Kinetics and Equilibrium of the Reversible Alprazolam Ring-Opening Reaction

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Abstract □ Alprazolam underwent a facile 1,4-benzodiazepine ring-opening reaction in an acidic aqueous solution to form a benzophenone compound. The reaction was demonstrated by means of UV, IR, and ^{1}H- and ^{13}C-NMR spectroscopy. Its reverse cyclization reaction to alprazolam occurred when an acidic solution was neutralized. Both the ring-opening and the cyclization rate constants were obtained from the overall rate constant measured at 25° over a pH range of 0.5–8.0; the latter was measured by monitoring the UV spectral change of the reaction. Although the equilibrium was favored for the benzophenone compound in acidic solutions, it was possible to directly measure the cyclization rate at three acidic pH values by providing a sink condition for the product, alprazolam, using a biphasic reaction system. The bell-shaped cyclization rate pH profile was interpreted in terms of a change in the rate-determining step. The pH profile of the ring-opening rate showed an inflection point indicating a different reactivity of mono- and dicationic alprazolam. The apparent equilibrium between alprazolam and the benzophenone compound at a given pH was estimated from the rate constants for the ring-opening and cyclization reactions. The results agree with the apparent pK_a measured by a conventional UV spectrophotometry and a titration technique. The pK_a of monocationic alprazolam, the reactive species for the covalent hydration, was determined from the pH dependence of the initial absorbance when an alprazolam solution is acidified.

Keyphrases □ Alprazolam—kinetics and equilibrium of reversible ring-opening reaction □ Kinetics—alprazolam, reversible ring-opening reaction □ Equilibrium—alprazolam, reversible ring-opening reaction

The relationship between structure and chemical reactivity is an important research subject in physical organic chemistry. In heterolytic reactions involving nucleophilic reagents, an activated electrophile is often chosen as a model substrate and its reaction with a variety of nucleophiles studied. The nucleophilicity is determined from the observed reaction rates, preferably in the form of linear free-energy relationships. Such studies are well documented for substrates containing carbonyl groups (1–7) and to a lesser extent for substrates containing imino (C=N—) bonds (8). A systematic structural change in a series of analogous substrates toward a given nucleophile such as water has not been incorporated as commonly in mechanistic studies, presumably because of a limited choice of substrates. This practice is usually the case, especially when looking for a series of substrates containing an activated imino bond (9).

BACKGROUND

Analogues of 1,4-benzodiazepine provide an excellent opportunity to investigate the structural influence on the lability of a C=N— bond toward a given nucleophile, since many 1,4-benzodiazepines undergo nucleophilic covalent hydration across the C(5)—N(4) double bond, which is followed by ring opening to the corresponding benzophenone compounds (10–21).

A detailed kinetic study on the reversible ring-opening reaction of alprazolam (8-chloro-1-methyl-6-phenyl-4*H*-*s*-triazolo[4,3-*a*][1,4]benzodiazepine)[1] was undertaken. In addition to the azomethine bond in the seven-membered ring, alprazolam contains other weakly basic centers on the 1,2,4-triazole. The opened-ring compound carries a primary amino function; thus, the observed rate constant (which is the sum of the ring opening and the cyclization rate constants) was a composite function of several microscopic rate constants. The present study attempted to determine these constants separately. Comparison of these constants with the corresponding rate constant of other benzodiazepines may eventually clarify the structure–activity relationships associated with an imino bond.

The facile reversibility of the ring-opening reaction required solution of the multiple equilibria involved. An attempt was made to solve these equilibria in terms of pH change through experimentally determined ionization constants and the apparent equilibrium between the two compounds engaged in the reversible reaction.

EXPERIMENTAL

Materials and Equipment—A buffer system of low ionic strength (0.01 *M*) (22) was used throughout the kinetic study to avoid any possible buffer catalysis. At pH <2, the ionic strength was inevitably >0.01 *M*; however, only hydrochloric acid was present in the system. For the pH ranges of 2.0–3.8, 4.0–6.0, 6.0–7.0, and 7.0–8.5, chloroacetate, succinate, phosphate, and tromethamine buffers were used, respectively. The buffer components were analytical grade.

A UV spectrophotometer[2] with a cell compartment between thermoplates was used throughout the kinetic study. A constant temperature of 25 ± 0.1° was maintained by a circulating water bath[3]. When UV scanning was necessary, a different spectrophotometer[4] equipped with an automatic repetitive scanning device was used. Titration for pK_a determination as well as pH measurement was done using an automatic titrator[5]. When continuous flow of a reaction system through a spectrophotometer was required, a two-piston pump with a low dead volume[6] was used for circulation.

NMR Studies—All spectra were reported on a 200-MHz NMR spectrometer[7] with a magnetic field strength of 4.7 tesla. The instrument was operated in the FT mode and the lock was provided by the deuterium signal from the solvent.

^{1}H-NMR spectra were run at 200 MHz at ambient probe temperature (20.5°). Typical instrument settings were: sweep width, 2600 Hz; acquisition time, 1.5 sec; data points, 8K (with zero filling); and pulse width, 5 μsec. Typically, <100 transients were needed for a good signal-to-noise ratio.

^{13}C-NMR spectra were run at 50.3 MHz at ambient probe temperature (21.5°). Typical instrument settings were: sweep width, 10,000 Hz; acquisition time, 0.720 sec; data points, 16K (with zero filling); and pulse width, 5 μsec (30° tip angle). Typically, 20,000 transients were needed for a good signal-to-noise ratio.

Samples for ^{1}H- and ^{13}C-NMR spectra were prepared by dissolving 250 mg of alprazolam (~0.8 mmole) or compound *C* (Scheme I; ~0.75 mmole) in 2.0 ml of deuterated chloroform[8]. To this was added 158 μl of trifluoroacetic acid[9] (~2 meq) and 50 μl of deuterated water[8].

Reversible Reaction Kinetics—The overall reaction was initiated either by acidifying a neutral solution of alprazolam to a desired pH or by neutralizing an acidic solution which previously had been equilibrated at a pH close to 1.0. In the former case, ~160 μl of a stock solution of alprazolam in methanol (~10 mg/25 ml) was injected directly into a 1.0-cm

[1] The numbering system is in accordance with that of *Chemical Abstracts*. In the remainder of the present report, however, the numbering system of the parent 1,4-benzodiazepine is used exclusively.

[2] Gilford model 250 spectrophotometer.
[3] Lauda model B-2 waterbath.
[4] Zeiss model DMR-21 spectrophotometer.
[5] Radiometer model PHM-26 equipped with models ABU-12, TTA 60, and TTT-116.
[6] Milton Roy minipump.
[7] Varian model XL-200 NMR spectrometer.
[8] Merck Sharp and Dohme. Deuterated chloroform containing 1% tetramethylsilane was passed through a silica column to eliminate trace hydrochloric acid.
[9] Aldrich (used as received).

