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Practical Kinetics III: Benzodiazepine Hydrolysis

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Abstract □ The velocity constants for chlordiazepoxide hydrolysis were measured by independent techniques. A quantitative TLC kinetic procedure is compared with an extractive method. The data derived from both processes are in approximate agreement, further exemplifying the feasibility of TLC for rapid stability evaluation of liquid formulations as well as solution kinetic studies. In the extractive procedure, benzodiazepine–substrate was separated from the lactam product by methylene chloride extraction of acidic aqueous solution. The TLC procedure consisted of separation on silica gel plates followed by elution and subsequent analysis. The log k–pH relationship for the hydrolysis representing water addition coupled with expulsion of methylamine is presented. This function is characterized by water and hydroxide-ion attack on monoprotic species along with specific hydrogen-ion catalysis at higher hydronium-ion concentrations, and the rate law for the decomposition of chlordiazepoxide is given. Through several half-times (pH 0.15–11.5, 79.5°), this hydrolytic reaction generating lactam predominated; however, more benzophenone was formed as the pH decreased. Velocity constants were invariant over a 200-fold concentration range. The subsequent acid-facilitated cleavage of lactam to benzophenone was not further investigated. Both general acid catalysis and general base catalysis were evidenced, with borate, acetate, formate, and phosphate buffers accelerating the conversion of chlordiazepoxide to lactam. At pH values below neutrality, nonlinear dependency of the rate constant on buffer concentration was observed. This finding may be explained by a change in the rate-determining step as buffer concentration varied.

Keyphrases □ Benzodiazepines—hydrolysis of chlordiazepoxide, velocity constants, conversion to lactam, TLC and extraction procedures compared □ Chlordiazepoxide—hydrolysis, velocity constants, conversion to lactam, TLC and extraction procedures compared □ Hydrolysis—benzodiazepines, velocity constants for chlordiazepoxide hydrolysis, TLC and extraction procedures compared □ Kinetics—benzodiazepine hydrolysis

This investigation was undertaken to study the kinetics of chlordiazepoxide hydrolysis as well as to compare pertinent rate data concerning breakdown of the compound using both classical and quantitative TLC analytical techniques.

TLC techniques have been utilized in these laboratories for rapid determination of solution stability of diverse classes of medicinal agents. TLC is applicable when separations of reactant–product(s) are available, coupled with satisfactory analytical procedures for eluted compounds.

The benzodiazepines were of interest because they represent prototypes giving mutual substrate–product interference in the UV region, limiting straight UV spectroscopy in their study (1). Therefore, a kinetic study of the hydrolysis was instituted using both quantitative TLC and spectrophotometric (following separation of reactants from products) methods. The aim, besides delineation of the kinetic profile of chlordiazepoxide hydrolysis, was to compare the agreement between techniques.

In the spectrophotometric procedure, the lactam formed by hydrolysis was readily removed from acidic solutions of the parent, chlordiazepoxide, by partitioning into methylene chloride, allowing a comparative means for determination of velocity constants.

EXPERIMENTAL

Materials—Glass-distilled water was used in all experiments. Reagent grade inorganic salts were employed without further purification.

Chlordiazepoxide reference standard[1], 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide reference standard[1], and 2-amino-5-chlorobenzophenone[1,2] were used.

Kinetic Measurements—Stock Solution 1A was prepared containing 0.90 mg/ml chlordiazepoxide. Two milliliters of this solution was pipetted into 200-ml flasks containing 198 ml of appropriate buffer solution previously equilibrated at the specified temperature, giving a final concentration of 2.98×10^{-5} M. Ten-milliliter samples were periodically withdrawn by pipet and then acidified below pH 1.1 by dropwise addition of concentrated hydrochloric acid.

The aqueous acidic solution was extracted with 3 × 10-ml portions of reagent methylene chloride (methylene chloride was discarded) in a separator, and the aqueous layer was read in the UV at 244 (or 305) nm. Residual absorbances were generally less than 5% of the initial readings and were subtracted for calculation of velocity constants. First-order rate constants were readily evaluated from:

$$k_{obs} = 0.693/t \qquad \text{(Eq. 1)}$$

The observed rate constants derived in this manner were generally reproducible within ±10%.

Stock Solution 2A of chlordiazepoxide (1–2 mg/ml) in appropriate buffer solutions was prepared and placed in constant-temperature baths, 79.5°; aliquots were withdrawn periodically by pipet. A 0.25-ml sample was streaked[3] by means of a streaker apparatus[4] on 20 × 20-cm, 250-μm, silica gel fluorescent plates[5]. One sample was placed on each half of the scored plate with drying by warm air. The plate was developed in *fresh* benzene–dioxane–ethanol–ammonia (50:44:5:1), and the silica gel band corresponding to the

[1] NF reference standards.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Applied Science Laboratories Catalog No. 17710, Hamilton syringe, Hamilton Co., Whittier, Calif.
[4] Catalog No. 1700, Applied Science Laboratories, Inc., State College, Pa.
[5] Analtech, Newark, Del.

