Technical Report

An Improved Method for Determination of Phosphatidylcholine Hydroperoxide

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Summary
An improved colorimetric method for the determination of phosphatidylcholine hydroperoxide is described. Phosphatidylcholine hydroperoxide was allowed to react with potassium iodide in the presence of acetylchloride at room temperature, and the liberated iodine was measured colorimetrically at 550 nm after the addition of starch in 10% aqueous acetic acid. A linear response was observed between 5 and 40 nmol.

Key words: colorimetric assay, phosphatidylcholine hydroperoxide, hydrochloric acid, acetyl chloride

Introduction

One cause of ischemic injury of organs during preservation or reperfusion is considered to be peroxidation of phospholipids present in the cell membranes1). To confirm this assumption, a simple and reliable method is necessary to determine phosphatidylcholine hydroperoxide.

Recently, Miyazawa et. al. developed a specific detection system applying chemiluminescence-high performance liquid chromatography (CL-HPLC) to determine phosphatidylcholine hydroperoxide (PC-OOH) in biological fluids2). Since standard PC-OOH is not commercially available, it is necessary to quantitate the hydroperoxide concentration.

The colorimetric methods for determination of lipid hydroperoxide are carried out by measuring the amount of liberated iodine3,4). However, there are some problems, especially concerning reproducibility and sensitivity because of the high reactivity of aluminum chloride.

Herein, we describe an improved assay of PC-OOH using acetylchloride instead of aluminum chloride.

Materials and Methods

Reagents
Phosphatidylcholine from porcine liver was purchased from Serdary Research Laboratories. Acetylchloride, acetic acid, potassium iodide and soluble starch were obtained from Wako Pure Chemical Company. Cumene hydroperoxide and trimethoxyborane were products of Aldrich Chemical Company.

Synthesis of photooxygenated PC-OOH
Photooxygenation was conducted by irradiating a sample solution in a Pyrex reactor, cooled in ice-water, with a 100 watt halogen lamp (USHIO, ICV 100-200GS). Oxygen gas was bubbled into the reaction mixture.

A solution of L-α-phosphatidylcholine (1 ml, 5 mg/ml) in dichloromethane and 0.1 M methylene blue (2.5 ml) in methanol was irradiated for 30 min. The suspension was chromatographed on silica gel (0.25 g, methanol) to give a fraction of the ox-
ygenation product. This fraction was concentrated, diluted with dichloromethane (5 ml) and then stocked at -20°C.

Procedure for colorimetric determination of standard cumene hydroperoxides
Cumene hydroperoxides (5, 10, 20, and 40 nmol) in dichloromethane were transferred to a test tube which was protected from light. The solvent was evaporated under nitrogen, and the hydroperoxides were dissolved in ethanol (50 µl). To this solution were added, potassium iodide (250 mg) in ethanol (50 µl) and acetylchloride (1 mmol) in ethanol (50 µl). The mixture was vortexed and maintained at room temperature. After 10 min, we added 10% aqueous acetic acid (1.5 ml) and the starch solution (50 µl) which had been prepared by dissolving soluble starch (1 g) and sodium chloride (20 g) in water (100 ml) heated at 60°C until it became clear, cooled and finally filtered through a 0.45-µm millipore filter. The mixture was vigorously shaken, and the absorbance was measured at 550 nm by a UVIDEC a-1 (JASCO). The standard curve was prepared with stock solutions of known concentrations of cumene hydroperoxide.

Effects of various acids instead of acetylchloride
Under the colorimetric assay condition, 2% (w/v) aluminum chloride in ethanol, 0.07% (w/v) of 37% aqueous hydrochloric acid in ethanol, and 2% (w/v) trimethoxyborane in ethanol were used instead of acetylchloride.
Cumene hydroperoxides (5, 10, 20, and 40 nmol) were dissolved in ethanol (50 µl). The potassium iodide solution (50 µl) and each acid in ethanol (50 µl) were added. The mixture was treated as described above.

Measurement of photooxygenated PC-OOH
The dichloromethane solution of the photooxygenated product of phosphatidylcholine was measured as described above. The time course of the absorbance development was plotted against the time from 0 to 30 min after the beginning.

Results and Discussion
Modification of reaction conditions
The reproducibility for the conventional colorimetric assay of lipid hydroperoxide using aluminum chloride as catalyst is inadequate. This is probably caused by the excessively high reactivity of the hydrochloric acid generated.
To inhibit the generation of excess hydrochloric acid, we used acetic acid as the solvent for the starch-iodide reaction instead of hydrochloric acid. The concentration of acetic acid in the aqueous solution was 10% (v/v) and the pH values (2.30) of this solution were equal to that of the aqueous solution of hydrochloric acid in the conventional methods.
The reaction time for this procedure performed at room temperature was 10 min. The absorbance of the starch-iodide reaction mixture with photooxygenated PC-OOH (420 nmol/ml) reached the maximum and constant values at or after 10 min (Fig.1).

Choice of acetylchloride as catalyst
The reactivity and reproducibility in the reaction with aluminum chloride, hydrochloric acid, trimethoxyborane, or acetylchloride as catalyst were examined by ap-

![Fig. 1 Time course of the absorbance development of phosphatidylcholine hydroperoxide](image)
Each value is the mean ± SD (n=5).

plying various concentrations of cumene hydroperoxide as described above (Fig. 2).

The starch-iodide reaction with 0.07% (w/v) of 37% aqueous hydrochloric acid in ethanol demonstrated high sensitivity but poor reproducibility. On the other hand, no changes in absorbance were observed in the reaction using trimethoxyborane, a stable Louis acid which does not produce hydrochloric acid in the reaction system. These findings suggested that the reaction of hydroperoxides with potassium iodide is catalyzed by hydrochloric acid.

The reaction with aluminum chloride at room temperature was reproducible. However, the reaction hardly occurred at a low concentration of cumene hydroperoxide, i.e., 5 and 10 nmol.

The reaction of potassium iodide with cumene hydroperoxide catalyzed by acetylchloride was reproducible and showed a linear calibration between 5 and 40 nmol (Fig. 3). The detection limit was 5 nmol.

Under optimal conditions, the concentration of phosphatidylcholine photooxygenated product as described in the experimental section was 420 nmol/ml (38% yield) and the relative standard deviation of the absorbance value was 11.0%. The lowest concentration of PC-OOH that could be determined was 5 nmol. The present study clearly showed that the concentration of PC-OOH can be calculated using the standard curve determined by the improved colorimetric method. This method is applicable to the measurement of other lipid derivatives of PC-OOH with a standard curve and to determinations of a small amount of phosphatidylcholines of microbiological materials or fluids by the CL-HPLC system. However, under this reactive condition, slightly dissolved lipids in the starch solution can not be measured correctly because of turbidity. Further improvement of this method is necessary to accommodate measurement of various other lipids.

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