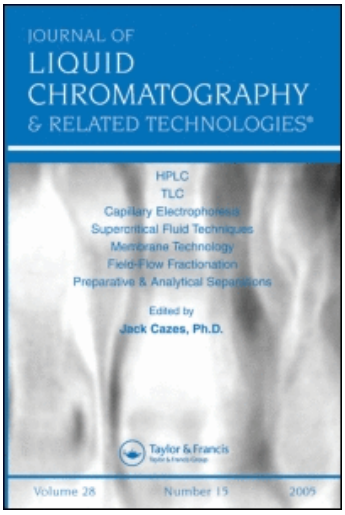

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HPLC of Fluorescent Products of Acetaminophen Reaction with Peroxidase

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Original Article

HPLC OF FLUORESCENT PRODUCTS OF ACETAMINOPHEN REACTION WITH PEROXIDASE

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ABSTRACT

HPLC was utilized in this work to follow the progress of the reaction of acetaminophen and hydrogen peroxide in the presence of horseradish peroxidase. Several fluorescent as well as ultraviolet-detectable compounds where detected and separated by HPLC on a reversed-phase column. These reaction products are polymers of acetaminophen. The compounds are stable and fluoresce directly over a wide pH range without the need for further chemical reactions. Surfactants enhance the fluorescence intensity. The fluorescent compounds have several potential practical applications as sensitive tests for: hydrogen peroxide, peroxidase activity and also as an indicator reaction for coupled enzymatic assays such as glucose and uric acid.

INTRODUCTION

Acetaminophen (4'-hydroxy-acetanilide) is a commonly used analgesic drug. Intermediates in the **in vivo** metabolism of this compound have been implicated in the hepatic and renal damage in man which accompany large or chronic doses. However, in the cat, acetaminophen toxicity is manifested by severe hematologic

changes (1). Several previous studies (2,3) have demonstrated that the action of peroxidase (EC 1.11.1.7) on acetaminophen in the presence of hydrogen peroxide produces oxidation and polymerization products which absorb in the ultraviolet region.

Here we describe that the addition of hydrogen peroxide and horseradish peroxidase to acetaminophen also produces fluorescent compounds. We demonstrate that HPLC is a valuable tool for separating and characterization of these compounds and for investigating the mechanism of their formation. The importance of these reaction products lies in their stability and their fluorescence over a wide range of pH and their potential application as sensitive indicator reactions for hydrogen peroxide as well as coupled kinetic assays.

MATERIALS AND METHODS

Chemicals- Acetaminophen (4´-hydroxy-acetanilide) was obtained from Eastman Kodak Co. (Rochester, N.Y.). Horseradish peroxidase (EC1.11.1.7) was obtained from Sigma Co. (St. Louis, MO.). Triton 405, ARW 7, and Brij 35 were obtained from Technicon Instruments (Tarrytown, NY). Triton X100 was obtained from Fisher Scientific (Fairlawn, NJ).

Solutions - Acetaminophen was dissolved in 25 mM Tris buffer, pH 7.5 to give solutions of 1 or 5 g/L.

Methods: For demonstration of enzymatic breakdown of acetaminophen 1 ml of the 5 g/L solution was mixed with 600 ul of hydrogen peroxide and 400 ul of peroxidase solution (1 g/L, 22

units) . In general, samples were measured at room temperature 30 minutes after initiation of the reaction, unless indicated otherwise. For characterization the capacity factor (k´) vs. pH, 1 ml of acetaminophen (5 g/L) , was mixed with 600 ul of hydrogen peroxide 3% (in water) and 20 ul (1.1 units) of horseradish peroxidase. After 20 min incubation at 37 C the samples were injected on the column.

Surfactants, at different concentrations in phosphate buffer (7 mmol/L , pH 8), were added to the fluorescent products of the reaction after it proceeded for 10 min as described earlier. The fluorescence intensity was measured in a Gilson Fluorometer (366 nm excitation and 420 nm emission) as described later .

For demonstration the feasibility of glucose analysis in a coupled reaction we incubated 500 uL of different concentration of glucose (40-200 mg / L) with following compounds (at final concentration), glucose oxidase 1 mg/ml, acetaminophen 1mg/ml, and 0.3 U of peroxidase in phosphate buffer 50 mmol/pH 6.0 . The reaction left for 20 min at 25 C. This reaction, has not been optimized for routine analysis.

