Lipoperoxides in Plasma as Measured by Liquid-Chromatographic Separation of Malondialdehyde—Thiobarbituric Acid Adduct

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This assay of plasma lipoperoxides involves hydrolysis in dilute H$_3$PO$_4$ at 100 ºC; complexation of malondialdehyde (MDA), a hydrolysis product, with thiobarbituric acid (TBA); methanol precipitation of plasma proteins; fractionation of the protein-free extract on a C$_18$ column; and spectrophotometric quantification of the MDA–TBA adduct at 532 nm. The detection limit was 0.15 µmol of MDA per liter of plasma. Run-to-run precision (CV) averaged 8 to 13%. Analytical recovery of MDA after addition of tetrathoxypropane standards to 21 specimens of human or rat plasma averaged 98% (SD 7%). Lipoperoxide concentrations (as MDA) averaged 0.60 (SD 0.13) µmol/L in plasma specimens from 41 healthy persons and 1.4 (SD 0.3) µmol/L in plasma specimens from 12 control rats. Mean lipoperoxide concentrations were 1.5 to 2.3 times as great in plasma sampled from rats one to three days after subcutaneous administration of N$l$C$_2$ at dosages (250 to 750 µmol per kilogram body wt) previously shown to induce lipid peroxidation in lung, liver, and kidney.

Additional Keyphrases: toxicology · nickel · rats · oxygen free-radicals · reference interval

Lipid peroxidation, a general mechanism whereby oxygen free-radicals induce tissue damage (1–3), has been implicated in diverse pathological conditions, including atherosclerosis, aging, rheumatic diseases, cardiac and cerebral ischemia, respiratory distress syndrome, various liver disorders, irradiation, thermal injury, and toxicity induced by certain metals, solvents, pesticides, and drugs (4–12). Clinical-chemical tests to assess lipid peroxidation include (a) fluorometry of lipofuscin-like substances in serum (13); (b) spectrophotometry of conjugated dienes in lipid extracts of plasma (14, 15); (c) gas chromatography of ethane, pentane, and other alkanes in exhaled breath (16, 17); and (d) hydrolysis of plasma lipoperoxides to form malondialdehyde (MDA; CHO$\cdot$CHO), which reacts with thiobarbituric acid (TBA) to yield a red MDA–TBA adduct, which is measured by spectrophotometry or fluorometry, usually after extraction into butanol (7, 18–22).

Bird et al. (23) and Yu et al. (24) described “high-performance” liquid chromatographic (HPLC) techniques for determining MDA in which the MDA–TBA adduct is separated from interfering substances by chromatography on a column of octadecyl silica gel. Here we have adapted these HPLC techniques to measurements of plasma lipoperoxides, after acid hydrolysis of lipoperoxides and formation of the MDA–TBA adduct under the reaction conditions used by Mihi et al. (25) and Sunderman et al. (26) for analysis of MDA precursors in tissue homogenates. The new HPLC procedure is more specific, sensitive, and reproducible than earlier techniques. It provides a practical method for assessing lipid peroxidation in both human subjects and experimental animals.

Principle

Plasma lipoperoxides are hydrolyzed by boiling in dilute phosphoric acid. MDA, one of the hydrolysis products, is reacted with TBA to form MDA(TBA)$_2$ adduct (24, 27). Plasma proteins are precipitated with methanol and removed from the reaction mixture by centrifugation. The protein-free extract is fractionated by HPLC on a column of octadecyl silica gel, to separate the MDA–TBA adduct from interfering chromogens. The MDA–TBA adduct is eluted from the column with methanol/phosphate buffer and quantified spectrophotometrically at 532 nm. Plasma lipoperoxide concentrations are computed by reference to a calibration curve prepared by assays of tetrathoxypropane (TEP), which undergoes hydrolysis to liberate stoichiometric amounts of MDA (28).

