Communications to the Editor

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FORMATION OF N-ACETYL-p-BENZOQUINONE IMINE, THE WELL-KNOWN TOXIC METABOLITE OF ACETAMINOPHEN, BY THE REACTION OF ACETAMINOPHEN WITH NITRITE UNDER MODEL STOMACH CONDITIONS

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The main reaction product of acetaminophen (4 mM) with nitrite (1 mM) under model stomach conditions was identified as p-benzoquinone. HPLC analysis of the reaction mixture with an electrochemical detector revealed the presence of N-acetyl-p-benzoquinone imine, the toxic metabolite of acetaminophen, as an intermediate in p-benzoquinone formation. About 14% of acetaminophen was estimated to be transformed into this intermediate when the reaction was carried out at pH 3 for 1 h at 37 °C.

KEYWORDS — acetaminophen; paracetamol; nitrite; p-benzoquinone; N-acetyl-p-benzoquinone imine

Phenolic compounds react with nitrite at gastric pH to give C-nitroso and C-nitro derivatives and diazoquinones. Of these products, the diazoquinones are mutagenic, and their formation from tyramine,[1] bamethane,[2] phenol[3] and acetaminophen (AP)[4] have been reported. But the previous works on bamethan[2] and phenol[3] and our recent study on AP[4] indicate that diazoquinones were formed only in the presence of a four-molar excess of nitrite. This suggests that phenolic compounds, especially in high concentration, seldom form diazoquinones in the digestive tracts of normal subjects. During the course of the study of nitrosation of AP, we recognized that lower salivary nitrite levels favored the formation of an unknown product. This paper identifies the unknown product as p-benzoquinone (BQ) and the probable involvement of N-acetyl-p-benzoquinone imine (ABQI), which is considered to be an ultimate toxic metabolite in AP overdose,[5-7] as an intermediate in BQ formation.

Figure 1 shows the HPLC chromatogram of two reaction mixtures obtained under different conditions. The AP concentration (4 mM) in the reaction system was decided on the basis of the usual dose and stomach content. The AP disappeared almost completely when treated with 16 mM of nitrite, and 2-nitro-4-acetylaminophenol, 2,6-dinitro-4-acetylaminophenol, 4-acetylamino-6-diazo-2,4-cyclohexadienone and the unknown (retention time, 4.2 min) were formed (Fig. 1a). On the other hand, the unknown was formed apparently as a main product when the reaction was carried out with 1 mM of nitrite (Fig. 1b). The AP was completely stable in the absence of nitrite. The nitrite levels in saliva and gastric juice are reported to be 0.26-1.57 mM[8] and 0.02-0.46 mM,[9] respectively, suggesting that this unknown may be formed in the stomach.

The unknown was isolated by successive extraction of the reaction mixture with diethylether and chloroform and silica-gel chromatography (2 X 15 cm) of the

