# Hydrolysis of Temazepam in Simulated Gastric Fluid and Its Pharmacological Consequence

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**Abstract** □ Temazepam (TMZ), a hypnotic and anxiolytic drug, underwent hydrolysis in simulated gastric fluid (SGF; pH 1.2). The hydrolysis reaction of TMZ in acetonitrile:SGF (1:19, v/v) at 37 °C was an apparent first-order reaction, with a half-life of 5.47 ± 0.17 h (i.e., ~12% of the remaining TMZ was hydrolyzed per hour). The predominant hydrolysis product (2′-benzoyl-4′-chloro-*N*-methyl-2-amino-2-hydroxyacetanilide) and a minor hydrolysis product [2-(methylamino)-5-chlorobenzophenone], derived from acid-catalyzed reaction of TMZ in an aqueous solution, were characterized by ultraviolet–visible absorption mass, infrared, and proton nuclear magnetic resonance spectra analyses. The kinetics of the hydrolysis reaction were studied as a function of acid concentration, temperature, and ionic strength and in deuterated solvent. Results indicated that the predominant hydrolysis reaction at pH ≈ $pK_a$ (1.46) was caused by protonation at N4, followed by a nucleophilic attack by water at C5 of the C5–N4 iminium ion and a subsequent ring-opening reaction. Pharmacological activity tests in mice indicated that the predominant hydrolysis product of TMZ was inactive. The results suggest that a fraction of an orally taken TMZ may be inactivated by hydrolysis in the highly acidic gastric fluid.

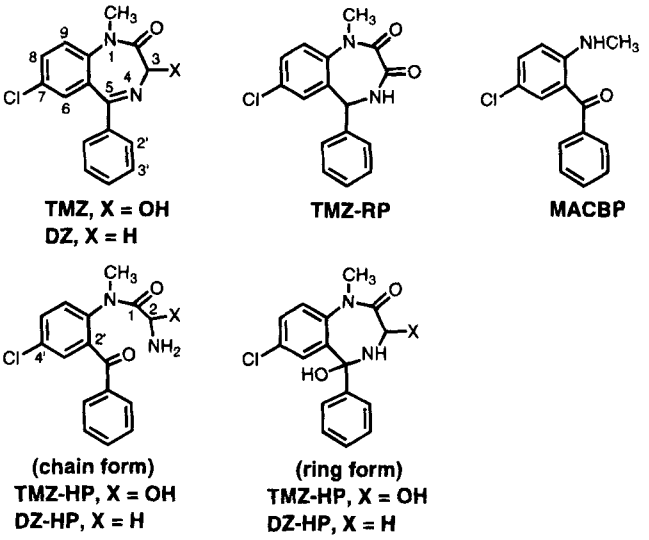


**Figure 1**—Structures and abbreviations of diazepam (DZ), temazepam (TMZ) and its base-catalyzed rearrangement product (TMZ-RP),[3,4] the ultimate hydrolysis product of DZ and TMZ (MACBP), two possible acid-catalyzed hydrolysis products (DZ-HP) of DZ, and two possible acid-catalyzed hydrolysis products (TMZ-HP) of TMZ.

## Introduction

Temazepam (TMZ; Figure 1) is clinically used in the treatment of anxiety and insomnia and as an adjuvant for anesthesia.[1] TMZ is also an active metabolite of diazepam (DZ), a frequently prescribed hypnotic/anxiolytic drug.[2] Although TMZ is commonly administered as an oral preparation (capsule, tablet, or elixir), the stability of TMZ in gastric fluid has heretofore not been reported. TMZ undergoes a rearrangement reaction to form TMZ-RP (Figure 1) in strongly alkaline media.[3,4] For the purpose of detection and structural confirmation, a 1,4-benzodiazepine contained in clinical samples such as blood and urine was often hydrolyzed under strongly acidic conditions (e.g., 6 M HCl at 100 °C for 1 h) to a benzophenone derivative.[5–10] The pathway(s) involved in acid-catalyzed hydrolysis of a 1,4-benzodiazepine to a benzophenone derivative has heretofore not been reported.

This study shows that TMZ undergoes a significant hydrolysis reaction in simulated gastric fluid (SGF; pH 1.2) and HCl solutions to form primarily a 2′-benzoyl-4′-chloro-*N*-methyl-2-amino-2-hydroxyacetanilide (TMZ-HP). TMZ-HP is then slowly hydrolyzed to form a 2-(methylamino)-5-chlorobenzophenone (MACBP). The mechanism of the acid-catalyzed hydrolysis and its pharmacological consequence have been studied and are the subjects of this report.

