Preface

Free radicals and reactive oxidizing agents were once ignored as biochemical entities not worth close scrutiny, but are now recognized as causes or contributing factors in dozens, if not hundreds, of disease states. In addition, free radical metabolisms of xenobiotics have become increasingly important to pharmacologists. Accordingly, the need has arisen to accurately quantify reactive oxygen species and their byproducts.

Methods in Biological Oxidative Stress is practical in scope, providing the details of up-to-date techniques for measuring oxidative stress and detecting oxidizing agents both in vitro and in vivo. The contributors are recognized experts in the field of oxidative stress who have developed novel strategies for studying biological oxidations.

The chapters of *Methods in Biological Oxidative Stress* cover widely used standard laboratory techniques, often developed by the authors, as well as HPLC–electrochemical measurement of protein oxidation products, particularly nitrotyrosine and dityrosine, and HPLC–electrochemical detection of DNA oxidation products. Additionally, recently developed techniques are presented to measure lipid oxidation and nitration products such as 5-NO_2-γ-tocopherol and isoprostanes, using HPLC-electrochemical/photodiode array methods and mass spectrometry as well as electron paramagnetic resonance (EPR) techniques.

In scope, presentation, and authority therefore, *Methods in Biological Oxidative Stress* was designed to be an invaluable manual for clinical laboratories and teaching institutions now conducting routine measurements of biological oxidants and biological oxidative stress or implementing new programs in this vital area of research. As a reference work, this collection of techniques and methods will prove useful for many years to come.

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Analysis of Aldehydic Markers of Lipid Peroxidation in Biological Tissues by HPLC with Fluorescence Detection

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1. INTRODUCTION

Increasing evidence supports a role for oxidative stress in the neuronal degeneration observed in a spectrum of neurological disorders including stroke, amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), head trauma, and Alzheimer's disease (AD) (reviewed in ref. *1*). Of particular interest is the role of lipid peroxidation and the aldehydic by-products of lipid peroxidation in the pathogenesis of neuron degeneration in these diseases. Peroxidation of lipids leads to aldehyde formation including C_3–C_{10} straight-chain aldehydes and a series of α,β-unsaturated aldehydes including acrolein and 4-hydroxynonenal (HNE). Although the straight-chain aldehydes have no discernable toxicity, acrolein and HNE are neurotoxic and could potentially play a role in the pathogenesis of AD among other diseases. The most common methods of measuring lipid peroxidation center around measurement of aldehydic by-products, including the use of ultraviolet (UV)-Vis spectrometry to measure the heat mediated-condensation products of aldehydes with thiobarbituric acid in the thiobarbituric acid-reactive substances (TBARs) assay. Although this method will provide an overall measure of aldehyde levels, including malondialdehyde (MDA), it is plagued with interferences from nonlipid derived aldehydes from sugars, amino acids, and DNA, and species resulting from chemical interaction of thiobarbituric acid with nonlipid molecules during the assay *(2)*. Another method that allows measurement of aldehydes is through the use of high pressure liquid chromatography (HPLC) for separation and UV-Vis or fluorescence detection to

analyze the individual aldehydes. For use with UV-Vis, aldehydic by-products are reacted with 2,4 dinitrophenylhydrazine (2,4 DNPH), whereas detection by fluorescence uses derivatization with 1,3 cyclohexanedione. Comparison of the two detection methods indicates that use of HPLC with fluorescence detection provides the greatest sensitivity and detection limits for aldehydic markers of lipid peroxidation.