Table I—Observed First-Order Rate Constants[a,b] (k_{obs} in hr^{-1}) for Hydrolysis of Chlordiazepoxide (2.98×10^{-5} *M* at 79.5 ± 0.1°, Ionic Strength 0.5)[c]

pH[d]	Buffer, Molarity		k_{obs}, hr^{-1}[e]			
	[HCl]					
0.14	1.0			1.51	—	
0.24	0.8			1.14	—	
0.38	0.6			0.91	—	
0.46	0.5			0.82	—	
0.55	0.4			0.64	—	
0.84	0.2			0.36	0.31[f]	
1.12	0.1			0.26	0.20[f]	
1.40	0.05			0.18	0.16[f]	
1.76	0.02			0.14	0.12[f]	
2.05	0.01			0.12	0.11[f]	
	[H_3PO_4]	[$H_2PO_4^-$]	0.25[g]	0.5[g]	0.75[g]	1.0[g]
1.70	0.3	0.2	0.34	0.49	0.61	0.72
1.76	0.25	0.25	0.36	0.48	0.60	0.70
2.0	0.2	0.3	0.31	0.39	0.47	0.57
2.5	0.1	0.4	0.27	0.36	0.43	0.52
	[HCOOH]	[$HCOO^-$][h]	0.1[i]	0.2[i]	0.3[i]	0.5[i]
3.0	—	—	0.28	0.38	0.46	0.65
3.5	—	—	0.25	0.32	0.39	0.53
3.75	—	—	0.25	0.33	0.40	0.55
4.0	—	—	0.23	0.28	0.33	0.38
	[CH_3COOH]	[CH_3COO^-][h]	0.1[i]	0.2[i]	0.3[i]	0.5[i]
4.0	—	—	0.37	0.52	0.67	0.94
4.5	—	—	0.29	0.39	0.47	0.63
5.0	—	—	0.25	0.28	0.32	0.36
5.4	—	—	0.24	0.26	0.29	0.35
	[$H_2PO_4^-$]	[HPO_4^{-2}]				
6.0	0.2875	0.0605	0.29[j]	0.36[j]	0.44[j]	0.53[j]
7.1	0.020	0.0579	0.23[k]	0.24[l]	—	—
7.9	0.006	0.06	0.23[k]	0.22[l]	—	—
	[H_3BO_3]	[NaOH]		0.1[m]	0.5[m]	
8.9	1.0	0.5		0.24	0.27	
10.9	—	0.02		0.23	0.22[f]	
11.5	—	0.10		0.22	0.23[f]	
—	—	Water —		0.18[n]	0.23[o]	

[a] Analyses consisted of acidification with concentrated hydrochloric acid (dropwise) to pH about 1.1 of a 10-ml aliquot of chlordiazepoxide (2.98×10^{-5} *M*) in appropriate buffer solution followed by extraction with 3 × 10 ml of methylene chloride. The aqueous solution was monitored at either 244 or 307 nm. Acidic solutions were extracted directly with methylene chloride and read. [b] The k_{obs} value was reproducible ±10% from run to run. [c] Ionic strength was made to 0.5 *M* with sodium chloride except with 0.5 *M* HCl or where otherwise specified. [d] The pH values of buffers were measured at 79–80° on a Metrohm pH meter standardized at this temperature. The pH of hydrochloric acid and sodium hydroxide solutions were calculated from pH = $-\log r\pm$ [HCl] and pKw = pH + pOH_s where pOH = $-\log r\pm$ [NaOH]. Activity coefficient, $r\pm$, and pKw data were available in the literature (2). [e] Velocity constants were determined at least in duplicate. Values given are averaged. [f] These constants were determined without addition of sodium chloride to 0.5 *M*. [g] Four columns represent $[H_3PO_4]/[H_2PO_4^-]$ at 1/4, 1/2, 3/4, and 1 × molar concentrations of that stated under buffer heading. Buffer was prepared by addition of 1 *N* HCl to 0.5 *M* $NaH_2PO_4 \cdot H_2O$. [h] Formate and acetate buffers were made by dissolving 0.5 *M* NaOH in water followed by addition of formic or acetic acid to the desired pH and then distilled water to 1 liter. [i] Runs made in 0.1, 0.2, 0.3, and 0.5 *M* acetate and formate, respectively. [j] Carried out at 0.35, 0.21, 0.14, and 0.07 *M* with regard to total buffer. [k] Ionic strength = 0.2. [l] Run at 0.5 *M* total buffer. [m] Run in 0.1 and 0.5 *M* with regard to each buffer component. [n] Chlordiazepoxide hydrochloride in distilled water. [o] Chlordiazepoxide base in distilled water.

starting material was marked under UV light.

The silica gel containing the chlordiazepoxide was removed by scraping and extracted with 25 ml of methanol, followed by filtration through a sintered-glass filter. The filtrate was made up to 50 ml and read[6] at 305 or 244 nm. Log absorbance was plotted *versus* time, and k_{obs} values were obtained using Eq. 1. Rate constants were reproducible within ±10%. Residual absorbance was nil at both wavelengths.

The pH determinations were made[7] at specified temperatures standardized with phthalate and borate buffers (pH 4 and 9.2, respectively) (2). The pH value of hydrochloric acid and sodium hydroxide solutions was determined from pH = $-\log a_{HCl}$ and pKw + pOH where pOH = $-\log a_{NaOH}$. The values for the activity coefficients are available from the literature (3).

Buffers were prepared as stated in Table I. Sodium chloride was added to ionic strength 0.5 unless otherwise specified.

Product Analysis—The products of the hydrolysis of chlordiazepoxide are the corresponding lactam and benzophenone and, occasionally, a spot of unknown origin. These are separated from one another readily by TLC, employing *fresh* solutions of benzene–dioxane–ethanol–ammonia (50:44:5:1 or 60:30:5:1). The following R_f values were obtained from two systems:

	50:44:5:1	60:30:5:1
chlordiazepoxide	0.5	0.65
lactam	0.4	0.55
benzophenone	0.95	0.95
unknown	0.7	0.8

These R_f determinations were made by spotting 0.25 ml of a 2-mg/ml solution on silica gel fluorescent plates[5]. The R_f values may change slightly from day to day and run to run but their relationship to each other remains relatively constant.