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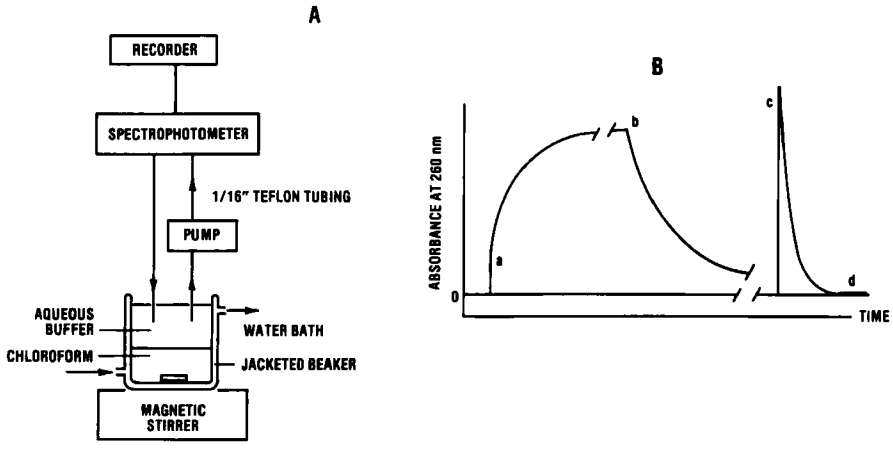


Figure 1—*(A) Experimental setup for monitoring irreversible process of cyclization of the opened-ring compound to alprazolam. (B) A typical tracing of absorbance at 260 nm as a function of time: a to b, reversible overall (largely ring-opening) reaction; b to c, cyclization reaction only (alprazolam is continuously extracted to chloroform layer); c to d, alprazolam partitioning process at given hydrodynamics.*

cell containing 3.0 ml of a given buffer. A Hamilton microsyringe (250 μl) was used for introducing the alprazolam stock solution. After rapid mixing, the absorbance increase at 260 nm was continuously monitored. The concentration of alprazolam and methanol in the final reaction system was 6.2×10^{-5} *M* and 5.0% (v/v), respectively.

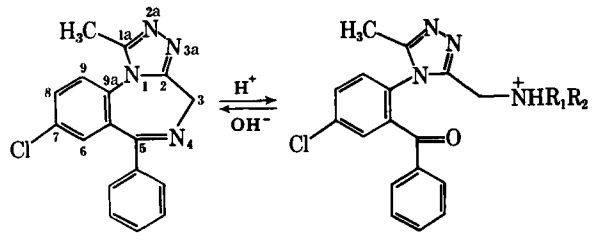
At pH >3.5, the absorbance change was so small that the overall reaction rate constant was obtained by neutralizing an acidic solution. A typically acidic stock solution of alprazolam was prepared by dissolving ~10 mg of alprazolam in 25 ml of 0.2 *N* HCl containing 20% (v/v) methanol. The solution was left at room temperature for at least 10 min but not more than 30 min prior to use. This stock solution (160 μl) was introduced into a cell containing 3.0 ml buffer at the desired pH. The reaction was then initiated by rapidly injecting 160 μl of 0.2 *N* NaOH. After mixing, the absorbance increase at 240 nm was continuously recorded. The concentration of alprazolam in the final reaction mixture was the same as in the first case; however, the methanol concentration was only ~1.0% (v/v). The final ionic strength was no longer 0.01 *M*; it was calculated to be ~0.02 *M*. A separate series of experiments established that a methanol concentration of ≤5% (v/v) did not influence the observed rate constant within experimental error.

In both of the described procedures, the pH determined at the end of the reaction was considered to be the pH maintained during the reaction. The observed rate constant (k_{obs}) was calculated in a conventional manner, *i.e.*, from the slope of the plot of $\log(A_\infty - A_t)$ *versus* time, where A_t is absorbance at a given time t and A_∞ is that at equilibrium. In general, excellent first-order kinetics were observed over three half-lives. Under extremely acidic pH, however, a small but significant deviation from linearity was observed after the reaction was more than 75% completed.

Kinetics of Irreversible Cyclization Reaction—At pH 2.41, 2.75, and 3.35 the rate of cyclization of the opened-ring compound to alprazolam was measured by providing a sink condition for the product. Approximately 0.7 ml of an alprazolam stock solution in methanol (~1.38 mg/ml) was added to 50 ml of a given buffer solution which had been placed in a jacketed beaker at 25°. The absorbance change at 260 nm was followed immediately by circulating the reaction mixture through a 1.0-cm flow-through cell at a flow of ~18 ml/min. After the equilibrium was established and k_{obs} was obtained, 50 ml of chloroform previously saturated with the buffer solution and kept at 25° was gently added. The binary mixture was vigorously stirred with a magnetic stirrer while the aqueous upper layer was being continuously circulated through the spectrophotometer.

Every 5–10 min, 20 ml of the chloroform layer was replaced with fresh chloroform to make sure that a perfect sink condition for alprazolam was maintained. The decrease in UV absorption 260 nm from the aqueous layer was recorded continuously down to zero. To be sure that under the hydrodynamic conditions adoped the observed rate did not merely represent the transport rate across the interface, the following test was made at the end of a kinetic run. The pH of the aqueous layer remaining on top of chloroform was readjusted to 8.0–8.5 by adding a concentrated solution of tromethamine. To this, ~0.7 ml of the alprazolam stock solution in methanol was introduced and the disappearance was monitored. Throughout this operation, the hydrodynamics remained the same as previously. The experimental setup and a typical absorbance tracing are shown in Fig. 1.

