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High-performance liquid chromatography with postcolumn derivatization for simultaneous determination of organic peroxides and hydrogen peroxide

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Abstract

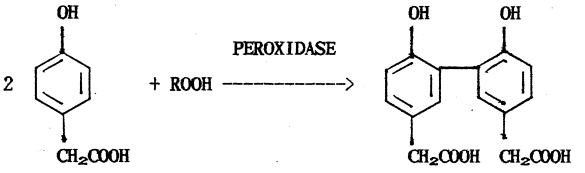
A reversed-phase HPLC procedure with enzyme mediated postcolumn derivatization and fluorescence detection has been developed for the simultaneous analysis of organic peroxides such as 1-((1-hydroperoxycyclohexyl)-dioxy)-cyclohexanol (HDC), *t*-butyl hydroperoxide (BHP) and cumene hydroperoxide (CHP), as well as hydrogen peroxide. The detection limits are 6.0 ng for H_2O_2, 0.15 μg for HDC, 3.7 μg for BHP and 7.0 μg for CHP respectively. Detection limits may be enhanced by preadsorption of the organic peroxides on a mixture of XAD-4 and XAD-8 resins with methanol elution. The method was used to detect a metastable peroxide intermediate from the ozonation of hexadeca-9-enoic acid.

Keywords: Derivatization, LC; Hydrogen peroxide; Peroxide; Hexadeca-9-enoic acid

1. Introduction

Organic peroxides have been identified as intermediates and by-products in the fields of atmospheric and stratospheric chemistry, polymerization, biochemical synthesis and metabolism [1]. The most common method for analysis of peroxides is based on oxidation of iodide [2,3]. Some other spectrophotometric methods such as complexation with titanium and so on are also available [2,4,5]. However, these methods are still limited for the quantitative analysis of organic peroxides.

A fluorescent method based on horseradish peroxidase-coupled oxidation has been successfully used for determining hydrogen peroxide in air and water [6–8]. In the presence of a hydrogen donor molecule such as 4-hydroxyphenylacetic acid (POHPA), horseradish peroxidase enzyme catalyzes the reduction of peroxides via the overall reaction:



(POHPA) (A)

The dimeric product (A) fluoresces with a peak excitation wavelength of 320 nm and peak emission

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wavelength of 410 nm, and the fluorescence intensity is proportional to the peroxide concentration. The method has been automated for the analysis of H_2O_2 with a detection limit of approximately 1×10^{-8} mol/l [6], but the method is not very sensitive to organic peroxides which react with the enzyme much slower than H_2O_2. Nevertheless, it will be used for organic peroxides in this paper.

In view of the fact that many peroxides are thermally unstable and many are relatively non-volatile, HPLC is the method of choice for their analysis, if a preseparation method is required. In the past, there were a number of publications which used HPLC as a tool for organic peroxides analysis. Most of these used reversed-phase HPLC with UV detection [9–15], with electrochemical detection [9,16–18], or with iodide derivatization [19] and alkylation derivatization [20]. Normal-phase HPLC has also been used [21–24].

In the course of studies on the by-products of the ozonation process in water, we have developed a TLC method for the analysis of some organic peroxides [25]. In this paper, we report a postcolumn derivatization method for the simultaneous determination of organic peroxides and hydrogen peroxide by HPLC with fluorescence detection, which has better selectivity than UV detection. Chemiluminence detection may be a potentially good method for peroxides, but there is still some difficulty in the analysis of aqueous organic peroxides.

2. Experimental

2.1. Solvents and reagents

Acetonitrile, water and methanol were HPLC grade. Chloroperoxidase (Sigma Chemical Co. #C0278, 1250 units per mg of protein) and horseradish peroxidase (Sigma Chemical Co. #P8375, 300 units per mg of protein) were used as received. The sources of the peroxides were as follows: H_2O_2 (Fisher Chemical, #H327); *t*-butyl hydroperoxide (BHP) (Aldrich Chemical, #21312-8); cumene hydroperoxide (CHP) (Aldrich, #24750-2); 1-((1-hydroperoxycyclohexyl)-dioxy)-cyclohexanol (HDC) (Aldrich, #28908-6). Hexadeca-9-enoic acid (Aldrich, #28692-3) and 4-hydroxyphenylacetic acid (POHPA) (ICN Biochemicals, Inc. #08583X) were used as received. Ozone was generated on-site with an OREC generator using oxygen gas feed at about 1 l/min. Ozone output was 15 mg/min. Stock solutions of the organic peroxides were made up in methanol by gravimetry, then dissolved into laboratory grade water to prepare calibration standards using volumetric techniques. The hydrogen peroxide was standardized by the AOAC iodine titration method before dilution.