## Experimental Section

**Materials**—TMZ ($\epsilon_{230}$ = 30.2 cm$^{-1}$ mM$^{-1}$, MeCN) was generously provided by Sandoz Pharmaceuticals Corp. (East Hanover, NJ). DZ was generously provided by Hoffmann La Roche Inc. (Nutley, NJ). Deuterated $CDCl_3$ (99.8 atom % D containing 0.03% TMS), deuterium oxide ($D_2O$, 99.9 atom % D), $D_2SO_4$ (99 atom % D), and 2-amino-5-chlorobenzophenone (ACBP; $\epsilon_{236}$ = 21.3 cm$^{-1}$ mM$^{-1}$, MeCN) were obtained from Aldrich Chemical Co. (Milwaukee, WI). MACBP ($\epsilon_{236}$ = 19.8 cm$^{-1}$ mM$^{-1}$, MeCN) was prepared by methylation of ACBP with dimethyl sulfate in MeCN:10 N NaOH (99:1, v/v) at room temperature.[4,11] MACBP may also be prepared from ACBP by phase-transfer catalysis.[12] The base-catalyzed rearrangement product of TMZ (TMZ-RP, recrystallized in ethanol; $\epsilon_{250}$ = 10.9 cm$^{-1}$ mM$^{-1}$, MeCN) was prepared from TMZ as described.[4] HCl solutions were diluted from 12 M HCl (Fisher Scientific, Clifford, NJ). Pepsin (Pepsin A, EC 3.4.23.1, 570 units/mg protein) and metrazol were obtained from Sigma Chemical Co. (St. Louis, MO).

SGF was prepared with a pH of 1.2 as described.[13] Briefly, 0.2 g of NaCl and 0.32 g of pepsin were dissolved in 0.7 mL of concentrated HCl (12 M), and distilled water was added to make a final volume of 100 mL.

For spectral analysis, TMZ-HP ("HP" abbreviates hydrolysis product; $\epsilon_{250}$ = 8.54 cm$^{-1}$ mM$^{-1}$, MeCN) was prepared by dissolving 20 mg of TMZ in 10 mL of MeCN:0.2 M HCl (1:1, v/v) and the mixture was kept at 60 °C for 5 h. TMZ-HP, a minor amount of MACBP, and unreacted TMZ in the solution were extracted with 20 mL of ethyl acetate. The ethyl acetate phase was washed three times with 0.1 M phosphate (pH 7.0) and twice with water, subsequently dehydrated with anhydrous $MgSO_4$, and evaporated to dryness by blowing with a gentle stream of nitrogen. The residue was redissolved in MeCN and the TMZ-HP was purified by reversed-phase HPLC.

**HPLC**—HPLC was performed using a Waters Associates (Milford, MA) Model M45 solvent pump and a Model 441 absorbance detector (254 nm). The system was fitted with a Vydac C18 column (5 μm particles, 4.6 mm i.d. × 25 cm, catalog no. 201TP54; The Separations Group, Hesperia, CA). For sample analysis in kinetic experiments, MeCN:0.02 M phosphate buffer (pH 7.0) (1:1, v/v) was used as the mobile phase at a flow rate of 1 mL/min. For purification of TMZ-HP for spectral analyses, MeCN:$H_2O$ (45:55, v/v) was used as the

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mobile phase, also at 1 mL/min. HPLC analysis was conducted at an ambient temperature (23 ± 1 °C). Samples were injected via a Shimadzu (Shimadzu Corp., Kyoto, Japan) Model SIL-9A automatic sample injector equipped with a water-jacketed sample rack. The temperature of the sample rack was maintained by passing constant-temperature water from a thermostated water circulator. The actual temperature of the solution in the sample vial was measured with a portable digital thermometer fitted with a detachable probe (Thomas Scientific, Swedesboro, NJ). The temperature variation was ±0.1 °C. The detector signal was recorded with MacIntegrator (a software and hardware package from Rainin Instruments Co., Inc., Emeryville, CA) on a Macintosh SE computer (Apple Computer, Cupertino, CA).

**Acidity Constant**—The absorbance values at 223, 258, and 292 nm were recorded for solutions containing an identical concentration of TMZ (25.3 μM) in MeCN:$H_2O$ (1:20, v/v) and various concentrations on HCl at ambient temperature (23 ± 1 °C). The wavelengths for monitoring absorbance changes as a function of acid concentration were determined by a difference spectrum[14] between the acidic and neutral forms of TMZ. Following the absorbance vs [HCl] plot, the $K_a$ value (acidity constant or acid dissociation constant) was determined by a curve fitting program in SigmaPlot (Jandel Scientific, Corte Madera, CA).