Materials and Methods

Apparatus

Polypropylene test tubes, 13-mL capacity, with screw caps (no. 60-541-685; Walter Sarstedt, Inc., Princeton, NJ).

Polypropylene microtubes, 2-mL capacity, with screw caps that contain O-ring seals (no. 72-693; Walter Sarstedt, Inc.).

High-speed microcentrifuge, to accommodate 2-mL microtubes.

HPLC apparatus. Solvent-delivery module and injection system (Model 112, Beckman Instruments, Inc., Fullerton, CA) with 50-µL sample loop. A guard column, 3.9 mm (i.d.) × 2.3 cm, packed with Bondapak Corasil C$18$ (37- to 50-µm particle diameter; Waters–Millipore Corp., Milford, MA). A 3.9 mm (i.d.) × 30 cm chromatographic column packed with µBondapak C$18$ (10-µm particle diameter; Waters–Millipore). The flow rate of the mobile phase is 2 mL/min.

Spectrophotometric monitor, variable wavelength (Model 3000; LDC/Milton Roy Co., Riviera Beach, FL), with 14-µL flow-cell volume, 1-cm optical pathlength, set at 532 nm and a sensitivity of 0.02 absorbance unit (A). Although the voltage output is nominally 10 mV, we connect the spectrophotometric monitor to a strip-chart recorder with 1 mV full-scale range (Model 9176: Varian Corp., Palo Alto, CA), so that the actual recorder sensitivity is 0.002 A full-scale. Recorder chart-speed is 1 cm/min.

Reagents

Phosphoric acid solution, 0.44 mol/L. Dilute 10 mL of “ultra-pure” H$_2$PO$_4$ reagent (relative density 1.69, 44.0 mol/L, 850 g/L; J. T. Baker Co., Phillipsburg, NJ) to 1 L with
water (distilled water is used throughout).

**TBA solution, 42 mmol/L.** Dissolve 0.8 g of 4,6-dihydroxy-2-thiopyrimidine (no. T-5500, molecular mass 144.2 Da; Sigma Chemical Co., St. Louis, MO) in approximately 50 mL of water, with stirring on a hot-plate (50–55 °C). Cool the solution to 25 °C and dilute to 100 mL with water. Stored at room temperature, this reagent is stable for two weeks.

Ethanol, 400 mL/L solution. Dilute 420 mL of 95% ethanol to 1 L with water.

**TEP standards solutions.** In a 25-mL volumetric flask, dilute 50 μL of 1,3,3,3-tetraethoxypropane reagent (anhydrous, relative density 0.91, molecular mass 220.3 Da, purity 98%+, no. T-9889; Sigma Chemical Co.) to the mark with 400 mL/L ethanol solution. Prepare freshly each month and store at 4 °C. For an intermediate standard, pipet 0.5 mL of this TEP stock standard solution into a 100-mL volumetric flask and dilute to the mark with 400 mL/L ethanol solution. Prepare freshly each fortnight and store at 4 °C. To prepare TEP working standard solutions (0.61, 1.22, 2.43, and 4.86 μmol/L), pipet into four 25-mL volumetric flasks 0.375, 0.75, 1.5, and 3.0 mL, respectively, of TEP intermediate standard solution and dilute the contents to the mark with water. Prepare these weekly and store at 4 °C.

**Methanol–NaOH solution.** Pipet 4.5 mL of 1 mol/L NaOH solution into a 50-mL volumetric flask and dilute to the mark with methanol ("HPLC" grade; J. T. Baker Co.). Stored in a polyethylene bottle at 4 °C, this is stable indefinitely.

**Potassium phosphate buffer, 50 mmol/L, pH 6.8.** Dissolve 13.6 g of anhydrous KH₂PO₄ in approximately 1.6 L of distilled water and titrate to pH 6.8 with 1 mol/L KOH solution, monitoring constantly with a pH meter. Dilute to 2 L with water and filter through a polycarbonate filter (0.45-μm pore diameter; Waters–Millipore).