RESULTS AND DISCUSSION

The chemistry and kinetics of hydrolysis of several benzodiazepines were reported previously (1). The reaction under consideration is the transformation (Scheme I) of neutral or cationic chlordiazepoxide (I or IH^+) to the lactam (II). Further decomposition to

[6] Cary 14 recording spectrophotometer.
[7] Metrohm pH meter.

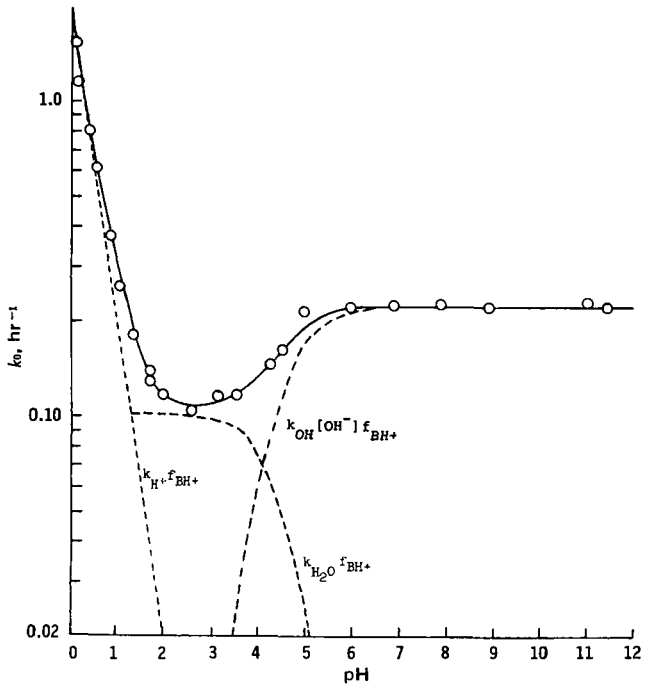


Figure 1—*Rate–pH profile for the hydrolysis of chlordiazepoxide at 79.5° and ionic strength 0.5* M. *Values of* k_0, *except those in hydrochloric acid and sodium hydroxide solution, were obtained from extrapolation of plots of* k_{obs} *against buffer concentration. The circles indicate actual data points or the extrapolated value. The three dashed-line segments are indicative of* k_{H^+}, k_{H_2O}, *and* k_{OH^-}, *with the solid line illustrating a summation of the three.*

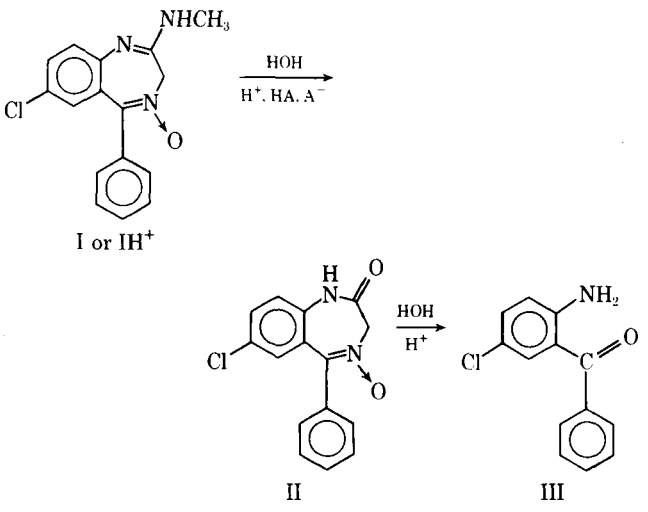
the yellow benzophenone (III) occurred in all of the more acidic reaction mixtures, as previously reported (1).

Intermediates with the diazepine ring opened were reported but were apparently not present in appreciable amounts under the conditions of this study (1).

The benzophenone was not observed to cause interference with the extractive analytical method, because it was separable along with lactam from aqueous acidic solutions containing the starting material.

The hydrolysis of chlordiazepoxide followed strict first-order kinetics in each run. The observed first-order rate constants, k_{obs}, were evaluated utilizing Eq. 1 or:

$$\log(A_t - A_\infty) = \log(A_0 - A_\infty) - k_{obs}t/2.303 \qquad \text{(Eq. 2)}$$



Scheme I

Table II—Effect of Ionic Strength on Hydrolysis of Chlordiazepoxide in Acidic Solution at 79.5°

[HCl]	pH[a]	k_{obs}, hr^{-1} Additional Ionic Strength from Sodium Chloride, M[b] 0.0	0.17	0.43	0.96
0.01 *N*	2.05	0.110	0.113	0.119	0.097
0.02 *N*	1.76	0.120	0.125	0.131	0.134
0.04 *N*	1.49	0.136	0.148	0.158	0.167
0.06 *N*	1.32	0.154	0.173	0.205	0.198
0.10 *N*	1.12	0.207	0.253	0.284	0.312

[a] Measured pH values with hydrochloric acid added where necessary to bring solutions in each set to constant pH value. [b] Total ionic strength for each experiment is total of the concentration of hydrochloric acid in the left column plus additional ionic strength by sodium chloride addition. The exact amounts added were 2, 5, and 11 g of NaCl/200 ml of reaction solution.

where A_t, A_0, and A_∞ represent absorbances at times t, 0, and infinity, respectively.

With the TLC procedure, A_∞ is 0, thus simplifying Eq. 2. Plots derived from Eq. 2 exhibited linearity over three or more half-times, with residual absorbances in the vicinity of 3% of the initial value for the extractive procedure.

The λ_{max} 245 nm was employed for spectrophotometric readings in this work, although the maximum at 305 nm is equally suitable from a theoretical as well as practical point of view.

Rate constants were invariant over a 200-fold chlordiazepoxide concentration range.