**Kinetics of Hydrolysis**—In kinetic studies of the hydrolysis reaction, a sample vial containing 1.5 mL of either SGF or HCl solution was equilibrated to the temperature under study. TMZ (150 μg in 75 μL of MeCN) was then added to the vial. The solution was thoroughly mixed and placed in a sample well of the autosampler's thermostated sample rack. The first sample was injected for analysis after the sample vial had been placed in the sample well for 5 to 10 min to allow the temperature of the solution to reach equilibrium. A sample (15 μL) was subsequently injected for analysis by reversed-phase HPLC at each sampling interval (ranging from 6 to 30 min). The error involved in repetitively injecting a constant volume (15 μL) of samples was ≤2%. Hydrolysis $t_{1/2}$ was determined by a curve fitting program in CA-Cricket Graph III (Computer Associates International, Inc., Islandia, NY) following plotting of log(peak area of TMZ) vs time. Hydrolysis $t_{1/2}$ (=0.693/$k$ for pseudo-first-order reactions, where $k$ is the rate constant) values were averages of at least three determinations.

**Spectral Analysis**—UV–vis absorption spectra of samples were determined using a 1-cm path length quartz cuvette on a Model DW2000 spectrophotometer (SLM Instruments, Urbana, IL). Mass spectral analysis was performed on a Finnigan mass spectrometer system (Model 4500 or Model 4600; Finnigan MAT, San Jose, CA) with a solid probe by either electron impact at 70 eV or chemical ionization ($NH_3$); the ion source was maintained at 105 °C. The Fourier transform proton NMR spectrum was obtained with a Model XL 300 MHz spectrometer (Varian Associates, Palo Alto, CA). The sample was dissolved in $CDCl_3$ with a trace of tetramethylsilane (TMS). Chemical shifts are in ppm relative to TMS. The FT-IR spectrum was determined with a Model FTS-45 spectrometer (Bio-Rad Laboratories, Hercules, CA).

**Pentobarbital-Induced Sleep Time in Mice**—Adult Swiss Webster mice (male, body weight of 19-21 g), obtained from the National Cancer Institute (Bethesda, MD), were allowed free access to food and water except during the experimental test period. Pentobarbital-induced sleep time in mice was determined as the time between loss of righting reflex and the moment mice regained this reflux.[15] Each chemical was suspended in 1.5% sodium (carboxymethyl)cellulose and administered by gastric gavage 30 min before intraperitoneal injection of pentobarbital (sodium salt in saline, 50 mg/kg). Groups of 10 mice were used for each chemical and each dose.

**Antimetrazol Activity in Mice**—Adult Swiss Webster mice (male, body weight 20–24 g) were used. Antimetrazol activity was determined according to the method of Goodman et al.[16] Each chemical was suspended in 1.5% sodium (carboxymethyl)cellulose and administered by gastric gavage 30 min before the administration of metrazol. Seizures were elicited by rapid intraperitoneal injection of an aqueous solution of metrazol (110 mg/kg of body weight). The volume of metrazol solution injected into each mouse did not exceed 0.25 mL. The effects of metrazol in the presence and absence of the second drug were (1) tail straight up, (2) hindleg tonic extensor seizure, (3) survival time, and (4) mortality. Groups of 10 mice were used for each chemical and each dose.

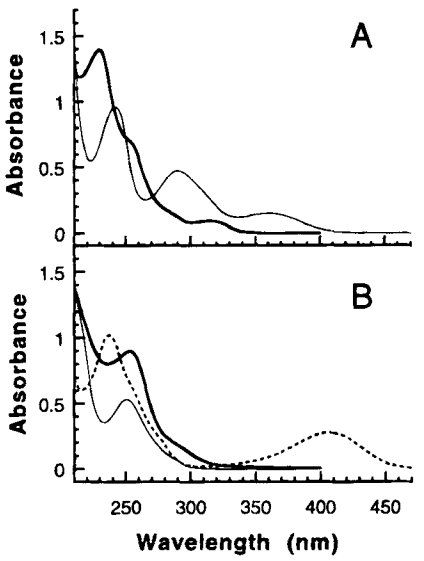



**Figure 2**—Ultraviolet–visible absorption spectra of TMZ in MeCN (A, thick line curve, 49 μM), TMZ in MeCN:2 M HCl (1:20, v/v) (A, thin line curve, 49 μM), TMZ-HP in MeCN (B, thick line curve, 105 μM), TMZ-RP in MeCN (B, thin line curve, 48 μM), and MACBP in MeCN (B, – –, 50 μM).