2. MATERIALS

1. HEPES buffer, pH 7.4, containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH$_2$PO$_4$, 0.6 mM MgSO$_4$, (0.7 µg/mL) pepstatin, (0.5 µg/mL) leupeptin, (0.5 µg/mL) aprotinin, and (40 µg/mL) phenylmethylsulfonyl fluoride (PMSF) for tissue homogenization.
2. 1.43 µM heptanal in HPLC-grade methanol (internal standard).
3. Cyclohexanedione reagent consisting of 10 g ammonium acetate, 10 mL glacial acetic acid, and 0.25 g 1,3 cyclohexanedione dissolved in 100 mL distilled/deionized water.
4. C$_{18}$ Sep-Pak Plus solid-phase extraction columns preconditioned with 10 mL HPLC-grade methanol followed by 10 mL distilled/deionized water.
5. HPLC-grade chloroform.
6. Dual-pump HPLC system equipped with a C$_{18}$ analytical column and a fluorescence detector operated at an excitation wavelength of 380 nm and an emission wavelength of 446 nm.
7. HPLC-grade tetrahydrofuran (THF) and filtered distilled/deionized water both thoroughly degassed.
8. Pierce bicinchoninic (BCA) or Lowry protein-detection kit.
9. Tissue samples for aldehyde analysis should be frozen immediately after autopsy and be stored at –80°C.

3. METHODS

1. Tissue homogenization: 100 mg frozen tissue is homogenized in 5 mL N$_2$-purged HEPES buffer using a modified Potter-Elvehjem motor-driven homogenizer or a chilled Dounce homogenizer. The homogenization is carried out on ice and all samples are maintained on ice. Use of 5 mL of homogenization buffer allows a large enough volume for enzyme assays from the same sample of tissue.
2. The method of aldehyde determination is that of Yoshino et al. *(3)* with modification *(4)*. Duplicate 500 µL aliquots of homogenate are added to glass test tubes (to prevent leaching of potential interferences from plastic) along with 500 µL 1.43 µM heptanal in HPLC-grade methanol. Heptanal was chosen as an internal standard because there is no detectable heptanal present in samples analyzed. The samples are vortexed for 30 s to extract aldehydes from the tissue homogenate and are centrifuged at 850g for 10 min. Aliquots (20 µL) of

    tissue homogenate are taken for protein content determination using the Pierce BCA method (Sigma).

3. After centrifugation, 500 µL of supernatant is mixed with 1 mL of 1,3 cyclohexanedione reagent and heated 1 h in a 60°C water bath.

4. After cooling to room temperature, 1 mL of the reaction mixture is added to preconditioned Sep-Pak C_{18} solid-phase extraction columns using virgin 1- mL plastic syringes.

5. The columns are washed with 2 mL distilled/deionized water to remove excess ammonium acetate and the derivatized aldehydes are eluted with 2 mL HPLC-grade methanol.

6. The samples are evaporated to dryness using a speed-vac or freeze-dryer.

7. Samples are dissolved in 1 mL HPLC-grade chloroform and centrifuged at 800*g* for 5 min to pellet any remaining ammonium acetate.

8. An 800-µL aliquot of the supernatant is removed and evaporated to dryness.

9. Before HPLC analysis, the residue is redissolved in 500 µL 50/50 HPLC grade methanol/water and 250 µL subjected to HPLC analysis using a dual-pump system equipped with a C_{18} analytical column. The elution conditions are 10:90 THF/water to 40:60 THF/water from time 0–30 min followed by 40:60 THF/water to 100% THF from 30–40 mins. THF (100%) is maintained for 5 min and initial elution conditions are reestablished from 46–49 min.

10. Detection of derivatized aldehydes is via fluorescence detection at an excitation wavelength of 380 nm and an emission wavelength of 446 nm.

11. Quantification of aldehyde levels is based on comparison of the peak area of interest to the peak area of heptanal (internal standard).

