standard, which is reflected in the improved precision achieved.

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References

Nonchromatographic Assay for Malondialdehyde–Thiobarbituric Acid Adduct with HPLC Equivalence, N. R. Badcock,* G. D. Zoanetti, and E. S. Martin (Dept. of Chem. Pathol., Women’s and Children’s Hosp., 72 King William Rd., North Adelaide, South Australia 5006, Australia; * author for correspondence: fax 61-8-8204 7100, e-mail Baddockn@wch.sa.gov.au).

Reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) or its diethyl derivative has been applied widely to assess lipid peroxidation in biological material [1]. The reaction yields a red MDA-TBA adduct, the product of 2 mol of TBA plus 1 mol of MDA [2]. The colored complex can be quantified spectrophotometrically from its visible absorbance ($A_{\text{max}}$ 532 nm) [3, 4] or by spectrofluorometry (exc 532 nm, em 553 nm) [5, 6] and is readily extractable into organic solvents such as butanol [7]. Preanalytical factors aside [8–10], interfering chromogens include several other ordinary side-products of lipid autooxidation, alkanals, alkenals, and aldehydes as well as bile pigments, cyclic peroxides, carbohydrates, and amino acids [11–18].

Isolation and quantification of the MDA-TBA adduct by HPLC reportedly largely eliminates the interfering chromogens [19, 20]. HPLC methods generally yield lower values than those based on direct measurements, and this would seem to reinforce their specificity. However, mean reference values for lipoperoxide concentrations in plasma of healthy adults ($0.60 \pm 0.13 \mu\text{mol}/L; n = 41$) were found by Wong et al. [21], who used a specific HPLC method, to be consistent with those of Francesco et al. [22] ($0.61 \pm 0.11 \mu\text{mol}/L; n = 20$), without chromatographic separation. Similarly, Fukunaga et al. [23] reported but failed to explain a close correlation ($r = 0.972, n = 14$) between their specific HPLC assay ($2.51 \pm 0.75 \mu\text{mol}/L$; MDA values taken from correlation plot) and the traditional direct Yagi method [5] ($2.21 \pm 0.54 \mu\text{mol}/L$, similarly calculated). In a discipline where published reference values can vary 100-fold [24], these examples represent remarkable concordance and question the ability of HPLC to reduce spectrophotometric interference to the MDA-TBA adduct.

Various approaches have been proposed to minimize analytical interference in direct methods without resorting to HPLC. These approaches include spectrophotometric correction formulas [24, 25], derivative spectrophotometry [26], and synchronous fluorescence [27]. By their strong correlation and close resemblance to values from HPLC methods [21, 28], these direct assays are also shown to be as effective as HPLC in minimizing interference.

Why are HPLC methods in reality seemingly neither more discriminatory nor more valid than several traditional assays, despite almost unanimous claims to the contrary? HPLC methods are certainly less practical and less readily adapted to screening studies, and the red adduct stains the HPLC equipment, resulting in lengthy clean-up procedures. If no substantial advantages are offered, why adopt a more time-consuming and tedious technique? The answer for the lack of discrimination became apparent during trials to incorporate an internal standard, similarly derivatized by TBA, into the HPLC procedure. No published HPLC assay of the MDA-TBA chromogen has used an internal standard. Essential to selection of an internal standard was noncontribution from pseudo-MDA activity through coelution of an indistinguishable 532-nm-absorbing chromogen from the introduced internal standard.