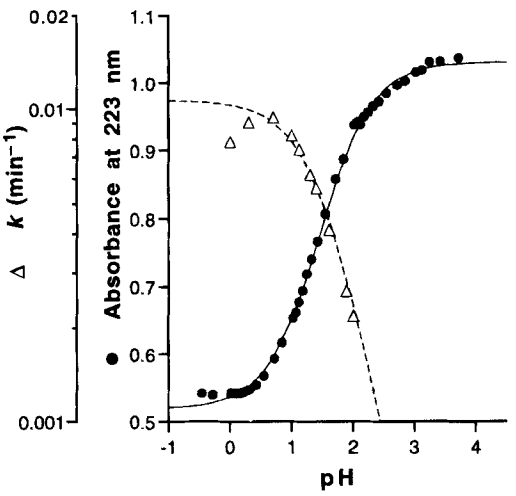



**Figure 3**—Acid concentration-dependent absorption changes of TMZ in MeCN:$H_2O$ (1:20, v/v) at 223 nm (●) and dependence of hydrolysis rates of TMZ on acid concentration in MeCN:$H_2O$ (1:20, v/v) at 50 °C (△). Absorbance measurements were performed at 23 ± 1 °C. The p$K_a$ value (1.46) was determined by a curve fitting computer software (—). The dashed line curve (– –) was generated by the equation $k = K'[H^+]/([H^+] + K_a)$, where $K'$ (0.01066 $min^{-1}$) is the intrinsic rate constant and $K_a$ is the acidity constant. Since $K_a$ was not determined at 50 °C, a value of 0.035 M, determined at ambient temperature (23 ± 1 °C), was used.

## Results and Discussion

**Acidity Constant**—Due to protonation at the N4 position in strongly acidic solutions,[17] the UV–vis absorption spectrum of the acidic form of TMZ was different from that of the unprotonated neutral form (Figure 2A).[10] The changes in absorptions were instantaneous and protonation at N4 appeared to be diffusion-controlled. Several wavelengths (*e.g.*, 223, 258, and 292 nm) were suitable for monitoring absorbance changes as a function of acid concentration. The acid concentration-dependent absorption of TMZ in MeCN:$H_2O$ (1:20, v/v) at 223 nm is shown in Figure 3. The acidity constant $K_a$ (0.035 M [$H^+$], p$K_a$ = 1.46) was determined by fitting the data with curve-fitting computer software. $K_a$ values determined at 258 and 292 nm (not shown) were essentially the same as that determined at 223 nm. The result in Figure 3

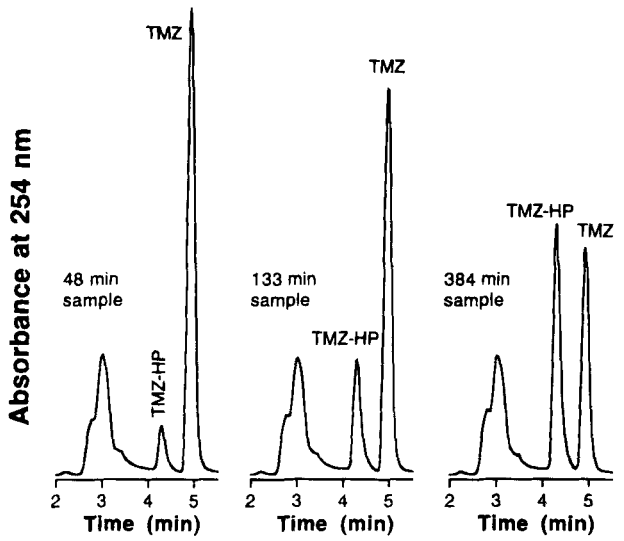



**Figure 4**—Sample chromatograms in the analysis of TMZ-HP formed in the hydrolysis of TMZ (0.1 mg/mL) in MeCN:SGF (pH 1.2) (1:19, v/v) at 37 °C. Samples (15 μL each injection) were taken for analysis at 48, 133, and 384 min following the initiation of the hydrolysis reaction. The hydrolysis $t_{1/2}$ of this reaction was 5.28 ± 0.01 h ($n$ = 3).

indicated that 77.2% and 22.5% of TMZ were protonated in MeCN:$H_2O$ (1:20, v/v) at pH 1 and 2, respectively. The $K_a$ values of TMZ in 1:9, 1:3, and 1:1 volume ratios of MeCN:$H_2O$ at ambient temperature were 0.057, 0.085, and 0.20 M [$H^+$], respectively.[11]