Determination of pK_a—The pK_a of the primary amino function present in the opened-ring compound was determined from a rapidly obtained titration curve. An accurately known amount of alprazolam between 50 and 100 mg was dissolved in 2.50 ml of methanol. To this, 15



A *B*: $R_1 = R_2 = H$
C: $R_1 = CH_3$ and $R_2 = H$
D: $R_1 = R_2 = CH_3$

Scheme I

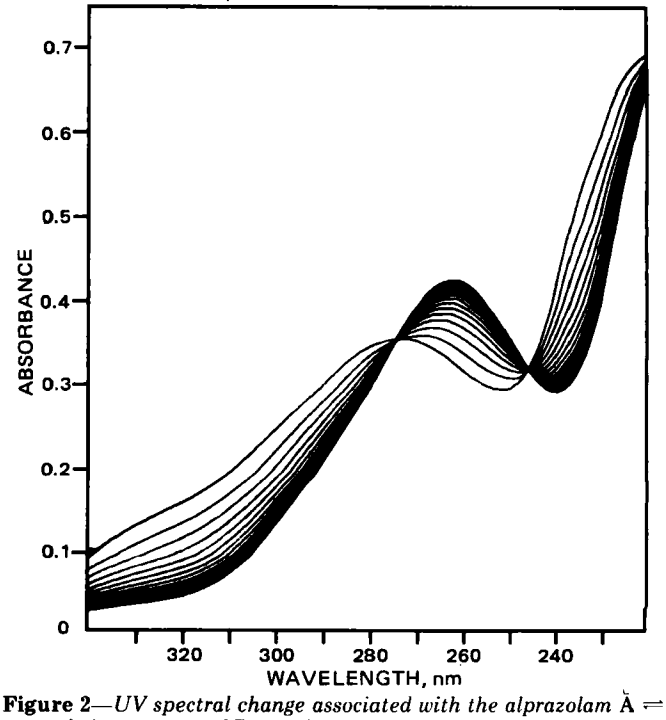


Figure 2—*UV spectral change associated with the alprazolam* A ⇌ *opened-ring-compound* B *reaction at pH 1.17 and room temperature;* A + B = 4.02×10^{-5} M; *methanol, 10% (v/v); and repetitive scanning speed, 300 nm/min.*

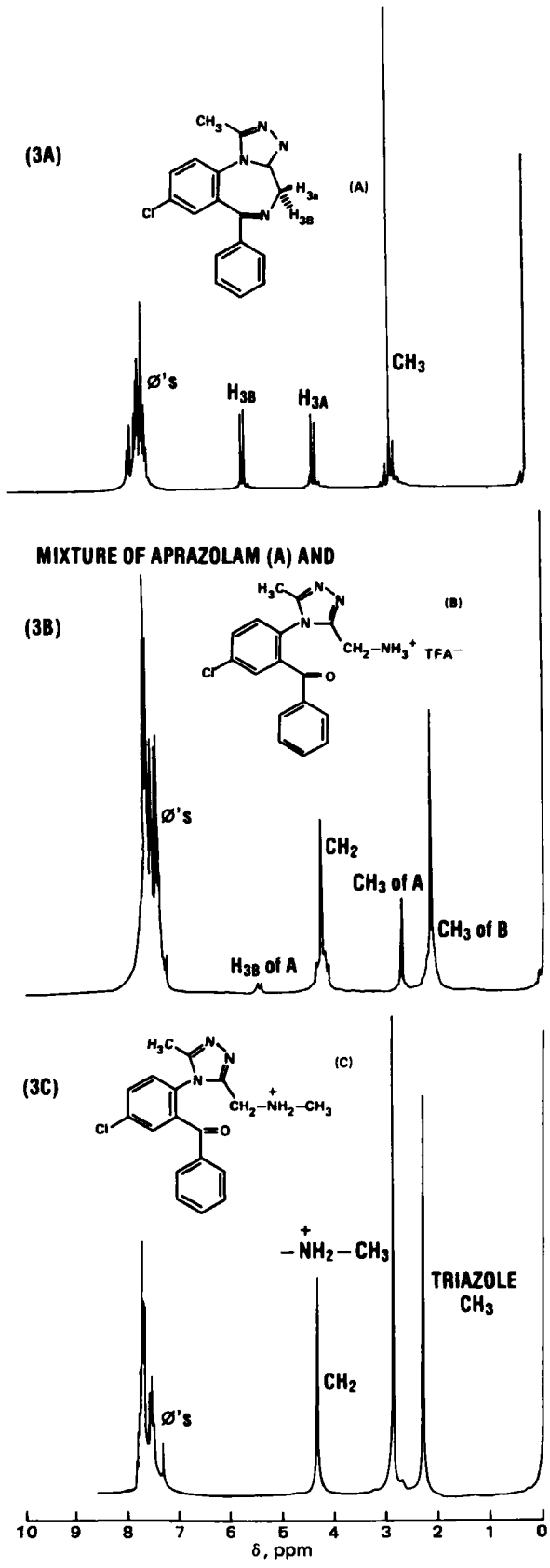


Figure 3—^1H-*NMR spectrum of alprazolam in deuterochloroform, A; a mixture in* $CDCl_3$ *of alprazolam and compound* B *(~1:3) which was formed* in situ *upon addition of trifluoroacetic acid and deuterated water, B; and compound* C *in* $CDCl_3$ *containing* TFA *and* D_2O, *C. In samples B and C, the amounts of* TFA *and* D_2O *added were 2.0 meq of alprazolam or compound* C *and 50 μl, respectively. Spectra were all obtained from a sample size in the range of 250 mg.*

