Specific Determination of Malonaldehyde by *N*-Methyl-2-phenylindole or Thiobarbituric Acid

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ABSTRACT: Reactivity of a commercially available test kit (LPO-586), based on *N*-methyl-2-phenylindole, toward aldehydes was characterized and compared with that of thiobarbituric acid (TBA). In hydrochloric acid, LPO-586 produced a violet pigment with malonaldehyde (MA) but not with other tested aldehydes. In methane sulfonic acid, LPO-586 produced the violet pigment with MA and 4-hydroxynonenal (HNE), but not with other tested aldehydes. Pigment formation with MA was not inhibited by other aldehydes, but that with HNE was inhibited by alka-2,4-dienals. TBA produced a red pigment with MA but not with other tested aldehydes in hydrochloric acid or in acetate with ethylenediaminetetraacetic acid (EDTA). Both the LPO-586 test in hydrochloric acid and the TBA test in hydrochloric acid or in acetate with EDTA can be used for specific measurement of MA in oxidized lipid samples.
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KEY WORDS: Alka-2,4-dienal, 4-hydroxyalkenal, lipid oxidation, malonaldehyde, *N*-methyl-2-phenylindole, thiobarbituric acid.

Many aldehyde species are generated during lipid oxidation and are used as indices for evaluating stages of lipid oxidation (1). Since the thiobarbituric acid (TBA) test was first introduced by Kohn and Liversedge in 1944 (2), it has attained wide use in lipid oxidation studies (3–8). The test produces a red (532-nm absorbing) pigment by heating a mixture of TBA and sample in a strongly or mildly acidic medium. The concentration of red pigment produced from the same test sample varies depending on the test procedure because of different reactivities of TBA toward aldehydes (8). While the pigment can be formed from alk-2-enals and alka-2,4-dienals, depending on the test procedures, the pigment is quantitatively produced from malonaldehyde (MA) and this reaction depends little on test procedures (8). MA can be specifically determined by TBA reaction in acetate (pH 3.5) with ethylenediaminetetraacetic acid (EDTA) (9,10).

A reagent kit based on *N*-methyl-2-phenylindole (LPO-586) has been made available. The test instructions indicate that the contents of MA and 4-hydroxy-alkenals in samples are differentially determined by the reagent. The reagent produces a violet 586-nm absorbing pigment with a trimethine structure from MA in both hydrochloric acid and methane sulfonic acid, and from 4-hydroxyalkenals only in methane sulfonic acid. However, because of a lack of scientific information on the characteristics of this reagent, its use is limited.

In the present study, reactivity of *N*-methyl-2-phenylindole toward aldehyde species was characterized and compared with that of TBA. We found that both LPO-586 and TBA tests may be useful for specific determination of MA by selection of appropriate reaction solvents.

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EXPERIMENTAL PROCEDURES

Materials. The LPO-586 kit was obtained from Bioxytech S.A. (Bonneuil sur Marne, France), and contained 11.4 mM *N*-methyl-2-phenylindole in acetonitrile, 10.4 M methane sulfonic acid, 10 mM 4-hydroxynonenal (HNE) diethylacetal in acetonitrile, and 10 mM tetramethoxypropane (TMP) in 20 mM Tris-HCl buffer (pH 7.4). Thiobarbituric acid (TBA) was obtained from Merck (Darmstadt, Germany). *trans*-2-Hexenal (hexenal) and *trans*,*trans*-2,4-heptadienal (heptadienal) were obtained from Aldrich Chemical Company (Milwaukee, WI).

LPO-586 test. The LPO-586 test in hydrochloric acid or methane sulfonic acid was performed according to the reagent instructions (Bioxytech S.A.). Into a test tube with a screw cap, 200 μL of a solution of an aldehyde at the indicated final concentration of the reaction, 650 μL of 8.6 mM *N*-methyl-2-phenylindole in acetonitrile/methanol (3:1, vol/vol), and 150 μL of concentrated hydrochloric acid (final pH of the reaction mixture: –0.2) or 10.4 M methane sulfonic acid (final pH of the reaction mixture: 0.2) were placed in this order. The mixture in hydrochloric acid was heated at 45°C for 60 min, and the mixture in methane sulfonic acid at 45°C for 40 min. The absorption spectrum of each mixture was recorded.

