

An Improved Method for the Measurement of Malondialdehyde in Biological Samples

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An improved method was developed for measuring malondialdehyde (MDA) as its thiobarbituric acid (TBA) complex. Samples were initially incubated with 1% potassium iodide and 0.1% butylated hydroxytoluene at 50°C for 20 min, and then with 0.4% TBA at 60°C for 60 min. The MDA-TBA complex formed was extracted with isobutyl alcohol and measured by high-performance liquid chromatography with fluorescence detection. The improved method allows for a more specific determination of MDA present in biological samples.

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Due to its relative simplicity and high sensitivity, measurement of malondialdehyde (MDA) as thiobarbituric acid (TBA) adduct has been extensively used to assess the extent of lipid peroxidation in a variety of pathological conditions (1). In recent years, many improved procedures for measuring the MDA-TBA chromophore have been reported (1-3). Factors, such as reaction time, temperature and pH, as well as the presence of transition metals, antioxidants and reducing agents can markedly affect the formation of both the TBA-MDA chromophore and of artifacts during sample processing. We previously reported a selective and sensitive method for measuring MDA in biological samples using high-performance liquid chromatography (HPLC) with fluorescence detection (4). In the procedure described here, the possibility of artifact formation was further decreased by reacting the sample with KI prior to the formation of the MDA-TBA adduct. Thus, a more specific quantitation of MDA present in the assay system can be achieved. In addition, the quantitative relationship between conjugated dienes, hydroperoxides and MDA was examined in peroxidized fatty acids.

MATERIALS AND METHODS

Chemicals and reagents. Butylated hydroxytoluene (BHT), TBA, linoleic acid, linolenic acid, 1,1,3,3-tetramethoxypropane (TMP), KI, hydrogen peroxide (30%) and soybean lipoxygenase (type I) were purchased from Sigma Chemical Company (St. Louis, MO). Boric acid and isobutyl alcohol were from Mallinckrodt (St. Louis, MO); methanol (HPLC grade), glacial acetic acid and hydrochloric acid were from Fisher Scientific (Fair Lawn, NJ); tetrabutylammonium dihydrogen phosphate was from Aldrich Chemical Company (Milwaukee, WI); and ethanol (95%) was from Midwest Grain Products of Illinois (Pekin, IL).

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Abbreviations: BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography; LOH, hydroxy fatty acid; LOOH, fatty acid hydroperoxide; MDA, malondialdehyde; TBA, thiobarbituric acid; TMP, 1,1,3,3-tetramethoxypropane.

Preparation of standards. The stock solution of MDA was prepared by dissolving 0.1 M TMP in 0.02 N HCl. Hydrogen peroxide, 0.1 μ M, was prepared in distilled water. Working standards for MDA and hydrogen peroxide, ranging from 0.1-10.0 μ M, were prepared fresh before use.

Enzymatic oxidation of fatty acids. Stock solutions of linoleic acid and linolenic acid (0.05 M) were prepared with 20% ethanol in 0.1 M potassium borate buffer, pH 8.0. The stock solution (0.5 μ L) was mixed with 2.5 mL of 0.1 M borate buffer. Samples were oxidized by adding 3,000 units of lipoxygenase (5) at room temperature. The contents were then aliquoted before and 10 min after the addition of lipoxygenase for measuring the levels of lipid peroxidation products, MDA, lipid hydroperoxides and conjugated dienes.

Measurement of lipid peroxidation products. For MDA measurements, aliquots of the enzymatically peroxidized fatty acids (50-200 μ L) and MDA standards (0.1-1.0 nmol) were added to test tubes that contained 0.1 mL of 0.1% BHT in 95% ethanol and 0.5 mL of 1% KI in methanol/acetic acid (2:1, vol/vol). The test tubes were then incubated at 50°C for 20 min. After adding 1 mL 0.4% TBA in 0.02N HCl, the contents were incubated at 60°C for 60 min, cooled to room temperature and extracted with isobutyl alcohol. The concentration of MDA was measured by HPLC with fluorescence detection using a C-18 reverse-phase column as described previously (4). Tetrabutylammonium dihydrogen phosphate, 0.5%, was added to the mobile phase (methanol/water, 1:1, vol/vol) as ion-pairing reagent.

The levels of diene conjugation in fatty acids were measured at 233 nm (6). Lipid hydroperoxides were quantified by the triiodide procedure described by Gebicki and Guille (7) using hydrogen peroxide as a standard.