Instrumentation- Fluorescent and ultraviolet reaction products were analyzed by reversed-phase HPLC on a Nova 5-um C18 column (Waters Associates, Milford MA) either with isocratic solvent 5% acetonitrile in 7mM phosphate buffer, pH 6.5 or as a gradient of phosphate buffer (7 mmol/L pH 6.5)/ acetonitrile (0- 60 % over 20 min period) as indicated later, at a flow rate of 1.2 ml/min. The analytical system consisted of a Beckman 420

microprocessor and 110 A pumps (Beckman Instruments, Fullerton CA) and Model 420-C fluorescence detector (366 nm excitation and 420 nm emission) and Model 440 ultraviolet detector, 254 nm, (Waters Associates).

RESULTS AND DISCUSSION

Horseradish peroxidase catalyze the reaction of acetaminophen and hydrogen peroxide with the formation of several unknown compounds as measured by the ultraviolet detector at 254 nm using an acetonitrile gradient (3), Fig. 1, top. However, monitoring the effluent simultaneously with a fluorescent detector also revealed the presence of a major unknown fluorescent peak in addition to several minor ones (Fig. 1, bottom). Early in the course of reaction, at 5 minutes, a small fluorescent peak emerged. It increased in size with progress of the reaction (at 10 min). Upon further incubation for 20 minutes, 2 or 3 secondary peaks evolved, with a concomitant decrease in height of the major peak indicating that the secondary peaks were probably formed from the primary peak. The major fluorescent peak appeared to correspond to one of the primary ultraviolet peaks. It had a maximum absorption at 243 nm by scanning spectrophotometry (Fig. 2), very close to the parent compound which had an absorption maximum at 245 nm. The fluorescent compound has an excitation maximum at about 340 nm and emission maximum at about 430 nm, Fig 3.

The effect of pH on the capacity factor (k') of the fluorescent compounds is shown in Figure 4 . The k' of the major

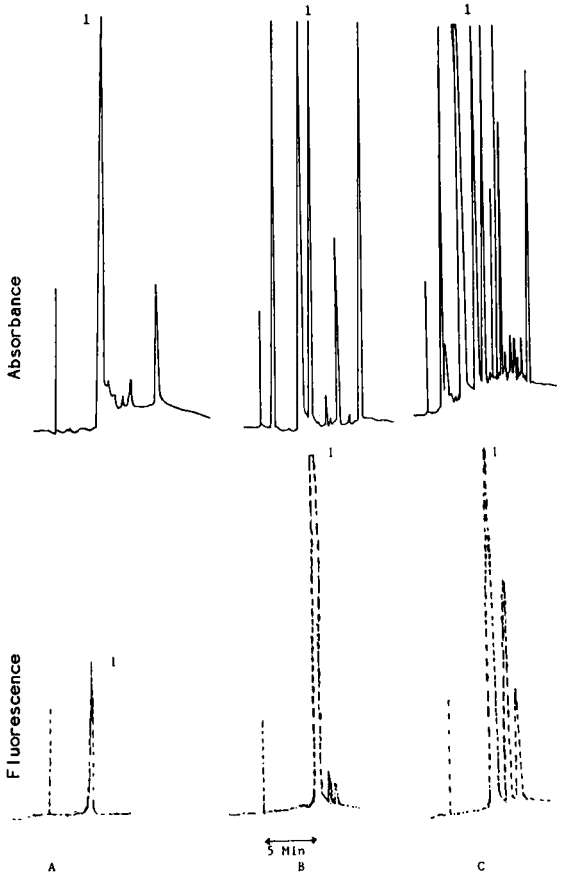

Figure 1. Production of fluorescent compounds by the action of peroxidase and hydrogen peroxide on acetaminophen with time (1 ml of 1 g/L dissolved in 7 mM phosphate buffer, pH = 6.5) with 200 uL of 3% H_2O_2 in the presence of peroxidase (0.3 units). A - 5 min B - 10 min C - 20 min at 37 C . The separation was performed by acetonitrile : water gradient 0- 60 % over 20 min at a flow rate of 1.2 ml/min.