Catalysis by Hydrogen Ions and Solvent—The pH dependencies of the observed first-order rate constants, k_{obs}, in the absence of general acids and bases, k_0, is shown in Fig. 1. The k_0 plotted in Fig. 1 was derived from reaction mixtures containing only hydrochloric acid (Tables I and II) or from the intercepts of graphs of k_{obs} *versus* total buffer concentrations of 0.02–0.1 *M* between pH 2.5 and 5.0 (Fig. 2 and Table III). The estimation of k_0 between pH 6 and 11.5 was obtained from sodium hydroxide solutions or by extrapolation of k_{obs} to zero buffer concentration (Table I).

Chlordiazepoxide normally exists in two ionic forms under the conditions studied (4). The log k_0–pH profile (Fig. 1) consists of three portions. These segments are hydrogen-ion-catalyzed hydrolysis of protonated substrate and the interaction of water and hydroxide with the same substrate (5). A second alternative for hydroxide reacting with protonated substrate is the kinetically equivalent interactions of water with neutral chlordiazepoxide, thus producing a curve of the shape previously reported (6). The expression:

$$k_0 = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-])f_{BH^+} \qquad \text{(Eq. 3)}$$

where $k_{OH^-}[OH^-]f_{BH^+}$ is kinetically equivalent to $k'_{H_2O}f_B$, is characterized in Fig. 1. The terms f_{BH^+} and f_B are the fractions of chlordiazepoxide present in the protonated and neutral forms, with a_{H^+} being the hydrogen-ion activity. The second-order veloci-

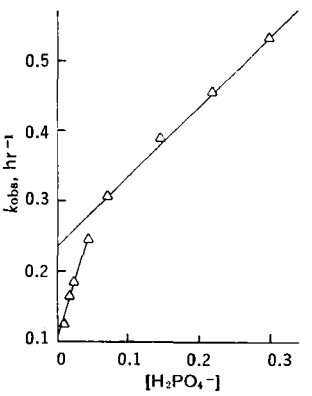


Figure 2—*Influence of pH 1.9 phosphate buffer on hydrolysis of chlordiazepoxide at 79.5° and ionic strength 0.5* M. *The ratio of* H_3PO_4–$H_2PO_4^-$ *is 2:3 in all solutions. Extrapolation of results below and above 0.1* M [$H_2PO_4^-$] *obviously leads to two different* k_0 *values. If one line were drawn connecting all points, it would be curved. This nonlinear dependency is illustrative of a change in the rate-determining step or the presence of at least two rate constants over the range of buffer concentrations represented.*

Table III—Rate Constants (k_{obs} in hr^{-1}) for Hydrolysis at Low Total Buffer Concentrations (0.02–0.10 *M*) at 79.5° and Ionic Strength 0.2

pH	Buffer	k_{obs}, hr^{-1} M^a 0.01	0.02	0.03	0.05	k_{app}[b]
1.90	$[H_3PO_4][H_2PO_4^-]$	0.121	0.158	0.180	0.222	1.2
2.62	$[ClCH_2COOH][ClCH_2COO^-]$	0.115	0.149	0.166	0.230	1.3
4.55	$[CH_3COOH][CH_3COO^-]$	0.169	0.195	0.244	0.312	1.7
		M^c 0.005	0.0075	0.015	0.02	
4.25	$[HCOOH][HCOO^-]$	0.169	0.204	0.241	0.249	1.3
3.1	$[CH_3CH_2OCH_2COOH][CH_3CH_2OCH_2COO^-]$	0.152	0.181	0.186	0.194	1.3

[a] Represents equimolar quantities of each buffer component. [b] Calculated from slope of plots of k_{obs} *versus* total buffer concentrations; measured in M^{-1} hr^{-1}. [c] Represents molarity of acidic component. Molarity of basic component = 3 × molarity of acidic component in each case.

ty constants are $k_{H^+} = 2.05\ M^{-1}\ hr^{-1}$ and $k_{OH^-} = 2.3 \times 10^7\ M^{-1}\ hr^{-1}$, with the first-order constants being $k_{H_2O} = 0.10$ and $k'_{H_2O} = 0.223\ hr^{-1}$ at 79.5°, where 55.5 *M* water is incorporated into the constant.

Since f_{BH^+} and f_B may be written as $[H^+]/([H^+] + K_a)$ and $K_a/(K_a + [H^+])$, respectively, Eq. 3 can be stated as:

$$k_0 = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-])([H^+]/([H^+] + K_a)) \quad \text{(Eq. 4)}$$

The individual constants comprising the rate–pH profile (Fig. 1) were dissected in the following manner. The k_{H^+} was evaluated at high acidities (pH 0.15–1.0), while the k_{OH^-} was established from the results over the 6–11.5 pH region. The k_{H_2O} could be obtained from dilute acidic solutions (about 0.005 *N* HCl) at pH ≈ 1.5–3.0, where neither k_{H^+} nor k_{OH^-} was appreciably operative, or by the difference: $k_0 - (k_{H^+}a_{H^+} + k_{OH^-}[OH^-]) = k_{H_2O}$ in the 1.5–3.0 pH region. The pKa estimated at half-neutralization of a solution of chlordiazepoxide in 50% polyethylene glycol 300–water was 4.6–4.8 at 80°; Fig. 1 illustrates the amount contributed by the individual constants k_{H^+}, k_{H_2O}, and k_{OH^-}.

A surprising, although not unprecedented, feature of the rate–pH profile is the apparently lower rate constant for the spontaneous rate of hydrolysis of protonated species relative to the neutral molecule[8]. This phenomenon appears to argue against a predominantly nucleophilic role for the water reaction with chlordiazepoxide. Were nucleophilic attack the case, one would anticipate a higher velocity constant for the protonated base as compared to the neutral molecule.

