nologies; on the other hand, there is still a rather wide discrepancy among methods, probably related to differences in the extraction and preparation of the antigen. One CD patient in our study had negative results with all four assays with IgA anti-tTG concentrations even lower than those of controls in two of the tested ELISAs. This might be tentatively accounted for by the occurrence of autoantibodies directed against an antigen different from tTG, yet leading to EmA positivity (19).

Moreover, for each package a gray zone within the reference limits renders borderline values difficult to interpret: they should be considered according to the clinical setting. In this study, we tested only samples drawn from EmA-positive CD patients; performances of anti-tTG ELISAs need further evaluation in a prospective cohort of patients referred to a malabsorption clinic, including those with EmA-negative results and a larger number of controls affected by diverse pathologies. This might avoid the need to use borderline values. At present, we are unable to recommend the complete replacement of EmAs with recombinant human anti-tTG ELISAs for CD screening purposes, although the combination of the two methods can be proposed, especially when faced with borderline values that fall in the gray zone.

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References


Determination of Malondialdehyde as Dithiobarbituric Acid Adduct in Biological Samples by HPLC with Fluorescence Detection: Comparison with Ultraviolet-Visible Spectrophotometry, Jens Lykkefeldt (Department of Pharmacology and Pathobiology, Royal Veterinary and Agricultural University, 9 Ridebanevej, DK-1870 Frederiksberg C, Copenhagen, Denmark; fax 45-35-35-35-14, e-mail jopl@kvl.dk)

Living cells are constantly exposed to reactive oxygen species, some of which are capable of initiating lipid peroxidation by abstraction of an allylic proton from a polyunsaturated fatty acid. This process, by multiple stages leading to the formation of lipid hydroperoxides, is a known contributor to the development of atherosclerosis (1).

The thiobarbituric acid (TBA) test is an easy and quick assay for the assessment of lipid peroxidation in which malondialdehyde (MDA) is derivatized. The rationale and methodology have been discussed in detail elsewhere (2, 3) and have rightfully been criticized for low specificity and artifact formation because only a fraction of the MDA measured was generated in vivo (1,3). Furthermore, the TBA derivatization procedure itself leads to the formation of several MDA-unrelated ultraviolet (UV)-absorbing and fluorescent species, the latter as shown in Fig. 1, A–C. Despite this fact, the method remains one of the most useful and commonly used measurements of oxidative damage because of its simplicity. In recent years, several HPLC-based TBA assays have evolved with increased specificity (4–7), but nevertheless the spectrophotometric methods remain commonly used. In the present report, MDA was quantified in plasma, erythrocytes, and liver homogenates from 3-month-old guinea pigs by either our “modern” TBA test based on HPLC with fluorescence detection, which quantifies only the genuine MDA(TBA)2 adduct, or by the original less specific and less sensitive spectrophotometric method, which measures the total absorbance of several species. The purpose was to evaluate the use of the original TBA test by today’s standards by assessing the overestimation and sensitivity in the various applications that are routinely performed in several laboratories.

All compounds were of the highest quality available. Specifically, butylated hydroxytoluene (BHT), tetrame-
thoxypropane, TBA, and phosphotungstic acid were from Fluka.

Sample preparation was modified from Yagi (8) when analyzing plasma and from Ohkawa et al. (9) when analyzing tissue or cells. The modifications described here use smaller amounts, thus allowing the assays to be performed conveniently in microcentrifuge tubes. In addition, the use of the chain-breaking antioxidant BHT has been incorporated to suppress the peroxidation known to occur during the assay itself. All samples were stored at −80 °C until analysis in duplicate.

