investigated, only in some cases have the apparent constants of the ring-opened–ring-closed equilibrium been determined.[3,4,7,8]

The aim of this paper is to propose a method which would make possible a quantitative investigation of equilibria between all species actually present in the solution. At the same time the paper offers a verification of the method developed for flurazepam and its comparison with the pH metric method which is based on function formation. The pH metric method was proposed in a previous paper[9] for determining flurazepam hydrolysis constants. In addition, this paper describes the determination of acidity constants, equilibrium constants in a heterogeneous system and buffer characteristics of homogeneous and heterogeneous flurazepam systems.

Experimental

Apparatus and Reagents.—UV spectrophotometric measurements were carried out with a Beckman DU-50 spectrophotometer equipped with 1 cm silica cells. A PHM-62 pH meter (Radiometer) with a glass calomel electrode assembly was used for pH measurements. The interpretation of the pH values measured is described in a previous paper,[10] where it was found that $pc_H = -\log[H_3O^+] = pH - 0.04$ for $I = 0.1$ mol dm^{-3} (NaCl) and $T = 25$ °C.

Flurazepam monohydrochloride was produced by Hoffmann La Roche (Basle, Switzerland). Other reagents (NaCl and HCl) were of analytical reagent grade (Merck). All solutions were prepared in twice distilled water. Standardization of HCl solutions was done pH metrically.

Method.—Details of the specific procedure applied have been given previously[9] and may be summarized as follows. The spectrum of flurazepam monohydrochloride (BH^+), was obtained from its aqueous solution ($pc_H = 5.8$). The spectrum of the diprotonated form (BH_2^{2+}) was recorded (fast scan) from its solution in 1 mol dm^{-3} HCl (scan speed: 750 nm min^{-1}). Solutions of flurazepam in the pc_H range 1–4 were prepared by mixing rapidly standard flurazepam and hydrochloric acid solutions of known concentration. For the verification of K_{a1} values for flurazepam,[9] the spectra of these solutions were recorded immediately on preparation, whereas for the determination of the hydrolysis constants the spectra were taken after equilibration was complete (4 h). pc_H Values of the working solutions were determined on the basis of measured pH values, whereas in solutions with $pc_H < 2$, the pc_H values were calculated from the concentration of HCl.

