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A sensitive and reliable method for the detection of lipid peroxidation in biological tissues

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Abstract

A simple, accurate and cost effective method has been designed for the determination of lipid peroxidation in biological tissue samples. The method was a modification and improvement on existing methods available for lipid peroxidation determination. Solid-phase extraction was used to separate the thiobarbituric acid–malondialdehyde complex from thiobarbituric acid-reactive substances and HPLC was performed using a C_{18} (Waters Spherisorb, 5 μm, 250 $\times$ 4.6 mm i.d.) column to achieve isolation of the complex. The procedure was validated with respect to linearity of calibration (0.998), precision, sensitivity and limits of quantitation (1 nmol mL^{-1}) and detection (0.5 nmol mL^{-1}). Resorcinol was used as an external standard. The method was tested by inducing free radical generation with a known free radical generator, quinolinic acid, in rat brain homogenate. The results showed that the method presented allowed detection of lipid peroxidation products at concentrations in the nanomolar (nM) range compared with the micromolar (μM) range detected by other methods, thus rendering it suitable for use with biological samples. In addition, the modified method allowed for detection of the purified lipid peroxidation products, thus eliminating the possibility of simultaneous detection of impurities that absorb at the same wavelength.

Introduction

There is considerable interest in the role played by lipid peroxidation and other free radical reactions in human disease and toxicology (Gutteridge & Halliwell 1990). Although aerobic lifestyles are advantageous in many ways, the utilization of oxygen by cells for many biochemical reactions results in the formation of highly reactive free radical species (Ottino & Duncan 1997). These oxygen-derived reactive species are able to induce irreversible and reversible damage to macromolecular targets including proteins, nucleic acids and cellular membranes (Voltera et al 1994; Ottino & Duncan 1997).

The measurement of putative elevated end products of lipid peroxidation in animal material is probably the evidence most frequently quoted in support of free radical-induced tissue damage (Gutteridge & Halliwell 1990). The thiobarbituric acid test is the most widely used assay for measuring lipid peroxidation (Gutteridge & Halliwell 1990). The assay involves the reaction between malondialdehyde, an end product of lipid peroxidation, and thiobarbituric acid to yield a pink chromogen which is measured colorimetrically at 532 nm using a spectrophotometer (Gutteridge & Halliwell 1990; Southgate & Daya 1999). However, this simplistic method is subject to several sources of error.

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The main limitation of this assay is that the malondialdehyde content of many materials of plant and animal origin is inflated by pigments which absorb in the same region as the thiobarbituric acid–malondialdehyde complex (Draper & Hadley 1990). The fact that other aldehydic compounds other than malondialdehyde can react with thiobarbituric acid to form a complex that absorbs in the same region as the thiobarbituric acid–malondialdehyde complex has led to the term thiobarbituric acid-reactive substances (Draper & Hadley 1990). In this report we have addressed this and other problems with the assay, and have suggested a modified method that offers a reliable indication of lipid peroxidation. Our proposed method was tested using a biological application, where quinolinic acid, a known neurotoxin and free radical inducer (Southgate et al 1998; Southgate & Daya 1999; Stone 2000), was used to induce peroxidation of rat brain homogenate in-vitro.

Materials and Methods

Chemicals and reagents

Quinolinic acid, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane (98%), and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Corporation (St Louis, MO). Methanol (HPLC grade) was purchased from BDH Laboratory Supplies (Poole, UK). Trichloroacetic acid and resorcinol were purchased from Saarchem (PTY) Ltd (Krugersdorp, South Africa). IsoluteJ C_{18} solid-phase extraction (SPE) columns were obtained from International Sorbent Technology (Mid Glamorgan, UK).

Animals

Adult male Wistar rats (250–300 g) were housed in a controlled environment, with a 12-h light:dark cycle, with food and water freely available. The Rhodes University animal ethics committee approved the experiments.

Instrumentation

Samples were analysed on a modular, isocratic high-performance liquid chromatographic (HPLC) system. The chromatographic system consisted of a Spectraphysics Iso Chrom LC Pump, a Linear UVIS 200 Detector, and a Perkin Elmer 561 Recorder. Samples were introduced into the system using a Rheodyne fixed loop injector, fitted with a 20-μL loop. Evaporation of elution solvents following sample extraction was performed using an N-EVAP analytical evaporator.

Table 1 Assay validation data.

Concn malondialdehyde (nmol mL^{-1})	Relative standard deviation (%)	
	Intraday (n = 3)	Interday (n = 3)
5	3.03	3.38
10	1.64	2.11
20	1.43	1.64
25	0.96	0.90

Chromatographic conditions

Analytical separation was achieved using a C_{18} (Waters Spherisorb, 5 μm, 250 × 4.6 mm i.d.) column. Following injection, samples were filtered using an in-line 2-μm pre-column filter (Upchurch Scientific). The mobile phase composition for the analysis was 14% methanol in Milli-Q water and was degassed using a 0.45-μm membrane filter before use. The mobile phase flow rate was 1.2 mL min^{-1} and the chart speed on the recorder was 5 mm min^{-1}. The detector sensitivity was set at 0.05 aufs (absorbance units full scale) and the thiobarbituric acid–malondialdehyde complex was detected at 532 nm. The analytical procedure was validated by assessment of linearity of calibration (5–25 nmol mL^{-1}), repeatability, sensitivity, precision (Table 1), and limits of quantitation and detection. Resorcinol (0.05 mg mL^{-1} in water) was used as an external standard. It is important for each laboratory to validate and optimize the HPLC analytical procedure to suit their requirements before use.