**Mobile phase.** Prepare the HPLC mobile phase daily, just before use. Mix 400 mL of HPLC-grade methanol and 600 mL of potassium phosphate buffer solution in a side-arm suction flask; then de-gas by reducing the pressure for 20 min, with vigorous magnetic mixing.

**Methanol–water mixtures.** Into two side-arm suction flasks, transfer 400 and 800 mL of HPLC-grade methanol and 600 and 200 mL, respectively, of distilled water that has been filtered through a polycarbonate filter. Before use for HPLC, de-gas these solutions under reduced pressure for 20 min, with vigorous magnetic stirring.

**Additional materials.** We also used nickel chloride (NiCl₂·3H₂O; Alfa Inorganics Division, Ventron Corp., Beverly, MA), quality-control sera of human origin ("Decision Level One Control Serum" from Beckman Instruments, Inc., Brea, CA; "RIA Reference Serum" from Wien Laboratories, Inc., Succasunna, Nj), six standard reference materials (glucose, cholesterol, bilirubin, urea, uric acid, and creatinine, all from the U.S. National Bureau of Standards, Gaithersburg, MD), and 17 samples of common drugs, obtained from the respective pharmaceutical manufacturers. Erythrocyte hemolysate was prepared for interference tests by mixing 3.5 mL of heparinized whole blood from a healthy adult with 3 mL of a leukocyte separation medium ("Monol-Poly Resolving Medium," cat. no. 16-980-49; Flow Laboratories, Inc., McLean, VA), which contains a mixture of polysaccharide (Ficol-400) and sodium metrizoate (Hypaque-86) having a density of 1.114 kg/L. After centrifuging these samples at 300 × g for 30 min in a swinging-bucket rotor, we removed the supernatant layers of plasma, leukocytes, and platelets. The sedimented erythrocytes were washed with isotonic NaCl solution, hemolyzed by addition of five volumes of distilled water, and centrifuged at 2000 × g for 20 min to sediment erythrocyte envelopes. The concentration of hemoglobin in the hemolysate was measured by spectrophotometry (29).

**Procedures**

**Blood collection from humans.** Collect the blood samples by venipuncture into 7-mL evacuated tubes containing EDTA solution as anticoagulant (10.5 mg of K₂EDTA in 70 μL of water). Centrifuge the blood (900 × g, 20 min) and remove the supernatant plasma, being careful to avoid contamination with platelets. Store the plasma at 4 °C for no longer than 24 h before analysis.

**Blood collection from rats.** Induce vasodilation of the tail by placing the rat in a warmed cage (approximately 35 °C) for 10 min. After transferring the rat to a plastic restraining cone warmed with a heating pad, excise 5 mm from the tip of the tail with a scalpel; discard the first drops of blood. When the blood flows freely, collect 0.5 mL in a 0.5-mL microtube containing 0.75 mg of dry K₂EDTA and two plastic beads (2-mm diameter) to facilitate mixing. Centrifuge the microtube as described for human blood, and pipet duplicate 50-μL aliquots of plasma directly into the 13-mL polypropylene test tubes for lipoperoxide assay. Discard any samples with visible hemolysis.

**Lipoperoxide hydrolysis and TBA reaction.** In each analytical run, assay—in duplicate—reagent blanks, TEP working standard solutions, plasma specimens, and a quality-control specimen. Pipet 0.75 mL of the phosphoric acid solution into each 13-mL polypropylene test tube. Using a piston-displacement pipettor, pipet 50 μL of TEP standards, plasma specimens, and quality-control specimen into the respective tubes and vortex-mix. Add 0.25 mL of TBA solution to each tube. Add distilled water (0.50 mL for reagent blanks, 0.45 mL for TEP standards, plasma, samples, and quality-control samples) to adjust the final volume to 1.5 mL. Cap the tubes tightly and place them in a boiling water bath (100 °C) for 60 min, then in an ice-water bath (0 °C) until the HPLC analyses are performed.

