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Note

Determination of plasma lipoperoxides by high-performance liquid chromatography

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The importance of free radicals in the alteration of many biological systems explains the numerous reports concerning the determination of lipoperoxides in tissues and biological fluids. Various techniques have been described, including the thiobarbituric acid method [1–3], conjugated dienes measurement [4] and the determination of expired hydrocarbons [5] and chemiluminescent and fluorescent substances [6,7]. The most widely accepted standard method is Yagi's technique [1], which is based on the reaction of malonaldehyde (MDA), generated from lipoperoxides with 2-thiobarbituric acid (TBA). Nevertheless, some problems occur with this assay because several types of compound other than MDA give a positive TBA test [8,9]. To resolve these difficulties, high-performance liquid chromatography (HPLC) has been used and recent reports describe HPLC methods for quantified free MDA [10,11] or the TBA–MDA adduct [12,13]. The determination of free MDA is time-consuming, needs costly columns and requires large amounts of plasma or tissue. Methods using the TBA derivative of MDA prior to the injection into the HPLC system do not have these disadvantages and are more sensitive.

For these reasons, and in order to determine plasma lipoperoxides levels, a simple, sensitive and selective HPLC method using visible and spectrophotometric fluorescence detection is described. The results obtained were correlated with those from Yagi's method.

EXPERIMENTAL

Reagents and chemicals

Acetonitrile, ethyl acetate, ethanol and methanol were HPLC grade (Merck, Darmstadt, F R.G.). HPLC-grade water was prepared by passing deionized water

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through a Millipore membrane (0.45 μm) and all the solvents and products were degassed before use. Ethanolamine and diethylthiobarbituric acid (DETBA) were purchased from Sigma (St. Louis, MO, U.S.A.). DETBA (10 m*M*) was dissolved in phosphate buffer (0.1 *M*) adjusted to pH 3 with phosphoric acid.

1,1,3,3-Tetraethoxypropane (malonaldehyde bis or diethyl acetal, Merck) was dissolved in water to prepare a stock solution (250 μ*M*). This stock solution was kept at 4 °C and was stable for several months. Tetraethoxypropane (TEP) standard solution and a blank were prepared freshly each day (from 25 to 250 pmol/ml). TEP was used to generate malonaldehyde in situ under acidic conditions.

Analytical and extraction procedure

Duplicate samples were analysed by HPLC procedure and by the fluorescence micromethod using the TBA reaction [1].

Plasma or serum (50 μl) was transferred to a screw-capped tube and 1 ml of 10 m*M* DETBA in phosphate buffer (0.1 *M*, pH 3) was added. The mixture was mixed for 5 s. After 60 min at 95 °C, the tubes were placed in ice for ca. 5 min, and 1 ml of ethyl acetate was then added. The mixture was shaken for 1 min to extract the DETBA–MDA adduct. The ethyl acetate extraction efficiency was found to be as good as the usual butanol extraction, described previously in Yagi's method [1]. To break down the emulsion, 1 ml of diethyl ether was added and the mixture was centrifuged at 2500 *g* for 10 min. The organic layer was transferred to another tube and evaporated under nitrogen at 40 °C. The residue was treated again with 125 μl of methanol, and 50 μl were injected in duplicate on a reversed-phase chromatographic column.

Chromatography

The chromatographic material consisted of an LKB HPLC system including a Rheodyne injector with a 50-μl sample loop, a 250 × 4.6 mm I.D. Merck LiChrosorb RP-18 (5 μm) analytical column and a LKB UV–visible detector. A 40 × 4 mm I.D. precolumn (Merck) with Hibar C_{18} reversed-phase packing was used to protect the analytical column. The mobile phase (flow-rate, 0.7 ml/min) was 0.1% ethanolamine aqueous solution–acetonitrile (71:29, v/v). The DETBA–MDA adduct (red pigment) was quantified by either visible spectrophotometry (539 nm) or, in a few cases, by fluorescence detection (excitation wavelength 515 nm, emission wavelength 553 nm). A Hewlett-Packard calculator integrator was used to treat the intensity of the signal.