Homogenate preparation

Rats were killed by cervical dislocation and the brains were rapidly excised and homogenized (10% w/v) with 0.1 M phosphate-buffered saline (PBS), pH 7.4. The homogenate was frozen in liquid nitrogen and stored at −70°C until needed. All samples were used within seven days of homogenate preparation.

Assay

Homogenate (1 mL) containing varying concentrations (0, 0.25, 0.5, 1 mM) of quinolinic acid was incubated in an oscillating water bath for 1 h at 37°C. At the end of the incubation period, 0.5 mL BHT (0.5 mg mL^{-1} in

methanol) and 1 mL trichloroacetic acid (15 % in water) were added to the mixture. The tubes were sealed and heated for 15 min in a boiling-water bath to release protein-bound malondialdehyde. To avoid adsorption of malondialdehyde onto insoluble protein, the samples were cooled and centrifuged at 2000 *g* for 15 min. Following centrifugation, 2 mL of the protein-free supernatant was removed from each tube and 0.5 mL thiobarbituric acid (0.33 % in water) was added to this fraction. The tubes were sealed and incubated in a boiling-water bath at acidic pH for 30 min. This step was not necessary when generating the calibration curve.

After cooling, thiobarbituric acid–malondialdehyde was separated from other possible thiobarbituric acid-reactive substances using an IsoluteJ C_{18} solid-phase extraction (SPE) column that was pre-washed with 2 mL methanol followed by 2 mL distilled water. The sample (1 mL) was loaded onto the column, which was subsequently washed with 2 mL distilled water. The thiobarbituric acid–malondialdehyde complex was eluted with 1 mL methanol. The methanol was then evaporated using an N-EVAP analytical evaporator at 60°C under a gentle stream of nitrogen. The residue (pink) was dissolved in distilled water (0.5 mL) containing 0.05 mg mL^{-1} resorcinol. These samples were analysed by HPLC as described above. The malondialdehyde levels were obtained from a calibration curve generated using 1,1, 3,3-tetramethoxypropane as described above. The ratio of the peak height of thiobarbituric acid–malondialdehyde to the peak height of resorcinol (external standard) was plotted against the concentration of malondialdehyde in the complex injected. Protein estimation was performed by the method described by Lowry et al (1951). Final results were expressed as nmol malondialdehyde (mg protein)$^{-1}$. The data were analysed statistically using the Tukey-Kramer multiple comparisons test. The level of significance was accepted at $P < 0.05$.

Results

The retention time for the thiobarbituric acid–malondialdehyde complex and the external standard (resorcinol) was approximately 2.4 and 6.5 min, respectively. Regression analysis showed that concentration and peak height ratio were linear over the range 5–25 nmol mL^{-1} ($r^2 = 0.998$). Each point on the calibration curve was based on triplicate determinations. The sensitivity of the method was evaluated by determining the lowest reproducible concentration of thiobarbituric acid–malondialdehyde detectable. The limit of detection was attained with samples containing 0.5 nmol mL^{-1} thiobarbituric acid–malondialdehyde and the limit of quantitation was 1 nmol mL^{-1}. The precision (intraday and interday) was reported as relative standard deviation (Table 1). The interday precision ranged from 0.96 % to 3.03 %, while the intraday precision ranged from 0.9 % to 3.38 % for the concentration range studied.

Table 2 Concentration-dependent effect of quinolinic acid on lipid peroxidation in rat brain homogenate.

Quinolinic acid concn (mM)	Malondialdehyde (nmol (mg protein)$^{-1}$)
0	0.67 ± 0.09
0.25	1.09 ± 0.09*
0.5	1.86 ± 0.14*
1.0	2.09 ± 0.05*

Values are the mean ± s.e.m. of triplicate determinations. *$P < 0.05$ compared with control, Tukey-Kramer multiple comparisons test.

As shown in Table 2, exposure of rat brain homogenate to various concentrations (0–1 mM) of quinolinic acid increased lipid peroxidation in a concentration-dependent manner.