**Kinetics of Hydrolysis in SGF**—TMZ-HP, formed by hydrolysis of TMZ in either MeCN:SGF (1:19, v/v) at 37 °C or HCl solutions, was readily separated from TMZ by reversed-phase HPLC (Figure 4). Under the chromatographic conditions, MACBP had a retention time of 17.4 min. In all kinetic experiments, the amount of MACBP formed was insignificant relative to those of TMZ and TMZ-HP. Sample chromatograms illustrating the time-dependent changes in the peak areas of TMZ and TMZ-HP at 254 nm are shown in Figure 4. The major unidentified acid-catalyzed product (designated as U1) reported earlier,[11] formed in aqueous ethanol solutions, is now established to be TMZ-HP. Absorption peaks eluted at 3 ± 0.5 min in Figure 4 were due to pepsin contained in SGF and these were minor in samples using HCl solutions. The kinetics of hydrolysis of TMZ in SGF indicated apparent first-order reactions and were essentially independent of the substrate concentration. At 0.1, 0.2, and 0.3 mg/mL of TMZ, the hydrolysis $t_{1/2}$ values in MeCN:SGF (1:19, v/v) at 37 °C were 5.64 ± 0.06 ($n$ = 3), 5.51 ± 0.11 ($n$ = 3), and 5.28 ± 0.01 ($n$ = 3) h, respectively.

Under identical experimental conditions, a product with a UV absorption spectrum similar to that of TMZ-HP was formed from DZ. However, this product was readily converted to DZ upon neutralization. This result suggested that the intermediate formed from DZ in acidic aqueous solution was similar to that of TMZ-HP or its immediate precursor.

**Characterization of TMZ-HP**—The UV–vis absorption spectrum of TMZ-HP in MeCN was characteristically different from that of TMZ in either MeCN or HCl (Figure 2A) and similar to that of TMZ-RP in MeCN (Figure 2B). TMZ-HP was readily converted to MACBP in an alkaline solution at ambient temperature and was slowly converted to MACBP in an acidic solution at elevated temperatures. No TMZ was detected when TMZ-HP was dissolved in either neutral or acidic aqueous solution for an extended period of time. Chemical ionization (CI, $NH_3$) mass spectral analysis of TMZ-HP revealed a weak ion at $m/z$ 302 (loss of OH), $MH^+$ at $m/z$ 319, $(MH + NH_3)^+$ at $m/z$ 336, and the associated chlorine

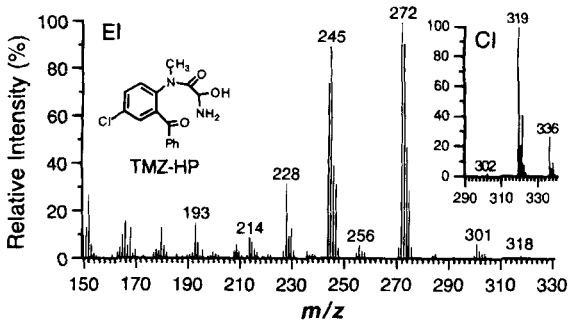



**Figure 5**—Abbreviated electron impact (EI) and chemical ionization (CI, $NH_3$; inset) mass spectra of TMZ-HP.

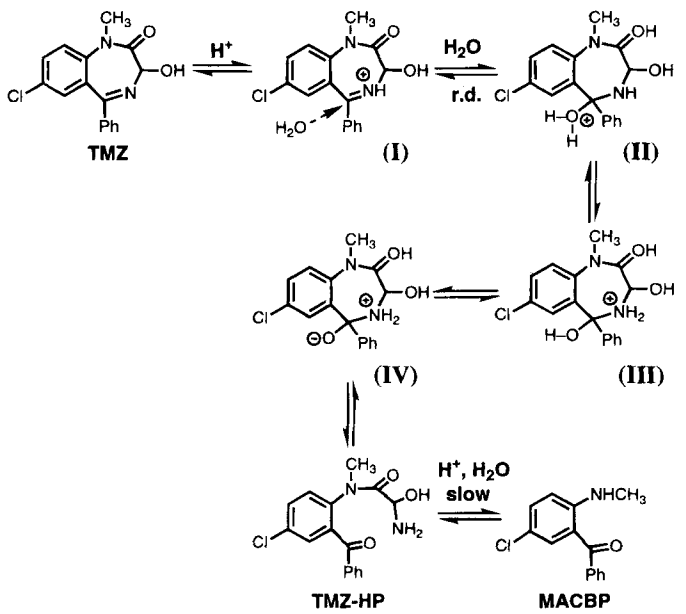
isotope ions (inset of Figure 5). The result established that TMZ-HP was formed from TMZ by the addition of a water molecule. The electron impact (EI) mass spectrum of TMZ-HP gave a very weak $M^+$ at $m/z$ 318 (Figure 5) and this was often not detectable in repeated analyses. Readily recognizable fragment ions were: 302, 301, 273, 272, 256, 255, 245, 244, and 228. The fragment ion at $m/z$ 245 and those derived from it ($m/z$ at 244, 228, 209, and 193)[10] indicated that a fragment of MACBP was formed.