TBA test. The TBA test in hydrochloric acid or acetate was carried out according to the modified procedure previously reported (9). Into a test tube with a screw cap, 200 μL of a solution of an aldehyde to give the indicated final concentration of the reaction, 650 μL of a mixture of 0.20 mL of 5.2% sodium dodecyl sulfate solution, 50 μL of 0.8% butylated hydroxytoluene solution in glacial acetic acid, 1.50 mL of 0.8% TBA solution in water, 1.70 mL of water with or without EDTA at the final concentration of 2 mM, and 150 μL of 1 M HCl (final pH of the reaction mixture: 0.8) or 20% acetate buffer (pH 3.5) were placed in this order. The mixture was kept at 5°C for 60 min and then heated at 100°C for 60 min.

The mixture was extracted with 1.0 mL of a mixture of 1-butanol/pyridine (15:1, vol/vol). The absorption spectrum of the extract was recorded.

RESULTS

The LPO-586 test of TMP (a malonaldehyde precursor), HNE diethylacetal (an HNE precursor), heptadienal or hexenal, at a final concentration of 10 μM, was performed in hydrochloric acid or in methane sulfonic acid. The absorption spectrum of the reaction mixture of TMP (Fig. 1A) showed that a single pigment with a maximum at 586 nm was produced in hydrochloric acid but two pigments with maxima at 500 and 586 nm were produced in methane sulfonic acid. The absorption spectrum of the reaction mixture of HNE diethylacetal (Fig. 1B) showed that a large amount of two pigments with maxima at 500 and 586 nm were produced in methane sulfonic acid, while only small amounts of pigments were produced in hydrochloric acid. The absorption spectrum of the reaction mixture of heptadienal (Fig. 1C) showed that a small amount of a pigment with a maximum at 500 nm was produced in methane sulfonic acid, while no pigments were produced in hydrochloric acid. The absorption spectrum of the reaction mixture of hexenal (Fig. 1D) showed that no pigments were produced in either medium. Hence, TMP produced a single peak (586 nm) in hydrochloric acid. However, both TMP and HNE diethylacetal produced the pigment in methane sulfonic acid. Absorbance at 586 nm increased linearly as the concentration of TMP was increased up to 20 μM in both hydrochloric acid [absorbance = 0.101 TMP concentration (μM), correlation coefficient $r = 0.999$] and methane sulfonic acid [absorbance = 0.101 TMP concentration (μM), $r = 0.999$]. Absorbance at 586 nm increased linearly as the concentration of HNE diethylacetal was increased up to 20 μM in both hydrochloric acid [absorbance = 0.0067 HNE diethylacetal concentration (μM), $r = 0.998$] and methane sulfonic acid [absorbance = 0.100 HEN diethylacetal concentration (μM), $r = 0.999$]. Reproducible relationships were obtained between absorbance and TMP or HNE diethylacetal concentration.

Formation of a 586-nm absorbing pigment from TMP in hydrochloric acid or methane sulfonic acid was not affected by the presence of HNE diethylacetal, hexenal, or heptadienal. Pigment formation from HNE diethylacetal in methane sulfonic acid was not affected by hexenal (Fig. 2A), but pigment formation was inhibited by heptadienal (Fig. 2B). Pigment formation from HNE diethylacetal was also inhibited by other alka-2,4-dienals. We found that the LPO-586 test in hydrochloric acid medium reflected MA but not other aldehydes.

TBA tests of TMP, HNE diethylacetal, heptadienal, and hexenal were performed in hydrochloric acid or in acetate with or without EDTA. TMP at a final concentration of 8 μM gave the same amount of red 532-nm absorbing pigment in both solvents (Fig. 3A). HNE diethylacetal and heptadienal at 40 μM produced a small amount of pigment in acetate without EDTA but produced no pigments in hydrochloric acid or in acetate with EDTA (Figs. 3B and C). Hexenal at 40 μM did not produce pigment under any conditions. Absorbance at 532 nm increased linearly as the concentration of TMP was increased up to 10 μM in hydrochloric acid [absorbance = 0.120 TMP concentration (μM), $r = 0.999$], in acetate with EDTA [absorbance = 0.120 TMP concentration (μM), $r =$

