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Note

One-step separation of free fatty acids and phospholipids in brain tissue extracts by high-performance liquid chromatography

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The accumulation of free fatty acids (FFA) and the degradation of phospholipids are among the characteristics found in injured and edematous brains [1–5]. In order to study the mechanisms involved, a high-performance liquid chromatographic (HPLC) method which can separate and monitor FFA and various phospholipids simultaneously would be very useful. Although there are a number of studies describing the separation of phospholipid classes by HPLC [6–12], a suitable method cannot be found that would also allow one to monitor FFA simultaneously.

Phospholipids and FFA can be detected in the low UV region, mainly by the presence of their isolated double bonds. There are basically two UV transparent solvent systems developed for the separation of phospholipids: one is the acetonitrile—methanol—water system first developed by Jungawala et al. [6]; the other is the hexane–isopropanol—water system developed by Geurts van Kessel et al. [7]. In both systems, FFA eluted near the solvent front together with neutral lipids (NL) and cholesterol. In the acetonitrile–methanol–water system, the phosphatidylinositol (PI) peak is very close to the solvent front where FFA elute, and further separation is difficult to achieve. Thus we chose to modify the hexane–isopropanol–water system.

Schlager and Jordi [8] have separated a number of species of fatty acids, triglycerides and cholesterol using the hexane—isopropanol—water system. However, this system was difficult to adopt owing to the use of three solvent systems, the problem with immiscibility of hexane with the final solvent mixture, and a large shift in the baseline.

Previously we employed a hexane–isopropanol–water system and were able

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to detect a FFA peak close to the NL region [13]. Although the method is routinely used in our laboratory for quick monitoring of FFA and phospholipid changes, we find that the FFA peak is often contaminated with a small amount of NL as determined by thin-layer chromatography (TLC). In this paper, we describe a modification of the method that allows one to completely separate FFA from NL with simultaneous separation of major phospholipids. The application of this method to monitor phospholipase A_2 (PLA_2) activity, which was hypothesized as a key element in the development of brain injury and edema [14], is also described.

EXPERIMENTAL

Chemicals and reagents

Phospholipid standards of bovine brain origin, fatty acids, cholesterol and cholesterol esters, bee venom phospholipase A_2 were all obtained from Sigma (St. Louis, MO, U.S.A.). Phosphatidylinositol of bovine liver was obtained through Calbiochem-Behring (San Diego, CA, U.S.A.). Neutral lipids and fatty acid methyl esters were purchased from Supelco (Bellefonte, PA, U.S.A.). 5-Hydroxy-6,8,11,14-eicosatetranoic acid (5-HETE) was a gift from Dr. J. Pike of Upjohn (Kalamazoo, MI, U.S.A.). Labelled lipid standards were from New England Nuclear (Boston, MA, U.S.A.). Solvents were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Distilled water was filtered through a Millipore Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Silica gel H plates were purchased from Analtech (Newark, DE, U.S.A.).

High-performance liquid chromatography

Standards or tissue lipid extracts were separated by a Hewlett-Packard 1082 B liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) using a prepacked silica column of 5 μm particle size (Micro-Pak Si-5, 30 cm $\times$ 4 mm I.D., Varian Assoc., Palo Alto, CA, U.S.A.). The neutral lipids, free fatty acids and various phospholipids were separated by a stepwise gradient containing 1, 4 and 9% water in hexane—isopropanol (3:4). The gradient began with an initial mobile phase of 1% water in hexane—isopropanol and was increased to 4% at 10 min and to 9% at 20 min. The gradient was maintained at 9% for another 10 min and was returned to 1% afterwards. The flow-rate was maintained at 1 ml/min. The elution was monitored at 206 nm with recorder response set at 0.6 absorbance units full scale (a.u.f.s.).

The peaks eluted from the HPLC system were collected and rechromatographed by TLC for further identification. HPLC fractions along with standards were applied to 250-μm silica gel H plates and chromatographed with the following solvent systems: heptane–diethyl ether–formic acid (90:60:4) for neutral lipids and fatty acids [15] and chloroform–methanol–water (65:35:5) for phospholipids. Iodine vapor and 2,7-dichlorofluorescein spray were used as the method of detection.