RESULTS AND DISCUSSION

Fig. 1a shows representative chromatograms from DETBA–MDA standard adduct and from two patients with normal (Fig. 1b) and high lipoperoxide levels (Fig. 1c). The retention time of the DETBA–MDA adduct peak is ca. 7–7.5 min. The peaks eluted prior to this are not identified. The addition of ethanolamine improved the chromatogram and the reproducibility of the retention time for a

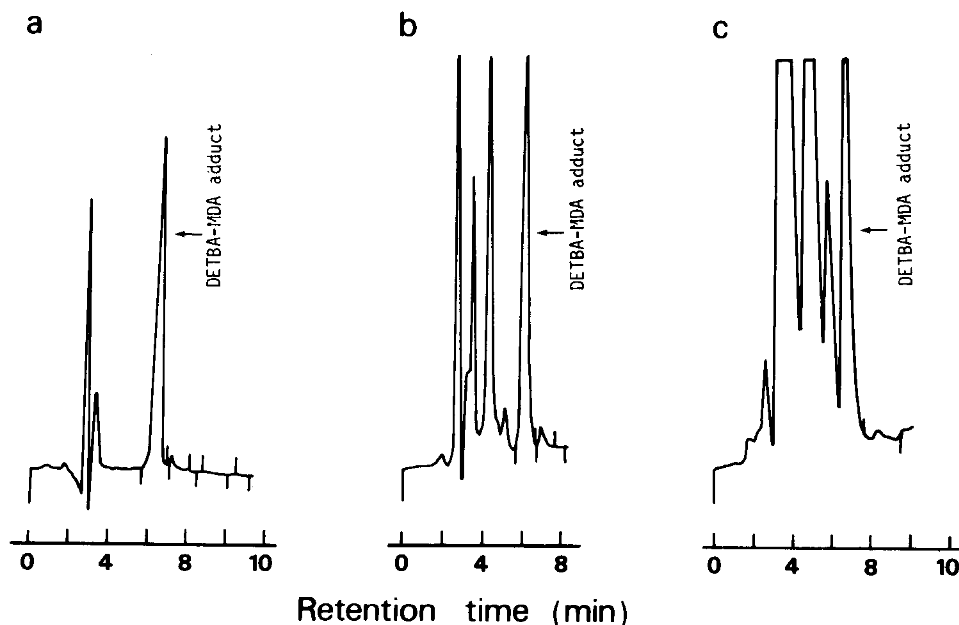

Fig. 1. Chromatograms of plasma lipoperoxides by HPLC. (a) Standard adduct; (b) control subjects; (c) cholestatic patient. The peaks eluted before the DETBA-MDA adduct peak are not identified.

series of measurements. Ethanolamine permits the saturation of the free silanol groups on the stationary phase.

Within the range of concentrations studied, the standard curve for the DETBA-MDA adduct was linear ($r = 0.99$) between 10 and 460 pmol per tube, and the detection limit was 2 pmol. The same column was used for at least 120 analyses without a decline in performance. The ethyl acetate extraction and the rapid evaporation of the organic layer increase the sensitivity of this method.

The coefficient of variation was 4% for a plasma control (mean 0.57 nmol/ml) and 9% for plasma from a cholestatic patient (mean 2.87 nmol/ml). We checked the possibility of various interferences using visible and fluorescence spectrometric analysis of the efflux fraction at a retention time of 7.5 min. The comparison between these spectra and those obtained with a standard adduct prepared as previously described [14] did not give any significant difference.

Comparison of the HPLC and Yagi methods

The technique of Yagi is one of the most useful methods in clinical research, and the spectrometric fluorescence measurement greatly increases the sensitivity of the technique. We have studied the lipoperoxide values of eighteen healthy adult subjects. The mean ($\pm$S.D.) of the MDA plasma concentration was 1.27 ± 0.98 nmol/ml by the HPLC method and 2.72 ± 1.32 nmol/ml by the method of Yagi. The coefficient of correlation between the results obtained by the two methods was 0.85 (Fig. 2). In twenty cholestatic children, the mean of the MDA levels was 3.43 ± 3.60 nmol/ml by the HPLC method and 6.92 ± 3.97 nmol/ml by Yagi's method, with a correlation coefficient of 0.63 (Fig. 3).