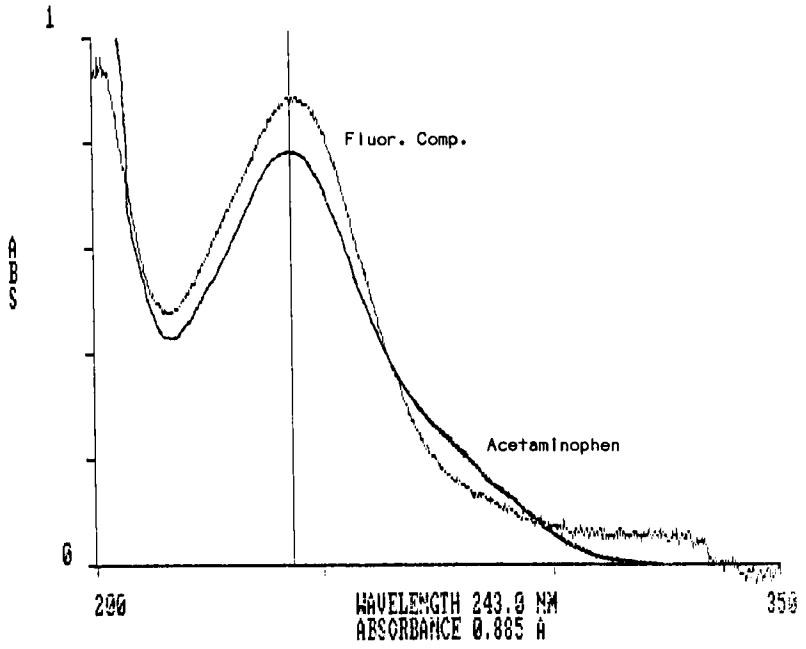

Figure 2. The ultraviolet spectra of the fluorescent compound.

fluorescent peak did not change very much between pH 4.0 to pH 6.5. However, it decreased with increasing pH between 6.5 to 8.0 indicating the compound has an acidic hydroxy phenolic group. The minor fluorescent peaks eluted only at pH 7.0 and above. Their capacity factor also decreased with increasing pH from 7.0 to 8.0.

Through mass spectrometry and NMR (3), it has been previously shown that the ultraviolet compounds resulting from the action of peroxidase on acetaminophen are different polymers arising from the formation of phenoxy free radicals which rapidly undergo dimerization and polymerization(4). Chemical desorptive

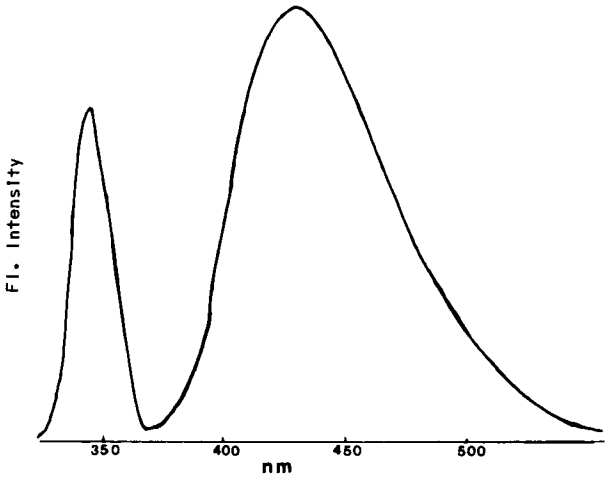

Figure 3. The emission and absorption spectra of the fluorescent compound.

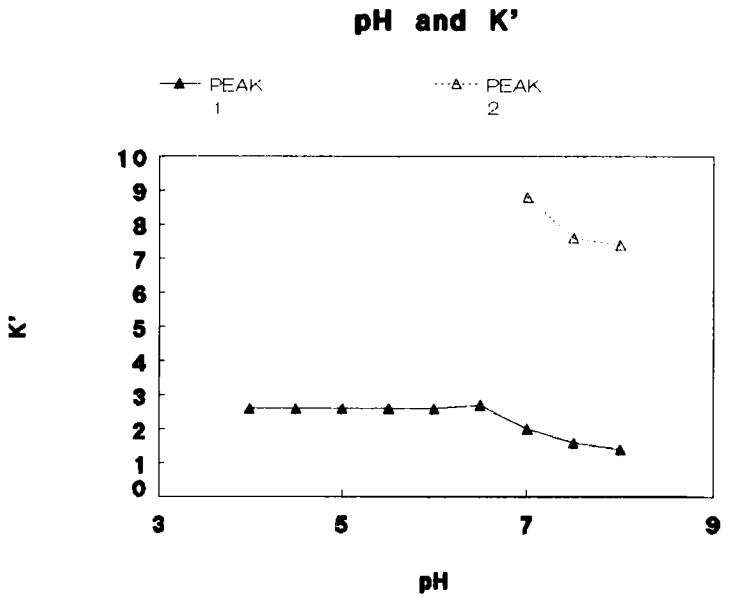

Figure 4. k' against pH for the fluorescent compound.

ionization mass-spectrometry was performed on the fluorescent peak after collecting the appropriate fraction. The fragment in highest abundance (about 80 %) has a mass of 391 . This indicates that the fluorescent compound is a polymer composed of at least three acetaminophen rings probably with a loss of one side chain. However, since the peak was not checked thoroughly for purity, it is difficult to deduce a specific chemical structure for the compound at the present time.