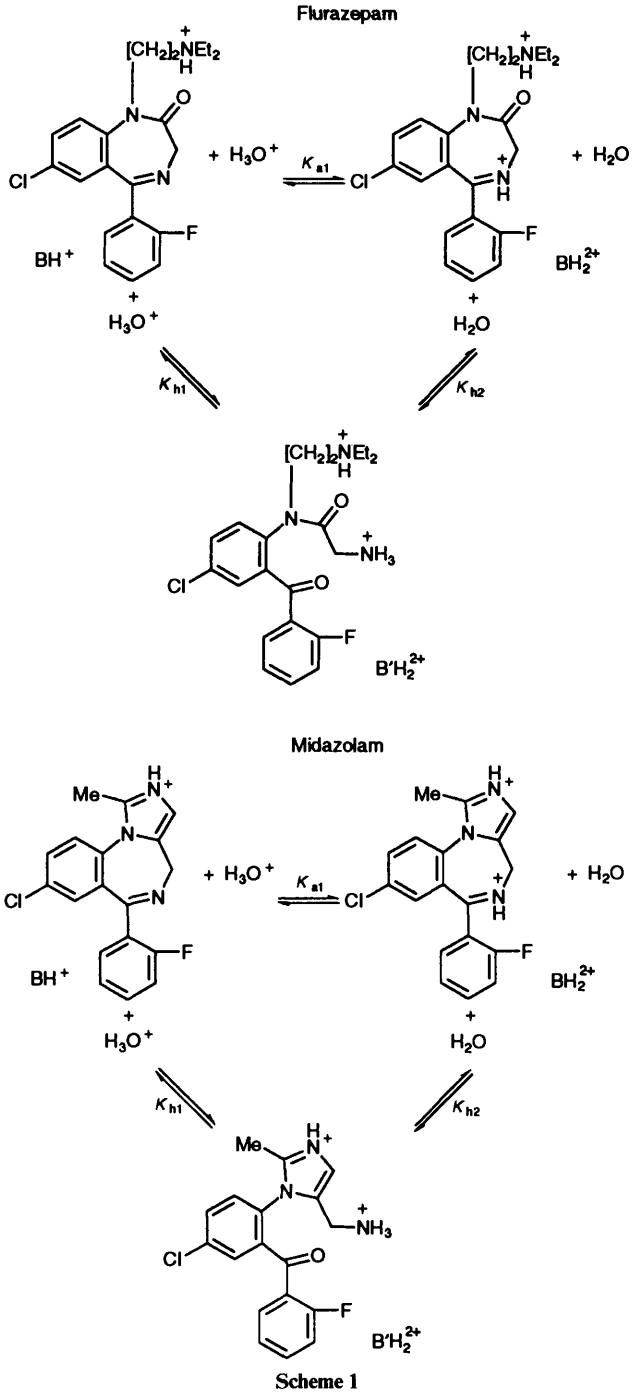
Results and Discussion

In previous papers it has been established that in acidic solutions (pH < 4) of flurazepam[9] and midazolam,[11] respectively, two processes occur simultaneously: protonation of the azomethine nitrogen (simple acid–base reaction) and hydrolysis of the 4,5-azomethine bond. Regardless of the complexity of the mechanisms of these processes, when equilibrium is reached the resulting effect may be depicted as shown in Scheme 1.

pK_{a2} values of the triethylamine group in flurazepam (8.37) and imidazole nitrogen in position 2 of midazolam (5.90) as well as the amine group of the ring-opened compound whose pK_a was estimated to *ca.* 7–8 (ref. 3) show that at pH < 4 the corresponding functional groups do not participate in acid–base reactions and that at equilibrium only three species are present in the solution.

In general, equilibrium constants of the processes shown above can be defined in eqns. (1)–(3). In fact, equilibrium

$$K_{a1} = \frac{[BH^+][H_3O^+]}{[BH_2^{2+}]} \quad (1)$$



Scheme 1

$$K_{h1} = \frac{[B'H_2^{2+}]}{[BH^+][H_3O^+]} \quad (2)$$

$$K_{h2} = \frac{[B'H_2^{2+}]}{[BH_2^{2+}]} \quad (3)$$

constant K_{h1} is complex and is equal to the quotient K_{h2}/K_{a1}. Introduction of this constant makes it possible to use the experimental data at pH values at which spectrophotometric measurements are the most accurate. In addition, if there is a pH interval over which BH^+ and $B'H_2^{2+}$ species are dominant in the solution relative to BH_2^{2+}, it is possible to determine an approximate value for this constant from A – pH data within that interval.[9] However, as that interval is relatively narrow on account of the overlapping equilibria, in order to avoid approximations, a method is proposed which takes into account the pH interval over which all three species are present in the solution.

As in the case of all spectrophotometric methods for the determination of equilibrium constants,[12] the proposed method is based on the fact that absorbance is additive in the region within which the Lambert–Beer's law is valid.

$$A = l(A_{BH^+} + A_{BH_2^{2+}} + A_{B'H_2^{2+}}) \quad (4)$$

i.e.

$$A = l\{\varepsilon_{BH^+}[BH^+] + \varepsilon_{BH_2^{2+}}[BH_2^{2+}] + \varepsilon_{B'H_2^{2+}}[B'H_2^{2+}]\} \quad (4a)$$

In eqns. (4) and (4a) l denotes the length of the cell, A_{BH^+}, $A_{BH_2^{2+}}$ and $A_{B'H_2^{2+}}$ are the absorbances, and ε_{BH^+}, $\varepsilon_{BH_2^{2+}}$ and $\varepsilon_{B'H_2^{2+}}$ represent the molar absorptivities of the corresponding species. If the stoichiometric concentration of benzodiazepines is designated as C, it follows that the balance mass equation for acidic solution is eqn. (5). Combining eqns. (1)–(5), on condition

$$C = [BH^+] + [BH_2^{2+}] + [B'H_2^{2+}] \quad (5)$$

that the length of the cell is 1 cm and that the stoichiometric concentration is constant during the course of measurements, we obtain eqn. (6) in which $K_{a1}K_{h1} = K_{h2}$.

$$A = \frac{K_{a1}A_{BH^+} + [H_3O^+]A_{BH_2^{2+}} + K_{a1}K_{h1}[H_3O^+]A_{B'H_2^{2+}}}{[H_3O^+] + K_{a1}K_{h1}[H_3O^+] + K_{a1}} \quad (6)$$

In principle, the equilibrium constants in eqn. (6) can be determined from A – pc_H data by the application of the non-linear curve-fitting analysis (it is not necessary to know the molar absorptivities of pure species) or by solving a set of linear equations (whose minimum number corresponds to the number of unknowns).[12] However, both methods require the use of complex computer programs.