The ring form of TMZ-HP has the same molecular weight as that of the chain form (Figure 1). The ring form of TMZ-HP was eliminated as a possibility because (1) it is expected to lose a water readily to produce a fragment ion at $m/z$ 300 (molecular weight of TMZ) and the fragment ions derived from it in EI mass spectral analysis[10] and (2) it is not expected to produce the observed fragment ions at $m/z$ 272 [loss of CH(OH)$NH_2$ from $M^+$] and 273 [reprotonated fragment ion following loss of CH(OH)$NH_2$ from $M^+$] in EI mass spectral analysis (Figure 5). Furthermore, the lack of an absorption band at 1605 $cm^{-1}$ due to the C=N stretch (see below), observed in the FT-IR spectrum of TMZ,[18] also eliminated the ring form of TMZ-HP (Figure 1) as a possibility.

FT-IR spectral analysis of TMZ-HP indicated a medium strength absorption band at 1102 $cm^{-1}$ (C–O), a strong absorption band at 1668 $cm^{-1}$ (C=O), and a strong and broad absorption band at 3400 $cm^{-1}$ (O–H) with a shoulder at 3300 $cm^{-1}$ (N–H). The absorption band at 3300 $cm^{-1}$ due to N–H stretch was absent in the IR spectrum of TMZ.[18] The characteristic absorption band at 1605 $cm^{-1}$ due to C–N stretch of the diazepine ring in TMZ[18] was absent in the IR spectrum of TMZ-HP. FT proton NMR spectral analysis of TMZ-HP (in $CDCl_3$; the proton of $CHCl_3$ was at 7.263 ppm) indicated three protons (*N*-methyl, singlet) at 3.214 ppm and one proton (C3-H, singlet) at 9.351 ppm.

Taken together, the results established the structure of TMZ-HP as 2′-benzoyl-4′-chloro-*N*-methyl-2-amino-2-hydroxyacetanilide (the chain form in Figure 1). Thus, the acid-catalyzed hydrolysis of TMZ occurs by cleavage of the N4-protonated C5–N4 imine bond, following a water attack at C5. The resulting TMZ-HP is further hydrolyzed to form MACBP at a much slower rate by hydrolytic cleavage of the N1–C2 amide bond.

**Factors Influencing Hydrolysis Rates**—The rates in acid-catalyzed hydrolysis of TMZ were further studied using MeCN:$H_2O$ (1:20, v/v; containing various concentrations of HCl) as the solvent at 50 °C. The hydrolysis $t_{1/2}$ values were 316 ± 26 ($n$ = 3, 0.01 M HCl), 263 ± 11 ($n$ = 3, 0.013 M HCl), 168 ± 7 ($n$ = 3, 0.025 M HCl), 124 ± 4 ($n$ = 3, 0.04 M HCl), 112 ± 5 ($n$ = 3, 0.05 M HCl), 93 ± 5 ($n$ = 3, 0.075 M HCl), 84 ± 3 ($n$ = 4, 0.1 M HCl), 74 ± 2 ($n$ = 3, 0.2 M HCl), 76 ± 3 ($n$ = 3, 0.5 M HCl), and 88 ± 5 ($n$ = 3, 1 M HCl) min. Similar to that in SGF, the hydrolysis in HCl solutions was also an apparent first-order reaction. At [HCl] ≤ 0.2 M, the hydrolysis rate increased with increasing concentrations of HCl and




**Scheme 1**—Proposed mechanism in the formation of TMZ-HP in acid-catalyzed hydrolysis of TMZ and the subsequent hydrolysis of the N1–C2 amide bond to form MACBP. The formations of TMZ-HP from **IV** and of MACBP from TMZ-HP were essentially irreversible. See the text for discussion.

appeared to be parallel to the degree of protonation at the N4 position (Figure 3). The results suggested that protonation at N4 of TMZ was required for the hydrolysis reaction. At [HCl] ≥ 0.2 M, the hydrolysis rate slightly decreased with increasing concentrations of HCl. This decrease in hydrolysis rate may be due to a decreased concentration of the dipolar ion intermediate **IV** at pH ≪ p$K_a$ (see Scheme 1 and discussion below).

