Fluorometric Determination of Lipid Peroxides in Tissues with 1,3-Diphenyl-2-thiobarbituric Acid


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Lipid peroxides in tissues were fluorometrically determined using 1,3-diphenyl-2-thiobarbituric acid (DPTBA) as a fluorogenic reagent, and the results were compared with those obtained by the DPTBA-visible method, thiobarbituric acid (TBA)-fluorescence method, or TBA-visible method. The optimum conditions for determining lipid peroxides were analyzed by using brain homogenate. The optimum pH for the reaction of brain homogenate with DPTBA was 2.5. The amount of DPTBA suitable for the reaction with 100 µl of a 1% brain homogenate was 0.12 M. A linear relationship was observed between the fluorescence intensity and the amount of malondialdehyde (MDA) in the range of 1.25-12.5 nmol/ml. The detection limit of the DPTBA-fluorescence method was 0.006 nmol/g wet weight at a signal-to-noise ratio of 3. The sensitivity of this method was 5.5 times higher than that of the TBA-fluorescence method. Lipid peroxides in tissues could be easily determined without separation from glucose and bilirubin which interfere with TBA method. Lipid peroxides could be determined more accurately, precisely, and quickly using a smaller amount of liver, heart and brain homogenates by the DPTBA-fluorescence method than by other methods.

Keywords 1,3-Diphenyl-2-thiobarbituric acid, lipid peroxide, tissue, fluorometry, malondialdehyde

Several reports have been made on arteriosclerosis, cancer and many diseases caused by lipid peroxidation, which occurs under the aerobic conditions and impairs physiological function.1-4 Many methods have been reported for analysis of the lipid peroxides in serum, e.g. thiobarbituric acid (TBA)5, hemoglobin-Methylene Blue6 and cyclooxygenase methods.7 We previously reported a method for sensitive determination of lipid peroxides in serum using 1,3-diphenyl-2-thiobarbituric acid (DPTBA)6. Nakashima et al.9 developed a colorimetric method for the determination of lipid peroxides with DPTBA instead of TBA.10 Herein, we report a method for assaying lipid peroxides in the tissues using fluorescence detection instead of visible detection.

Experimental

Materials  
Six-week-old male mice of the ddY strain each weighing 20 – 30 g obtained from Tokyo Laboratory Animals Science Co., Ltd., Japan, were used. All chemicals were of analytical grade and used without further purification. Deionized water was further purified by the Milli-Q System for reagent grade water. DPTBA was prepared using thiocarbanilide, malonic acid and acetyl chloride.11

Equipment  
A Shimadzu spectrofluorophotometer model RF-540, a Kubota Medical Appliance Supply Corporation KR-20000T centrifuge, and a Shimadzu double-beam spectrophotometer UV-140–02 were used.

Procedure  
The recommended standard procedure was as follows. The mice were not fed for 24 h before the experiments. Brain, heart and liver were excised from the mice killed by decapitation, weighed and chilled in ice-cold 0.9% sodium chloride. After washing with 0.9% sodium chloride, tissue homogenates were prepared in a ratio of 1 g of wet tissue to 99 ml of 0.9% sodium chloride in a Teflon homogenizer. To a mixture of 1.5 ml of 0.4 M hydrochloride-sodium acetate buffer solution (pH 2.5) and 0.1 ml of a 0.12 M DPTBA-dimethyl sulfoxide (DMSO) solution in a 10 ml glass-stoppered test tube, 100 µl of 1% tissue homogenate chilled in ice was added rapidly, and the mixture was shaken vigorously for 30 s. The mixture was heated in a water bath at 95°C for 40 min using a
glass condenser. After cooling for 5 min in water, 5.0 ml of methyl isobutyl ketone (MIBK) was added and the mixture was shaken vigorously. After centrifugation at 3000 r.p.m. at 2°C for 15 min, the fluorescence intensity of the supernatant layer was measured at 556 nm with excitation at 520 nm.

The DPTBA-visible method was as follows. To a 2.5 ml of Walpole buffer solution (pH 2.0) and 0.1 ml of 0.12 M DPTBA in DMSO was added 100 µl of 10% tissue homogenate. The well-mixed solution was heated for 30 min in a water bath at 95°C, chilled for 5 min in water, and then shaken with 4.0 ml MIBK by a shaker. After centrifugation at 3000 r.p.m. for 15 min at 2°C, the absorbance of the organic layer was measured at 538 nm against MIBK.

The TBA-fluorescence and -visible methods were as follows. To a mixture of 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of a 0.8% aqueous solution of TBA was added 100 µl of 10% tissue homogenate. The mixture was adjusted to 4.0 ml with deionized water, and then heated in a water bath at 95°C for 60 min using a glass fall as a condenser. After cooling, 1.0 ml of deionized water and 5.0 ml of a mixture of 1-butanol and pyridine (15:1 v/v) were added and the mixture was shaken vigorously. After centrifugation at 3000 r.p.m. at 2°C for 15 min, the organic layer was taken and its fluorescence intensity was measured at 550 nm with an excitation at 514 nm. The TBA-visible method was carried out in the same way as the TBA-fluorescence method. The absorbance of the final organic layer was measured at 532 nm against 1-butanol.