Measurement of MDA in biological samples. To evaluate the applicability of the procedure to biological samples, freshly obtained human plasma was assayed for MDA levels. One-tenth to one-half mL of plasma was pipetted into a test tube that contained 0.5 mL of 1% KI in methanol/acetic acid (2:1, vol/vol) and 0.1 mL of 0.1% BHT in 95% ethanol. After incubation at 50°C for 20 min, the contents were mixed with 0.5 mL 0.4% TBA in 0.02N HCl and incubated at 60°C for 60 min. The samples were then extracted with 2 mL of isobutyl alcohol and measured for relative fluorescence by HPLC as described above. The differences between the sample means were analyzed using analysis of variance followed by Honest's multiple comparison test (Turkey Honest Significant Difference Test). A 95% ($P < 0.05$) of confidence level was used to determine statistical significance.

RESULTS

KI and BHT, individually or combined, had no effect on the formation of MDA-TBA chromophore over the

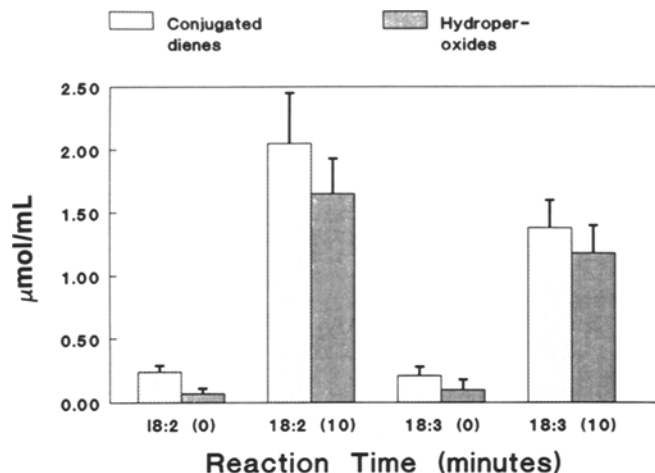


FIG. 1. Effect of KI and butylated hydroxytoluene on the formation of conjugated dienes and hydroperoxides. Linoleic acid (18:2) and linolenic acid (18:3) were incubated with lipoxygenase for 0 or 10 min. The reaction mixture consisted of 25 μmol fatty acid and 3,000 units of lipoxygenase in 3.0 mL of 0.1 M borate buffer (8.33 μmol fatty acid/mL). Results are expressed as μmol of conjugated dienes or hydroperoxides/mL of reaction mixture. The values represent means ± standard deviation of five experiments.

concentration range of 0.10–10 μM. The same was observed when the concentrations of KI and BHT were doubled or halved (results not shown).

The formation of conjugated dienes and hydroperoxides from linoleic acid and linolenic acid following reaction with lipoxygenase is shown in Figure 1. At 0 time, an average of 0.24 and 0.21 μmol/mL of conjugated dienes and 0.07 and 0.10 μmol/mL of hydroperoxides were detected for linoleic acid and linolenic acid, respectively. Ten minutes after the addition of lipoxygenase, an average of 2.05 and 1.38 μmol/mL of conjugated diene and 1.65 and 1.18 μmol/mL of hydroperoxides were found for linoleic acid and linolenic acid, respectively. This compares to less than 0.004 μmol/mL of MDA-TBA detected in the peroxidized

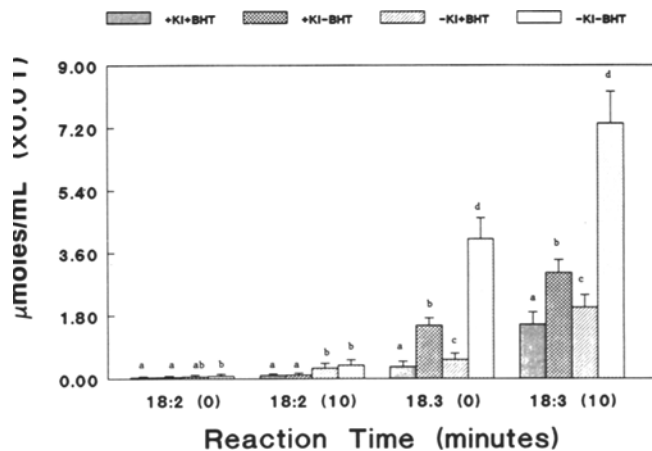


FIG. 2. Effect of KI and butylated hydroxytoluene (BHT) on the formation of malondialdehyde-thiobarbituric acid chromophore. Linoleic acid (18:2) and linolenic acid (18:3) were incubated with lipoxygenase for 0 or 10 min. The values represent the mean ± standard deviation of five experiments. See Figure 1 legends for additional experimental details. The means that have different lower case letters are significantly different ($P < 0.05$).