The temperature-dependent hydrolysis $t_{1/2}$ values of TMZ in MeCN:0.1 M HCl (1:20, v/v) were 268 ± 3 ($n$ = 3, 37 °C), 157 ± 3 ($n$ = 3, 43 °C), 84 ± 2 ($n$ = 3, 50 °C), 54 ± 2 ($n$ = 3, 55 °C), and 36 ± 2 ($n$ = 3, 60 °C) min. The thermodynamic parameters, determined by an Arrhenius plot of the temperature-dependent reaction $t_{1/2}$ (slope = 3.911, $r^2$ = 1.000), were energy of activation ($E_{act}$) = 17.9 kcal/mol and, at 25 °C, $\Delta H^{\ddagger}$ = 17.3 kcal/mol, $\Delta S^{\ddagger}$ = −22.8 cal/K per mol, and $\Delta G^{\ddagger}$ = 24.1 kcal/mol. The energy of activation was relatively small, indicating that a small energy was required to form the transition state. The relatively large negative $\Delta S^{\ddagger}$ indicated a gain of orderliness in the transition state. The large negative $\Delta S^{\ddagger}$ also suggested that the transition state was extensively solvated.[19]

The hydrolysis reaction was further studied using MeCN: 0.25 M $H_2SO_4$ in $H_2O$ (or $D_2SO_4$ in $D_2O$) (1:20, v/v) as the solvent at 37 and 50 °C. The reaction became slower when $H_2SO_4/H_2O$ was replaced by $D_2SO_4/D_2O$. The $t_{1/2}$ values were 207 ± 4 min ($n$ = 3; 37 °C) and 61 ± 1 min ($n$ = 3; 50 °C) in $H_2SO_4/H_2O$ and 421 ± 5 min ($n$ = 3; 37 °C) and 124 ± 1 min ($n$ = 3; 50 °C) in $D_2SO_4/D_2O$. Thus the isotope effects ($k_H/k_D$) were 2.03 at both 37 and 50 °C. At 0.25 M $H_2SO_4$ (pH = 0.6), the reaction rate is in the plateau region of the rate–pH profile (Figure 3). These results indicated that the rate-determining step of the hydrolysis reaction at [HCl] ≤ 0.2 M required a proton transfer.[19]

The mechanism of the hydrolysis reaction was further probed by studying the dependence of the hydrolysis rate on ionic strength. In MeCN:0.1 M HCl (1:20, v/v) containing 0, 0.01, 0.05, and 0.1 M of NaCl, the $t_{1/2}$ values of TMZ hydrolysis at 50 °C were 77.6 ± 3.9 ($n$ = 7), 76.7 ± 1.7 ($n$ = 3), 77.4 ± 0.5 ($n$ = 3), and 79.3 ± 2.0 ($n$ = 3) min, respectively. The

**Table 1—Prolongation of Pentobarbital-Induced Sleep Time and Antimetrazol Activity of TMZ, TMZ-Hp, and TMZ-RP in Mice[a]**

| Drug and Dose | Sleep Time (min) | Seizure[b] (%) | Survival Time (min) | Mortality (%) |
|---|---|---|---|---|
| PB (50 mg/kg) | 31.8 ± 6.5 | | | 0 |
| TMZ (10.5 mg/kg) + PB (50 mg/kg) | 121.6 ± 37.2[c] | | | 0 |
| TMZ (5.25 mg/kg) + PB (50 mg/kg) | 65.9 ± 18.4[c] | | | 0 |
| TMZ-HP (11.1 mg/kg) + PB (50 mg/kg) | 38.0 ± 12.2[d] | | | 0 |
| TMZ-HP (5.56 mg/kg) + PB (50 mg/kg) | 30.6 ± 9.4[b] | | | 0 |
| TMZ-RP (10.5 mg/kg) + PB (50 mg/kg) | 44.4 ± 21.5[d] | | | 0 |
| TMZ-RP (5.25 mg/kg) + PB (50 mg/kg) | 34.5 ± 7.5[d] | | | 0 |
| Metrazol (110 mg/kg) | | 100 | 7.2 ± 2.0 | 100 |
| TMZ (10.5 mg/kg) + metrazol (110 mg/kg) | | 10[e] | >120 | 0 |
| TMZ (5.25 mg/kg) + metrazol (110 mg/kg) | | 60[e] | >120 | 0 |
| TMZ-HP (11.1 mg/kg) + metrazol (110 mg/kg) | | 100 | 5.2 ± 1.7[d] | 100 |
| TMZ-HP (5.56 mg/kg) + metrazol (110 mg/kg) | | 100 | 6.1 ± 1.9[d] | 100 |
| TMZ-RP (10.5 mg/kg) + metrazol (110 mg/kg) | | 100 | 6.5 ± 1.4[d] | 80 |
| TMZ-RP (5.25 mg/kg) + metrazol (110 mg/kg) | | 100 | 6.8 ± 1.7[d] | 100 |

