Synthesis and Characterization of Acetaminophen Oxidation Products

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Background

Commonly Accepted Mechanism of Hepatotoxicity

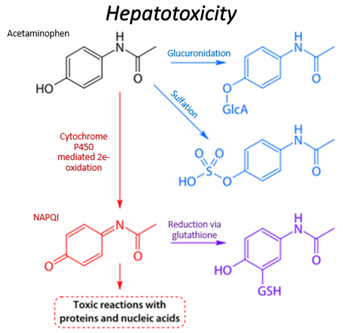


Figure 1: Current literature supports a toxicological mechanism of APAP overdose via a net 2e- oxidation of APAP into the deleterious metabolite, NAPQI.

Peroxidase Cycle

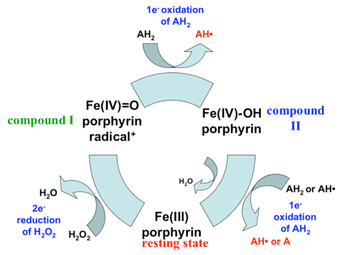


Figure 2: Cytochrome P450s are able to carry out two 1e- oxidations in addition to the commonly accepted one 2e- oxidation as illustrated to the left. This oxidation can be modeled using Horseradish peroxidase or electrochemical methods as shown below.

Alternative Acetaminophen Metabolism

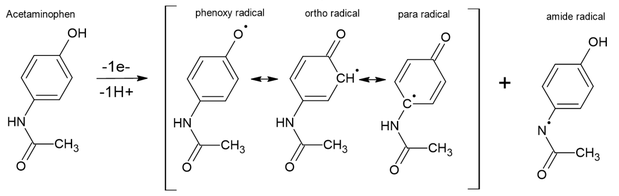
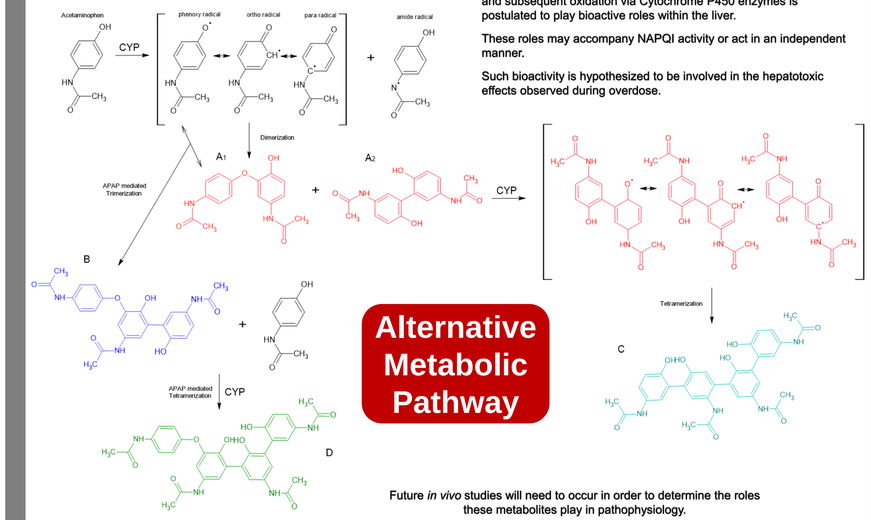


Figure 3: One electron oxidation of APAP yields four different radical intermediates as a result of oxidation at either the phenoxy or amide positions. A mechanism of radical polymerization could yield a variety of different metabolites based on the position of preference of the lone electron.

Motivations

Acetaminophen (APAP) is a common analgesic and an active ingredient in many painkillers such as Tylenol and Percocet. Upon overdose, APAP can lead to toxicity in the liver, which accounts for a striking proportion of acute liver failures in the United States annually. Considering the potential for APAP induced hepatotoxicity, our research group has analyzed APAP and its tendency to oxidize into reactive products through *in vitro* enzymatic methods, which were visualized through HPLC. Characterization of these APAP oxidation products were carried out using ESR, ESI-MS/MS, and H-NMR. Our findings provide structural insight into potentially deleterious APAP oxidation metabolites formed early in the liver during times of APAP overdose via a mechanism of radical polymerization.

Conclusions



The resulting metabolite formation following acetaminophen overdose and subsequent oxidation via Cytochrome P450 enzymes is postulated to play bioactive roles within the liver.

These roles may accompany NAPQI activity or act in an independent manner.

Such bioactivity is hypothesized to be involved in the hepatotoxic effects observed during overdose.

Alternative Metabolic Pathway

Future *in vivo* studies will need to occur in order to determine the roles these metabolites play in pathophysiology.