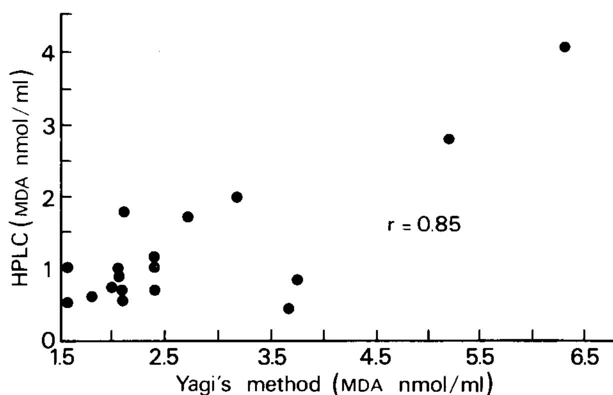

Fig. 2. Correlation between plasma lipoperoxide levels determined by HPLC and by Yagi's method in control subjects.

There was a good correlation between the two procedures for the control subjects; the lower mean value obtained by the HPLC method could be explained by a greater selectivity of this method in which lipohydroperoxide levels measure the DETBA–MDA adduct amount alone whereas Yagi's method is not as so selective. Thus, this latter technique measures other TBA adducts with other lipoperoxide breakdown products, such as 2,4-dienal or 2-enal aldehydes [9], and several biological products such as flavin or drugs [6]. Such discrepancies are not important if the results determined by each technique have a constant relationship. In some cases, principally in cholestatic patients, we found important differences in the ratios of concentrations. It is important to notice that in the two methods, MDA present in vivo or produced in situ from hydroperoxides can react with plasma protein amino groups to form Schiff's bases [6]. The spectrometric properties of Schiff's bases are similar to those of TBA–MDA adduct. This fact could explain the pink precipitate obtained in some cases after the reaction at 95°C with TBA or DETBA. This coloured material is extractable by ethyl acetate or

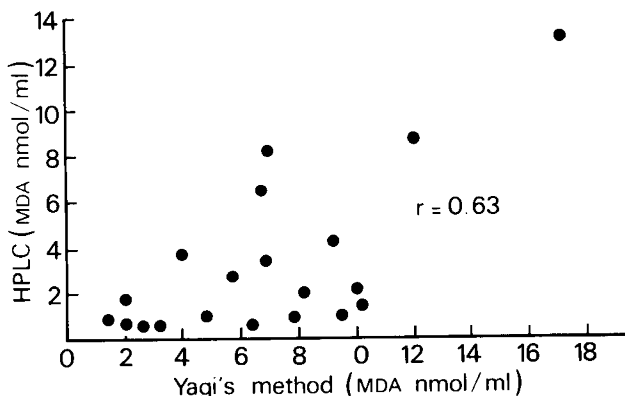

Fig. 3. Correlation between the plasma lipoperoxide levels determined by HPLC and by Yagi's method in cholestatic patients.

butanol but, in some cases, there is no correlation between the intense coloration and the MDA levels.

Comparison of two detection systems of HPLC efflux

HPLC is a selective method and allows a specific measurement of the column efflux by spectrophotometry or by other methods. In particular, the chromatograms obtained by the fluorescence method showed only one peak with the same retention time as in the visible detection. These results suggest that there are probably no substances coeluted with DETBA–MDA adduct. We have measured ten plasma samples in duplicate with these two modes of detection. The correlation coefficient was 0.91. The mean of the MDA values was 1.24 ± 0.61 nmol/ml with visible spectrophotometric detection and 1.35 ± 0.61 nmol/ml with spectrofluorescence detection. With the material used the sensitivity of detection was similar in the two methods.

CONCLUSION

We report a rapid and simple method of plasma lipid peroxidation. The use of diethylthiobarbituric acid as reagent and ethyl acetate as extraction solvent, and the addition of ethanolamine to the mobile phase, contribute to the good sensitivity and reproducibility of the technique. The association of the HPLC separation and fluorescence detection could resolve many problems concerning the measurement of the lipoperoxides.

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