12. Identification of chromatographic peaks is by comparison to chromatograms of authentic standard compounds.

13. Results of the analyses are calculated as nmol aldehyde/mg protein, based on initial protein content measurements.

4. DISCUSSION

The elution conditions described previously are a modification of those used by Yoshino et al. *(3)* and allow the separation of straight chain and α,β-unsaturated aldehydic by-products of lipid peroxidation in biological samples. Fig. 1 shows a representative chromatogram of a mixture of standards including propanal, butanal, pentanal, hexanal, heptanal, and HNE and demonstrates adequate chromatographic separation of the individual aldehydes. Minimum detection limits of HNE are approx 0.1 pmol with shot to shot reproducibility of a standard HNE solution of 3–5%. The signal-to-noise ratio is on the order of 50 for standard solutions of HNE. Generally there are no significant interferences observed using this method, provided glass test tubes and HPLC-grade solvents are used for the derivatization reactions and chromatography. It is possible that fluorogenic compounds may be leached from plastics if used during derivatization. Based on the
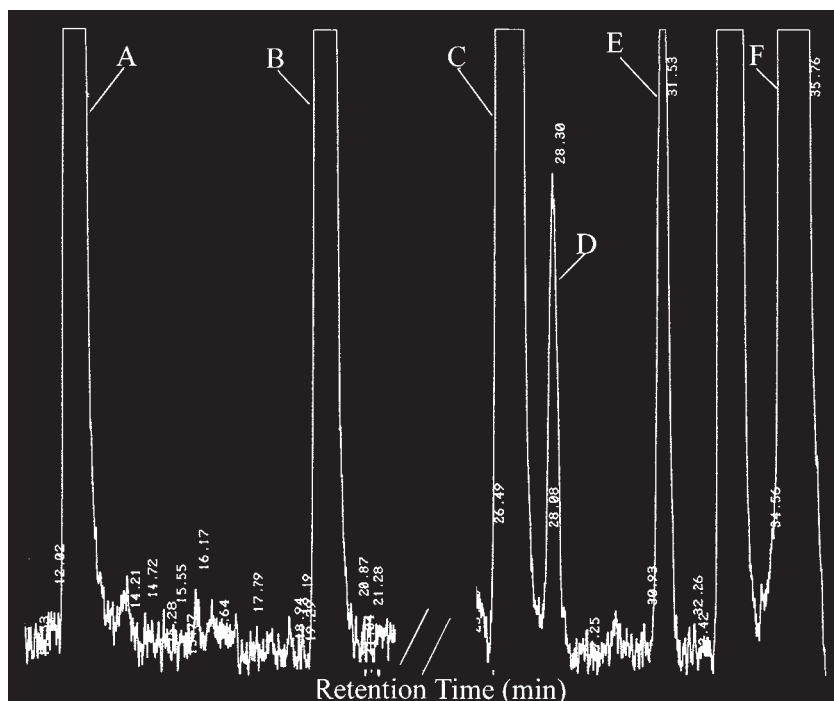
Fig. 1. Representative HPLC of a mixture of standard aldehydes. *A* Propanal. *B* Butanal. *C* Pentanal. *D* 4-hydroxynonenal. *E* Hexanal. *F* Heptanal.

extraction procedure described earlier, it is likely that the results of the assay reflect levels of both free and protein-bound aldehydes.

Another potential problem associated with the use of HPLC with optical detection is the coelution of other compounds with the aldehydes of interest. Because the derivatization process is dependent on the reaction between the aldehydic group and 1,3 cyclohexanedione (Fig. 2), it is unlikely that other compounds without aldehydic groups would be derivatized. Additionally, the possible aldehydic products are well-separated as shown in Fig. 1.

Overall, HPLC with fluorescence detection provides an effective analytical approach for the measurement of levels of the aldehydic by-products of lipid peroxidation.
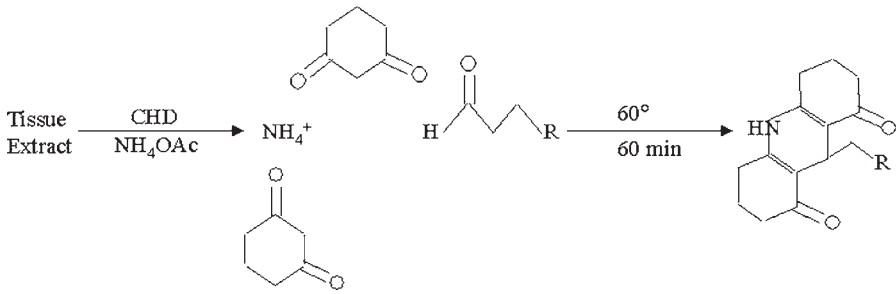
ACKNOWLEDGMENTS

Fig. 2. Reaction of 1,3 cyclohexanedione with a generic aldehyde.

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