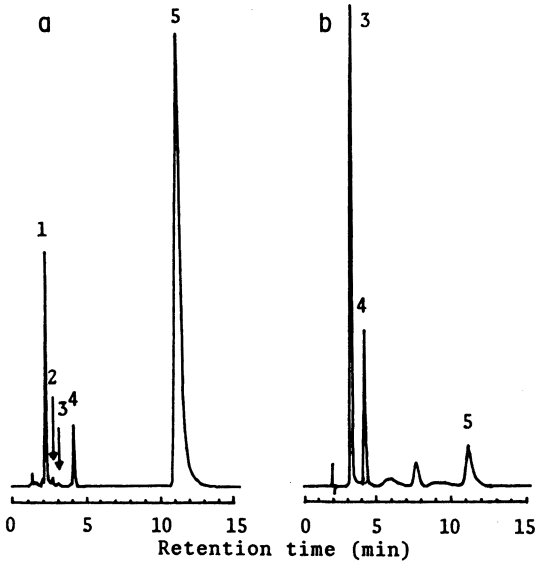


Fig. 1 Effect of Nitrite Concentration on Reaction Products

AP (4 mM) was treated with nitrite (a, 16 mM; b, 1 mM) at pH 3 for 1 h at 37 °C. HPLC conditions: Column, Spherisorb 5-ODS-I (4.6X150 mm); Mobile phase, 15%(v/v) acetonitrile/0.02 M phosphate buffer (pH 5.8); Flow rate, 1 ml/min; Detection, 254 nm. 1, 2,6-dinitro-4-acetylaminophenol; 2, 4-acetylamino-6-diazo-2,4-cyclohexadienone; 3, AP; 4, unknown; 5, 2-nitro-4-acetylaminophenol.

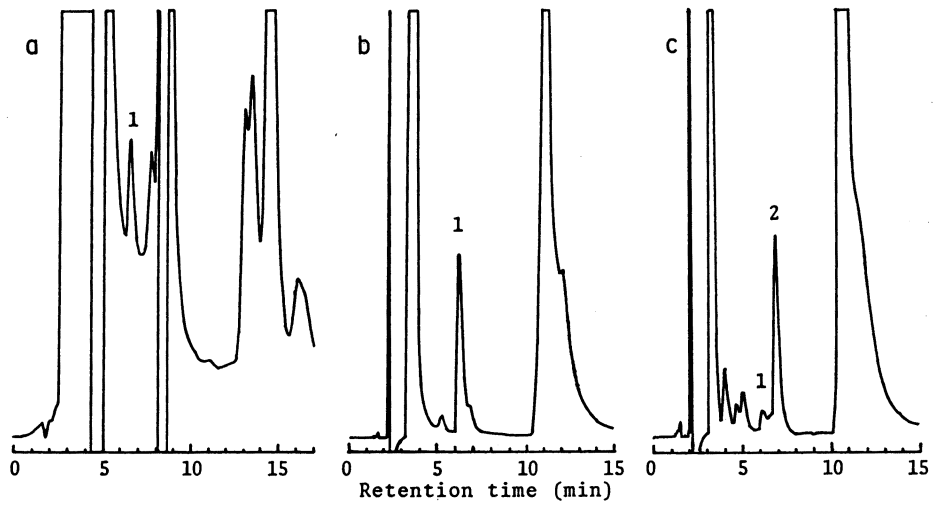
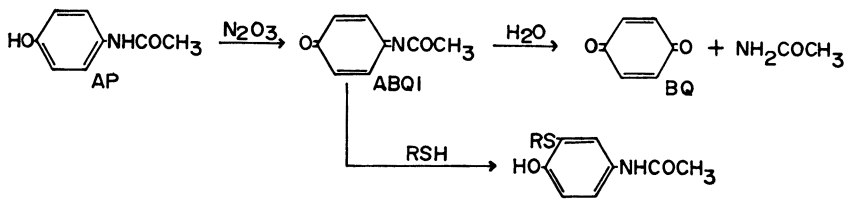


Fig. 2 HPLC Chromatographic Confirmation of ABQI in the Reaction Mixture

a: AP (4 mM) was treated with nitrite (1 mM) at pH 3 for 1 h at 37 °C, and analyzed by the same HPLC system as in Fig. 1 except for the use of 14% (v/v) acetonitrile/0.1 M phosphate buffer (pH 5.8) as a mobile phase and of a TOA ICA-3060 electrochemical detector (-0.24V). b: AP (0.4 mmole) was stirred in 25 ml of 8 mM nitrous acid/chloroform at 37 °C for 1 h, and analyzed by the same HPLC system as in Fig. 1 except for the use of 14% (v/v) acetonitrile/0.02 M phosphate buffer (pH 5.8). c:The reaction mixture obtained in b (0.6 ml) was shaken with an equal volume of water containing 20 µl of 20% methanethiol/methanol, and the organic phase was analyzed as in b (AUFS sensitivity increased twice). 1, ABQI; 2, 2-methylthio-4-acetylaminophenol.

concentrated chloroform extract using chloroform as solvent. The UV absorption spectrum ($CHCl_3$) showed an absorption maximum at 244 nm. The ^{1}H-NMR ($CDCl_3$) spectrum showed only one aromatic proton (δ 6.76). The EI-MS spectrum showed a molecular ion peak at 108 m/z and fragment ion peaks at 82 ($M^+-C_2H_2$) and 54 m/z ($M^+/2$). These spectral data coincided with those of an authentic BQ, and the above unknown was identified as BQ.