A considerably less complicated procedure is based on the linearization of the A – pc_H curves [eqn. (6)] and on establishing different variants of linear dependences, based on experimentally available data. A survey of linear dependences obtained by transforming eqn. (6), along with conditions under which individual variants can be applied, are presented in Table 1. Comparative analysis of all variants of the method proposed shows that only in the case of the first variant is it necessary to know the molar absorptivities of all pure species at the equilibrium state. In fact, this variant is not applicable, since direct determination of the molar absorptivitiy of hydrolytic product $B'H_2^{2+}$ is not possible in view of the fact that there is no pH range in the solution over which $B'H_2^{2+}$ is the sole species present. For the same reasons, at the state of complete equilibrium, direct determination of $A_{BH_2^{2+}}$ is not possible. However, because there is a difference between the rates of the corresponding parallel processes (fast simple protolysis and slow hydrolysis), the absorbance of the pure BH_2^{2+} form can be determined by a 'rapid procedure' (*cf.* Experimental). Therefore, all the other variants exclude only the knowledge of $A_{B'H_2^{2+}}$. Although it is necessary in the case of both variant 2 and variant 3 to know the absorbances of the same pure species, BH_2^{2+} and BH^+, nevertheless they differ from each other. Variant 2 can be applied at all analytically suitable wavelengths, but requires a fast procedure for the determination of $A_{BH_2^{2+}}$. For variant 3, determination of $A_{BH_2^{2+}}$ is not affected by time, since at the wavelength of the isosbestic point for which $\varepsilon_{BH_2^{2+}} = \varepsilon_{B'H_2^{2+}}$ there is no change in the absorbance with time.

Variants 4–6 can be applied at wavelengths corresponding to isosbestic points whereby it is necessary to know the absorbance of only one pure species. As A_{BH^+} can be determined almost

Table 1 Survey of the variants of the method for the spectrophotometric determination of the hydrolysis constants obtained by the linearization of $A - pc_H$ curves [eqn. (6)]

Variant	Linear dependence	Eqn.	Wavelength	Necessary data
1	$\underbrace{\frac{A_{BH_2^{2+}} - A}{A - A_{BH^+}}[H_3O^+]}_{y} = K_{a1} + K_{h2}\underbrace{\frac{A - A_{B'H_2^{2+}}}{A - A_{BH^+}}[H_3O^+]}_{x}$	(7)[a]	All except $\varepsilon_{BH_2^{2+}} = \varepsilon_{B'H_2^{2+}}$	$A_{BH^+}, A_{BH_2^{2+}}, A_{B'H_2^{2+}}$
2	$\underbrace{(A_{BH_2^{2+}} - A) + \frac{K_{a1}(A_{BH^+} - A)}{[H_3O^+]}}_{y} = \underbrace{K_{h2}A}_{x} - K_{h2}A_{B'H_2^{2+}}$	(8)	All	$A_{BH_2^{2+}}, A_{BH^+}$
3	$\underbrace{\log\frac{A_{BH^+} - A}{A - A_{BH_2^{2+}}}}_{y} = \log\left(K_{h1} + \frac{1}{K_{a1}}\right) - \underbrace{pc_H}_{x}$	(9)	Isosbestic point $\varepsilon_{BH_2^{2+}} = \varepsilon_{B'H_2^{2+}}$	$A_{BH_2^{2+}}, A_{BH^+}$
4	$\underbrace{A}_{y} = A_{BH^+} - \left(K_{h1} + \frac{1}{K_{a1}}\right)\underbrace{(A - A_{BH_2^{2+}})[H_3O^+]}_{x}$	(10)	Isosbestic point $\varepsilon_{BH_2^{2+}} = \varepsilon_{B'H_2^{2+}}$	$A_{BH_2^{2+}}$
5	$\underbrace{A}_{y} = A_{BH_2^{2+}} + \left(K_{h1} + \frac{1}{K_{a1}}\right)^{-1}\underbrace{\frac{A_{BH^+} - A}{[H_3O^+]}}_{x}$	(11)	Isosbestic point $\varepsilon_{BH_2^{2+}} = \varepsilon_{B'H_2^{2+}}$	A_{BH^+}
6	$\underbrace{(A - A_{BH^+})\left(1 + \frac{K_{a1}}{[H_3O^+]}\right)}_{y} = K_{h2}A_{B'H_2^{2+}} - \underbrace{K_{h2}A}_{x}$	(12)	Isosbestic point $\varepsilon_{BH_2^{2+}} = \varepsilon_{BH^+}$	A_{BH^+}

[a] $K_{h2} = K_{h1}K_{a1}$.