Discussion

The thiobarbituric acid test (Gutteridge & Halliwell 1990) has many limitations. The main limitation to the colorimetric assay is the interference by other thiobarbituric acid reactants and pigments in the reaction mixture. These thiobarbituric acid-reactive substances inflate the malondialdehyde content of many materials. Therefore, the isolation of the thiobarbituric acid–malondialdehyde complex should be a standard part of the thiobarbituric acid method for malondialdehyde determination (Draper & Hadley 1990). Our proposed method involved the separation of the thiobarbituric acid–malondialdehyde complex from thiobarbituric acid-reactive substances using solid-phase extraction. This procedure was short, simple and cost effective as the extraction columns could possibly be used up to three times. In addition, our methanol evaporation procedure was carried out under nitrogen as opposed to air (Draper & Hadley 1990), thus preventing any further oxidation of the complex. The thiobarbituric acid–malondialdehyde complex was then isolated using a validated HPLC procedure. This method made use of

an external standard to which the thiobarbituric acid–malondialdehyde complex was compared, providing an accurate assessment of malondialdehyde levels in the material being analysed. In addition, the complex was detected in the visible range as opposed to other HPLC methods where spectrofluorometry is used (Londero & Lo Greco 1996; Fukunaga et al 1998). Thus, although the proposed method appeared to be equally sensitive to that of other HPLC methods with visible detection (Templar et al 1999), it allowed for purification of the thiobarbituric acid–malondialdehyde complex from extraneous substances, allowing less interference in the detection of lipid peroxidation.

Materials of animal origin usually contain large amounts of protein, to which malondialdehyde may be bound (Draper & Hadley 1990). It is therefore imperative to release the protein-bound malondialdehyde, achieved by adding trichloroacetic acid and heating the subsequent extract. This is more effective than some of the other methods used (Southgate & Daya 1999), where the extract is not heated (results not shown). It is also important to prevent adsorption of the thiobarbituric acid–malondialdehyde complex onto insoluble protein. This is facilitated by the removal of any solid material by centrifugation. Our method involved the use of PBS as the buffer in which the samples were homogenized. Other methods (Southgate & Daya 1999) use Tris-HCl buffer. Tris-HCl buffer is important to stabilize membranes, but by itself it is a hydroxyl radical scavenger. Yamato & Tang (1996) showed that Tris buffer (20 mM) reduced peroxidation levels by 15% compared with samples homogenized in distilled water. PBS does not scavenge free radicals (results not shown).

This modified method for the determination of lipid peroxidation was tested to determine whether it could be used to detect different levels of peroxidation. Rat brain homogenate was incubated with different concentrations of quinolinic acid, previously shown to increase lipid peroxidation (Southgate & Daya 1999). Quinolinic acid produced a marked increase in lipid peroxidation (Table 2). Therefore, the modified method can be used to determine lipid peroxidation in biological samples, with satisfactory results.

In conclusion, the method designed in our laboratory provided a more accurate assessment of lipid peroxidation than the conventional colorimetric method. The HPLC method was simple, cost effective and did not require fluorometric detection. The method satisfactorily reduced the sources of error to which the colorimetric method is prone. The procedure is suitable for tissue samples and can be modified depending on the material being analysed. The short retention time of the thiobarbituric acid–malondialdehyde complex also reduced total analysis time.

References

Draper, H. H., Hadley, M. (1990) Malondialdehyde determination as an index of lipid peroxidation. In: Packer, L., Alexander, A. N. (eds) *Methods in Enzymology*. 186, Academic Press, San Diego, pp 421–431

Fukunaga, K., Yoshida, M., Nakazono, N. (1998) A simple, rapid, highly sensitive and reproducible quantification method for plasma malondialdehyde by high performance liquid chromatography. *Biomed. Chromatogr.* **12**: 300–303

Gutteridge, J. M. C., Halliwell, B. (1990) The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem. Sci.* **15**: 129–135

Londero, D., Lo Greco, P. (1996) Automated high performance liquid chromatography separation with spectrofluorometric detection of a malondialdehyde–thiobarbituric adduct in plasma. *J. Chromatogr. A* **729**: 207–210

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 193–265

Ottino, P., Duncan, J. R. (1997) Effect of α-tocopherol succinate on free radical and lipid peroxidation levels in BL6 melanoma cells. *Free Radic. Biol. Med.* **22**: 1145–1151

Southgate, G. S., Daya, S. (1999) Melatonin reduces quinolinic acid-induced lipid peroxidation in rat brain homogenate. *Metab. Brain. Dis.* **14**: 165–171

Southgate, G. S., Daya, S., Potgieter, B. (1998) Melatonin plays a protective role in quinolinic acid-induced neurotoxicity in the rat hippocampus. *J. Chem. Neuroanat.* **14**: 151–156

Stone, T. W. (2000) Development and therapeutic potential of kynurenic acid and kynurenine derivatives for neuroprotection. *Trends Pharmacol. Sci.* **21**: 149

Templar, J., Kon, S. P., Newman, D. J., Raftery, M. J. (1999) Increased plasma malondialdehyde levels in glomerular disease as determined by a fully validated high performance liquid chromatography method. *Nephrol. Dial. Transplant.* **14**: 946–951

Volterra, A., Trotti, D., Tromba, C., Floridi, S., Racagni, G. (1994) Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. *J. Neurosci.* **14**: 2294

Yamato, H., Tang, H. (1996) Preventive effect of melatonin against cyanide-induced seizures and lipid peroxidation in mice. *Neurosci. Lett.* **207**: 89–92