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Automated high-performance liquid chromatographic separation with spectrofluorometric detection of a malondialdehyde–thiobarbituric acid adduct in plasma

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Abstract

An automated HPLC separation method for the detection of malondialdehyde (MDA) in plasma samples of a healthy population is described. The procedure involves a first step of acidic hydrolysis and complex formation with thiobarbituric acid (TBA), then a protein precipitation step, and finally the separation of TBA–MDA adducts through a complete HPLC apparatus with spectrofluorometric quantification. This procedure is very useful for a routine laboratory because of its rapidity (t_R = 1.7 min), simplicity and applicability, and the method gives very good results with respect to linearity (0.1–12 μmol/l), precision (within-assay C.V.%: 8, between-assay C.V.%: 10) and accuracy (recovery average: 91.66%).

Keywords: Lipid peroxidation; Malondialdehyde; Thiobarbituric acid

1. Introduction

Lipid peroxidation is a free-radical-mediated degradative process, propagated by chain reactions, to which the molecules of polyunsaturated fatty acids are particularly subject, causing the formation of lipid hydroperoxides [1]. In biological systems these hydroperoxides are broken down into a variety of carbonyl secondary products (aldehydes and ketones).

Hydroperoxides and carbonyl groups increase the permeability of membranes, because of the presence of new hydrophilic centres induced in phospholipids. This explains the following phenomena: passive swelling of mitochondria, vesiculation of endoplasmic reticulum, leakage of enzymes and coenzymes, lysis of red blood cells, alterations of protein structure and function, all of which lead to cell and tissue damage.

Measurement of carbonyl secondary products is an index of degree of lipid peroxidation [2]. Malondialdehyde (MDA) is one of these secondary products and its detection is useful as an indicator of the extent of tissue damage. The most widely employed method for the determination of MDA in biological materials is based on its reaction with thiobarbituric acid (TBA) [3,4]. The specificity of this assay was improved by using HPLC techniques [5–8]. Here we have described a rapid, sensitive and highly selective HPLC assay with spectrofluorometric detection of MDA–TBA adducts in human plasma.

2. Experimental

2.1. Reagents

TBA and tetramethoxypropane (TMP) were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC-grade methanol was obtained

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from Carlo Erba Reagenti (Milano, Italy). All other chemicals were of the highest commercial grade available.

2.2. Analytical procedure

We measured MDA plasma levels in a healthy population of blood donors ($N = 104$). Blood samples were collected by venipuncture in Vacutainer tubes containing EDTA as anticoagulant. For the calibration curve, MDA was obtained by acid hydrolysis of 1,1,3,3-tetramethoxypropane [9]. The TBA test was performed optimizing Wong's hydrolysis conditions [6]: 500 μl of a distilled water–reagent blank, TMP working standard solutions, plasma specimens were pipetted into new screw-capped glass tubes; finally, 750 μl of 0.44 *M* phosphoric acid solution and 250 μl of 42 m*M* TBA solution were added. The tubes were placed in a boiling water bath (100°C) for 60 min, then they were cooled at 0°C; 500 μl of the boiled samples were extemporaneously neutralized with 500 μl of a 1 *M* methanol–NaOH mixture (45.5:4.5, v/v); after vortex mixing and centrifugation, 30 μl of supernatant were chromatographed in the HPLC system, at room temperature.

2.3. HPLC analysis

HPLC separation of MDA–TBA adducts was performed with a complete GOLD System apparatus (autosampler 507, programmable solvent module 116, analog interface module 406, detector, IBM PS2-Beckman Instrument) with a Shimadzu spectrofluorometric detector RF551 set at 532 nm excitation and 553 nm emission. The mobile phase consisted of 40:60 (v/v) 0.05 *M* methanol–potassium phosphate buffer, pH 6.8; the flow rate was 1.5 ml/min with an average retention time of 1.7 min; the column used for the separation was a 5 μm, 125 mm × 4.6 mm I.D. C_{18} reversed phase filled in our laboratory with Nucleosil (Riedel-De Haen AG Seelze Hannover, p/n 39842). The detection limit was 0.8 pmol per 30 μl injected (i.e. 0.10 μmol per litre of plasma).