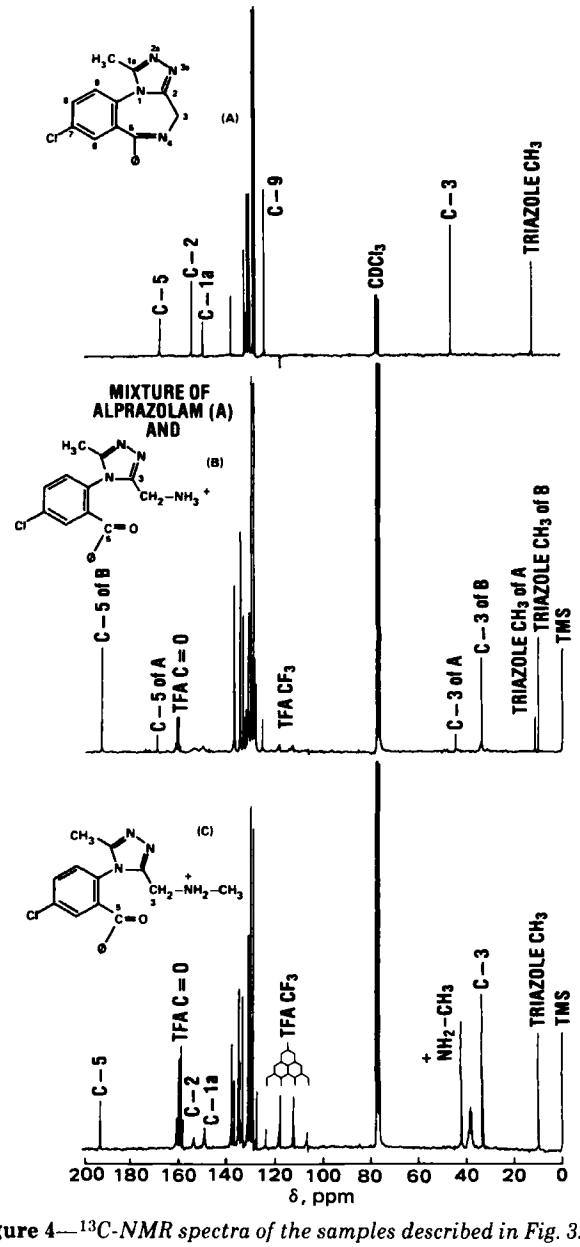


Figure 4—^{13}C-*NMR spectra of the samples described in Fig. 3.*

ml of water and 3.0 ml of 0.1 *N* HCl were added. The solution was left for at least 30 min, and the pH was accurately measured (2.0–2.5). Immediately after adding 3.0 ml of 0.1 *N* NaOH, the solution was rapidly titrated with 0.1 *N* HCl. The starting pH was ~8.5. The titration was repeated three times, and it took <4 min on the average to complete the titration. The pH observed at half of the titration end point was taken as the pKa of the primary amino group of the benzophenone compound. Since less than 2.0 ml of the titrant was consumed at the titration end point, the concentration of alprazolam and methanol near the pKa was on the order of 0.01 *M* and 10% (v/v), respectively. Although the system contained as much as 10% methanol, precipitation was observed at ~5–10 min after completion of titration (pH ~2.75), but not during the titration.

An attempt was made to measure the pKa of protonated alprazolam (the reactive species) from the pH-dependence of the initial absorbance at a given wavelength immediately after acidification of a series of alprazolam solutions at a given concentration. The experimental procedure was essentially the same as the procedure in which k_{obs} was measured. Into a 1.0-cm UV cell containing 3.0 ml of a given buffer, 100 μl of an alprazolam stock solution in methanol (0.719 mg/ml) was rapidly introduced through a 250-μl Hamilton syringe. Absorbance at 310 nm was monitored immediately and the initial absorbance value was estimated from extrapolation to the time of mixing. At a certain pH, the ring-opening reaction occurred quite rapidly (*i.e.*, $t_{1/2} \simeq 2$ min at pH 1.5). In such a case, the absorbance at the moment of mixing was estimated from the first-order kinetic plot by extrapolation to $t = 0$.

The conventional UV spectrophotometric pK_a determination (23) was carried out by scanning a series of alprazolam solutions at a given concentration (6.13×10^{-5} *M*) after the solutions were left at room temperatures for at least 30 min. The methanol concentration in these solutions was 5.0% (v/v). Each UV scan was made against the corresponding buffer as a reference.

RESULTS AND DISCUSSION

System Characterization—At an early stage of drug development, it was found that alprazolam undergoes the reversible reaction shown in Scheme I; the opened-ring compound *B* was favored in an acidic solution and alprazolam was favored in a neutral or alkaline solution. The identity of the reaction was established by means of IR, UV, and ^{1}H- and ^{13}C-NMR spectroscopy.

The solid product obtained immediately after neutralizing an acidic solution of alprazolam contained two compounds: alprazolam and a compound with an IR spectrum closely resembling that of compound *C* (benzophenone C=O absorption band at 1665 cm^{-1}). Attempts to isolate this second component in free form were not successful. Exhaustive crystallization only produced alprazolam. A similar difficulty in obtaining the opened-ring compound was reported previously for a related 1,4-benzodiazepine system (10).

On the other hand, when an alprazolam solution was acidified, the UV spectrum changed continuously and rapidly with two isosbestic points at 246 and 274 nm (Fig. 2). At pH ~1.0, the equilibrium UV spectrum (λ_{max} 262 nm and λ_{min} 239 nm) was found to be virtually identical to that of compound *C* or *D* (λ_{max} 263 nm and λ_{min} 238 nm; ϵ_{263} 1.01×10^4 and ϵ_{238} 5.80×10^3). Compounds *C* and *D* do not cyclize to form a 1,4-benzodiazapine ring. Similarly, when an acidic solution of alprazolam was neutralized with a concentrated sodium hydroxide solution, the UV spectrum slowly changed back to that of the neutral alprazolam species (λ_{max} 222 nm and λ_s 245 nm; ϵ_{222} 4.27×10^4 and ϵ_{245} 1.86×10^4).

In the ^{1}H-NMR spectrum, the nonequivalent protons at C-3 of alprazolam appeared at 5.4 and 4.10 ppm (Fig. 3A). These doublets merged into a singlet at 4.10 ppm when trifluoroacetic acid and deuterated water were added (Fig. 3B). In addition, the singlet at 2.60 ppm, which corresponds to the methyl group on the triazole moiety, underwent an upfield shift to 2.00 ppm. The resulting ^{1}H-NMR spectrum of the compound produced from alprazolam in the presence of trifluoroacetic acid and deuterated water closely resembled the ^{1}H-NMR spectrum of a model compound *C* obtained under identical conditions (s at 4.35 ppm for 2H and s at 2.32 ppm for 3H) (Fig. 3C).

Changes in the ^{13}C-NMR of alprazolam (Fig. 4A) that occurred upon addition of trifluoroacetic acid and deuterated water provided more direct evidence for the reaction shown in Scheme I. A downfield shift took place for C-5, from 168 to 192 ppm, supporting the presence of a benzophenone carbonyl carbon (Fig. 4B). The upfield shift of C-3 from 46 to 34 ppm was also in accordance with the ring-opening reaction. As expected, the signal corresponding to the methyl group on the triazole moiety changed very little with an upfield shift of ~1.5 ppm.

Finally, the overall ^{13}C-NMR spectrum of the reaction product was nearly identical to that of compound *C* (Fig. 4C), with the benzophenone carbonyl at 193 ppm, C-3 at 34 ppm, and the triazole methyl at 10 ppm. This is consistent only if the reaction product has the same benzophenone-type structure as compound *C*.

The reaction that alprazolam undergoes in an aqueous acidic solution can be further characterized based on the aforementioned spectroscopic data. IR and NMR (particularly ^{13}C-NMR) data positively identify the reaction as that in Scheme I. The product *B* is favored under acidic conditions and *A* under neutral or alkaline conditions. The reaction is similar to the ring-opening reaction of other 1,4-benzodiazepines (10–21).

The presence of isosbestic points in the UV spectral change supports the conclusion that there were no significant side reactions, during the attainment of equilibrium. Under a strongly acidic condition (pH < 0.5), however, the isosbestic points were found to drift. The nature of this slow side reaction has not been established.

The UV spectrum of alprazolam cation ($\lambda_{max} \simeq 274$ nm; Fig. 2), which rapidly changes as the ring-opening reaction takes place, is different from that of neutral alprazolam (λ_{max} 222 nm and λ_s 245 nm).