Fatty acid analysis

The FFA eluted from the HPLC system were methylated with 5% hydrochloric acid in methanol and subsequently analyzed by gas–liquid

chromatography (GLC) (Hewlett-Packard Model 5830A) using a fused-silica capillary column (SP 2330, 30 m × 0.25 mm I.D., Supelco). The operation and the quantitative procedure were as described previously [5]. A known concentration of heptadecanoic acid (17:0) was routinely added to the homogenates as an internal standard.

Preparation of tissue extracts

Rat brain cortical slices were homogenized in Krebs–Ringer buffer (50 mg wet weight per ml) with a tissue homogenizer (Kontes, San Leandro, CA, U.S.A.) for 1 min at 4°C. The homogenate (2.5 mg/ml protein) was transferred to a 37°C bath and treated with PLA_2 (18 U/ml) for various time intervals. At the end of the incubation periods, a 1-ml aliquot was removed from each sample and extracted with 4 ml of chloroform–methanol (2:1) [16]. The lower phase was dried under nitrogen and resuspended in the HPLC starting solvent. Alternatively, extraction of samples using hexane- isopropanol (3:2) [17] produced similar results.

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC separation of a standard mixture of FFA (palmitic acid, arachidonic acid, docosahexaenoic acid), NL (trilinolein, diolein and monostearin) and phospholipids [phosphatidylethanolamine (PE), phospatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM)]. NL and FFA elute separately as single cluster peaks. NL elute near the solvent front, at 2.8 min. FFA elute between NL and PE at 9–10 min. The absorbance of the FFA peak is primarily due to the unsaturated double

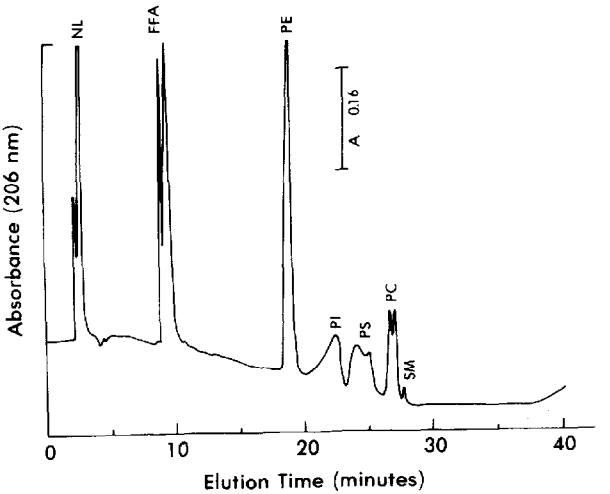

Fig. 1. HPLC separation of lipid and fatty acid standards. Chromatographic conditions are as given in Experimental. UV absorption was monitored at 206 nm at 0.6 a.u.f.s. The amounts of the lipid and free fatty acids injected into the column were: PE, 23 μg; PI, 8 μg; PS, 7 μg; PC, 23 μg; SM, 2 μg; trilinolein, 0.4 μg; diolein, 0.4 μg; monostearin, 0.4 μg; palmitic acid, 0.3 μg; arachidonic acid, 0.2 μg; docosahexaenoic acid, 0.4 μg. The gradient starts at 1% water in hexane–isopropanol and reaches 9% at the 20-min mark.

bonds of arachidonic acid (20:4) and docosahexaenoic acid (22:6). Palmitic acid (16:0) at 0.3 μg concentration contributes less than 1% of the absorbance of these peaks. The elution of palmitic acid and arachidonic acid in the same region was further confirmed by the use of radioisotopes. Over 98% of [^{14}C]-palmitate and [^{3}H]arachidonic acid was recovered between 9 and 10 min. The recovery of NL was close to 100%.

The elution times for the major phospholipids are: PE, 19.00 min; PI, 22.50 min; PS, 24.06 min; PC, 26.71, 27.07 min; SM, 27.72 min. Lysophosphatidylethanolamine (LPE) elutes between PI and PS at 23.10 min and lysophosphatidylcholine (LPC) elutes towards the end of the program at 37.68 min (not shown). Phosphorus determination [18] indicated that the recoveries for PE, PI, PS, PC and LPE were around 96, 99, 87, 94 and 100%, respectively. Recovery for labeled LPC was close to 98%.