Formation of BQ by the reaction of phenolic compounds and nitrite has been observed with 4-dimethylaminophenol, and a quinone imine is postulated to be the intermediate.[10] It is especially important in the present reaction to clarify whether a quinone imine is an intermediate in BQ formation, because the corresponding quinone imine, ABQI, is the well-known toxic metabolite of AP.[5-7] HPLC of the reaction mixture showed the presence of a peak corresponding to that of a synthesized ABQI[7] (Fig. 2a). Incubation of AP without nitrite did not give this peak. The peak was detected only with an electrochemical detector at -0.24 V, probably due to its scarcity in the reaction mixture. Since ABQI was easily hydrolyzed to BQ in aqueous solution but relatively stable in organic solvents,[7] AP was also treated with nitrous acid/chloroform.[11] The peak corresponding to ABQI was detected with a UV detector (Fig. 2b). This peak decreased greatly when the reaction mixture was treated with nucleophiles such as cysteine and methanethiol, and its decrease with methanethiol was followed by the appearance of a peak corresponding to a synthesized sample of 2-methylthio-4-acetylaminophenol[12] (Fig. 2c). These characteristics coincided with those of an authentic ABQI. The above results clearly indicate the formation of ABQI as an intermediate during the reaction of AP with nitrite under the simulated stomach conditions. This is the first time to show the occurrence of a quinone imine as a nitrosation product of phenolic compounds.



The transformation of AP into ABQI was determined on the basis of the acetamide formed, since BQ was likely to react with other products in the reaction mixture, and since acetamide formed during the hydrolysis of ABQI to BQ was expected to reflect the overall amount of ABQI. Acetamide was determined by passing the reaction mixture through a Sep-pak C18 cartridge and analyzing the eluent by means of the same HPLC system as in Fig. 1 except for the use of water as solvent and the detection at 210 nm. About 14% of AP was estimated to be transformed into ABQI when AP (4 mM) was treated with 1 mM of nitrite at pH 3 for 1 h at 37 °C. This is rather surprising in view of the fact that the bioactivation pathway is minor, 80-90% of AP being eliminated via the alternative pathway of conjugation with glucuronic acid and sulfate.[13] The ABQI formed by endogenous nitrosation of AP may be consumed by the hydrolysis to BQ and interaction with other substances in the stomach, but it may cause some injury to the digestive tract because of its high cytotoxicity[5] and the high yield described above. BQ may also be hazardous because of its toxicity.[14,15] Infiltration of red blood cells in gastric mucosa after large doses

of AP[16] and a significant association between chronic AP intake and gastric ulcer[17] have been reported. A cause-and-effect relationship between the present reaction and these facts should be clarified.

Another interesting aspect of the present reaction is the larger amounts of BQ and ABQI formed at lower nitrite concentration, 0.045 and 0.1 mM of BQ and 0.2 and 0.54 mM of ABQI (estimated on the basis of acetamide) by the reaction of 4 mM of AP with 16 and 1 mM of nitrite, respectively, for 1 h at 37 °C. On the other hand, the amount of 2-nitro-4-acetylaminophenol increased with increasing concentration of nitrite. These results suggest that AP is consumed rapidly through the formation of a larger amount of C-nitro derivatives at higher concentrations of nitrite, and that ABQI and BQ are accordingly formed in smaller amounts. Further investigation on the mechanism of this reaction is now in progress.

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