For plasma, 10 μL was placed in a microcentrifuge tube; 500 μL of 42 mmol/L H2SO4 was then added and mixed gently, after which 125 μL of 100 g/L phosphotungstic acid was added and vortex-mixed. After 5 min at room temperature, the mixture was centrifuged (3 min at 16 000g). MDA is associated with the lipoprotein, and consequently was contained in the pellet. The supernatant was discarded, and the precipitate was resuspended in 300 μL of 42 mmol/L H2SO4 and 45 μL of 100 g/L phosphotungstic acid, after which the sample was centrifuged (3 min at 16 000g). The supernatant was again discarded, and the pellet was resuspended in 350 μL of H2O. 50 μL of 0.7 mmol/L BHT in 200 mL/L ethanol, and 100 μL of TBA reagent consisting of 6.7 g/L TBA in H2O diluted 1:1 with glacial acetic acid. The mixture was heated immediately for 60 min at 95 °C and cooled on ice; the MDA(TBA)2 adduct was then extracted with 500 μL of n-butanol. The layers were separated by centrifugation (3 min at 16 000g).

For erythrocytes washed with phosphate-buffered saline (−25% hematocrit) or tissue homogenate prepared with 11.5 g/L KCl (9 mL/g of tissue), a 40-μL sample was diluted with 100 μL of H2O and mixed with 20 μL of 2.8 mmol/L BHT in ethanol, 40 μL of 81 g/L sodium dodecyl sulfate, and 600 μL of TBA reagent consisting of 8 g/L TBA diluted 1:1 with 200 mL/L acetic acid adjusted to pH 3.5 with NaOH. The mixture was immediately heated (60 min at 95 °C) and cooled with running water; 200 μL of H2O and 1000 μL of butanol-pyridine (15:1 by volume) were then added. After vigorous mixing, the organic layer was separated by centrifugation (3 min at 16 000g).

Calibration curves were constructed using tetramethoxypropane (0.1−5.0 μmol/L for plasma; 2.5−50 μmol/L for erythrocytes/tissue homogenates). Butanolic extracts were analyzed on an Agilent 8453 UV-Visible spectrophotometer fitted with an 80-μL flow cell (absorbance measured at 532 nm; reference wavelength, 600 nm) or on an automated HPLC gradient system consisting of the following Agilent Model 1100 units: Thermoast Autosampler (4 °C), Gradient Pump, Vacuum Degasser, Thermostated Column Compartment operated at 30 °C, and fluorescence detector (excitation, 515 nm; emission, 533 nm). All units were connected to a personal computer for control and for collection and analysis of data (UV-Visible ChemStation, Rev. A.08.01, or 2D ChemStation for LC, Rev. A.06.03; Agilent). Chromatographic separation was achieved on a Zorbax Eclipse C8 column [particle size, 5 μm; column size 150 × 4.6 mm (i.d.); Agilent] fitted with a SecurityGuard C8 guard column (Phenomenex). Mobile phase A consisted of 300 mL/L methanol in 50 mmol/L potassium dihydrogen phosphate buffer, pH 7.0; mobile phase B consisted of 800 mL/L methanol in the same buffer. Both mobile phases contained 0.2 g/L sodium azide to prevent bacterial growth. A gradient was included in the latter part of each HPLC run as a washing step to eliminate carryover and to extend column life. The gradient (percentage of solvent B) and flow rates were as follows: 0 min, 0% B, 1 mL/min; 4.5 min, 0% B, 1 mL/min; 5.5 min, 100% B, 2 mL/min; 8.5 min, 100% B, 2 mL/min; 9.5 min, 0% B, 2 mL/min; 10 min, 0% B, 2 mL/min; 11 min, 0% B, 1 mL/min. The injection volume was 5 μL, and the MDA(TBA)2 adduct eluted at 3.75 min.