The fluorescence intensity was dependent on the pH, increasing with an increase in buffer pH from 2.5 to 10, with a peak at about pH 8.0, Fig 5. In general, surfactants have been demonstrated to enhance the intensity of fluorescent compounds (5,6). Addition of surfactants to the reaction mixture increased the fluorescence intensity of the compounds (Fig 6). A five-fold increase in fluorescence intensity occurred with the addition of 50 ml/L of Triton X100. The fluorescence intensity of the compound decreased by 20% upon storage in the refrigerator in the first 24 hours; however, it remained stable at this level for the next two days. We did not attempt here to add the surfactants directly to the eluting solvent to enhance the fluorescence of the peaks in order to keep the system simple.

The reaction of peroxidase on acetaminophen can be used as a sensitive indicator reaction for the assay of different analytes including hydrogen peroxide, acetaminophen, and peroxidase. In addition, it can be used as an indicator reaction for coupled oxidative enzymatic assays measuring such analytes as glucose,

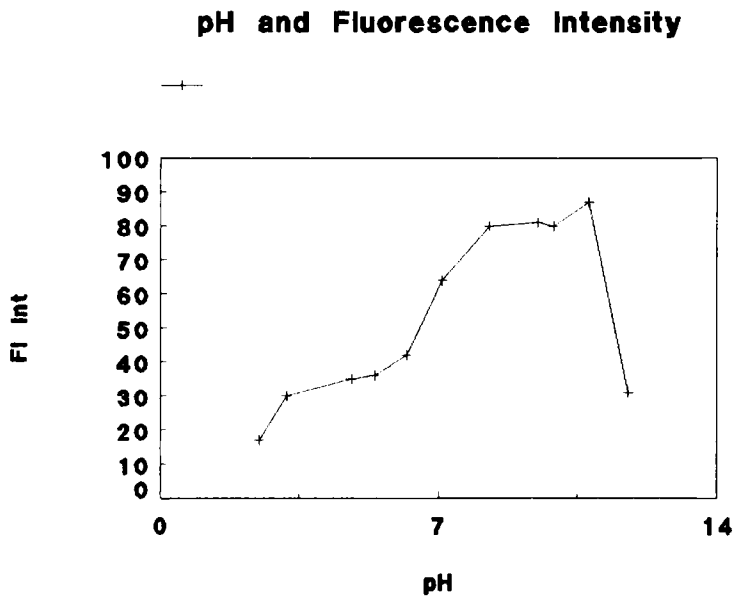

Figure 5. The effect of pH on the fluorescence intensity of the reaction products, excitation wavelength at 360 nm and emission at 455 nm. Peroxidase, 0.3 units was added to 1 ml of acetaminophen solution ,1 g/L, containing 200 ul of 3% hydrogen peroxide. An aliquot of 100 uL was added to 2 ml of 10 mM phosphate buffers.

cholesterol, uric acid, ethanol, and lactate. Any of these substrates with the appropriate oxidative enzyme (uricase, glucose oxidase, etc.) can generate hydrogen peroxide which can combine with acetaminophen and peroxidase to produce fluorescence. Althogh other fluorescent compounds are available for coupling reaction, these can be limited by the pH optimum for one of the enzymes (7,8). The need for a wide selection of different compounds with different fluorescence characteristics always exists because the difference between the pH optimum for

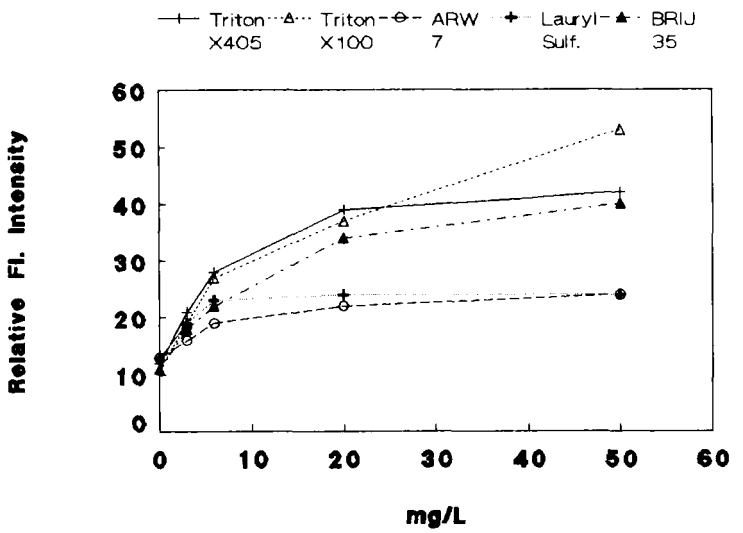

Figure 6. Effect of different surfactants (Brij 35, Triton X100, Triton 405, SDS, and ARW 7) on the fluorescence intensity of the reaction products of acetaminophen in phosphate buffer 7 mmol/L, pH 8.