This more rapid reaction of "water" with neutral relative to protonated species may represent a reaction of hydroxide ion with the conjugate acid as $k_{OH^-}[OH^-]\ f_{BH^+}$ and not the kinetically equivalent k_{H_2O}/f_B. Alternatively, it may exemplify rate-determining breakdown of the tetrahedral addition intermediate through a neutral transition state (*i.e.*, a transition state with no net charge). Examples of this phenomenon have been reported in the hydrolysis of imidate and thioimidate esters (7, 8).

A second point refuting solvent catalysis of the neutral substrate is the absence of observable buffer attack in the 7–11 pH region. Where water serves as the catalytic species, one might expect catalytic effect by HPO_4^{-2}, borate, *etc.* (9).

Catalysis by General Acids and Bases—General acids and bases are effective catalysts for the conversion of protonated chlordiazepoxide to the lactam (Scheme I) while only slight, almost negligible, catalysis of the neutral molecule is observed. Figures 3 and 4 illustrate plots of k_{obs} and k_{app} against buffer component (9) and percent acid (10), respectively, at total buffer concentrations greater than 0.1 *M*. Both methods are feasible for estimation of the specific catalytic constants (second-order rate constants) for catalysis by acidic and basic species comprising the buffer, k_{HA} and k_{A^-}. Table I lists k_{obs} values for different conditions; Table IV summarizes the second-order rate constants, k_{app}, for buffer catalysis at total buffer concentration >0.1 *M*.

Table III shows the k_{app} for lower concentrations of buffer which produce rate constants inconsistent with those found at higher buffer concentrations.

Figure 2 exemplifies catalysis over the entire realm of buffer concentrations employed for phosphate buffer at pH 1.9 and 79.5°. The overall slope is comprised of at least two separate rate constants, as previously reported (11–14). This nonlinear dependency

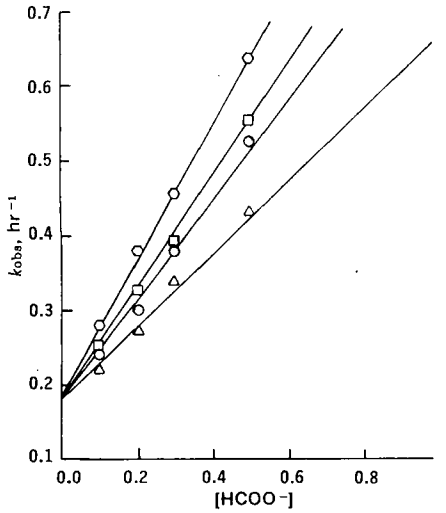


Figure 3—*Dependency of the observed first-order rate constant on the concentration of formate ion in the hydrolysis of chlordiazepoxide at 79.5° and ionic strength 0.5* M. *The extrapolation to zero buffer concentration leads to erroneous results* (k_0 *is apparently 0.18* hr^{-1}) *due to nonlinear dependency at formate concentrations of less than 0.1* M. *Key:* ○, *pH 3.0;* □, *pH 3.25;* ○, *pH 3.5; and* △, *pH 4.0.*

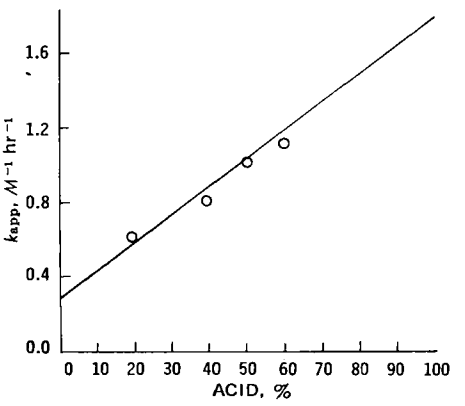


Figure 4—*Apparent second-order rate constants for catalysis of chlordiazepoxide hydrolysis by phosphoric acid–dihydrogen phosphate buffers at 79.5° as a function of the percentage of the acidic component in the buffer. The values* $k_{H_3PO_4} = 1.7\ M^{-1}\ hr^{-1}$ *and* $k_{H_2PO_4^-} = 0.3\ M^{-1}\ hr^{-1}$ *are obtained by extrapolation to 0 and 100% acid, respectively.*

[8] Dr. W. P. Jencks, personal communication.

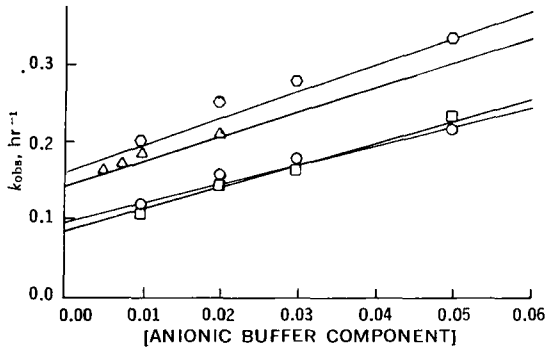


Figure 5—*Dependency of the velocity constants on buffer concentration at total buffer = 0.10* M *and below. Intercepts of plots with ordinate represent* k_0 *values utilized for rate–pH profile (Fig. 1). Key: ○, acetate, pH 4.55; △, ethoxyacetate, pH 3.1; ○, chloroacetate, pH 2.6; and □,* $H_2PO_4^-$*, pH 1.9.*

of the data on buffer concentration is probably brought about by more than one velocity constant being operative over the 0.01–1.0 *M* buffer range. This leads to erroneous k_0 values upon extrapolation (Figs. 3 and 4), unless results of 0.01–0.10 *M* buffers are included. The phenomenon is indicative of a change in the rate-limiting step as the buffer components in solution increase (11–13). This nonlinear relationship is particularly influential in the pH range where appreciable protonated chlordiazepoxide, BH^+, is present (pH 1.8–5.0). At pH values below 1.8, the phenomenon may hold, but it was not investigated with acid–anionic buffers because hydrochloric acid sufficed.