The HPLC profiles of a reagent blank, 1.0 μmol/L calibrator, and a plasma sample are shown in Fig. 1, A–C, respectively. The derivatized plasma sample (Fig. 1C) contains the MDA(TBA)2 adduct at 3.75 min at a concentration of ~0.5 μmol/L. The concentrations in tissue and erythrocyte samples were ~25-fold higher than the concentration in plasma. The reagent blank (Fig. 1A) contained a small constant background peak at 3.75 min attributable to the fluorescence of TBA itself, as described by Londero and Lo (4). The detection limit of ~0.1 μmol/L in the sample (10 fmol on the column) is adequate for routine quantification of MDA in plasma. Conversely, detection of MDA in plasma by spectrophotometry is not advisable because the detection limit of the latter method is ~0.5−1 μmol/L in the sample. Spectrofluorometry has the sensitivity needed for measuring the low concentrations of the MDA(TBA)2 adduct present in plasma samples. Substantially increased sensitivity and specificity compared with regular spectrophotometry are achieved by use of the bichromatic methodology of excitation and emission wavelengths. Nevertheless, the human plasma reference values reported for spectrofluorometry (8) are approximately fourfold higher than those found by others (4, 6) and by us (unpublished results) when measured by HPLC with fluorescence detection. Not surprisingly, it appears from Fig. 1 that species other than the MDA(TBA)2 adduct exhibit fluorescence at the chosen excitation/emission wavelengths, thereby justifying the use of HPLC for their separation.

Erythrocytes are vulnerable to lipid peroxidation because of their high hemoglobin and oxygen content combined with a membrane rich in polyunsaturated fatty acid side chains. Fig. 1D shows the correlation between MDA measured by HPLC and UV-visible spectrophotometry, respectively. In Fig. 1D, the UV-visible absorbance (○) was measured at 532 nm with 600 nm as the reference wavelength. The latter wavelength is used merely for baseline correction. A very poor correlation was observed with an r2 of only 0.28. This is expectable because the samples contained considerable amounts of presumably heme-derived chromophores, which interfere with the assay and cause gross overestimation of MDA. The Morton–Stubbs correction is a classic three-point correction procedure that allows a shoulder peak to be measured more accurately by correcting for some of the interference.
from other compounds as well as shifts in baseline (10). The background interfering absorbance at the measuring wavelength is estimated by linear regression based on the absorbance at predetermined wavelengths on opposite sides of the peak of interest (11). When we used the Morton–Stubbs correction, the samples showed increased correlation with the HPLC data (Fig. 1D, ●). The latter overestimation appears to be acceptable, whereas the observed variability still leads to an $r^2$ of only 0.49.

The correlation between MDA measured in guinea pig liver homogenates by HPLC and spectrophotometry, respectively, was also estimated ($y = 0.999x + 40.9; n = 60$; data not shown). In this case, excellent correlation between the measurements was observed ($r^2 = 0.96$). However, overestimation was still ~40% on average.

In conclusion, spectrophotometric determination of so-called TBA reactive substances is a frequently used method for estimation of lipid peroxidation. Despite the disadvantages outline above, the results presented here show that, for tissue samples, particularly in the comparison of groups of samples obtained from the same species, spectrophotometry offers valid data. In addition, measurement in erythrocytes may be acceptable, but not optimal, when the Morton–Stubbs correction is used. In all cases, however, substantial or even gross overestimation is observed compared with HPLC determination of the same samples; consequently, the actual concentrations reported from spectrophotometry-based experiments should be regarded only as rough estimates. On the basis of the data from this laboratory, determination of MDA in plasma by the TBA assay cannot be adequately performed using spectrophotometric detection. The validity of the TBA assay rationale in general, however, was not considered in the present study. One major problem that cannot be alleviated by any TBA-based assay is the well-known artificial formation of the genuine MBA(TBA)$_2$ adduct from sources other than lipid hydroperoxides. Far more specific, albeit complex, methods for the assessment of lipid peroxidation are available. However, these methods most often require the use of expensive and specialized instrumentation and rarely allow the high throughput of the TBA HPLC assay. Consequently, when properly carried out, the TBA assay remains a useful and easy screening technique for lipid hydroperoxides.

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References