fluorescence and catalysis for the enzymes. Unlike other substrates which may require additional steps for fluorescence or may not fluoresce at the appropriate pH for the primary or the indicator enzyme (7,8), acetaminophen reaction products fluoresce directly over a wide pH range , rendering these compounds potentially suitable for many continuous kinetic assays. Figures 7 and 8 demonstrate a glucose reaction and linearity for aqueous solutions based on the measurement of the fluorescence of a coupled acetaminophen/peroxidase reaction.

In conclusion, there are several advantages of utilizing the fluorescent products of acetaminophen, including their ability to

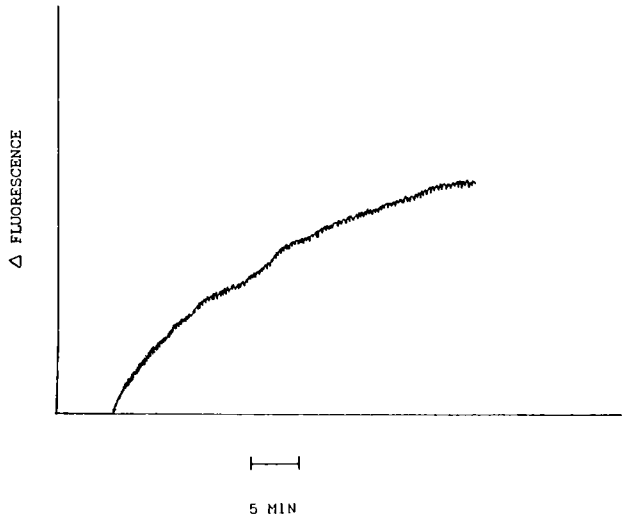

Figure 7. Kinetics of glucose (800 mg/L) reaction using acetaminophen, and peroxidase as indicator reaction with measurement of fluorescence intensity.

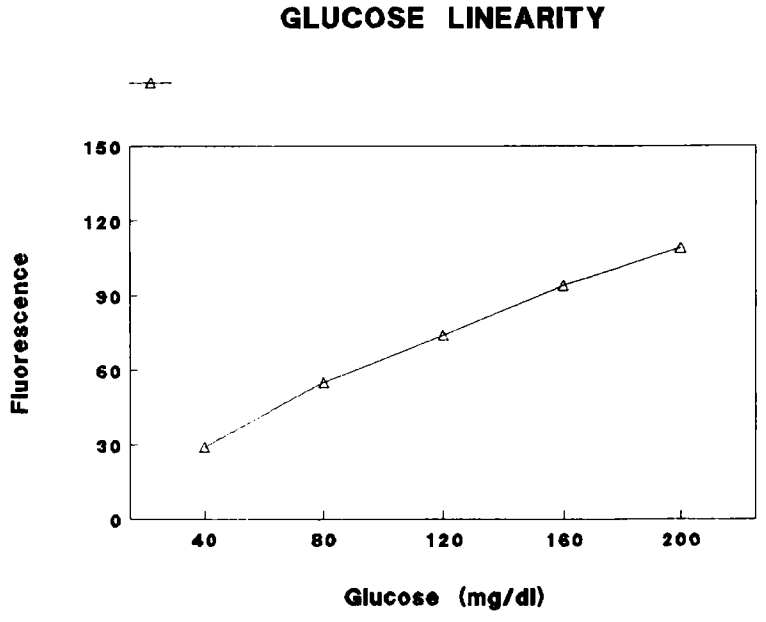

Figure 8. Fluorescence intensity against different glucose concentrations. Conditions as in Fig 7.

display fluorescence over a wide range of pH values and to exhibit stability without the need for further manipulation. More sensitive compounds probably can be synthesized based upon this information. HPLC helps to study the mechanism of peroxidase action on acetaminophen and might explain part of the toxicity of this drug on the hematologic system in cats (1), especially since hemoglobin possesses peroxidase activity (9). Futher studies are needed to isolate, characterize, investigate the mechanism of formation of these compounds and optimize methods for their utility in practical assays.

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