The water content of the starting solvent strongly influences the retention time of the lipid fractions (Fig. 2), with an increase in the initial solvent water content decreasing the retention time. FFA peaks shift significantly from 9–10 min to less than 3 min when the initial solvent water content is changed from 1 to 5%, suggesting that FFA probably co-eluted with NL and cholesterol in other HPLC systems [7, 8].

The initial solvent water content has very little influence on the mobility of PC and SM, with their elution times toward the end of the water gradient at 9%. The elution times of PS vary within a 3-min interval. PI and PE elute

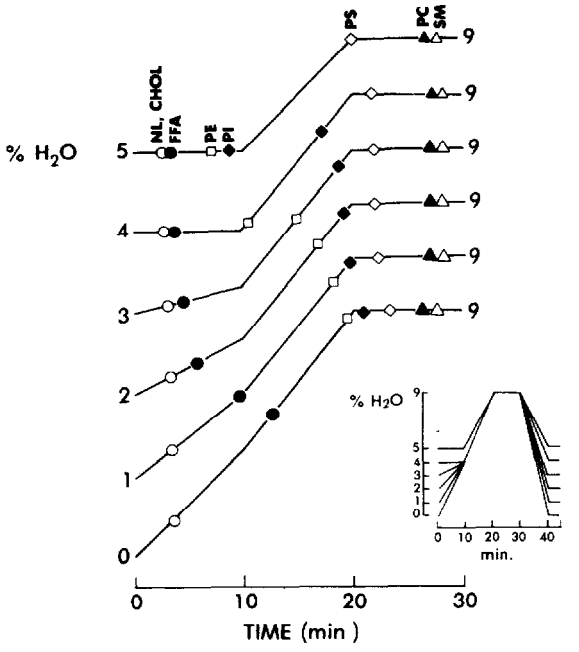

Fig. 2. Effect of initial solvent water content on lipid elution. Conditions were the same as in Fig. 1 except that the initial solvent water content was varied between 0 and 5%. Upper graph shows the changes in elution pattern of various lipids. (○) NL, cholesterol (CHOL); (●) FFA; (□) PE; (◆) PI; (◇) PS; (▲) PC; (△) SM. The insert shows the various gradient schemes used.

considerably earlier when the initial water content of the gradient is changed from 1 to 5%.

The program with an initial solvent water content of 1% was chosen because it appears that the free fatty acids were clearly separated from neutral lipids and phospholipids. In this system, cholesterol, and cholesterol esters elute at 2.4 min, arachidonic acid methyl esters at 3 min, cerebrosides elute between 4 and 6.5 min, cardiolipin comes out at 12.5 min, 5-HETE at 13.9 min, prostaglandins at 15—16 min, and phosphatidic acid at 24 min.

The enzyme PLA_2 is known to hydrolyze the polyunsaturated acyl moieties of phospholipids. Fig. 3 shows the HPLC profiles of PLA_2-treated brain membrane extracts. After 1 min of exposure to PLA_2, an abrupt increase of the FFA peaks (further confirmed as FFA by TLC) was observed, followed by gradual increases after 5 and 30 min treatment. The integrated areas of both PE and PC were significantly decreased at 1 min and 5 min. At 30 min, only a very small integrated area was observed for both PE and PC in the lipid elution profile. Furthermore, PS and PI were also completely degraded at 30 min suggesting the wide selectivity for phospholipids of bee venom PLA_2. The detection of LPE and LPC in the chromatogram further indicates the hydrolysis of PE and PC by PLA_2.