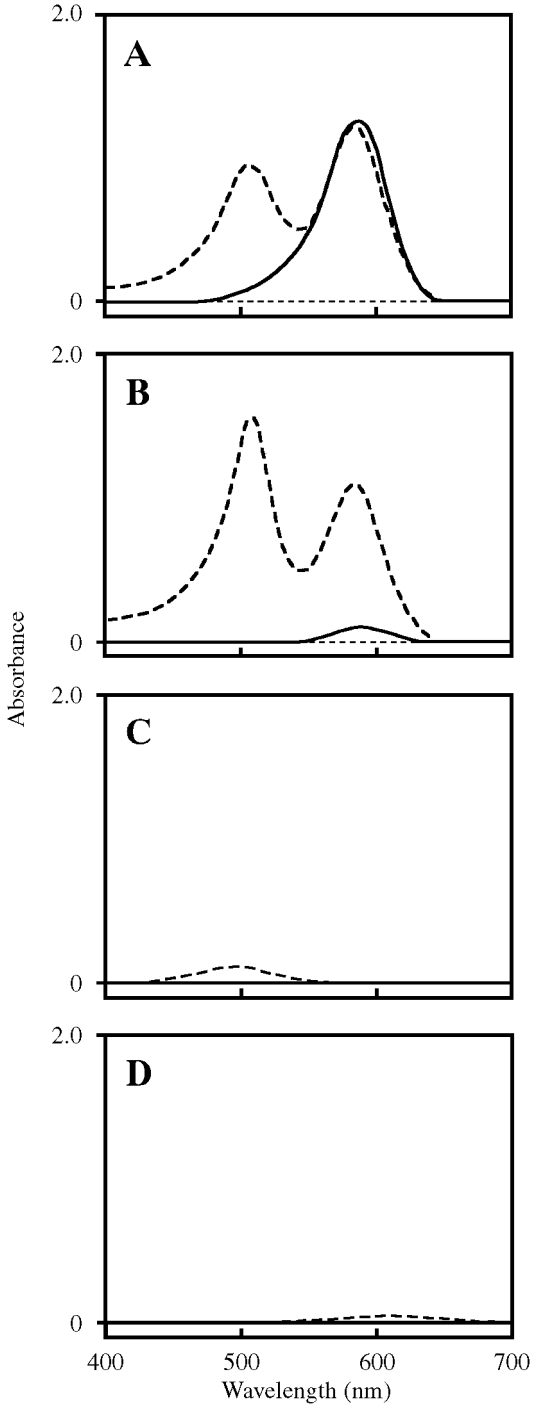

FIG. 1. Absorption spectra of the reaction mixtures of the LPO-586 test with 10 μM tetramethoxypropane (TMP) (A), hydroxynonenal (HNE) diethylacetal (B), heptadienal (C), and hexenal (D) in HCl (———) and in methane sulfonic acid (- - - - - -).

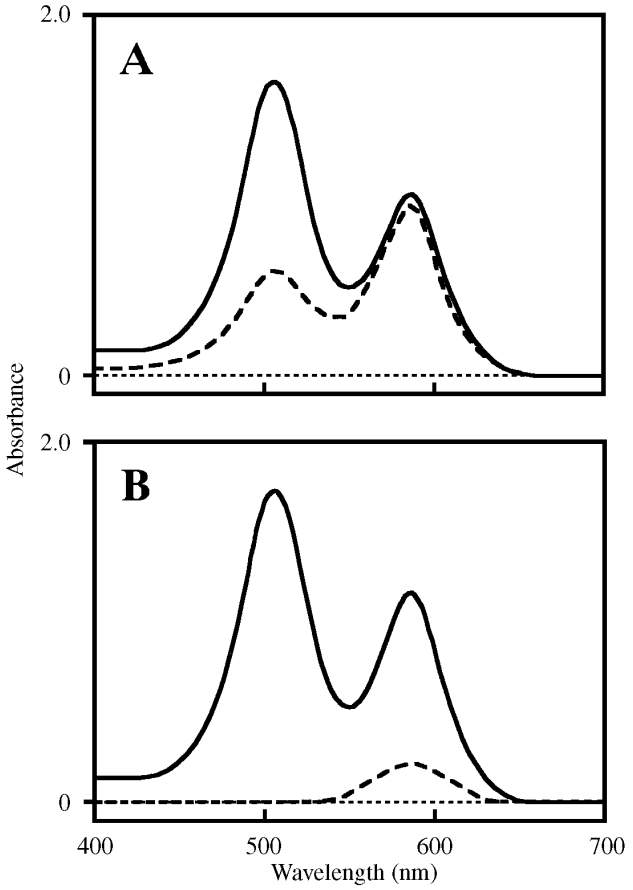

FIG. 2. Absorption spectra of the reaction mixtures of the LPO-586 test with 10 μM HNE diethyl acetal in the absence (———) and presence (- - - - - -) of 10 μM hexenal (A) and heptadienal (B) in methane sulfonic acid. See Figure 1 for abbreviation.