2.2. Post-column HPLC derivatization

Fig. 1 is a schematic diagram of the postcolumn reaction detector system. The HPLC was a Perkin–Elmer 410 BIO LC pump with Rheodyne 7125 injector, Micro Pac MCH-10 10 μm C18 column (30 cm×4 mm), P-E LS-3B fluorescence detector, and

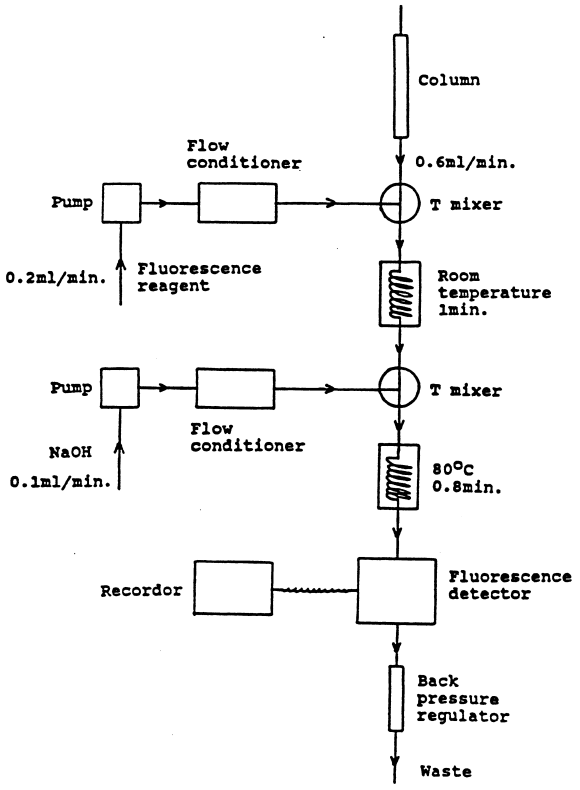

Fig. 1. Schematic diagram of the postcolumn reaction detector system.

P-E LCI-100 laboratory computing integrator. For post-column derivatization a system was assembled from components from Pickering Laboratories, Inc. (Mountain View, CA) consisting of a CRX 390 postcolumn reactor and FC 4870A flow conditioner. Two Beckman Model 110A reagent delivery pumps were used to pump the fluorescence reagent and sodium hydroxide solution into two mixing tees.

As shown in Fig. 1, the flow-rate of the mobile phase was nominally 0.6 ml/min, the flow-rate of fluorescence reagent (32 units of chloroperoxidase and 7.9×10^{-3} mol/l POHPA in 0.05 mol/l phosphate buffer) and 0.2 mol/l NaOH solution were 0.2 ml/min and 0.1 ml/min respectively. The eluate from the analytical column met the fluorescence reagent in the first tee mixer and then passed through a reactor coil made of 0.3 mm I.D. teflon tubing. After 1 min of retention time, the flow entered another T mixer and met sodium hydroxide, then passed through another reactor which was at an elevated temperature.

The retention time in the second reactor was approximately 0.8 min, after which the stream was passed through a 2 mm pathlength fused-silica cell. Fluorescence intensity was measured with excitation and emission wavelengths at 320 nm and 410 nm.

Injection volumes were in the range of 10–100 μl. Several gradient programs were tried and the best one was: 100% water for 10 min; linear gradient for 10 min to 50% acetonitrile; hold for 20 min.

2.3. Preconcentration by XAD resin

A glass column with teflon stopcock was plugged with glass wool and filled with 5 ml of equal amounts of XAD-4 and XAD-8 resins (Robin and Haas) which had thoroughly cleaned by Soxhlet extraction with methanol, ethyl ether, acetonitrile and methanol for 24 h each [26]. A sample of water was passed through the tube at a flow-rate of 4 ml/min. The resin bed was eluted with methanol at a flow-rate of 0.5 ml/min. The first 5 ml of water-rich eluate was discarded and the next 5 ml was collected for HPLC analysis.