The MDA—TBA adduct is stable at acid pH but dissociates slowly at neutral or alkaline pH; thus the boiled samples must be neutralized individually within 10 min before injection onto the HPLC column, as follows. Pipet 0.5 mL of each boiled sample into a polypropylene microtube that contains 0.5 mL of methanol–NaOH solution. Cap the tube and vortex-mix, then centrifuge the plasma and quality-control samples (9500 × g, 5 min) to sediment the precipitated plasma proteins.

**HPLC assay.** Equilibrate the HPLC apparatus by pumping mobile phase (methanol–phosphate buffer) at 2 mL/min for at least 30 min, until the recorder baseline is stable. Inject sequentially 50 μL of each blank, TEP standard, quality control, and protein-free plasma extract, recording the absorbance of each effluent at 532 nm for 10 min. Measure the peak height of the MDA–TBA adduct, which has an average retention time of 4.2 min, and prepare a calibration curve by plotting the peak heights of the blank and TEP standard samples. Determine the concentrations of the plasma lipoperoxides as MDA (μmol/L) from the calibration curve.

**Column regeneration.** At the end of each HPLC run, remove the guard column and flush the injection system
sequentially with 10 mL of distilled water, 10 mL of methanol, 10 mL of nitric acid solution (5 mol/L), 10 mL of distilled water, and 10 mL of methanol/water (40/60 by vol). Reconnect the HPLC column, without the guard column, and regenerate the column by sequentially pumping methanol–water (40/60, 80/20, and 40/60 by vol) for 30 min each, at 2 mL/min. Repack the guard column daily, after cleaning the frits, discs, and fittings by boiling them in 2 mol/L nitric acid solution for 30 min and then in distilled water for 30 min. Before the next run, re-assemble the HPLC apparatus and inject 10 mL of methanol–water (80/20 by vol) to rinse the injection port and sample loop, followed by 10 mL of mobile phase.

With these precautions, the HPLC column should yield chromatograms with undiminished sensitivity and resolution for more than one month of steady use (>30 samples per day).

Cleaning plasticware. To clean the 13-mL polypropylene tubes, we treat them with TBA–H3PO4 solution, prepared by mixing 300 mL of the dilute phosphoric acid, 100 mL of the TBA solution, and 200 mL of distilled water. The tightly capped tubes are filled, placed in a boiling water bath for 1 h, then rinsed several times with distilled water, filled with water, capped, and returned to the boiling water bath for 1 h. The tubes are drained, rinsed, and dried by evaporation.

With these precautions, the peak height for MDA–TBA in chromatograms of reagent blanks should be <4 mm.

Subjects

Our human subjects were 41 asymptomatic, adult residents of central Connecticut (20 men, 21 women, ages 21 to 56 years, medical school faculty, staff, and students), who were not receiving medications (including oral contraceptives) and who gave no history of heart disease, cancer, diabetes, or other serious illnesses. The subjects were not fasted. Blood was sampled between 08:00 and 11:00 h.

The experimental animals were 59 male Fischer-344 rats (body weight 175–225 g; Charles River Breeding Laboratories, Inc., North Wilmingtom, MA), housed in stainless-steel mesh cages and fed Purina rat chow and water ad libitum. The rats were fasted for 17 h before blood collection. Some of the rats received a subcutaneous injection of NiCl2 solution (250, 500, or 750 mmol per kilogram body wt) 24, 48, or 72 h before the blood was sampled, between 08:00 and 09:00 h. Control rats received a 0.2-mL subcutaneous injection of 140 mmol/L NaCl vehicle solution 24, 48, or 72 h before blood collection.

Statistical computations (means, standard deviations, coefficients of variation, detection limits, and probability estimations by the Mann–Whitney test) were performed according to Sachs (30).