Results and Discussion

Effect of interfering substances on the measuring of lipid peroxides and time course variation of lipid peroxides in the tissue homogenate

Substances in serum such as glucose, sialic acid and bilirubin have been reported to interfere with the measurement of lipid peroxides by TBA methods. However, analysis by the standard procedure using high performance liquid chromatography (HPLC) showed no products in the reaction of glucose or sialic acid with DPTBA. Bilirubin is absent in the tissues. Therefore, chromatographic separation of malondialdehyde (MDA) from the glucose, sialic acid, or bilirubin was not required for the measurement of lipid peroxides in the tissues.

Figure 1 shows the serial changes in the lipid peroxide level in the liver kept at -80, 7, or 23°C after homogenization. The lipid peroxide level in the liver homogenates kept at -80°C for 4 h increased slightly. At 7°C, the lipid peroxide level increased with time, and a marked increase was noted at 48 h. The level in the liver homogenates kept at 23°C for 4 h was 6 times higher than that determined immediately after homogenization. Consequently, the lipid peroxides in the tissues had to be determined immediately after preparation of tissue homogenates.

Optimization of analytical conditions

The brain homogenate was used to establish the optimum conditions for the determination of lipid peroxides. Figure 2 shows the relationship between the fluorescence intensity of the reaction products of brain homogenates with DPTBA and the pH in the range of 1.0 - 5.0, which was adjusted with a hydrochloride-sodium acetate buffer solution. The highest fluorescence intensity of brain was obtained at pH 2.5, Figure 3 shows the fluorescence intensity of the reaction products of MDA with DPTBA at pH 2.5. The fluorescence intensity increased with increasing volume up to 1.5 ml but was constant with a larger volume. The optimum volume of the buffer solution was therefore 1.5 ml.

Figure 4 shows the fluorescence intensity obtained by the reaction of 100 µl of a 1% brain homogenate with 0.1 ml of 0.006 - 0.24 M DPTBA. The fluorescence intensity increased with increasing DPTBA concentra-
DPTBA and 1% brain homogenate. The fluorescence intensity increased with increasing heating time up to 40 min, and then arrived at the plateau. Accordingly, the heating requirement for the reaction of the tissue homogenates with DPTBA was 95°C for 40 min.

Table 1 shows the extraction efficiency of the reaction products using MIBK, ethyl acetate, chloroform, 1-pentanol, 2-pentanol, 1-butanol-pyridine mixture solution (15:1), 2-butanol and 1-butanol. MIBK was the most efficient solvent for extraction and did not interfere with the measurement of lipid peroxides.

Figure 6 shows the excitation and emission spectra of the products formed by the reaction of the brain homogenate with DPTBA. The maximum wavelengths of the excitation and emission spectra were 543 and 556 nm, respectively. They are so close that the lipid peroxides cannot be measured accurately. Therefore, the excitation wavelength was set at 520 nm.

**Determination of lipid peroxides in tissues**

A linear relation passing through the origin was obtained between the fluorescence intensity and
amounts of MDA in the range of 1.25 - 12.5 nmol/ml ($y=18.51x$, $r=0.999$). The DPTBA-fluorescence method was compared with the DPTBA-visible method and TBA method as shown in Table 2. The sensitivity of the DPTBA-fluorescence method was 5.5 times higher than that of the TBA-fluorescence method, and 25 times higher than that of the TBA-visible method. A linear relation passing through the origin was obtained between the fluorescence intensity and wet weight of brain tissue in the range of 0.5 - 2.0 mg. In the recovery test, 100 µl of a 5 nmol/ml MDA standard solution was added to 50 µl of brain homogenate, and then the fluorescence intensity was measured by the standard procedure. The mean recovery rate was found to be 93.5% ($n=5$). The lipid peroxide level in the brain determined by the DPTBA-fluorescence method was 298.4 nmol/g wet weight. Lipid peroxide levels in the liver, heart and brain of mice measured by the DPTBA-fluorescence method were 239.5, 271.2 and 269.7 nmol/g wet weight, respectively. Lipid peroxides could be determined more accurately and precisely using a smaller amount of tissue homogenate by the DPTBA-fluorescence method than by other methods.

### Table 2 Comparison of DPTBA-fluorescence method with DPTBA-visible, TBA-fluorescence and TBA-visible methods

<table>
<thead>
<tr>
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<th>DPTBA Fluores.</th>
<th>TBA Fluores.</th>
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<tbody>
<tr>
<td>Publication</td>
<td>[Visible]</td>
<td>[Visible]</td>
</tr>
<tr>
<td>Detection limit (S/N=3) (nmol/g wet weight)</td>
<td>0.006 (0.310)</td>
<td>0.033 (0.148)</td>
</tr>
<tr>
<td>Sample size/mg</td>
<td>[5.0-20.0]</td>
<td>[5.0-25.0]</td>
</tr>
<tr>
<td>Analysis time/min</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>Precision (RSD, % ($n=5$))</td>
<td>5.59 [4.94]</td>
<td>5.58 [4.53]</td>
</tr>
<tr>
<td>Accuracy (Recovery, % ($n=5$))</td>
<td>93.5±5.4 [86.8±5.5]</td>
<td>95.5±5.8 [89.5±4.3]</td>
</tr>
<tr>
<td>Lipid peroxide level of brain (nmol/g wet weight)</td>
<td>298.4 [239.5]</td>
<td>271.2 [269.7]</td>
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References