Cyclization Kinetics—The UV spectrum of alprazolam changed very little when a neutral solution was acidified to pH 5 or above, indicating that alprazolam is the predominant species at a neutral or an alkaline pH. The spectral change that occurred when an acidic solution was neutralized to pH >6 reflected the cyclization reaction exclusively. On the other hand, the rate of UV spectral change on acidification of an alprazolam solution to a pH <5 reflected the rate of both the ring-opening and the cyclization reactions. To separately determine the cyclization rate for this pH range, a sink condition for the product (alprazolam) was provided by continuously replacing chloroform in the system. A critical assumption made here was that only alprazolam partitions into the chloroform layer in a quantitative manner. This assumption appears to have been warranted by the pK_a of the primary amino function of the ring-opened compound in the range of 7.0 (see below); the benzophenone compound should exist exclusively as a cation over the pH range of the determination. Alprazolam is extremely insoluble in water (~0.1 mg/ml at 37°) but freely soluble in chloroform, and the pK_a of monocation is 2.4.

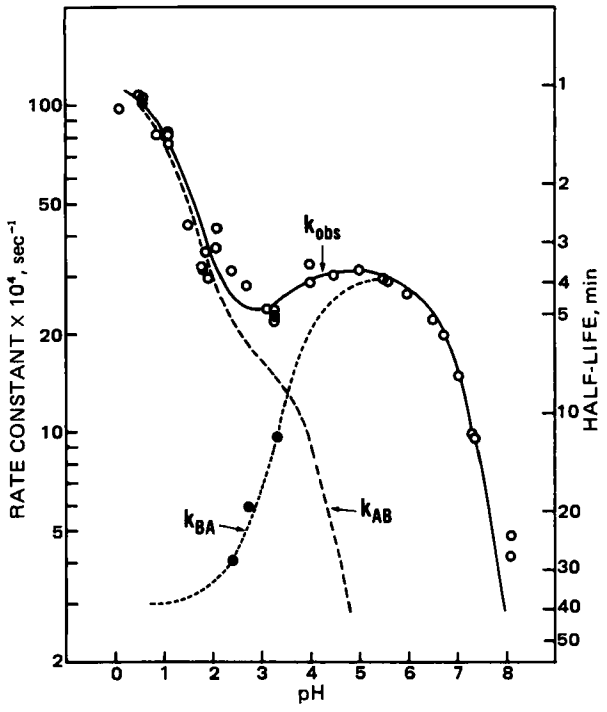


Figure 5—*pH Dependence of the overall alprazolam ring-opening and cyclization reaction rate constant (k_{obs}), ring-opening reaction rate constant (k_{AB}; dashed line), and cyclization rate constant (k_{BA}; dotted line up to pH 5.5 and descending solid line thereafter); all at 25°. The rates were also expressed in terms of half-life (right-hand side coordinate). Closed circles represent k_{BA} determined from an irreversible* B → A *reaction condition.*

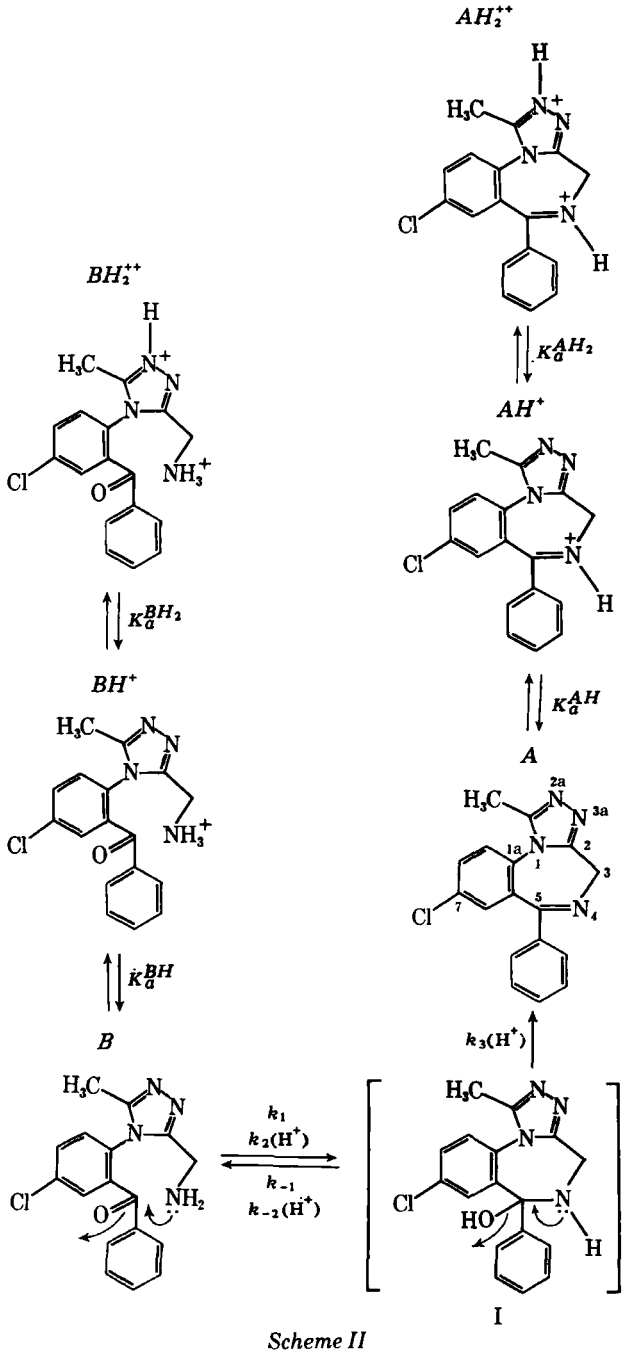
The three data points (Fig. 5) obtained from the described biphasic system together with the data points at pH values >5.5 resulted in a bell-shape pH-profile of the cyclization rate constant (Fig. 5). The cyclization reaction is analogous to a Schiff-base formation reaction, the kinetics of which have been thoroughly studied (24). The bell-shape pH-profile arises from a change in the rate-determining step with the pH change. Under acidic conditions, formation of the carbinolamine intermediate (*I* in Scheme II) is rate-controlling, since the concentration of kinetically reactive —NH_2 is extremely low and the dehydration of *I* is facilitated by protonation of the —OH group to form an excellent leaving group, H_2O. However, as the pH increases, approaching the pK_a of the primary amino group, the nucleophilic attack of the —NH_2 group on the benzophenone carbonyl group becomes faster than the dehydration of the carbinolamine intermediate *I*.