An example of this fallacious extrapolation is given in Fig. 3, where a value of $k_0 = 0.18\ M^{-1}$ is indicated for several pH values (acetate buffer) when the intercepts are actually considerably lower.

It is evident from the latter data in Table I that the degree of buffer catalysis is decreased or almost eliminated at pH >7, indicating an absence of buffer effect on neutral chlordiazepoxide.

The contribution of each buffer constituent of acid and acid anion to hydrolysis of protonated chlordiazepoxide, BH^+, may be estimated by plots of k_{obs} *versus* anion as shown in Fig. 3, utilizing the acidic dissociation constant of the buffer being investigated:

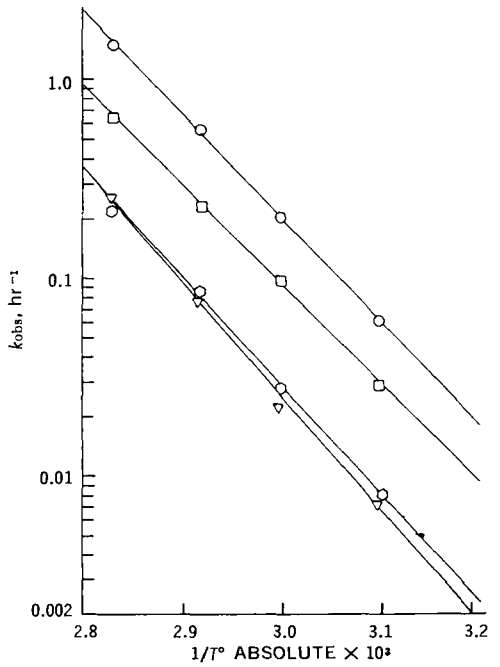


Figure 6—*Arrhenius parameters for hydrolysis of chlordiazepoxide. Key: ○, 1.0* N *HCl; □, pH 4.5 acetate buffer, ionic strength 0.5; △, pH 7 phosphate buffer, ionic strength 0.5; and ○, 0.1* N *NaOH.*

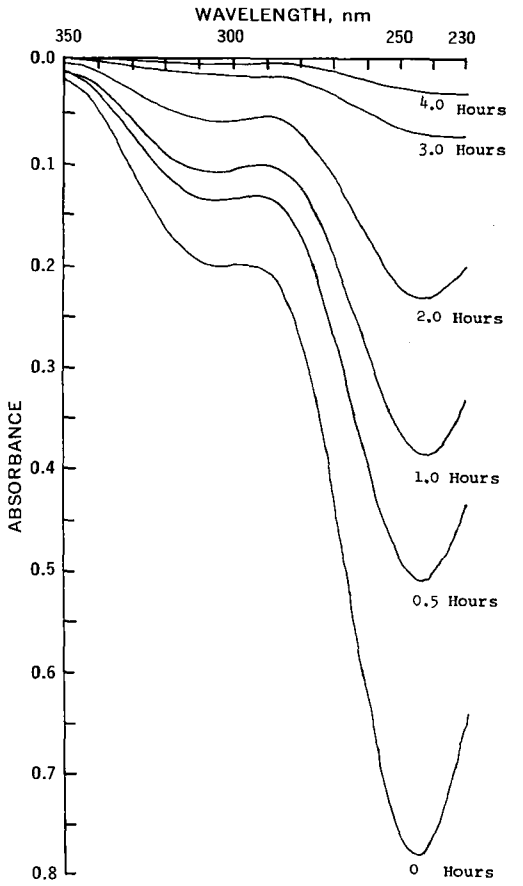


Figure 7—*Disappearance of absorbance as a function of time. Chlordiazepoxide was extracted from TLC plates at the hourly intervals listed on the spectrophotometric curves. The reaction conditions were 0.3* M *acetate, pH 4.0, ionic strength 0.5* M*, 79.5°. Peaks exhibit corresponding diminution.*

($K_a = [H^+][A^-]/[HA]$). The results presented in Figs. 3 and 4 are for total acidic and anionic concentrations of 0.1–1.0 *M*.

Equation 4 may then be written with the introduction of terms for the buffer where total buffer is less than 0.1 *M*:

$$k_{obs} = k_0 + (k_{HA}[HA] + k_{A^-}[A^-])(f_{BH^+}) \qquad \text{(Eq. 5)}$$

or:

$$k_{obs} = k_0 + \{k_{HA}([H^+]/K_a) + k_{A^-}\}[A^-] \qquad \text{(Eq. 6)}$$

where $f_{BH^+} \approx 1$; *i.e.*, pH < 3.5; pKa ≈ 4.6–4.8 at 80°.

Therefore, a plot of the $k_{obs} - k_0$ against [A−] where buffer concentration is >0.1 *M* gives a straight line with:

$$\text{slope} = k_{HA}([H^+]/K_a) + k_{A^-} \qquad \text{(Eq. 7)}$$

From a graph of Eq. 6 with slopes of a given buffer combination (*e.g.*, acetate–acetic acid) at several pH values, the slope obtained equals k_{HA} with an intercept of k_{A^-} (9). When the data of Fig. 3 are plotted utilizing Eq. 6, the values are: $k_{HCOOH} = 0.92\ M^{-1}\ hr^{-1}$ and $k_{HCOO^-} = 0.48\ M^{-1}\ hr^{-1}$ at 79.5°.