Electron Spin Resonance

Acetaminophen (APAP) radical

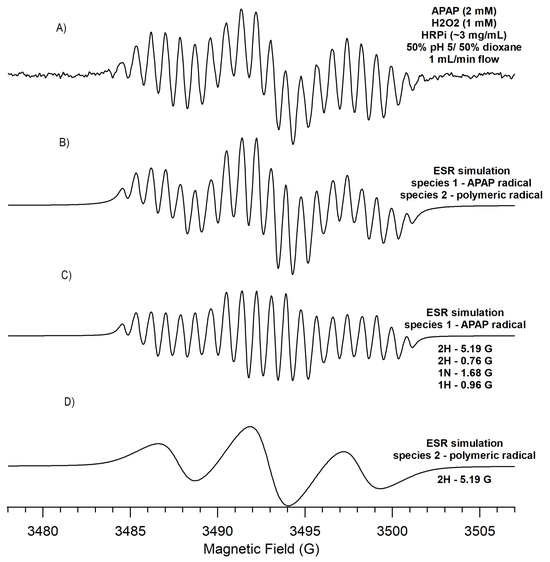


Figure 4: A) Detection of the APAP radical using an immobilized enzyme ESR flow method. The ESR spectra were collected using HRP covalently bound to AquiGel beads within a flat cell, 2mM APAP, 1mM H2O2, phosphate-citrate buffer (pH 5), 1 mL/min flow rate on a Bruker EMX spectrophotometer with an SHQ resonator (10 mm flat cell) operating at 9.8 GHz, 20 mW microwave power, 60 Gauss sweep width, and 0.5 Gpp modulation amplitude (100KHz). B) Spectrum A simulated using WINSIM software. C) Spectrum B with the broad polymeric radical signal (species 2) mathematically removed. D) Spectrum B with the species 1 signal mathematically remaining.

Computational Mapping

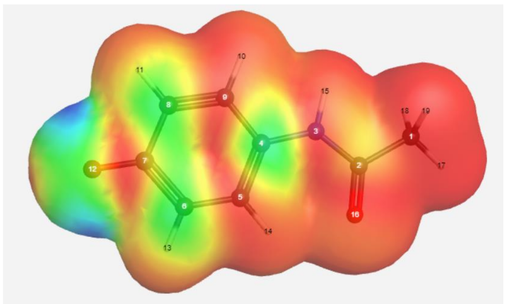


Figure 5: Electron density map of the acetaminophen radical using . The color corresponds with electron density in the order of blue > green > yellow > orange > red. The map is consistent with experimental ESR data concerning the preference of the lone electron to spend its time at the phenoxy and ortho positions.

APAP Oxidation

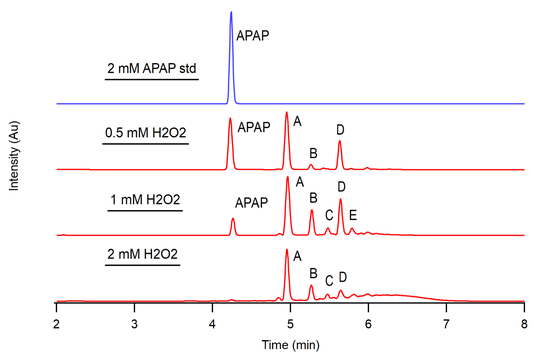


Figure 6: Reversed-phase HPLC separation of acetaminophen oxidation products as catalyzed by HRP with varying concentrations of H2O2 and constant acetaminophen levels. The reaction progress was monitored via pairing with a diode array detector (270 nm). A binary solvent system with a flow rate of 1.0 mL/minute containing Solvent A, 0.1% trifluoroacetic acid, and Solvent B, 100% acetonitrile, allowed for the separation and quantification of acetaminophen metabolites following oxidation. For chromatography, Solvent A was held at 100% for the first minute of the run, followed by a linear transition to 100% solvent B until minute 15 at which Solvent B was held constant for 3 more minutes.

Metabolite Purification

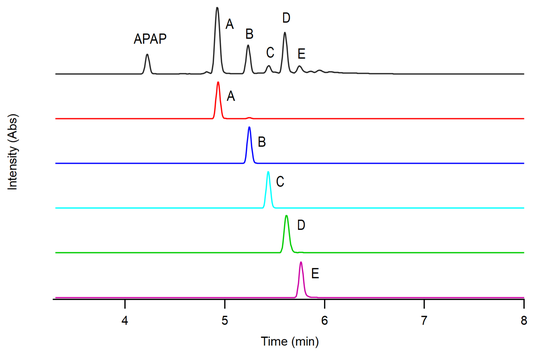


Figure 7: Metabolites were isolated utilizing a flash chromatography system from a scaled-up APAP/H2O2/HRP reaction mixture (black) containing 50 mM APAP, 25 mM H2O2, and 9.7 nM HRP. Separation was achieved through a linear gradient beginning at 100% solution A to 100% solution B over the course of 30 column volumes.