Closely following the kinetic treatment developed by Jenck (24), the following expression for the overall cyclization rate constant as a function of $[H^+]$ was derived:

$$k_{BA} = \frac{k_3[H^+](k_1 + k_2[H^+])}{k_{-1} + k_{-2}[H^+] + k_3[H^+] + k_3[H^+]} \times \frac{K_a^{BH}}{K_a^{BH} + [H^+]} \quad \text{(Eq. 1)}$$

Definitions of K_a^{BH} and several microscopic rate constants in Eq. 1 are shown in Scheme II. In this treatment, the dicationic species BH_2^{2+} does not need to be differentiated from the monocationic species BH^+ and that each of the rate constants can represent a kinetically equivalent reaction pathway; *e.g.*, the specific acid-catalyzed —NH_2 attack ($k_2[H^+]$ process) is equivalent to H_2O catalyzed *I* formation from BH^+.

Crude approximates of the rate constants were first obtained as described previously (24). These, together with the experimentally deter-



Scheme II

mined K_a^{BH} value, served as the first approximates in the final curve fitting by means of the SIMPLEX least-square computer program. Values for the individual microscopic rate constants and K_a^{BH} obtained using three data points at pH <3.4 and at 11 points at pH >5 are listed in Table I. The reliability of these values can be exemplified as follows: the experimentally determined pK_a^{BH} in the presence of 10% (v/v) methanol was 6.78 ± 0.02 (n = 3), whereas that from the numerical analysis of the pH profile was 7.02 with a range of 6.77–7.29 at a 95% confidence level. The bell-shaped curve, the dotted line up to pH 5.5 and the descending solid line thereafter (Fig. 5), was generated with the constants listed in Table I and Eq. 1. The positive deviation from a slope of +1 that one may expect on the log k_{BA}–pH profile when pH decreases from 3.5 was attributed to the steady increase in concentration of the protonated benzophenone carbonyl group, which should be extremely reactive toward the nucleophilic attack of —NH_2. This reaction pathway was implicitly included in the mathematical expression for k_{BA} (24).

Sites of Protonation on Alprazolam—Alprazolam contains four potentially basic nitrogens. It was challenging to determine the basicity ranking in an aqueous solution. In nonaqueous systems, three important pieces of evidence support that N-2*a* on the triazole ring is most basic:

Table I—Rate and Equilibrium Constants Associated with the *A* ⇌ *B* Reaction at 25°

Constants	Lower Limit[a]	Upper Limit[a]
$k_1 = 7.32\ sec^{-1}$	—	—
$k_2 = 4.26 \times 10^3\ sec^{-1}\ M^{-1}$	—	—
$k_{-1}/k_3 = 2.27 \times 10^{-4}\ M$	—	—
$k_2/k_3 = 0.343$	—	—
$K_a^{BH} = 9.51 \times 10^{-8}\ M (pK_a^{BH} = 7.02)$	5.13×10^{-8}	1.71×10^{-7}
$K_a^{BH} = 1.66 \times 10^{-7}\ M (pK_a^{BH} = 6.78)$[b]	—	—
$k_4 \cdot K \cdot K_a^{AH2}/K_a^{IH2} = 1.15 \times 10^{-2}\ sec^{-1}$	1.02×10^{-2}	1.30×10^{-2}
$k_4 \cdot K \cdot K_a^{AH2} = 9.02 \times 10^{-5}\ sec^{-1}\ M^{-1}$	2.67×10^{-5}	2.94×10^{-4}
$K_a^{IH2} = 7.84 \times 10^{-3}\ M (pK_a^{IH2} = 2.11)$	—	—
$K_a^{AH2} = 5.62 \times 10^{-2}\ M (pK_a^{AH2} = 1.25)$	3.16×10^{-2}	1.08×10^{-1}
$K_a^{AH} \cdot K_a^{AH2} = 3.95 \times 10^{-6}\ M^2$[c]	4.89×10^{-8}	4.27×10^{-5}
$K_a^{AH} = 7.03 \times 10^{-5}\ M (pK_a^{AH} = 4.15)$	—	—
$K_a^{AH} = 3.98 \times 10^{-3}\ M (pK_a^{AH} = 2.40)$[b]	—	—
$K_a^{BH2} = 3.16 \times 10^{-2}\ M (pK_a^{BH2} = 1.50)$[d]	—	—

[a] At a 95% confidence level. [b] Experimental data. [c] Not reliable (see the wide range of variation). [d] Estimated from the pKa of compound *C*.

(*a*), X-ray crystallographic study on the hydrogen bromide salt which was prepared from a nonaqueous system, indicated that a proton resides on N-2*a* (25); (*b*), ^{15}N-NMR spectrum of alprazolam in deuterated chloroform showed that N-2*a* undergoes a more profound upfield shift than any other nitrogen when trifluoroacetic acid is successively added to the system (26); and (*c*) in many chemical manipulations, N-2*a* proved to be the most nucleophilic and very likely the most basic; *e.g.*, simple alkylation preferably takes place at N-2*a* (27).

When the basicity of N-2*a* is compared with that of N-3*a*, the facts listed are in accordance with what is expected from the consideration of electronic effects; N-3*a* is linked with C(2)—CH_2(3)—N(4), whereas N-2a is linked with C(1*a*)—CH_3. The aniline N-1 should exert an identical electronic effect on both nitrogens. Thus, the electron-withdrawing effect of the aromatic azomethine bond, C(5)=N(4), should make the protonation at N-3*a* more difficult than at N-2*a*. On the other hand, N-1 is believed to be less basic than N-4 for reasons similar to that of medazepam (28). The latter is very similar to alprazolam structurally; instead of a triazole ring, a methyl group is attached to N-1. These two considerations leave either N-4 or N-2*a* to be the most basic center on the alprazolam molecule in aqueous solutions.

When alprazolam was methylated in methylene chloride at N-2*a* to form a quaternary ammonium compound, the UV spectrum hardly changed (27). Similarly, the UV spectrum of compound *C*, a compound containing a triazole ring but not a 1,4-diazepine ring, underwent a very small bathochromic shift of 4–5 nm when the pH of the sample solution was systematically changed from 4.0 to 0.3. Thus, it was apparent that protonation or methylation at N-2*a* is associated with little UV spectral change. This is perhaps because the lone-pair electrons on N-2*a* are not engaged in the aromaticity of 1,2,4-triazole. Although the opened-ring compound *C* underwent a very small bathochromic shift when dissolved in acidic solutions, it was possible to estimate the pK_a from the pH dependence of the absorbance at 280 nm. The pK_a of N-2*a* was found to be 1.5 or less.