2.4. Ozonation of hexadeca-9-enoic acid

The test compound was dissolved in 5 l of laboratory grade water at a concentration of 10 mg/l (0.04 mmol/l). Ozone was added through a bubbler at a dose rate of 10 mg/min. Four 1 l samples were taken at 0, 1.0, 2.0, and 3.0 min. Each sample was preconcentrated in a glass column packed with 5 ml of mixed XAD-4 and XAD-8 resins as described above and the second 5.0 ml of eluate collected for HPLC analysis.

3. Results and discussion

3.1. The consideration of the fluorescent reaction

The peroxidase mediated coupling of 4-hydroxyphenyl acetic acid (POHPA) induced by peroxidase is generally felt to involve the formation of an initial intermediate complex, Compound I, between the enzyme and the peroxide [27–29]. The possible structures of Compound I were discussed in the works of Hager et al. [30]. Compound I then reacts with ROOH again to produce another intermediate which oxidizes POHPA. The dimeric product (A) is ionized by addition of sodium hydroxide to a form with higher fluorescence quantum yield. Hydrogen peroxide and various organic peroxides due to their different sizes and structures react at different rates and therefore would have different sensitivities in the enzyme method.

3.2. Optimization of reaction temperature

We have found that heating the reaction mixture after sodium hydroxide addition increases the yield of the dimeric coupling product very substantially. Fig. 2 shows the effect of temperature on the fluorescence intensity of the three organic peroxides used in the study. The data suggests that a temperature of 60–80°C should yield a better increase in sensitivity of the method, although there is the potential hazard that labile peroxides may be lost. We have therefore adopted the procedure of increasing the temperature after addition of sodium hydroxide solution, presumably after the formation of Compound I.

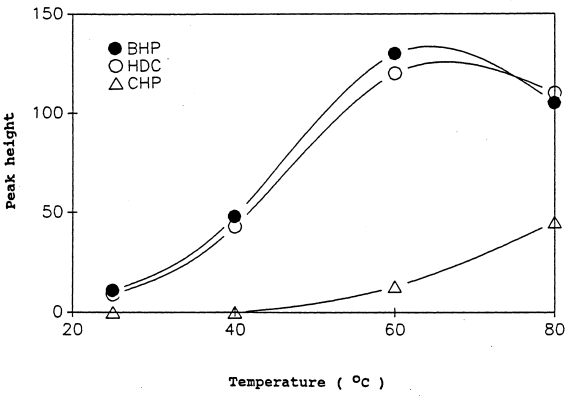

Fig. 2. Effect of increasing temperature of the HPLC eluate after addition of NaOH on the sensitivity HDC: 1.5×10^{-4}mol/l, BHP: 1.0×10^{-2}mol/l, CHP: 7.5×10^{-3}mol/l; injection volume: 30 μl.

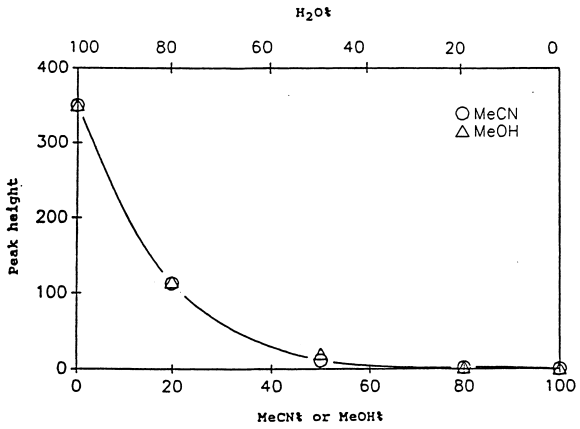

Fig. 3. Effect of mobile phase composition on the fluorescence intensity MeCN: acetonitrile; MeOH: methanol; HDC: 2×10^{-3}mol/l; injection volumn: 10 μl

3.3. Comparison of chloroperoxidase and horseradish peroxidase

This was done in order to take advantage of the fact that chloroperoxidase utilizes substituted peroxides such as peroxybenzoic acids and alkyl peroxides as substrates for oxygen evolution [30] and therefore, might be superior to horseradish peroxidase for the fluorescence coupling method for organic peroxides. The fluorescence intensities obtained with the horseradish peroxidase are somewhat higher than for the chloroperoxidase. Also it is noteworthy that horseradish peroxidase is less costly than chloroperoxidase by a factor of approximately 50%. On the other hand, the blank value for the chloroperoxidase system is about 1/6 of that of the horseradish peroxidase system.