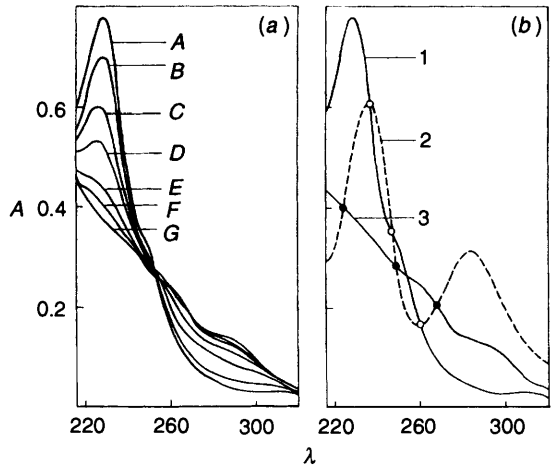


Fig. 1 (*a*) Absorption spectra of flurazepam in solution of various pc_H at the state of complete equilibration. pc_H: *A*, 5.8 (BH^+); *B*, 2.96; *C*, 2.40; *D*, 2.13; *E*, 1.60; *F*, 1.30; *G*, 0–1.17; $C_{tot} = 2.4 \times 10^{-5}$ mol dm^{-3}. (*b*) Absorption spectra of flurazepam relevant for the choice of optimum wavelengths: 1, BH^+; 2, BH_2^{2+} in 1 mol dm^{-3} HCl recorded at fast scan; 3, spectrum 2 after the equilibration was completed.

always without problem, proposed variants 5 and 6 are the simplest, except in cases, such as midazolam,[11] where hydrolysis and deprotonation of the BH^+ protolyte are not separated by a wider pH interval. When determining equilibrium constants several variants can most often be combined in order to avoid obtaining poor results which would be the case if the determination were performed at only one wavelength.

In addition, the proposed variants (except the first one) apparently require prior knowledge of acidity constant K_{a1}. However, owing to the fact that protolysis occurs instantaneously in a pH range which overlaps with that of hydrolysis, by optimizing the experimental procedure, both constants can be determined using the same starting solutions. This is achieved on the basis of $A - pc_H$ data obtained from fast scan spectra by the usual procedure[9,13] and $A - pc_H$ data obtained after complete equilibration.

Fig. 1(*a*) shows the spectra of acidic flurazepam solutions as a function of pc_H recorded after equilibration was completed. In Fig. 1(*b*) only those spectra that are relevant to the selection of optimum conditions for the determination of flurazepam hydrolysis constants are given. The spectrum of BH^+ species (1) was obtained using aqueous flurazepam hydrochloride solution, that of BH_2^{2+} species (2) was recorded at fast scan in 1 mol dm^{-3} HCl, while spectrum (3) corresponds to the spectrum of the same solution after completed equilibration. At the same time this spectrum is superimposable with spectra recorded in the pc_H interval from 0 to 1.2 [Fig. 1(*a*), *G*]. A detailed analysis of these and other spectra necessary for establishing the hydrolytic mechanism at the equilibrium state is described in a previous paper.[9]

Fig. 2 shows the linear dependence expressed by eqn. (8) obtained on the basis of experimental data for three optimal wavelengths.

Out of the three isosbestic points for BH_2^{2+} and $B'H_2^{2+}$ flurazepam species appearing at wavelengths of 224, 250 and 268 nm, a difference in absorbances sufficient for a reliable determination of equilibrium constants was observed only at 224 nm. On the basis of experimental data obtained at that wavelength and eqns. (9)–(11), straight lines shown in Fig. 3 were obtained. The intercept on the ordinate in Fig. 3(*b*) and (*c*) corresponding to the absorbances of pure BH^+ and BH_2^{2+} species are in good accordance with directly measured values.