Sucrose, fructose, other carbohydrates, several amino acids, and ascorbic acid, when exposed to hydroxyl radicals produced by ionizing radiation or metal-ion $H_2O_2$ systems, yield products that give a genuine MDA-TBA adduct on heating with TBA [29] and were not considered as potential candidates. Various alkanals such as formaldehyde, acetaldehyde, propanal, and hexanal, which have been shown to both reduce MDA recovery and likewise coelute with the MDA-TBA adduct [12] and N.R. Badcock, G.D. Zoanetti, and E.S. Martin, personal observation were not considered as potential candidates either. With the aforementioned precluded, we tested several other aldehydes, including 2-hexenal, cinnamic aldehyde, glycoaldehyde, furaldehyde, and 5-hydroxymethyl-2-furaldehyde, together with several compounds such as glycine-MDA, methionine-MDA, and dianil-MDA, which contain the MDA moiety [11]. All of these compounds reacted with TBA to form either yellow or red pigments with absorbance maxima at 450 or 532 nm, respectively. Importantly, all chromatograms indicated only two chromogens, the red, which always corresponded to the red MDA-TBA adduct irrespective of mobile phase...
[8, 20, 21, 23, 30], and the yellow reactive substance, which coeluted with a peak previously ascribed to an endogenous yellow TBA marker [30]. No other peaks were observed. In other words, despite HPLC, chromatographic separation of other red adducts from MDA-TBA could not be achieved.

The failure of HPLC to discriminate should not be totally unexpected. Examination of the majority of illustrative chromatograms generated from HPLC separation on C18 columns of the MDA-TBA adduct [8, 21, 23, 30–32] indicates a remarkable absence of those additional peaks that would be expected if HPLC separated the chromophore of interest from other interfering chromogens. The occasional exception to this clean HPLC single-peak elution profile is the yellow chromophore that elutes well before the MDA-TBA complex and has an absorbance maximum at 450 nm, and as such, is also well differentiated in the visible spectrum. Interestingly, Marcuse and Johansson [13] also reported the formation of only two pigments, a red one with a maximum absorbance at \( \approx 530 \text{ nm} \) and a yellow one with maximum at 450 nm.

With a reason for the clean tracings on HPLC came the explanation for their demonstrated equivalence with various direct methods, particularly those that exploit a correction for nonspecific background absorbance. However, although a correction factor is about equally effective in minimizing artifacts, we considered another approach to reducing analytical interference that did not assume linearity of background absorption. This involved the back-extraction of the acidic chromophores from the \( n \)-butanol organic phase (an end-point of most MDA-TBA extraction methods, including the one that we adopted, that of Lepage et al. [8]) into 200 \( \mu \text{L} \) of 4 mol/L NaOH. Tetraethoxypropane (always <20 \( \mu \text{L} \)) was added to plasma before reaction, in variance to published procedures, as an internal standard to eliminate bias in the assay from losses of any potential aldehyde trapped in protein as a Schiff base, coprecipitated, or absorbed onto any residual protein, and appropriate blanks were run in parallel to compensate for aldehydes present in solvents.

The back-extraction offered several advantages. The red MDA-TBA adduct was recovered completely by the NaOH extraction (99.5% ± 1.7%) for an overall extraction efficiency of >95%. Especially in samples of low MDA content, the cleanup and concentrating step improved analytical detection limits severalfold without resorting to time-consuming evaporation. In fact, the detection limit, determined as described by Gatautis and Pearson [33], was 0.3 \( \mu \text{mol}/\text{L} \) and near that of chromatographic methods with fluorescence detection. A bathochromic shift of 14 nm occurred with sodium hydroxide, giving a peak maximum at 546 nm for the red chromophore. This was noteworthy for two main reasons. Icteric plasma (containing bilirubin or biliverdin) had TBA reactivity with the resulting chromophore, having maximum absorptivity at 560 nm in acid solution and 600 nm in alkaline solution. In other words, any potential interfering chromogen from this source was removed further from the MDA-TBA chromophore, resulting in minimal background absorbance at 546 nm. Secondly, the alkaline extract exerted a marked hypochromic effect on the yellow chromogen, again minimizing interference without the need to include a correction factor. By adjusting the pH between 2.5 and 4.5 and heating the MDA-TBA mixture at 100 °C for 60 min, as recommended by Lepage et al. [8], the pyrolysis products of carbohydrates (sucrose, fructose, lactose), amino acids (\( l \)-tyrosine, \( l \)-methionine, \( l \)-tryptophan), and ascorbic acid at 100 \( \mu \text{mol}/\text{L} \) each were found not to interfere.