3.4. Effect of HPLC mobile phase on fluorescence intensity

In order to separate organic peroxides using reversed-phase HPLC it is generally necessary to use an organic solvent as a part of the mobile phase; however, the quenching effect of the solvent on the fluorescence yield of the POHPA dimer may be severe. Fig. 3 shows the quenching effect of acetonitrile and methanol in different proportions with water using a standard solution of HDC. It is apparent that one should use a solvent elution program that minimizes organic solvent, and if possible avoids rapid changes of solvent composition near the analyte peak.

3.5. Reversed-phase separation of peroxides

Fig. 4 is a typical chromatogram showing the separation and detection of the three model organic peroxides and hydrogen peroxide using the post-column derivatization system. The method appears to offer a satisfactory and specific method for the separation and detection of organic peroxides in the presence of hydrogen peroxide. Since hydrogen peroxide is well separated from the organic peroxides, the method also allows the quantitative analysis of H_2O_2 in a complicated sample containing other peroxides.

Instrumental detection limits were determined by injecting 30 μl of a mixture of H_2O_2, BHP, HDC and CHP seven times. The standard deviation of the integrator response was used to calculate detection limits by the method of Glaser et al. [31]. The values are: 6.0 ng for H_2O_2, 0.15 μg for HDC, 3.7 μg for BHP, and 7.0 μg for CHP. It is not clear why HDC is more sensitively detected than the other two peroxides; perhaps its alpha-hydroxy peroxide structure is more reactive towards the enzyme.

It was noticed that the use of an immobilized enzyme reactor can reduce consumption of expensive chemicals and reduce the complexity of the post-column detection system [32]. However, our system

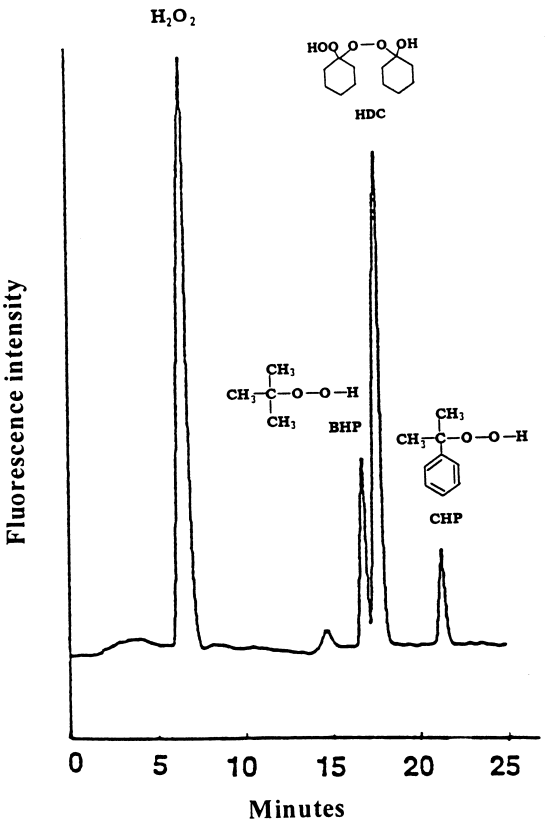

Fig. 4. Reversed-phase chromatogram of peroxides with post-column detection H_2O_2: 1.0×10^{-4}mol/l, HDC: 1.5×10^{-4}mol/l, BHP: 1.0×10^{-2}mol/l, CHP: 7.5×10^{-3}mol/l; injection volume: 30 μl.

is more flexible. It is easy to adjust reactor size, change temperature and reagent dose either for the first reactor or the second one. This is useful in getting the optimum conditions for the analysis of different interesting hydroperoxides. For instance, the *t*-butyl hydroperoxide can be easily measured in our system, but it gives no signal in the immobilized enzyme reactor system [32].

3.6. Preconcentration of aqueous samples with XAD resin

The limit of detection using the post-column method may be enhanced by preconcentration of the sample. We have used a method involving adsorption on a mixed bed XAD-4/8 resin followed by methanol elution. Recovery of HDC from two solutions at 1.0 and 0.05 μmol/l was 70 and 61% respectively. Recovery of hydrogen peroxide by this procedure is very low (less than one percent); therefore, one should analyze hydrogen peroxide by direct injection without preconcentration. Fortunately, the detection limit of H_2O_2 is two orders of magnitude lower than the typical organic peroxides in the direct method.