Results

HPLC analysis. Optimal conditions for separation of the MDA–TBA adduct were established by evaluating the components and sources of variation in the HPLC techniques of Bird et al. (23) and Yu et al. (24), including (a) trials of several commercial columns packed with octadecylsilica gel, (b) evaluations of a series of phosphate buffer–methanol and phosphate buffer–acetonitrile mixtures as the mobile phase, in lieu of the water–methanol mixtures used in the original techniques, and (c) varying the phosphate buffer concentration (from 50 to 200 mmol/L) and the proportion of phosphate buffer to organic solvent (from 80/20 to 60/40 by vol) in the mobile phase in order to achieve a narrow, symmetrical MDA–TBA peak in the chromatogram. Two satisfactory sets of operating conditions for HPLC fractionations were identified: (a) µBondapak C18 (10-µm particle diameter) as the column packing material and a 60/40 (by vol) mixture of phosphate buffer (50 mmol/L) and methanol as the mobile phase, with a solvent flow-rate of 2 mL/min; and (b) Chromsorb MC-C18 (5-µm particle diameter; E.S. Industries, Marlton, NJ) as the column packing material and a 80/20 (by vol) mixture of phosphate buffer (100 mmol/L) and acetonitrile as the mobile phase, with a solvent flow-rate of 1.5 mL/min. Operating condition "a" was selected for routine use. For 22 consecutive daily runs the retention time of the MDA–TBA adduct averaged 4.20 (SD 0.28) min.

Spectrophotometric and fluorometric detectors. In pilot studies we used a photometric monitor (Model 153; Altex Scientific, Inc., Berkeley, CA) with 8-µL flow-cell volume and 1-cm optical pathlength, set at 546 nm and a recorder sensitivity of 0.01 A full scale. The detection limit (as MDA) was 2.2 × 10⁻¹² mol per 50 µL of sample injected onto the HPLC column (equivalent to 2.7 µmol per liter of plasma), which proved inadequate to detect lipoperoxide concentrations in plasma from healthy subjects. To increase the sensitivity, we evaluated a fluorescence detector (Model FS-980; Kratos Analytical Division, Spectros, Inc., Ramsey, NJ) with 5-µL flow cell volume, set at excitation wavelength 625 nm, emission wavelength 550 nm, and recorder sensitivity = 0.1 µA full scale. The detection limit (as MDA) was 5.6 × 10⁻¹² mol per 50-µL sample injected onto the HPLC column (equivalent to 0.7 µmol per liter of plasma), which was insufficient to detect lipoperoxide concentrations in plasma samples from healthy subjects. Finally, a sensitive spectrophotometric detector was evaluated (Model 3000; LDC/Milton Roy) with 14-µL flow-cell volume and 1-cm optical pathlength, set at 532 nm, and modified by use of a 1-mV strip-chart recorder to provide 0.002 A full scale. The detection limit (as MDA) was 1.25 × 10⁻¹² mol per 50-µL sample injected onto the HPLC column (equivalent to 0.15 µmol of MDA per liter of plasma), which proved quite sufficient for quantification of plasma lipoperoxide concentrations in healthy persons. Under the specified operating conditions, the baseline noise level of the recorder tracing was 1.6 × 10⁻⁵ A and the baseline drift was 1.2 × 10⁻⁴ A/h. Illustrative chromatograms are shown in Figure 1.

Reaction with thiobarbituric acid. We established optimum reaction conditions for formation of the MDA–TBA adduct and precipitation of plasma proteins by systematically varying the concentration of each ingredient of the reaction mixture, the duration of boiling, and the pH and

![Fig. 1. Illustrative chromatograms for HPLC assay of plasma lipoperoxides, obtained by spectrophotometric monitoring at 532 nm](image-url)
methanol concentration for protein precipitation. A TBA concentration of 7 mmol/L in the reaction mixture was selected on the basis of experiments illustrated in Figure 2, in which chromatographic peak heights of the MDA–TBA adduct obtained with TEP standards and a plasma specimen are plotted vs TBA concentration. A 60-min boiling time was necessary for maximum yield of MDA–TBA adduct from plasma samples, although boiling for 30 min sufficed for the TEP standards. Varying the H₃PO₄ concentration in the reaction mixture from 0.33 to 0.66 mmol/L did not affect the yield of MDA–TBA adduct from TEP standards or plasma samples, so we selected a H₃PO₄ concentration of 0.44 mmol/L for routine use.