3. Results

3.1. Linearity

The calibration curve usually employed for MDA determination in human plasma ranged from 0.15 to 1.22 μmol/l of plasma, corresponding to 1.25–10.0 pmol/30 μl injected. The linearity of the method was also tested for calibration curves ranging from 0.15 to 12.2 μmol/l, corresponding to 1.25–100.0 pmol/30 μl injected. The equation of the regression line was: $y = -0.2331 + 17.686x$ ($n = 5$, $r = 0.999$, $R^2 = 0.9998$, standard error 0.049).

3.2. Precision and accuracy

We evaluated within-run and between-run precision by using aliquots from four different pools of plasma. Pooled plasma was prepared by mixing EDTA–plasma samples from ten different subjects; plasma samples were analyzed without delay for within-run test, and stored at −20°C for between-run test. No difference was found in MDA concentration of pooled plasma stored without addition of antioxidant and pooled plasma stored with the addition of 20 g/l butylated hydroxytoluene (BHT). Accuracy was determined by assessing the recovery in spiked samples: increasing amounts of standard solution were added to plasma samples whose MDA concentration was measured in the same run. All the results are shown in Tables 1 and 2.

3.3. Chromatograms

The average retention time of the MDA–TBA adduct was 1.7 min at a flow-rate of 1.5 ml/min. A typical chromatogram of the adduct is shown in Fig. 1, panel A. Fig. 1, panel B, shows a chromatogram of distilled water–reagent blank, thus demonstrating that no interference occurs in the test.

3.4. Physiological values

Normal values were established in 104 healthy blood donors, aged between 18 and 65 years. MDA reference values in our population were 0.85 ± 0.25 μmol per litre of plasma; smoking habits were taken

Table 1
Within-run (WR) and between-run (BR) precision determinations

	Mean (μmol/l)	S.D.	C.V. (%)
WR pool 1	0.40	0.03	7.5
WR pool 2	0.73	0.07	9.3
BR pool 3	0.38	0.02	5.2
BR pool 4	1.18	0.17	14.4

into account, but no significant differences were observed between smokers and non-smokers (Student's unpaired t-test, $P > 0.05$).

4. Discussion

The measurement of lipid peroxidation status in biological systems requires a sensitive and specific method, and HPLC analysis of MDA–TBA adducts is the most widely accepted. Furthermore, the requirement of a routine laboratory in which a large number of samples are analyzed every day is to have a rapid, simple and easily applicable technique. In order to reach these aims, we developed a method that joins a controlled reaction procedure with an automated HPLC detection. To optimize our reaction conditions, we checked all the assay steps. We collected blood in vacutainer tubes with EDTA as anticoagulant: in fact, EDTA plasma turned out to have lower MDA concentration values than sodium heparin–plasma or serum [10]. After collection, plasma samples were analyzed without delay. We used new screw-capped glass tubes to reduce the peak height of reagent blank; we tested boiling and temperature time and pH of reaction mixtures. As a result, we performed the hydrolysis step with a solution of 0.44 M H_3PO_4 for 60 min, confirming

Table 2
Analytical recoveries of standard added plasma

TMP added (μmol/l)	MDA detected (μmol/l)	Recovery (%)
0	0.40	–
0.30	0.65	93
0.61	0.88	87
1.22	1.54	95