The increased absorbance at 206 nm of FFA after PLA_2 treatment was

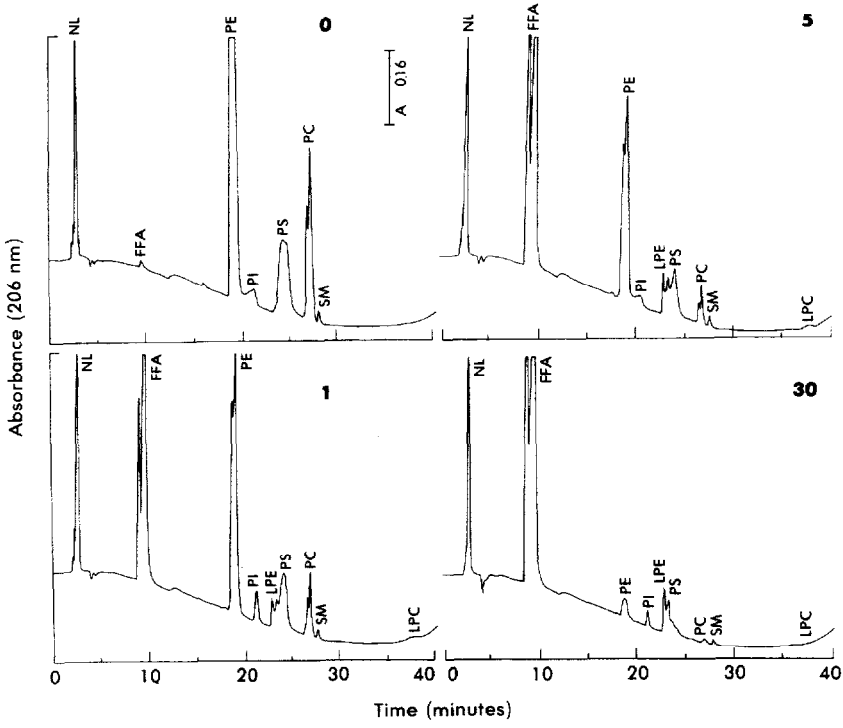

Fig. 3. HPLC separation of lipid extracts from brain homogenates treated with phospholipase A_2. Brain homogenates were prepared from rat cerebral cortex, treated with phospholipase A_2 for 0, 1, 5, 30 min and extracted as described in Experimental. Each chromatogram represents lipid extracts from 0.1 mg of membrane protein. Notice the appearance of LPE between PI and PS and LPC towards the end of the program.

TABLE I

EFFECTS OF PHOSPHOLIPASE A_2 ON FREE UNSATURATED FATTY ACID CONTENT OF BRAIN HOMOGENATES MEASURED BY HPLC AND GLC

The units for total unsaturated fatty acids were nmol per mg protein. These values represent the sum of the amount of 18:1, 20:4, and 22:6. The integrated area of free fatty acids was averaged from the HPLC profile of four different experiments (mean ± standard error of mean).

Incubation time (min)	Total unsaturated fatty acids		Integrated peak area × 10^3	
	Content	Increase (fold)	Content	Increase (fold)
0	0.32	—	24.3 ± 2.3	—
1	40.5	126	2634 ± 64	108
5	52.1	162	3856 ± 64	158
30	90.0	281	6042 ± 130	248

time-dependent. This increase was primarily due to the release of endogenous unsaturated fatty acids, i.e., oleic acid (18:1), arachidonic acid (20:4), and docosahexaenoic acid (22:6) as determined by GLC. Saturated palmitic acid (16:0) was increased moderately during the time course studies. Another saturated fatty acid, stearic acid (18:0) was not affected. The total unsaturated fatty acids increased 126-, 162- and 281-fold from control level at 1, 5, and 30 min, respectively, as determined by GLC (Table I). These increases are in accord with the increases of the integrated area of the free fatty acids detected at 206 nm in HPLC. The later was increased 108-, 158- and 248-fold, respectively, at 1, 5, and 30 min after the treatment of PLA_2. These data indicate the increased absorption in FFA was mainly due to the accumulation of unsaturated fatty acids. Thus, the HPLC chromatogram can usually give a first hand indication of changes in the lipid profile, particularly of the release of unsaturated FFA. However, the final quantitation of FFA and phospholipids requires collection of the HPLC eluent and subsequent analysis by GLC and by phosphorous determination.

Using the HPLC technique described, we were able to successfully monitor the changes in FFA and phospholipids in cold injured, free-radical treated and ischemic brains (data not shown). This study thus provides a simple tool to follow the FFA and phospholipid changes in injured brain and will be helpful in elucidating the mechanisms involved.

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