Calibration curves. Calibration plots (as MDA) were strictly linear up to 4.86 μmol/L. Based upon measurements of TEP standards in 13 consecutive analytical runs, the run-to-run coefficient of variation for slopes of the calibration line was 11%.

Precision and recovery. We evaluated run-to-run precision of lipoperoxide assays by analyses of commercial quality-control sera. As indicated in Table 1, replicate daily analyses yielded CVs of 12.6% and 8.0% for quality-control sera with lipoperoxide concentrations (as MDA) that averaged, respectively, 1.19 and 2.76 μmol/L. Analytical recovery of plasma at concentrations of 1.88 or 2.43 μmol/L averaged 98.1% (SD 7.1%) (range 88 to 114%), as listed in Table 2. When we increased the plasma sample volume from 50 μL to 100 μL, in an attempt to increase analytical sensitivity, this recovery was significantly smaller, ranging from 75 to 92%.

Anticoagulants and antioxidant. Plasma specimens collected with EDTA as the anticoagulant, as recommended by Lee (31), could be validly stored at 4 °C for at least 24 h without significant increase of lipoperoxide concentrations as compared with assays performed within 1 h after venepuncture. In contrast, lipoperoxide concentrations were increased by 1.5 to 2 times after similar storage of serum or of plasma specimens collected with heparin or citrate. Addition of EDTA anticoagulant to TEP standards, in the same quantity used for blood collection, did not affect the slope of the calibration line. Addition of butylated hydroxytoluene in amounts from 0.75 to 3.0 mg per 1.5 mL of reaction mixture did not affect the yield of MDA–TBA adduct from plasma samples that were collected with EDTA anticoagulant; therefore, butylated hydroxytoluene was not included in the reaction mixture.

Tests for interference. Addition of glucose, cholesterol, urea, uric acid, creatinine, or bilirubin (in albumin solution) to plasma specimens and TEP standard solutions at the concentrations listed in Table 3 did not interfere with the assays. Addition of erythrocyte hemolysate to plasma specimens caused positive interference in plasma lipoperoxide measurements, consistent with previous findings (32). When plasma hemoglobin concentrations were increased to 175 mg/L by addition of hemolysate, apparent plasma lipoperoxide concentrations were increased by two- to fourfold.

We added 15 commonly used drugs to plasma specimens and TEP standards at concentrations listed in Table 3 and assayed to test for interference. The only drug that substantially interfered was procarcinamide, which formed a colored compound that was eluted from the HPLC column 3.5 min after injection. N-Acetyprocainamide also formed a colored compound, but because its retention time was 5.5 min, it did not interfere. We confirmed the interference by procar-
Table 3. Tests for Interference in Plasma Lipoperoxide Assays

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc. added, per liter</th>
<th>Substance</th>
<th>Conc. added, per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>16 mmol</td>
<td>Theophylline</td>
<td>0.11 mmol</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10 mmol</td>
<td>Propranolol</td>
<td>1.9 μmol</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.1 mmol</td>
<td>Chlordiazepoxide</td>
<td>3.3 μmol</td>
</tr>
<tr>
<td>Urea</td>
<td>36 mmol</td>
<td>Diazepam</td>
<td>1.4 μmol</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.8 mmol</td>
<td>Amitriptyline</td>
<td>1.8 μmol</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.9 mmol</td>
<td>Nortriptyline</td>
<td>1.9 μmol</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>175 mg*</td>
<td>Desipramine</td>
<td>1.9 μmol</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.11 mmol</td>
<td>Imipramine</td>
<td>1.8 μmol</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>0.55 mmol</td>
<td>Phenytoin</td>
<td>80 μmol</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>0.33 mmol</td>
<td>Procaainamide</td>
<td>0.13 mmol*</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.17 mmol</td>
<td>N-Acetylprocainamide</td>
<td>0.11 mmol</td>
</tr>
</tbody>
</table>

*Substances that interfered in the assay when added to TEP standards or human plasma specimens at the specified concentrations.

manuscript in preparation).