[a] Experiments were conducted as described in the Experimental Section. [b] Appearance of tonic hindleg extensor component of the seizure. [c] $P < 0.001$. [d] $P > 0.05$. [e] Only appearance of tail straight up component of the seizure was observed.

results indicated that a change in the ionic strength of the solvent did not significantly alter the reaction rate. The results suggested that the rate-determining step was due to a reaction between a neutral molecule and a positively charged ion, a negatively charged ion, or a neutral molecule.[19] A negatively charged reactant is not expected to be present in a strongly acidic medium. Since the substrate is a positively charged ion in the strongly acidic aqueous solution, the second reactant must be a neutral water molecule.

**Mechanism of Hydrolysis**—On the basis of the results described above, a mechanism for the hydrolysis of TMZ in acidic media is proposed in Scheme 1. The reaction is initiated via a protonation at N4 to form an iminium ion (**I**). The rate-limiting step is the water attack at C5 of the iminium ion, which forms the tetrahedral intermediate **II**. **II** undergoes rearrangement via **III** to form the dipolar intermediate **IV**. A subsequent ring-opening reaction produces TMZ-HP. Under more acidic conditions (pH ≪ p$K_a$), the rate of the hydrolysis reaction is significantly reduced due to a decreased concentration of the dipolar intermediate (**IV**) and an increased concentration of **III**. The pathway **IV** → TMZ-HP (Scheme 1) became the rate-determining step at pH ≪ p$K_a$. TMZ-HP slowly undergoes an essentially irreverible hydrolytic attack at N1—C2 amide bond to form MACBP. The mechanism proposed in the formation of TMZ-HP is similar to that of the extensively studied acid-catalyzed hydrolysis of imines.[20] The relatively slow acid-catalyzed hydrolysis at the N1—C2 amide bond is also consistent with the extensively studied hydrolysis mechanism of chemicals with an amide bond.[21]

It is interesting to note that, under identical experimental conditions, DZ forms an unstable intermediate with UV absorption properties similar to those of TMZ-HP. This unstable intermediate is readily converted back to DZ upon neutralization of the reaction medium. The contrasting

properties of TMZ and DZ in acidic solutions suggest that the electron-withdrawing C3 hydroxyl group of TMZ facilitates the formation of TMZ-HP. In the dipolar intermediate (**IV**) of Scheme 1, an electron-withdrawing hydroxyl group at C3 decreases the nucleophilicity of the amine, thereby facilitating the cleavage of the C5—N4 bond. DZ does not have a C3 hydroxyl group. DZ-HP (a structure similar to either the ring form or the chain form of TMZ-HP, Figure 1), formed by hydrolytic attack of water at C5 of the N4-protonated iminium ion of DZ, readily reverted back to form DZ. This recyclization of either the ring or the chain form of DZ-HP to form DZ is expected since such cyclization reaction is commonly employed in the synthesis of a variety of 1,4-benzodiazepines.[22,23]

**Pharmacological Activity Tests in Mice**—TMZ significantly prolonged the pentobarbital-induced sleep time (Table 1). In comparison, neither TMZ-HP nor TMZ-RP had any effect. TMZ effectively protected the mice against metrazol-induced convulsion (Table 1). TMZ-HP had no antimetrazol activity. At 10.5 mg/kg, TMZ-RP did not protect the metrazol-induced convulsion, although it decreased the mortality rate (2 out of 10 mice survived). The slight reduction in mortality by TMZ-RP was not observed with a lower dose of metrazol (5.25 mg/kg). These results indicated that both the acid-catalyzed hydrolysis product (TMZ-HP) and the base-catalyzed rearrangement product (TMZ-RP) were pharmacologically inactive products of TMZ.

## Conclusions

TMZ undergoes a hydrolysis reaction under acidic conditions similar to those found in the stomach of humans to form a 2′-benzoyl-4′-chloro-*N*-methyl-2-amino-2-hydroxyacetanilide. At pH $\approx$ p$K_a$ (1.46), the reaction requires protonation at N4 and the rate-determining step involves a nucleophilic attack by water at C5 of a C5—N4 iminium ion. The initial hydrolysis product is further slowly hydrolyzed to form MACBP. Pharmacological activity tests in mice indicated that the predominant hydrolysis product is TMZ was inactive. The results suggests that a fraction of an orally taken TMZ may be inactivated by hydrolysis in highly acidic gastric juice.

## References and Notes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