Variant 6 [eqn. (12)] of the proposed method is not suitable for the determination of equilibrium constants because of analytically unsuitable wavelengths at which isosbestic points

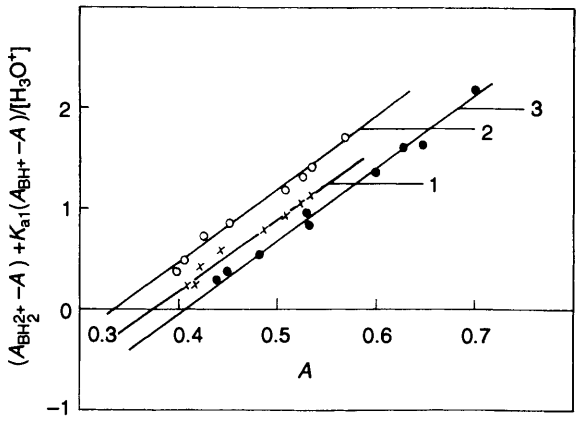


Fig. 2 Spectrophotometric determination of K_{h2} for flurazepam by the application of eqn. (8): 1, λ = 221 nm, C_{tot} = 2.0 × 10^{-5} mol dm^{-3}, A_{BH^+} = 0.590, $A_{BH_2^{2+}}$ = 0.290; 2, λ = 224 nm, C_{tot} = 2.0 × 10^{-5} mol dm^{-3}, A_{BH^+} = 0.650, $A_{BH_2^{2+}}$ = 0.340; 3, λ = 228 nm, C_{tot} = 2.4 × 10^{-5} mol dm^{-3}, A_{BH^+} = 0.800 and $A_{BH_2^{2+}}$ = 0.474

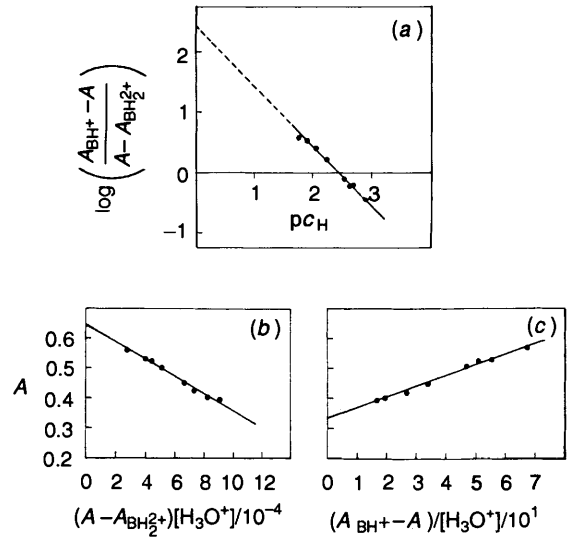


Fig. 3 Spectrophotometric determination of K_{h1} for flurazepam by the application of: (*a*), eqn. (9); (*b*), eqn. (10); (*c*), eqn. (11); C_{tot} = 2.0 × 10^{-5} mol dm^{-3}; A_{BH^+} = 0.650 and $A_{BH_2^{2+}}$ = 0.340

for BH^+ and BH_2^{2+} species appeared (238, 248 and 262 nm; Fig. 1).

The equilibrium constants determined by application of various variants of the proposed method are given in Table 2. By comparing the obtained values with those of pK_{h1} = −2.56 and pK_{h2} = −1.03 determined in a previous paper[9] by application of the pH metric method it is seen that a good accordance is achieved, which confirms the correctness of both methods proposed for quantitative investigation of the hydrolysis. However, since both methods require a previous knowledge of the hydrolysis mechanism at the equilibrium state, which can be determined directly spectrophotometrically, the spectrophotometric method has a considerable advantage over the pH metric method. In addition, another advantage of the spectrophotometric method is that experimental determination of all the equilibrium constants in acidic media (K_{a1}, K_{h1} and K_{h2}) can be performed using the same solutions. On the basis of the equilibrium constants determined by application of the proposed methods it is possible to determine the distribution of all species present in the solution, which hitherto has not been possible.

All aforegiven considerations can be applied also to the quantitative investigation of other benzodiazepines[11] and compounds which hydrolyse by an analogous mechanism.

Table 2 Stoichiometric hydrolysis constants for flurazepam; I = 0.1 mol dm^{-3} (NaCl); T = 25 °C

Eqn. applied[a]	λ/nm	pK_{h1}	pK_{h2}
(8)	221	−2.37 ± 0.05	−0.84 ± 0.03
	224	−2.39 ± 0.05	−0.86 ± 0.03
	228	−2.36 ± 0.05	−0.83 ± 0.02
(9)	224	−2.41 ± 0.02	−0.88 ± 0.05
(10)	224	−2.41 ± 0.03	−0.88 ± 0.06
(11)	224	−2.40 ± 0.02	−0.87 ± 0.05

[a] Eqns. (8)–(11) are given in Table 1.

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