The UV spectrum obtained immediately after acidifying an alprazolam solution to a pH below 0.5 shows an absorption maximum at 280 nm (Fig. 2). Appearance of this peak, which disappears again as the ring-opening reaction proceeds, amounts to a bathochromic shift of as much as 35 nm of the shoulder peak of alprazolam at 245 nm. The absorbance of this transient UV spectrum at a given wavelength was extrapolated to the time of acidification, and its dependence on the pH to which the alprazolam solution was acidified yielded an apparent pK_a of 2.40 (Fig. 6). This pK_a should correspond to that of N-4, and the large bathochromic shift observed reflects the resonance interaction of the positive charge with adjacent benzene rings.

The conclusion that N-4 should be more basic than N-2*a* in aqueous solutions is in contrast to many experimental evidences that N-2*a* is most basic in various nonaqueous environments. One possible explanation is based on the difference in steric hindrance that each nitrogen is subject to. In aqueous solutions, the proton donor is the relatively small hydronium ion. In nonaqueous systems in which N-2*a* was found to be more basic than N-4, the proton or alkyl group donors were all bulky: hydrogen bromide, trifluoroacetic acid, or trimethyloxonium fluoroborate (25–27). X-ray crystallographic data (25) indicate that N-1 and N-4 are subject to a higher degree of steric hindrance than the other two triazole nitrogens, making them less accessible to bulky proton donors. In this context

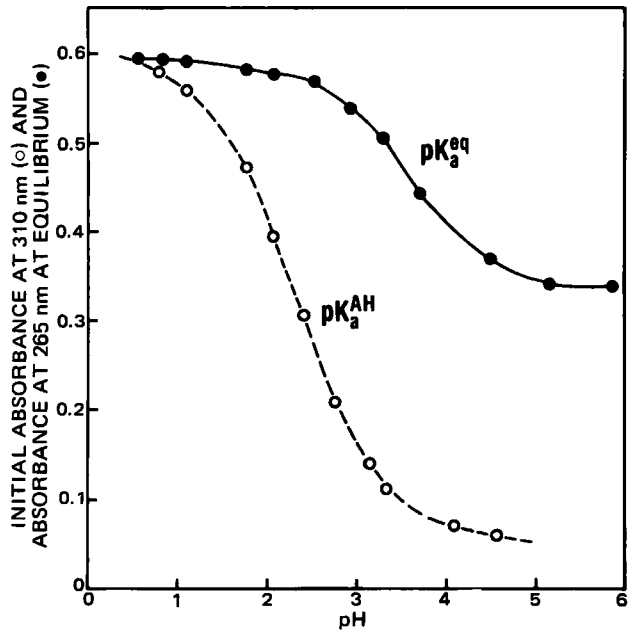
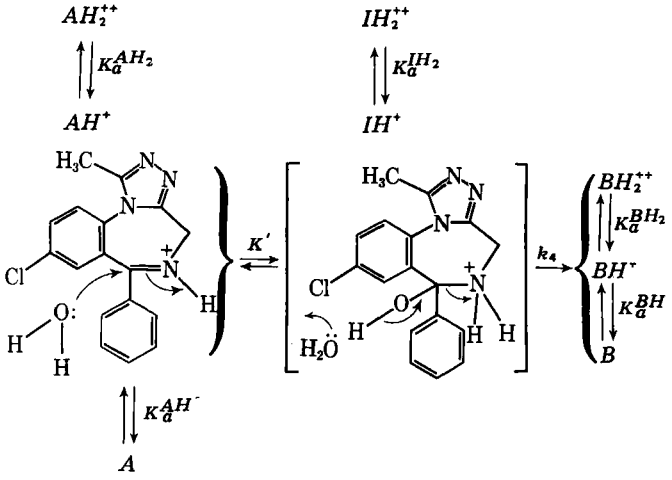


Figure 6—*Spectrophotometric determination of pK_a of monocationic alprazolam (pK_a^{AH}) and apparent equilibrium pK_a.*

it should be noted that the ^{15}N-NMR upfield shift associated with protonation of nitrogens in alprazolam with trifluoacetic acid was ordered N-2*a* > N-3*a* > N-4 > N-1 (26). It is not uncommon that the usual basicity order for a series of compounds can be completely inverted when bulky proton donors are used in the protonation (29).

Kinetics of Alprazolam Ring-Opening Reaction—The pH profiles of the ring-opening rate constant (k_{AB}) was obtained by subtracting k_{BA} from the spectrophotometrically determined overall rate constant (k_{obs}). That is, the value of k_{AB} was calculated at each pH where k_{obs} was measured in such a way that the sum of k_{BA} and k_{AB} best accommodated a total of 35 experimental data points of k_{obs}. In this manipulation, the pH dependence of k_{BA} was dictated by Eq. 2.

In deriving a mathematical expression for k_{AB} in terms of hydronium ion concentration, it was first assumed that the carbinolamine intermediate (IH^+) formation is much faster than the subsequent abstraction of a proton to form the benzophenone compound (BH^+; Scheme III) over the entire pH range studied. This is in accordance with the principle of microscopic reversibility applied to the cyclization reaction discussed earlier; however, it will create some error especially at a pH >4. The concentration of IH^+ can then be simply represented by an equilibrium constant, $K' = (I_T)/(A_T)$, and $d(B_T)/dt = k_4(I_T)$ by definition of k_4. Note that the only base which abstracts a proton from IH^+ is H_2O and, therefore, this step should be pH independent. In other words, the observed pH dependence of k_{AB} arises from the pH dependence of the apparent equilibrium constant K' in the kinetic scheme, and the equillibrium concentration of the intermediate at a given pH is ultimately governed by the reactivity of different ionic species of alprazolam present at that particular pH. This analysis simplifies the kinetic scheme tremendously, and the following expression for k_{AB} was derived, in which $K = (IH^+)/(AH^+)$, a pH independent equilibrium constant. Other constants included in the expression are all self-explanatory from Scheme III:



Scheme III

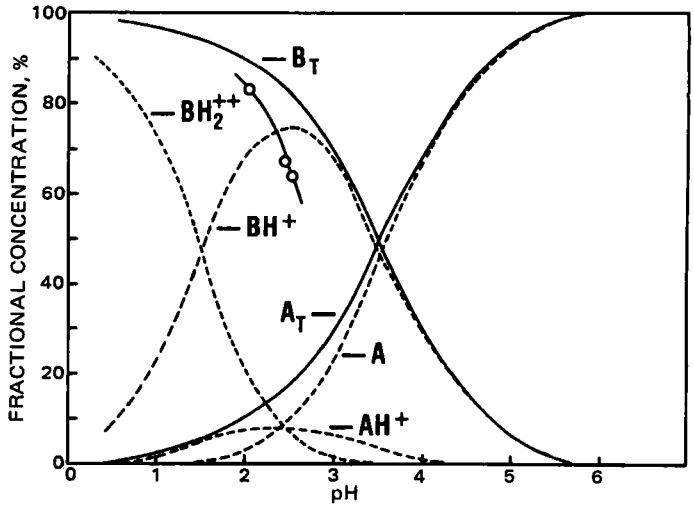


Figure 7—*Fractional concentration of various ionic species involved in the A ⇌ B reaction as a function of pH. Open circles represent the total concentration of the opened-ring compound determined by a rapid back-titration technique.*

$$k_{AB} = \frac{k_4K(K_a^{A2H}/K_a^{I2H})[H^+]^2 + k_4KK_a^{A2H}[H^+]}{[H^+]^2 + K_a^{A2H}[H^+] + K_a^{AH}K_a^{A2H}} \quad \text{(Eq. 2)}$$