Because pyrimidine solutions in alkali are often unstable because of ring-opening, stability of the red complex in the 4 mol/L NaOH extract was examined. After 10 min, there was no change in absorbance; thereafter absorbance decreased linearly with a half-life of 21 h. Correlation based on a least-squares linear regression between our nonseparative method and reversed-phase HPLC (Lepage et al. [8]), with the alkaline MDA-TBA adduct neutralized within 10 min of back-extraction for subsequent injection, gave a line of best fit with a slope of 0.8538 and a \( y \)-intercept of 0.116 \( \mu \text{mol}/\text{L} \) (Fig. 1). The correlation coefficient was 0.978 (\( P < 0.001 \)), and the mean of the 39 samples obtained from healthy adults (12 women and 12 men, mean age 36 years, range 22–52 years) and porphyric patients (6 female and 9 male, mean age 33 years, range 8–50 years) was 1.61 ± 1.06 \( \mu \text{mol}/\text{L} \) by our method and 1.51 ± 0.93 \( \mu \text{mol}/\text{L} \) by HPLC. Plasma MDA in healthy adult subjects averaged 0.89 ± 0.29 \( \mu \text{mol}/\text{L} \) by the direct assay and 0.88 ± 0.29 \( \mu \text{mol}/\text{L} \) by HPLC.

The method has been used to measure free radical generation in the cutaneous porphyrias both at diagnosis and in response to various treatment regimes. Porphyrias are phototoxic due to ultraviolet light, causing tissue damage by release of free oxygen radicals, which manifests as photosensitivity [34–36]. The resulting oxygen-related radicals have the potential to be especially cytotoxic in combination with the iron-induced oxidant stress of porphyria cutanea tarda (PCT) [37–39]. MDA

\[ \text{MDA} = \text{MDA}_{\text{direct assay}} + \text{MDA}_{\text{HPLC}} \]

\[ \text{MDA}_{\text{direct assay}} = 0.3 \text{ \mu mol/L} \]

\[ \text{MDA}_{\text{HPLC}} = 0.89 \text{ \mu mol/L} \]

\[ \text{PCT} (\bullet), \text{10 patients with PCT (\bigcirc)}, \text{and 5 patients with EPP (\bigast)}. \]
was increased substantially in plasma drawn from these porphyric patients at diagnosis [3.04 ± 0.30 and 2.29 ± 0.26 μmol/L for PCT and erythropoietic protoporphyria (EPP) patients, respectively, by direct assay], indicating oxygen-free radical inactivating capacity in these porphyrias (Fig. 1). Specific therapies [34], including chloroquine, β-carotene, charcoal, and cholestryamine, did not interfere. A detailed analysis of the concentrations and their relationship to plasma porphyrin amounts, photosensitivity, and other pathology will be reported elsewhere.

We conclude with the interesting and somewhat chastening observation that nonseparative methods that minimize background absorbance, either through correction factors, or, as in our case, back-extraction into NaOH, allow the MDA-TBA adduct to be measured with specificity comparable with HPLC methods.

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References


Improved Method for Genotyping Apolipoprotein E Polymorphisms by a PCR-Based Assay Simultaneously Utilizing Two Distinct Restriction Enzymes, Ariella Zivelin, Nurit Rosenberg, Hava Perez, Yonit Amit, Nurit Kornbrot, and Uri Seligsohn* (Inst. of Immunobiology and Hemostasis, Sheba Med. Center, Tel Hashomer, Israel; * author for correspondence: fax 972-3-5351658, e-mail zeligson@post.tau.ac.il)

Apolipoprotein E (apo E) is a protein that plays an essential role in lipid metabolism and distribution [1]. The apo E gene is polymorphic, and its three alleles code for isoforms E2, E3, and E4, which differ by single-amino-