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Note

Measurement of hydroperoxydocosahexaenoic acid in rat brain homogenates by reversed-phase high-performance liquid chromatography

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Free radical induced oxidation of polyunsaturated fatty acids has been implicated in the pathogenesis of tissue changes related to aging[1,2] and to trauma[3] of the nervous system. Because of their instability, the peroxides produced during this process have eluted direct quantitation. Docosahexaenoic acid (DHA), a polyunsaturated fatty acid, appears to be a candidate for oxygenation in the nervous sytem[4] and hydroperoxydocosahexaenoic acid (HPDHA) might be expected to accumulate in nervous tissue under oxidative stress. In this study, we have prepared HPDHA from DHA using soybean lipoxygenase, established its high-performance liquid chromatography (HPLC) characteristics and determined its recovery from brain homogenates. Then, we have attempted to measure the amount of HPDHA in homogenates prepared from rapidly frozen rat brains before and after a 60-min incubation at 37°C in the presence of room air, with or without the addition of the antioxidant butylated hydroxytoluene (BHT), or in the presence of nitrogen. Since HPDHA may be converted into hydroxydocosahexaenoic acid (HDHA), either in tissue or during isolation, we included this compound also in our studies.

MATERIALS AND METHODS

Soybean lipoxygenase [E.C. 1.13.11.12] was obtained from Sigma, St. Louis, MO, U.S.A. Sep-Pak™ silica cartridges were obtained from Rainin, Woburn, MA, U.S.A. and BHT, gold label, was purchased from Aldrich, Milwaukee, WI, U.S.A. Acetonitrile, hexane and water for HPLC were bought from B&J Minneapolis, MN, U.S.A. Reagent-grade glacial acetic acid and ammonium hydroxide, HPLC-grade phosphoric acid and isopropanol, and sodium borohydride were purchased from Fisher Scientific, Pittsburgh, PA, U.S.A. DHA was purchased from Nu Check Prep, Elysian, MN, U.S.A. HPDHA and HDHA were prepared by the method of Hamberg and Samuelsson[5] by using soyban lipoxygenase and purified by using the HPLC system described below. The purified HPDHA and HDHA were analyzed by gas chromatography–mass spectrometry (GC–MS) by using the procedure described previously[6]. Briefly: HPDHA and HDHA, collected during HPLC, were methylated with diazomethane and treated with hydrogen over platinum oxide. Trimethylsilyl derivatives of the reaction products were then analyzed by GC–MS on a Kratos MS-25

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instrument in the electron impact mode at 70 eV. Samples were separated on a 30 m × 0.32 mm I.D. fused-silica capillary column coated with a 0.25-μm film of DB-5. The column, operated in the split mode, was programmed from 200 to 280°C at 10°C/min.

Twelve-month-old, male, S/D retired breeder rats, weighing between 570 and 600 g (obtained from Harlan/Sprague-Dawley, Indianapolis, IN, U.S.A.) were decapitated into liquid nitrogen. Brains were removed while frozen and homogenized in 9 vol. equiv. of 0.05 *M* Tris–HCl. Lipids were extracted from 2 ml of the homogenates with 36 ml of hexane–isopropanol (3:2, v/v) containing 0.005% BHT, either immediately or after incubation for 1 h at 37°C in the presence of room air. In some experiments BHT (0.05%) was added to the homogenates before incubation; in others, nitrogen was substituted for room air.

Free fatty acid fractions were isolated from the hexane–isopropanol extracts by using silica Sep-Pak (TM) cartridges as previously described[7]. Isolated free fatty acid fractions were suspended in 100 μl of methanol and 10-μl aliquots were injected onto a 150 × 4.6 mm I.D. Econosphere™ 5-μm C_{18} cartridge (Alltech, Avondale, PA, U.S.A.). HPLC separations were performed on a Beckman chromatography system including a Model 167 variable-wavelength detector and a Model 506 autosampler. Two Model 110B pumps with a Model 406 analogue interface provided a concave (curve No. 5) gradient from 48 to 100% acetonitrile in 0.1% phosphoric acid over a period of 15 min post injection. The mobile phase was then held at 100% acetonitrile for an additional 5 min, returned to 48% in 2 min and allowed to equilibrate for 3 min before injecting the next sample. Data were collected and analyzed by using the System Gold (TM) software package on an IBM PS/2 Model 30 microcomputer. HPDHA and HDHA were detected at 235 nm. Peak areas were used for quantitation. A standard curve based on varying amounts (10–75 ng) of HPDHA injected onto the column and the corresponding peak areas at 235 nm were constructed (Fig. 1). The coefficient of determination for this curve was 0.98. For recovery experiments, since higher amounts of HPDHA (8.8 μg) were added to brain homogenates, the curve was extended to include amounts up to 216 ng of HPDHA injected onto the column. This curve had a coefficient of determination of 0.99. These curves were then used for quantitation of HPDHA in extracts from homogenates, and the results expressed as μg HPDHA per