The k_{HA} and k_{A^-} values may be determined utilizing plots of k_{app} against the percentage acid in the buffers, as given by Sander and Jencks (10) and exemplified in Fig. 4.

The following results were obtained from Fig. 4: $k_{H_3PO_4} = 1.7\ M^{-1}\ hr^{-1}$ and $k_{H_2PO_4} = 0.3\ M^{-1}\ hr^{-1}$ at 79.5°. These are in close proximity to the values obtained from Eqs. 6 and 7.

Table III summarizes some k_{obs} and k_{app} values for lower buffer concentrations (0.02–0.1 *M*). Figure 2 also depicts the influence of buffer on the observed first-order rate constant, with at least two slopes being obvious. Extrapolations of results in Table III were utilized in the determination of the k_0 shown in Fig. 1. Figure 5 illustrates the dependency of velocity constants (Table III) on the anionic component at lower buffer concentrations.

Table IV—Values of Apparent Second-Order Rate Constants[a] (k_{app} in M^{-1} hr^{-1}) for Buffer-Catalyzed Hydrolysis of Chlordiazepoxide at 79.5° and Ionic Strength 0.5 M

Acid	pH[b]	Acid[c], %	Concentration Range[d], M	k_{app}, M^{-1} hr^{-1}[e]
H_3PO_4	1.70	60	0.125–0.50	0.89
	1.76	50	0.125–0.50	0.90
	2.0	40	0.125–0.50	0.49
	2.5	20	0.125–0.50	0.36
HCOOH	3.0	—	0.1–0.5	0.92
	3.5	—	0.1–0.5	0.71
	3.75	—	0.1–0.5	0.68
	4.0	—	0.1–0.5	0.49
CH_3COOH[f]	4.0	—	0.1–0.5	1.92
	4.5	—	0.1–0.5	0.90
	5.0	—	0.1–0.5	0.38
	5.5	—	0.1–0.5	0.30
$H_2PO_4^-$	6.0	80	0.085–0.34	1.03

[a] Measured as stated under *Experimental* utilizing 2.98×10^{-5} M chlordiazepoxide. [b] The pH values were measured at 79° on a pH meter standardized at that temperature. [c] Percentage acid is not listed for acetate and formate solutions become they were prepared by addition of the appropriate acid to 0.5 M NaOH with dilution to 1 liter. [d] Concentration refers to total buffer, except for formate and acetate where acetate and formate ions were used in the calculation because total buffer was not known from the preparation method except by calculation from pH and pKa of the appropriate solutions. [e] Obtained from slopes of plots of k_{obs} against total buffer concentration, except in cases of acetate and formate buffers where anionic component was utilized for abscissa. [f] Chlordiazepoxide has pKa of about 4.6–4.9 at 79°; therefore, both protonated and nonprotonated species are present in the acetate buffer region.

At pH 4 and above, both appreciable cationic and neutral substrates are present so a mixture of rate constants is actually given in which the effects of various acids and their anions, namely acetate buffer, on both protonated and nonprotonated chlordiazepoxide should be considered for rigorous analysis.

Arrhenius Parameters—Estimates of the apparent activation energies were made from the Arrhenius equation:

$$\log k_{obs} = \log P - Ea/2.303RT \qquad \text{(Eq. 8)}$$

Values derived from the preceding expression vary between 23.0 and 25.2 kcal/mole under the condition shown in Fig. 6. This information corroborates the analytical method employed as well as giving the *Ea* determinations.

Ionic Strength Effects—In the acidic region where hydronium-ion catalysis on cationic substrate is evident, it may be expected that the Bronsted–Bjerrum equation (15) might hold in some modified form (16) or at least that a qualitative agreement might be seen. This was seemingly the case as may be seen from the results in Table II, which shows a slight increase in the velocity constants.

Reaction Products—Over the entire pH range investigated, the reaction sequence given in Scheme I predominated. Other products of the hydrolysis at higher temperatures were reported (1, 17) but only a fourth trace spot was evidenced with the TLC systems employed. Formation of the benzophenone was increased as the pH scale was descended.

Quantitative TLC and Comparisons—An original aim of the work was to investigate the rate constant discrepancies taking place when chlordiazepoxide was subjected to quantitative thin-layer kinetic analysis. For this reason, a second analytical method (extraction of acidified aqueous substrate with methylene chloride) was applied as a check. It is now evident that buffer catalysis along with the previously mentioned nonlinear dependency of rate constants on buffer concentrations contributed to the difficulties encountered.

Figure 7 illustrates a typical absorbance loss for chlordiazepoxide (80°, pH 4, Ac^- = 0.3 M) when subjected to quantitative TLC. The k_{obs} is 0.77 hr^{-1}, in comparison to 0.80 hr^{-1} determined by the extractive procedure. Figure 8 shows the reaction rate constant at pH 2 (phosphate buffer) and 80° plotted at both wavelengths (244 and 305 nm), with linearity at both wavelengths as determined by quantitative TLC. Table V lists data derived from both techniques and illustrates a reasonable degree of correlation between the two

Table V—Comparative Results of k_{obs} for Chlordiazepoxide Hydrolysis of Quantitative TLC Method Relative to the Extractive Procedure at 79.5°

pH	Buffer	k_{obs}, hr^{-1} Quantitative TLC	k_{obs}, hr^{-1} Extraction[a]
0.46	0.5 N HCl	0.74	0.82
1.12	0.1 N HCl	0.23	0.20
1.40	0.05 N HCl	0.17	0.16
2.0	Phosphate[b]	0.41	0.36
3.0	Formate[c]	0.61	0.65
4.0	Acetate[c]	0.91	0.74
4.5	Acetate[c]	0.58	0.63
5.0	Acetate[c]	0.41	0.36
6.0	Phosphate[d]	0.29	0.29
7.1	Phosphate	0.20	0.23
11.5	0.1 N NaOH	0.21	0.22

[a] Taken from Table I. [b] Buffer contains 0.125 M of each component at ionic strength 0.5. [c] 0.5 M acetate and formate ion. [d] See Table I, pH 6.0 and 7.1 for buffers.

sets. It was concluded that the quantitative thin-layer method works well for this benzodiazepine as well as certain congeners.