3.7. Ozonation of an unsaturated fatty acid in water

Previous studies [33] have shown that hexadec-9-enoic acid (HDA) is a constituent of water from the Los Angeles Aqueduct, the major source of water for the city of Los Angeles. At the municipal plant Aqueduct water is treated by a process which begins with ozonation at a dose of 1 mg/l. Under these conditions, HDA has been shown to be oxidized, both in field studies and in a laboratory ozonation system. The stable products are 1-heptanal, 1-heptanoic acid, 9-oxononanoic acid and nonanedioic acid [33], just as one would expect from the Criegee mechanism for cleavage of olefins by ozone [34]. However, the Criegee mechanism postulates an intermediate, 1-hydroxy-hydroperoxide, formed by addition of water to the carbonyl oxide intermediate. The postcolumn method for peroxides was used to search for this intermediate.

Fig. 5 shows the formation of a peroxide intermediate (the peak at 20 min) which grows as the ozonation process proceeds. Fig. 5 also shows that as the peak diminishes overnight, hydrogen peroxide (the peak at 6.7 min) is observed to form. This system is being investigated in more detail in order to confirm the identity of the (apparent) peroxide and to determine the effect of matrix constituents on its formation and stability.

4. Conclusion

A postcolumn derivatization method in HPLC directed on fluorescence detection for simultaneous determination of organic peroxides and hydrogen peroxide was proposed in this paper. In order to improve the efficiency of the enzymatic reaction for organic peroxides, the authors made several approaches: (1) to design a two reactor system and to

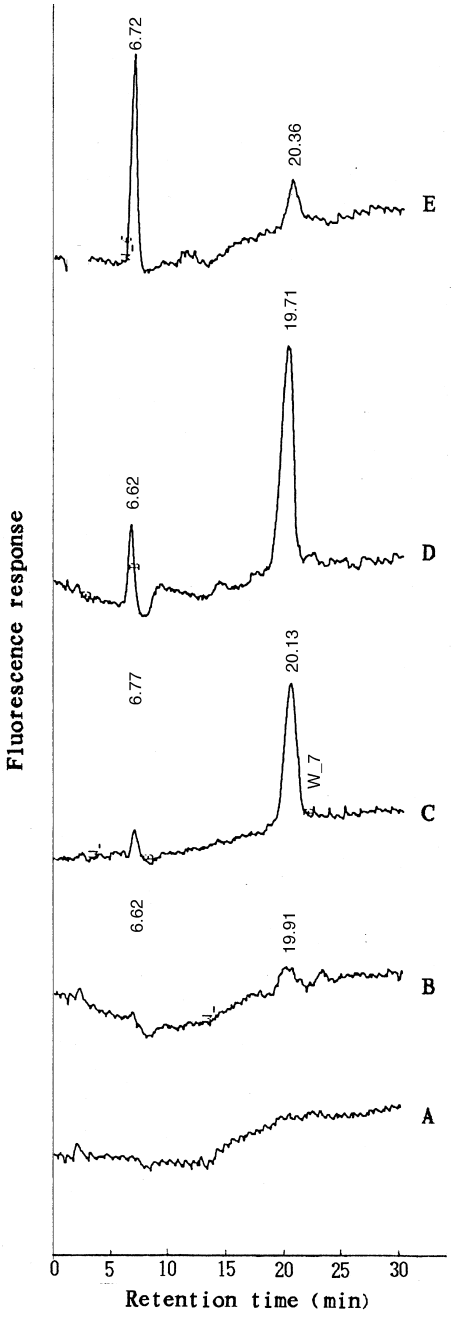

Fig. 5. Reversed-phase HPLC/postcolumn analysis of organic peroxide produced from aqeous ozonation of hexadec-9-enoic acid (HDA) A: 0 min; B: 1 min; C: 2 min; D: 3 min; E: sample D (ozonation for 3 min) measured after 24 h at room temperature.

raise the temperature in the second reactor, (2) to use chloroperoxidase instead of horseradish peroxidase and increase the enzyme concentration, (3) to optimize the gradient program. The developed system was applied into an ozonation by-product study in drinking water treatment and a peroxide intermediate was detected from ozonation of hexadec-9-enoic acid. Nevertheless, the detection limit of the method for some of the organic peroxides is to be further improved.

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