Lipoperoxides in plasma of control and NiCl₂-treated rats. Plasma lipoperoxide concentrations (as MDA) in vehicle-treated control rats averaged 1.4 μmol/L (SD 0.3, range 0.9 to 1.8 μmol/L). Mean lipoperoxide concentrations were increased by 1.5 to 2.3 times in plasma sampled from rats 24, 48, and 72 h after subcutaneous administration of NiCl₂ at dosages previously shown to induce lipid peroxidation in lung, liver, and kidney (26, 33) (Table 4).

Discussion

Since 1986, when Yagi et al. (34) first applied the reaction of TBA with MDA and related chromogens to estimate lipoperoxide concentrations in human serum, the assay has been used to study circulating lipoperoxides in a wide spectrum of diseases, such as myocardial ischemia and infarction (35), cerebral ischemia and stroke (8, 18, 19), atherosclerosis (7, 36–38), diabetes mellitus (39, 40), pregnancy and pre-eclampsia (41), multiple sclerosis (42), muscular dystrophy (18), various liver disorders (4, 12, 43, 44), burns (9), and parquat poisoning (45). Although assays of serum lipoperoxides by the MDA–TBA reaction have become popular in Japan, they have achieved limited clinical acceptance elsewhere because of concerns about analytical specificity, sensitivity, reproducibility, recovery, specimen instability, and drug interference.

Our reference values for plasma lipoperoxide concentrations in healthy adults are consistent with those of Francesco et al. (8) but substantially lower than values for serum reported by previous investigators (Table 5). We attribute the lower reference values to (a) use of plasma instead of serum, to avoid release of lipoperoxides from platelets during blood coagulation (36); (b) acidification with H₃PO₄, as recommended by Mihara et al. (25), instead of trichloroacetic or phosphotungstic acids, which were used in most previous assays; and (c) greater specificity, because HPLC separation of the MDA–TBA adduct minimizes spectrophotometric interference. The improved specificity, sensitivity, reproducibility, and analytical recovery provided by the present assay should increase the acceptance of plasma lipoperoxide determinations for clinical research and diagnosis. We believe that the present method overcomes the drawbacks of previous techniques and will prove useful as a diagnostic test for lipid peroxidation in a wide spectrum of diseases. However, the technique requires stringent precautions to prevent hemolysis; to minimize platelet contamination; to remove traces of lipids and detergents from pipets, test-tubes, and HPLC fittings; and to monitor the purity of analytical reagents and solvents.

Others (47, 48) have used HPLC analysis with spectrophotometry at 267 to 270 nm to measure the production of MDA from polyenes fatty acids during incubation in vitro with hepatic microsomes. Close correlations were noted between results for MDA as determined by HPLC and by conventional TBA assays. Analytical sensitivity of the direct HPLC techniques, without prior formation of the MDA–TBA adduct, evidently was insufficient for plasma lipoperoxide analysis, because the reported detection limits for MDA were 7 × 10⁻¹¹ mol (47) and 5 × 10⁻¹² mol (48), respectively. For comparisons, the MDA detection limits were 1.25 × 10⁻¹² mol in the present study, and 1 × 10⁻¹⁴ mol as obtained by Yu et al. (24) with a double monochromator spectrophotometric detector. Others (49, 50) described

