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Communications to the Editor

Synthesis, Decomposition Kinetics, and Preliminary Toxicological Studies of Pure *N*-Acetyl-*p*-benzoquinone Imine, a Proposed Toxic Metabolite of Acetaminophen

Sir:

The compound *N*-acetyl-*p*-benzoquinone imine (NAPQI, 1) is of interest as a proposed ultimate toxic metabolite in acetaminophen (2) overdose.[1,2] NAPQI was initially prepared by lead tetraacetate oxidation of acetaminophen, although the only characterized product was a Diels–Alder adduct.[3] Dehydration of *N*-hydroxyacetaminophen also yields NAPQI, which further reacts with the remaining *N*-hydroxyacetaminophen to form several products.[4-6] While Blair et al.[7] have described a synthesis resulting in stable benzene solutions of NAPQI, and Miner and Kissinger[8] have developed electrochemical methods for its generation in buffer, reports of the synthesis of pure NAPQI have yet to appear. Since the availability of purified material is of considerable importance in confirming its structure and its role in acetaminophen toxicity, we describe here a method for the preparation of NAPQI in crystalline form. In addition, the results of preliminary decomposition kinetic studies are reported.

NAPQI was synthesized by oxidation of acetaminophen (4′-hydroxyacetanilide) with freshly prepared silver oxide.[7] Acetaminophen (1.0 g, 6.6 mmol) and silver oxide (1.0 g, 4.3 mmol) were stirred in 50 mL of dry chloroform for 25 min at room temperature. Activated charcoal was added and filtered through Celite into a receiver containing approximately 1.0 mg of the radical scavenger, butylated hydroxytoluene. The chloroform was rotary evaporated under reduced pressure to a volume of approximately 1.0 mL and placed on ice. The crude NAPQI solution was then eluted through a 3.6 × 14 cm column of dried silica gel (70–325 mesh) with anhydrous ethyl ether. NAPQI was collected as a distinct yellow band and evaporated to give 265 mg of product: 42% yield relative to the limiting silver oxide. The NAPQI was sublimed (45–50 °C at 0.07 mmHg) to give yellow cubic crystals: mp 74–75 °C; IR (KBr pellet) ν_{max} 3050 (m), 2990 (w), 1700 (s, CH_3CO), 1651 (s, CO), 1620 (s), 1579 (s, CH), 1430 (m), 1362 (m), 1210 (s), 1103 (m), 885 (s) cm^{-1}; ^1H NMR (C_6D_6) δ 1.78 (s, 3 H), 6.00 (d, 2 H, J_{BA} = 9.0 Hz), 6.38 (d, 2 H, J_{AB} = 9.0 Hz); UV λ_{max} (*n*-hexane) 263 nm (ϵ 3.3 × 10^4 $M^{-1}cm^{-1}$), 376 (1.6 × 10^2). Anal. ($C_8H_7NO_2$) C, H, N. Purity was greater than 99% as determined by peak area comparison using normal-phase HPLC on a Partisil PXS 5/25 column with ethyl acetate as the mobile phase and UV detection at 254 nm. At a flow rate of 1.0 mL/min, the retention times were 4.5 min for NAPQI and 9.0 min for acetaminophen. The material is stable as the crystalline solid for at least 3 months if stored at 0 °C away from light and moisture.

A quantity of crystalline NAPQI was further purified by normal-phase HPLC using a Zorbax Sil (6.2 mm × 25 cm) column. With ethyl acetate as solvent and a flow rate of 2 mL/min, NAPQI was retained 7.0 min and acetaminophen was retained 15.0 min. A sample of this material was submitted to high-resolution mass spectral analysis on a VG 7070H Micromass instrument. The EI spectrum is characterized by a parent ion at *m/e* 149 with the fragments *m/e* 134 ($M^{+\cdot} - CH_3$), 107 ($M^{+\cdot} - CH_2CO$), 106 ($M^{+\cdot} - CH_3CO$), and 43 (CH_3CO^+); the elemental compositon of all ions was confirmed by high-resolution mass measurements. It is interesting to note that at lower source temperatures a significant M + 2 peak appears in the EI spectrum. This is common with quinonoid molecules[9] and appears to be caused by protonation of the imino quinone by traces of residual water in the ion source followed by rapid reduction. The injection of 1 μL of D_2O into the source shifts the M + 2 peak to M + 4, which is consistent with the proposed mechanism. In comparison, acetaminophen under the same conditions gives a different abundance pattern for the M + X ions, negating the possibility that the M + 2 peak arises from an acetaminophen impurity. High-resolution CI (methane) of NAPQI gives the quasimolecular ion *m/e* 150 and the addition products M + 29 = 178 and M + 41 = 190. A prominant M + 3 peak can be attributed to the quasimolecular ion of acetaminophen produced by the reduction of NAPQI in the ion source, as previously described. Some of the addition ions that are formed from NAPQI under CI conditions are unusual and apparently arise by radical trapping in the mass spectrometer ion source.[10]