Metabolite Oxidation

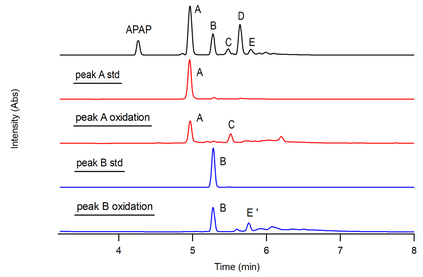


Figure 8: The scaled-up APAP/H2O2/HRP reaction seen left (black) serves as a product standard. Isolated metabolites were oxidized using the same H2O2/HRP system. 1 mM product A (red) and 1 mM product B (blue) were oxidized enzymatically with the addition of 0.5 mM H2O2. Oxidation of product A yielded in the formation of product C while the oxidation of product B yielded product E'.

Electron Spin Resonance (Metabolite A)

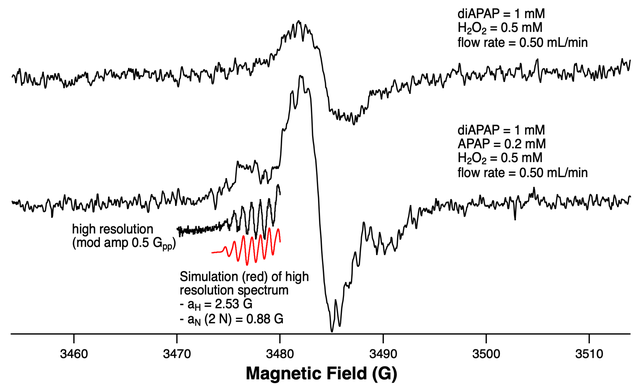
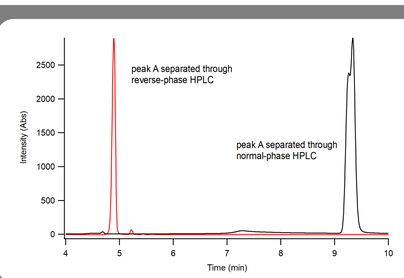


Figure 9: Detection of the diAPAP (metabolite A) radical using an immobilized enzyme ESR flow method as described earlier. The top spectrum was the signal associated with the oxidation of diAPAP. The following spectrum is the oxidation of diAPAP with the addition of a fractional amount of APAP. High resolution signal averaging was utilized to further characterize the second spectrum. The high-resolution experimental data is black and its simulation using WINSIM software is red.

Mass Spectrometry

Compounds	m/z (% relative abundance)
A	301.118 (100)
B	450.166 (100), 301.118 (7)
C	599.213 (100)
D	599.213 (41), 450.166 (100)
E	748.261 (44), 599.213 (100)

Figure 10: Electrospray ionization was utilized with a quadruple time-of-flight mass analyzer for the analysis of purified acetaminophen metabolites. The data represents a partial set of molecular ions as a result of the fragmentation of the metabolites. ESI-MS/MS data are m/z + 1 and contain relative abundance of each molecular ion.



Chromatography (Metabolite A)

Figure 11: The flash isolated compound A was analyzed via both normal phase (black) and reverse phase (red) HPLC. Reverse phase HPLC shows one distinct product peak while normal phase HPLC shows two chemically unique product peaks, which indicates a mixture of products.

Nuclear Magnetic Resonance (Metabolite A)

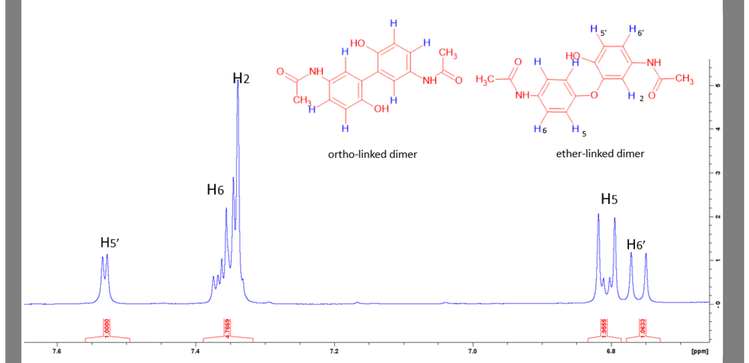


Figure 12: The H-NMR spectrum of product A (20 mg/mL in DMSO-d6) was obtained using a 400 MHz Bruker NMR spectrometer. The spectrum is zoomed in on the aromatic region.

Acknowledgements

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Knox College Department of Chemistry

Western Illinois University Department of Chemistry

References

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