Table 4. Lipoperoxide Concentrations in Plasma of NiCl₂-Treated Rats

<table>
<thead>
<tr>
<th>NiCl₂ dosage, μmol/kg</th>
<th>Hours after injection</th>
<th>No. of rats</th>
<th>Plasma lipoperoxide, as MDA, μmol/L*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>24–72</td>
<td>12</td>
<td>1.4 (0.3) 0.9–1.8</td>
</tr>
<tr>
<td>250</td>
<td>24</td>
<td>5</td>
<td>2.2 (0.3) 1.7–2.4</td>
</tr>
<tr>
<td>48</td>
<td>5</td>
<td>2.3 (0.3) 1.9–2.7</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>6</td>
<td>2.1 (0.4) 1.5–2.6</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>24</td>
<td>7</td>
<td>2.1 (0.2) 1.9–2.4</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>3.1 (0.7) 2.4–4.3</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>6</td>
<td>3.1 (1.4) 1.7–5.3</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>24</td>
<td>6</td>
<td>3.3 (0.4) 2.0–3.0</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>3.2 (0.8) 2.2–4.4</td>
<td></td>
</tr>
</tbody>
</table>

*All results for NiCl₂-treated rats differed significantly (p < 0.01) from controls by the Mann–Whitney test.

Table 5. Reference Values for Lipoperoxide Concentrations in Serum or Plasma of Healthy Persons

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Date</th>
<th>Method</th>
<th>No. of subjects</th>
<th>Mean lipoperoxide, as MDA, μmol/L, (and SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>1977</td>
<td>Fluorometry</td>
<td>82</td>
<td>3.42 (0.84)</td>
</tr>
<tr>
<td>45</td>
<td>1981</td>
<td>Fluorometry</td>
<td>8</td>
<td>3.74 (0.83)</td>
</tr>
<tr>
<td>41</td>
<td>1981</td>
<td>Fluorometry</td>
<td>19</td>
<td>3.27 (0.89)</td>
</tr>
<tr>
<td>35</td>
<td>1983</td>
<td>Spectrophotometry</td>
<td>94</td>
<td>47.2 (7.0)</td>
</tr>
<tr>
<td>46</td>
<td>1984</td>
<td>Fluorometry</td>
<td>10</td>
<td>1.7 (0.5)</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1985</td>
<td>Spectrophotometry</td>
<td>20</td>
<td>0.61 (0.11)</td>
</tr>
<tr>
<td>7</td>
<td>1986</td>
<td>Spectrophotometry</td>
<td>15</td>
<td>0.94 (0.09)</td>
</tr>
<tr>
<td>Present study</td>
<td></td>
<td>HPLC</td>
<td>41</td>
<td>0.60 (0.13)</td>
</tr>
</tbody>
</table>
HPLC techniques for analysis of serum lipoperoxides that involve acid hydrolysis of lipoperoxides and complexation of MDA with 1,3-diphenyl-2-thiobarbituric acid, extraction of the MDA-1,3-diphenyl-2-thiobarbituric acid adduct in acetone-water–pentane, separation of the adduct by HPLC on a column of LiChrosorb-RP18, with elution in acetone-water–water, and quantification of it by spectrophotometry at 537 nm (49) or fluorometry at 548 nm with excitation at 525 nm (50). Detection limits for this adduct were 1 × 10^{-12} mol by spectrophotometry (49), 2.4 × 10^{-14} mol by fluorometry (50).

In rats with lipid peroxidation induced by NiCl₂, the time-course and magnitude of hyperliperoxidemia found in this study are consistent with previous data showing abnormal concentrations of TBA chromogens in homogenates of lung, liver, and kidney (26, 23), increased excretion of ethane and ethylene in expired breath (51), and increased concentration of conjugated dienes in hepatic microsomal lipids (33). Thus, the present findings demonstrate the applicability of the plasma lipoperoxide assay for detection of lipid peroxidation induced by xenobiotic toxicity in experimental animals.

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References

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