Miner and Kissinger[8] have noted that the stability of

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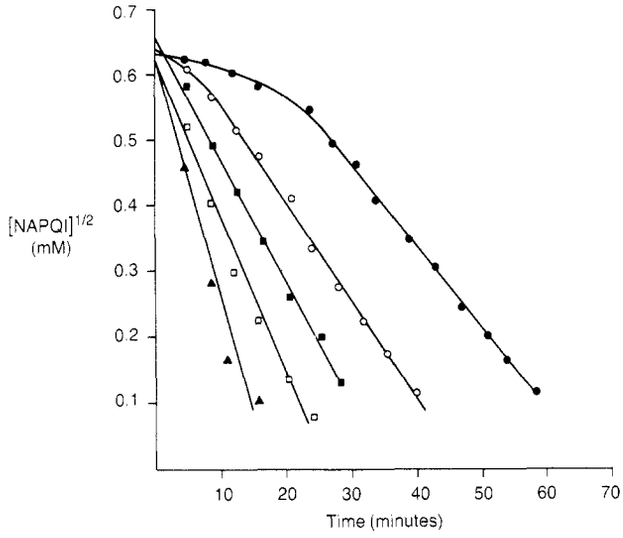
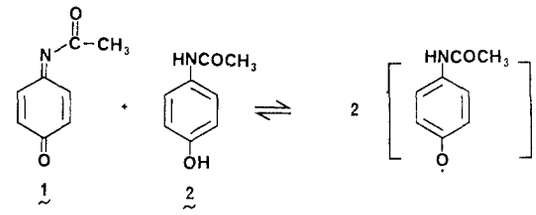


Figure 1. Decomposition rate plots for 0.4 mM NAPQI in 0.1 M sodium phosphate buffer, pH 7.4: NAPQI alone (●) and with inclusion of 8 (○), 20 (■), 40 (□), and 80 μM (▲) acetaminophen.

electrochemically generated NAPQI in buffer is dependent upon several factors, including pH, temperature, and buffer concentration, and that the decomposition kinetics appear to be half order. We have investigated the decomposition kinetics of pure NAPQI under similar conditions, monitoring the concentration over time by reverse-phase HPLC. A C_{18} μ-Bondapak column (3.9 mm × 30 cm) was used with a solvent system of 0.1 M citrate buffer, pH 7.2, and 20% methanol. At a flow rate of 2 mL/min, the retention time of acetaminophen and NAPQI were 2.5 and 3.3 min, respectively. Figure 1 shows the disappearance of 0.4 mM NAPQI in 0.1 M sodium phosphate buffer, pH 7.4, to be biphasic, with the second phase exhibiting half-order kinetics. An empirically derived rate constant was determined to be 1.1×10^{-4} $M^{1/2}$ s^{-1} with a half-life of approximately 11 min. The initial phase of the decomposition curve was nearly linear when plotted as second order with an apparent rate constant of 6.25×10^{-1} M^{-1} s^{-1}. The inclusion of increasing concentrations of acetaminophen significantly increased the rate of NAPQI disappearance with the elimination of the first slower phase. This may indicate a role for the semi imino quinone radical in the degradation of NAPQI, since the presence of acetaminophen would be expected to increase the formation rate of the radical by spontaneous comproportionation of acetaminophen and NAPQI. Additionally, the observation of half-order kinetics for the second phase is consistent with a free-radical decomposition process.[11]

(11) K. L. Laidler, "Reaction Kinetics", Volume I, Pergamon Press, New York, 1963, p 178.



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However, the decomposition of NAPQI in aqueous buffer appears to be quite complex and apparently involves reduction, hydrolysis, and radical coupling reactions. Analysis of decomposition mixtures by HPLC and EIMS has revealed the presence of acetaminophen as a reduction product of NAPQI, *p*-benzoquinone as a hydrolysis product of NAPQI, and small amounts of hydroquinone as a reduction product of *p*-benzoquinone. We also have preliminary evidence for the formation of an acetaminophen dimer. Further delineation of the decomposition kinetics of NAPQI will require complete characterization and quantification of the reaction products.

Preliminary toxicity studies have been conducted in male BALB/c mice with pure NAPQI. Results indicate that it is a significantly more potent toxin (LD_{50} of 20 mg/kg) than acetaminophen (LD_{50} of 500 mg/kg) as determined 24 h after ip injections of the respective compounds in propylene glycol (0.2 mL/20 g mouse). Liver histological scoring and SGPT levels were normal, indicating no hepatic necrosis; however, the blood was extensively coagulated. While it does not appear that death was due to liver damage, NAPQI added to suspensions of isolated hypatocytes proved to be substantially more cytotoxic than acetaminophen, requiring concentrations approximately one-tenth those of acetaminophen to cause equivalent extents of cell death. This would imply that NAPQI is so reactive and cytotoxic that little of it reaches the liver when administered by the ip route, reaction occurring in the intravascular space with which NAPQI initially comes in contact. If NAPQI were generated in the liver cell from acetaminophen, reactions leading to cellular damage clearly would be anticipated. Work is now in progress to determine more directly the possible role of NAPQI as the ultimate toxic metabolite of acetaminophen.

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