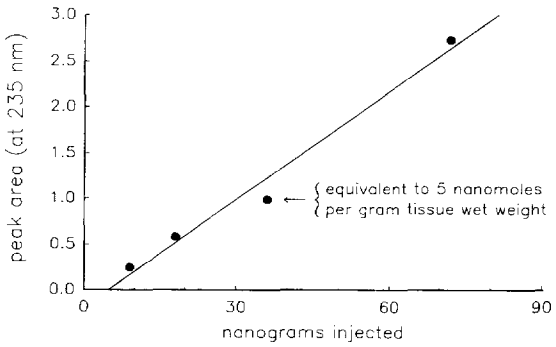

Fig. 1. HPDHA standard curve. Peak areas of absorbance at 235 nm produced by various amounts of HPDHA are plotted against the amount injected. The coefficient of determination for this curve was 0.98.

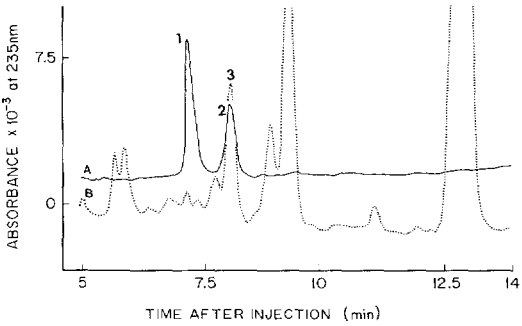

Fig. 2. Chromatograms, monitored at 235 nm of (A) HPDHA and HDHA standards, equivalent to 1.8 and 1.7 μg per gram tissue, wet weight, respectively and (B) the free fatty acid fraction isolated from rat brain homogenate after incubation in the presence of room air for 1 h at 37°C. Peaks: 1 = HDHA; 2, 3 = HPDHA.

gram of tissue, wet weight. This method could detect >0.5 μg of HPDHA per gram wet weight.

RESULTS

A representative HPLC separation of the HPDHA and HDHA standards prepared as described above and used in this study is shown in Fig. 2, tracing A. Under the described conditions adequate baseline separation of the two compounds was achieved. Since these standards were prepared in this laboratory, their identity was verified by GC–MS. The respective peaks were isolated by using HPLC, methylated and hydrogenated. Trimethylsilyl derivatives of these products behaved identically during GC–MS. The fragmentation pattern indicated that the common endproduct was 17-hydroxymethyldocosanoate.

For recovery experiments, 8.8 μg of HPDHA were added to 2 ml of 10% rat brain homogenate and the mixture kept on ice for 10 min prior to extraction of the lipids and their fractionation as described above. The recovery of HPDHA added directly to rat brain homogenate was 78%.

The free fatty acid fraction isolated after rat brain homogenates had been incubated for 1 h in the presence of room air showed a peak corresponding to HPDHA (Fig. 2, tracing B). No HPDHA was found before incubation. Homogenates incubated in the presence of room air for 1 h contained 3.1 ± 0.4 ($n = 5$) μg HPDHA per gram of tissue, wet weight. Homogenates incubated for 1 h in the presence of nitrogen or BHT did not contain any HPDHA. None of the samples studied contained any HDHA.

DISCUSSION

Fatty acid hydroperoxides have been considered either unstable[8] or to be immediately reduced after formation *in vivo*[9]. We[7] and others[10,11] found HPDHA to be sufficiently stable to permit its analysis by HPLC. In our previous study[7], where HPDHA (1 mg/ml) was added to brain lipid extracts, the recovery of HPDHA was determined to be 87%. The high extinction coefficient of HPDHA at 235 nm permits

the accurate quantitation of smaller amounts. We now show that the recovery of 8.8 μg HPDHA added to rat brain homogenates before the lipids were extracted is 78%. The somewhat lower recovery seen in the present study could be related to the smaller amounts of HPDHA used.

Our findings would then suggest that RPHPLC could be an important tool for quantitating small amounts of HPDHA. This tool could facilitate studies wherein brain homogenates are investigated for their ability to produce one oxidation product of DHA, namely HPDHA, during oxidative stress.

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