Various constants present in the equation for k_{AB} were obtained from the SIMPLEX least-squares program and are listed in Table I. The dashed line on Fig. 5 was generated from these values and Eq. 2.

The presence of an inflection point on the pH profile of k_{AB} implies that the AH_2^{2+} is somewhat more reactive than AH^+. Protonation on the triazole moiety would exert an electron-withdrawing inductive effect to make the C(5)=N(4) bond more susceptible to a nucleophilic attack at C-5. However, the position of the inflection point which will determine the kinetically measurable pKa of alprazolam is subject to a significant error in the present study in that the pH-profile of k_{AB} was obtained second hand. It was not surprising that the kinetically obtained pK_a^{AH} (4.15) is very different from that obtained experimentally (2.40). The pK_a of dicationic alprazolam ($pK_a^{A2H} \simeq 1.25$) is in a fair agreement with the pK_a of compound *C* (~1.5 or below).

Over the pH range where the ring-opening reaction takes place to significant extent, the rate-determining step is largely the breakdown of intermediate IH^+, and it was not possible to isolate the reactivity of the N(4)=C(5) bond toward the H_2O attack. This is believed to be the case for other 1,4-benzodiazepines; however, the pH-dependence of K' itself should result in some interesting structure–reactivity relationships among them.

Multiple Equilibria—In the context of the present discussion, the term "equilibrium" is defined as a state of the $A \rightleftharpoons B$ system after 5–10 reaction half-lives. This restricted definition is particularly important for strongly acidic aqueous solutions in which alprazolam and/or compound *B* undergoes another slow reaction which is yet to be identified.

Equilibrium UV spectra of alprazolam at different pH values tend to produce an isosbestic point at 254 nm for pH 0.5–6.0. This finding implies that there are only two predominant species in terms of UV absorption and the apparent equilibrium concentration of these two species is identical at pH 3.50 (*i.e.*, equilibrium $pK_a = 3.50$, Fig. 6). From the values of k_{AB} and k_{BA}, the pH dependence of the apparent equilibrium of $A \rightleftharpoons B$ can also be estimated (Fig. 7). These kinetically determined equilibrium constants also showed that at pH 3.50 the total concentration of *A* is equal to that of *B*.

Fractional concentrations of different ionic species of alprazolam and the opened-ring compound are then determined by the pK_a of the species involved. Since pK_a^{BH} (6.80, determined from the rapidly obtained titration curve) is much greater than 3.5, one can safely rule out the neutral species of the opened-ring compound as an important species at any pH. Similarly, dicationic alprazolam is also not included in the present analysis. The pK_a of dicationic opened-ring compound (pK_a^{BH2}) was

assumed to be 1.5, the pK_a of compound *C*. On the other hand, the pK_a of protonated alprazolam was determined from the initial absorbance at 310 nm after a methanolic solution was diluted to a given pH (Fig. 6); the pK_a^{AH} was 2.40. Note that at 310 nm the opened-ring compound or its analogue *C* absorbed very little UV energy (Fig. 2) and the pH dependence shown on Fig. 6 should reflect the protonation at N-4. From the values of equilibrium pK_a, pK_a^{AH}, and pK_a^{BH2}, the fractional concentration of important ionic species involved in the $A \rightleftharpoons B$ equilibrium was calculated (Fig. 7).

The concentration of the opened-ring compound was also estimated experimentally. An alprazolam solution in methanol was first acidified with HCl and left to attain equilibrium. The mixture was then neutralized with an equivalent amount of hydroxide ion and immediately back-titrated. The titration end point should represent the amount of the opened-ring compound which was generated from the acid treatment. A critical assumption was that during the neutralization and the titration no significant $A \rightleftharpoons B$ reaction occurred. Since the titration end point occurs at a pH betweeen 4 and 6, alprazolam was never protonated in the titration. Three determinations of the total opened-ring compound by this rapid titration technique are shown on Fig. 7 (open circles). Since the titration system contained as much as 10% (v/v) methanol, the total concentration of the opened-ring compound recovered at a given pH was expected to be much lower than in the absence of methanol. In this particular instance, not only should the activity of water have been lower but the pK_a^{AH} was also expected to decrease significantly to result in a displacement of pH profile for the fractional concentration of opened-ring compound toward lower pH.

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Factorial Designs in Pharmaceutical Stability Studies

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ABSTRACT □ An approach to analyzing and interpreting kinetic data from stability studies using factorial designs is presented. This may be useful for screening purposes or as an aid in identifying significant effects in complex systems. A typical 2^n factorial experiment is discussed, and methods of variance estimation and statistical testing are presented. An example of simulated data is used to demonstrate how typical results may be analyzed, as well as the potential and limitations of this design in interpretation and construction of kinetic models.

Keyphrases □ Factorial designs—in pharmaceutical stability studies, kinetic models, statistical analysis □ Stability studies, pharmaceutical—factorial designs, kinetic models, statistical analysis

Factorial designs are extremely useful in a wide variety of experimental situations, and applications of these designs to pharmaceutical problems have appeared in the recent literature (1–3). Factorial designs applied to stability studies of pharmaceuticals can be used for screening purposes or to help interpret complex systems. This paper deals with an approach to the design and statistical analysis of such experiments.

BACKGROUND

A factorial experiment considers the effects of various *factors* (*e.g.*, temperature, pH, drug concentration, buffer concentration) at several *levels* (*e.g.*, 2 pHs, one high pH and one low pH) where results of all combinations of the factor levels are observed. Modifications of the complete factorial design may be used in situations where it is not convenient or possible to do all of the combinations or trials (4). For this presentation, only experiments with all factors at two levels, a 2^n factorial design (where n is the number of factors, the effects of which are to be investigated), will be considered. The *main effect* is the difference in response (*e.g.*, rate constant) caused by the change in level of a factor (pH,

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