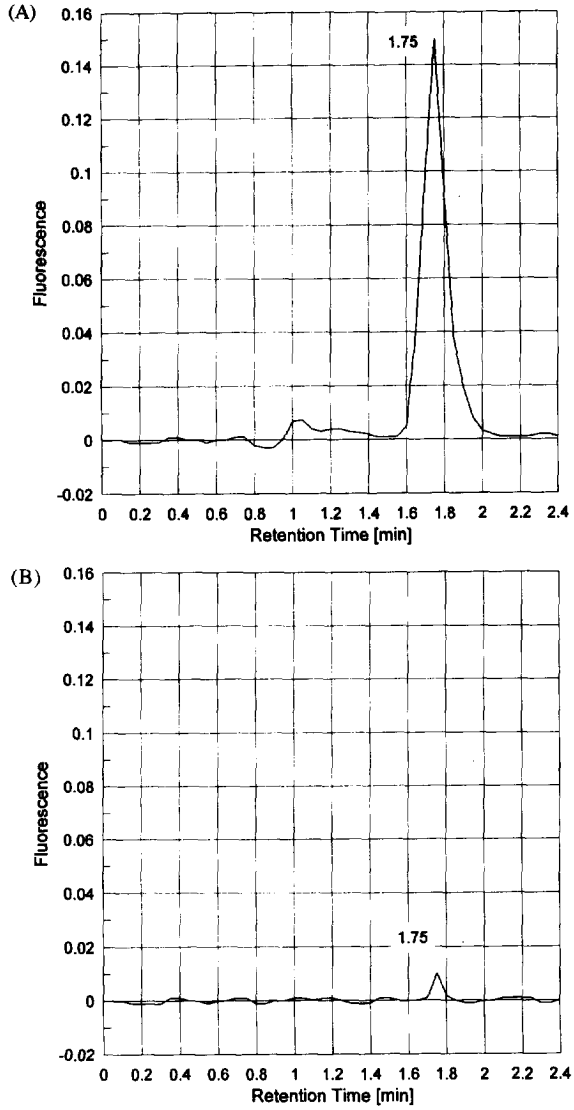

Fig. 1. Panel A: a typical chromatogram of a plasma sample; peak at 1.75 min is MDA–TBA adduct. Column, C_{18} RP (125 mm × 4.6 mm I.D., 5 μm); mobile phase, 0.05 M methanol–potassium phosphate buffer, pH 6.8 (40:60, v/v); flow-rate, 1.5 ml/min; detection wavelength, Ex: 532 nm; Em: 553 nm. Panel B: a chromatogram of distilled water–reagent blank. The peak at 1.75 min is due to the fluorescence of TBA itself.

the literature data [6]. To improve our resolution results we used a 5 μm, 125 mm × 4.6 mm I.D. C_{18} column connected to a complete GOLD System apparatus which enables the whole separation within about 2 min. The choice of a spectrofluorometric

assay quantification was made because we found it to be the best detector with respect to all analytical performances, including baseline noise level, baseline drift, signal-to-noise ratio, sensitivity and specificity. The comparison with spectrophotometric detection (532 nm) was favourable to use of spectrofluorometry.

In conclusion, we obtained a method that is satisfactory for the determination of MDA–TBA adducts in plasma samples with respect to sensitivity, specificity and reproducibility.

References

[1] J.M.C. Gutteridge, Free Radicals Res. Commun., 1 (1986) 173.
[2] H. Esterbauer and K.H. Cheeseman, Methods Enzymol., 186 (1990) 407.
[3] R.O. Sinnhuber, I.C. Yu and T.C. Yu, Food Res., 23 (1958) 620.
[4] R. Yagi, Biochem. Med., 15 (1976) 212.
[5] R.P. Bird, S.S.O. Hung, M. Hadley and H.H. Draper, Anal. Biochem., 128 (1983) 240.
[6] S.H.Y. Wong, J.A. Knight, S.M. Hopfer, O. Zaharia, C.N. Leach, Jr. and F.W. Sunderman, Jr., Clin. Chem., 33 (1987) 214.
[7] M.J. Richard, P. Guirard, J. Meo and A. Favier, J. Chromatogr., 577 (1992) 9.
[8] K. Fukunaga, T. Suzuki and K. Takama, J. Chromatogr., 621 (1993) 77.
[9] J.M. Gutteridge, Anal. Biochem., 69 (1975) 518.
[10] J.A. Knight, S.E. Smith, V.E. Kinder and H.B. Anstall, Clin. Chem., 33 (1987) 2289.