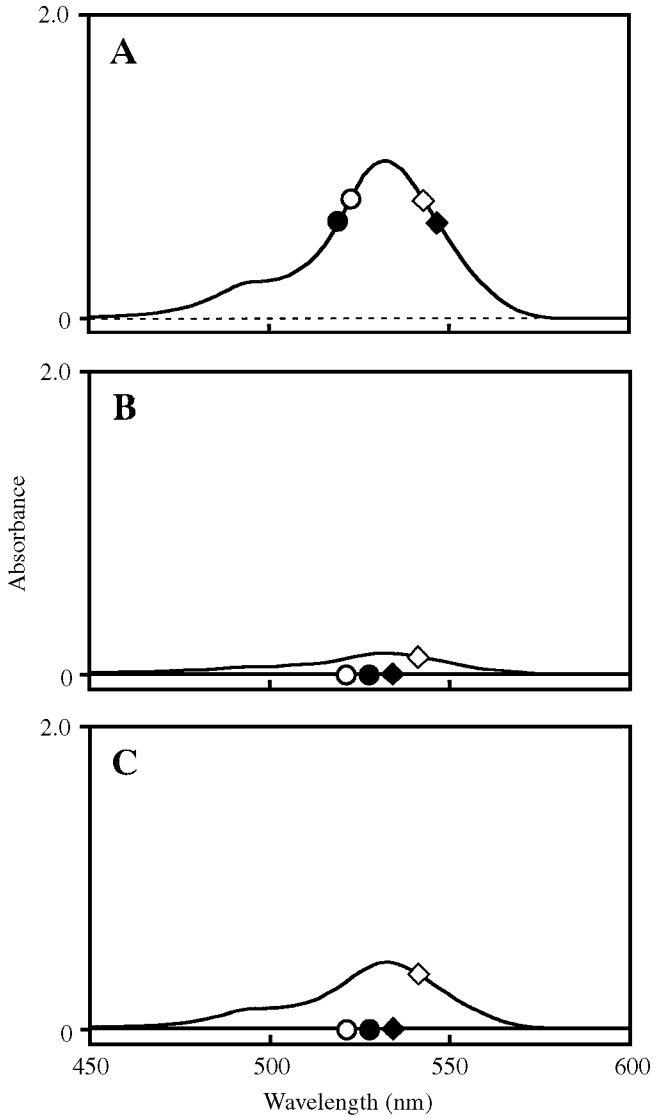

FIG. 3. Absorption spectra of the reaction mixtures of the thiobarbituric acid test with 8 μM TMP (A), 40 μM HNE diethylacetal (B), and 40 μM heptadienal (C) in HCl without (○) and with (●) ethylenediamine tetraacetic acid (EDTA), and in the acetate buffer (pH 3.5) without (◇) and with (◆) EDTA. See Figure 1 for other abbreviations.

0.999], and in acetate without EDTA [absorbance = 0.120 TMP concentration (μM), $r = 0.999$]. Absorbance at 532 nm increased as the concentration of HNE diethylacetal [absorbance = 0.005 HNE diethylacetal concentration (μM), $r = 0.991$] and heptadienal [absorbance = 0.012 heptadienal concentration (μM), $r = 0.995$] was increased to 40 μM in acetate without EDTA. Reproducible results were obtained. We found that the TBA test conducted in hydrochloric acid reflected MA but not other aldehydes.

DISCUSSION

When the LPO-586 test was conducted in hydrochloric acid, a single 586-nm absorbing violet pigment is produced only with TMP, and pigment formation was not disturbed by other aldehydes. When the LPO-586 test was conducted in methane sulfonic acid, equivalent amounts of violet pigment were produced from TMP and HNE diethylacetal. HNE diethylacetal gave two (500-nm and 586-nm absorbing) pigments in methane sulfonic acid, and pigment formation was suppressed by alka-2,4-dienals. In oxidized lipid samples, many kinds of aldehyde species, including alka-2,4-dienals, may be generated (1). Hence, the value obtained as the amount of 4-hydroxyalkenals from oxidized lipid samples by the test may be underestimated. Therefore, the LPO-586 test in hydrochloric acid may be useful for specific determination of MA in the test samples.

The present study supported the previous finding that MA can be specifically measured by TBA in acetate with EDTA (9,10). Furthermore, it clearly demonstrated that MA was specifically measured in hydrochloric acid. Hence, the TBA test in hydrochloric acid or in acetate with EDTA can be considered to be specific for MA.

In conclusion, the LPO-586 test in hydrochloric acid and the TBA test in hydrochloric acid or acetate with EDTA can

be similarly used for specific measurement of MA in oxidized lipid sample. The results of measuring lipid oxidation products in human red blood cells with the LPO-586 and TBA tests have been shown (11). Levels of MA determined by the LPO-586 test in hydrochloric acid are similar to those obtained by the TBA test in acetate with EDTA.

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