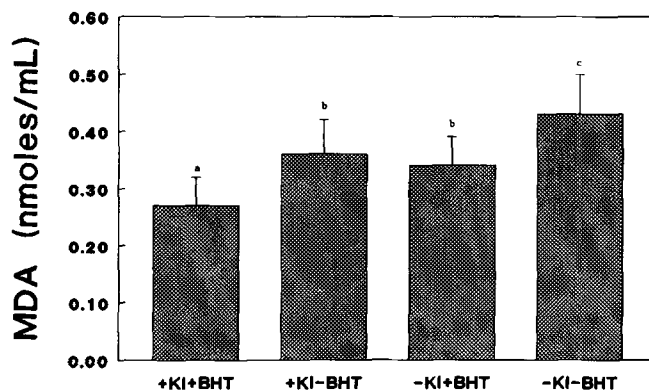


FIG. 3. Effect of KI and BHT on the measurement of MDA in human plasma. The values represent the mean ± standard deviation of fourteen samples. The means that have different lower case letters are significantly different ($P < 0.05$). See text for experimental details. Abbreviations: See Figure 2, and MDA, malondialdehyde.

linoleic acid and less than 0.08 μmol/mL in the peroxidized linolenic acid (Fig. 2) whether KI and/or BHT were included in the reaction mixture or not. As is also shown in Figure 2, the amounts of MDA-TBA found were highly variable depending upon the experimental conditions. The levels of MDA-TBA were decreased by the presence of BHT or KI in the reaction mixture; the addition of both KI and BHT had the greatest effect.

The applicability of the method to measuring MDA in biological samples was tested on human plasma samples. As is shown in Figure 3, the MDA values were significantly lower for plasma samples prepared with KI and/or BHT than for those without. The averaged values of 14 plasma samples were 0.27, 0.36, 0.34 and 0.43 nmol/mL for samples treated with +KI + BHT, +KI - BHT, -KI + BHT and -KI - BHT, respectively.

DISCUSSION

Due to its sensitivity and simplicity, the measurement of the MDA-TBA chromophore has been widely employed to assess the extent of lipid peroxidation. However, autoxidation of unoxidized lipids and generation of MDA-like compounds during sample processing are among the most critical and confounding factors limiting the accurate measurement of MDA (1–4). In biological samples, the assay is further complicated by the nature of the peroxidized intermediates generated, their reactivity with other components, and by the presence of MDA-like compounds and their precursors. In the present study, an improved method for assaying MDA was developed based on procedures previously reported by us (4) and others (8–10). The key feature of the assay is the prevention or reduction of artifact formation by both KI and BHT during sample processing. A classic procedure for measuring lipid hydroperoxides in fats and oils is based on the ability of KI to reduce lipid hydroperoxides (LOOH), except endoperoxides, to their corresponding alcohols (LOH) (Reaction I) (8,9). Iodometric procedures have been proposed for measuring lipid hydroperoxides in biological samples (7,9).



METHOD

The ability of KI to reduce lipid hydroperoxides also reduces free radical generation *via* homolytic cleavage. In addition, KI and/or released I₂ may saturate double bonds of unsaturated fatty acids (10) and thus further decrease the autoxidation potential of the fatty acids present. KI seems to complement the antioxidant property of BHT. In the presence of both KI and BHT, the generation of MDA or MDA-like substances from unoxidized fatty acids, hydroperoxides or other biomolecules can thus be prevented or minimized. However, it remains possible that not all types of artifacts formation are prevented under these experimental conditions.

As is shown in Figures 1 and 2, only small and highly variable amounts of lipid hydroperoxides were converted to MDA. Therefore, estimation of MDA *per se* is not a reliable measure of lipid hydroperoxides. Similarly, as is also shown in Figure 1, the levels of conjugated dienes vary widely depending on the state of oxidation; thus, estimation of conjugated diene is not a suitable measure of lipid hydroperoxides, either.

It has long been recognized that MDA and other aldehydes can be metabolized *in vivo* and *in vitro* by aldehyde dehydrogenases and related enzymes (6,11-13). In addition, MDA may react with various cellular components and thereby decrease cellular levels. Thus, the MDA levels present in fresh biological samples can be expected to be very low. The MDA levels measured as TBA values and reported for normal human plasma varied widely and ranged from 0.32 to 47.2 nmol/mL (3). Whether the average MDA levels in human plasma measured in the present study, 0.27 nmol/mL, represents the steady-state concentration remains to be seen. Nevertheless, by further decreasing the possibility of artifact formation during

sample processing, the present method is an improvement over the quite selective and sensitive method we reported previously (4), and affords a more specific measurement of MDA present in biological samples.

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