Problems initially arising were attributed to:

1. Influence of buffer catalysis when precautions were not taken to hold buffer concentration and anionic and basic components of the buffers constant at the given pH values.
2. Insolubility of the substrate in aqueous solutions as the pH increased. Concentrations of 1–2 mg/ml were used at pH 5. Whereas the solutions were homogeneous at 60–80°, they were prone to precipitate upon cooling to room temperature during the streaking process (see *Experimental*).
3. The nonlinear dependency of rate constants and buffer concentrations.

CONCLUSIONS

The hydrolytic degradation of chlordiazepoxide in aqueous solution *via* the lactam was studied over the 0.15–11.5 pH range at 79.5°. The lactam and benzophenone were the principal products formed under these conditions, so the reaction investigated was conversion of chlordiazepoxide to the corresponding lactam. Intermediates previously reported between the lactam and benzophenone (1) were not observed, with the exception of one of unknown origin seen by TLC. These intermediates may be transient in nature under the conditions employed.

This reaction is catalyzed by hydrogen and hydroxide ions, general acids, and general bases and undergoes a solvent reaction with

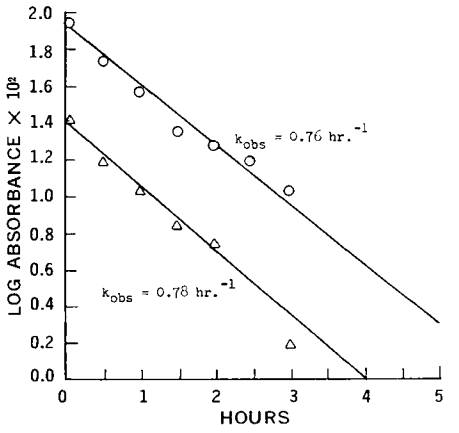


Figure 8—*Plots of results obtained from pH 2 phosphate buffer, H_3PO_4–$H_2PO_4^-$ = 0.3 M/0.45 M, ionic strength 0.5, temperature 80°. Data were obtained from samples periodically streaked and then separated by TLC, followed by elution from adsorbent and analysis at the pertinent wavelengths. Key:* O, *245 nm; and* △, *305 nm. See Fig. 7 for a typical plot of the absorbance diminution as function of time.*

protonated and neutral substrates. The following rate law may be written:

$$k_{obs} = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-] + k_{HA}[HA] + k_A[A^-])\, f_{BH^+} \quad \text{(Eq. 9)}$$

where f_{BH^+} and f_B represent the fractions of protonated and neutral chlordiazepoxide at a given hydrogen-ion concentration; pKa = 4.6–4.8 measured in 50% polyethylene glycol 300 at 80°.

The term $k_{OH^-}[OH^-]f_{BH^+}$, Eqs. 4 and 9, may be expressed as the kinetically equivalent $k_{H_2O}f_B$, as pointed out previously (8, 9).

Nonlinear dependency of the rate constant with buffer concentration in the 1.7–4.5 pH range was seen, which is likely the result of a change in the rate-limiting step with an increase of buffer in solution (11–14).

An extraction procedure was developed as a comparative method for the quantitative TLC process. The technique consisted of extraction of the decomposition products into methylene chloride from acidified chlordiazepoxide followed by UV analysis of the acidic aqueous layer.

The quantitative TLC method worked well for chlordiazepoxide, with initially discovered discrepancies being the consequence of a lack of control of the buffer concentration and occasional precipitation of the substance from solution upon cooling during the streaking procedure. When these factors were controlled, results were obtained in accord with those from the extraction procedure.

The work presented here corroborates applicability of the TLC technique to molecules that do not lend themselves to kinetic analysis by standard differential UV techniques. Thus, the delays occasionally encountered in assay development may be circumvented. Although this procedure is not the ultimate in simplicity, it affords ready availability of stability results to the formulator as well as to the kineticist.

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Simultaneous Determination of Phenylbutazone and Oxyphenbutazone in Plasma by High-Speed Liquid Chromatography

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Abstract □ A sensitive, specific, high-speed liquid chromatographic procedure is described for the simultaneous determination of phenylbutazone and its metabolite, oxyphenbutazone, in plasma. Acidified plasma is partitioned with cyclohexane–ether (1:1) containing the 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde as an internal standard. The organic extract is reduced to dryness, the resulting residue is redissolved in chloroform, and aliquots of this solution are chromatographed on an adsorption column, using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in *n*-hexane at 35°. Use of a UV detector permits quantitative analysis of samples containing less than 0.25 μg/ml of phenylbutazone or oxyphenbutazone.

Keyphrases □ Phenylbutazone–oxyphenbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Oxyphenbutazone–phenylbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Liquid chromatography, high speed—simultaneous determination in plasma of phenylbutazone and oxyphenbutazone

Several spectrophotometric procedures (1–6) and a GLC method (7) have been reported for the determination of phenylbutazone in biological fluids; however, fewer methods have been described for the estimation of two known metabolites (1) of this drug, oxyphenbutazone (1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine) and hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine].

The classical method of Burns *et al.* (1) is not of sufficient sensitivity to estimate phenylbutazone and oxyphenbutazone in biological fluids following single