The High-Performance Liquid Chromatographic Analysis for the Peroxidized Phospholipids in Equine Erythrocytes and Skeletal Muscle

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(Received 16 January 1991/Accepted 30 March 1991)

KEY WORDS: horse, HPLC, phospholipid peroxidation.

The exercise-induced hemolysis and acute myopathy so-called "tying-up syndrome" in horses have been thought to be related to the free radical mediated lipid peroxidation [3, 5]. The phospholipid peroxidation in cell membranes would induce the alteration in their fluidity and permeability [11] as well as the degradation of membrane-bound proteins [10].

Since the phospholipid bilayers of cell membrane are maintained by the heterogenous phospholipid classes, the peroxidized species in each phospholipid class should be determined to understand the in vivo oxidative changes in membranes.

For the determination of the oxidative changes, several high-performance liquid chromatographic (HPLC) methods have been introduced [7, 8, 18, 14, 20, 22]. However, these methods were used in the analysis of foodstuffs and not of tissue materials. Therefore, one of the HPLC methods, reported by Terao et al. [20], was modified to make it possible to determine the peroxidized phospholipids in the equine erythrocytes and skeletal muscle.

Blood samples were collected from the jugular vein in 4 clinically healthy adult thoroughbred horses. Erythrocytes were prepared as described in the previous report [15]. The tissue specimen of the middle gluteal muscle in 5 cm depth from the skin was biopsied from the same horses by using a percutaneous needle as reported by Snow and Guy [18]. A 300 μl of packed erythrocytes or 100–150 mg of tissue specimens were extracted by the Bligh-Dyer method [2]. A 2.5 ml of Bligh-Dyer’s chloroform phase obtained was filtrated with a membrane filter (DISMIC-13J, Advantec, Tokyo, Japan) and stocked at −80°C until the assay with 0.1% (w/v) tert-butylhydroxytoluene (Sigma, St. Louis, MO, U.S.A.).

The detection of the peroxidized phospholipids was carried out by using a model L-6200 HPLC system (Hitachi, Ltd., Tokyo, Japan) with a 4.6 mm × 25 cm silica gel column (Ultrasphere Si, Beckman, San Ramon, CA, U.S.A.). The column temperature was held at 35°C in a model CTO-2A column oven (Shimazu, Kyoto, Japan). For the separation of phospholipid classes, a gradient elution [16] was employed with a slight modification. Briefly, 1% water in acetonitrile was eluted isocratically for the first 10 min and then eluted with a linear gradient from 1 to 17% water in acetonitrile for 15 min. After that 17% of water in acetonitrile was held constant for 15 min. The flow rate was maintained at 1.2 ml/min. A 20 μl of the sample solution was applied to the column with a manual loop injector (RHEODYNE 7125, Cotati, CA, U.S.A.) and the eluates were monitored at 233 nm and 206 nm with model L-4200 and L-4000 variable-wavelength detectors (Hitachi, Ltd., Tokyo, Japan), respectively. The chromatograms were recorded with 2 model C-R1A data processors (Shimadzu, Kyoto, Japan). Finally, the ratio of peak areas (A233 nm/A206 nm) of each phospholipid class was calculated, since the peak-ratios showed a good linear relationship with the iodometric-determined peroxide values in the PE- and PC-standards [22]. For the re-equivalence of the column, 60 ml of 1% water in acetonitrile was eluted. Each phospholipid class was identified by the co-chromatography with the standards (phosphatidylinositol; PI, P-2517, bovine liver, phosphatidylserine; PS, P-7769, bovine brain, phosphatidylethanolamine; PE, P-9137, bovine brain, phosphatidylincholine; PC, P-5763, egg yolk, sphingomyelin; SPM, S-7004, bovine brain; Sigma, St. Louis, MO, U.S.A.). All solvents used in this study were commercial HPLC grades (Wako Pure Chemical, Osaka, Japan).

The typical chromatograms of the phospholipids in the equine erythrocytes and skeletal muscle, recorded at 206 nm and 233 nm, are shown in Figs. 1 and 2, respectively. Five phospholipid classes, PI, PS, PE, PC and SPM, were detected at 206 nm in the erythrocytes. However, only 2 classes, PE and PC, were detected in the skeletal muscle, that might resulted from the relatively higher concentration of PE and PC than the other phospholipid classes in the skeletal muscle. The peroxidized species, recorded at 233 nm, were detected with almost the same retention times to those of the corresponding phospholipid classes recorded at 206 nm. The SPM in the erythrocytes was not detected at 233 nm. The reproducibility of the retention times of each phospholipid class was shown in Table 1. The adequate reproducibility was also shown in the ratio of peak areas (A233 nm/A206 nm) from PI, PS, PE and PC in the erythrocytes (the ratio of peak area: 1.0×10⁻¹, 8.8×10⁻¹, 8.9×10⁻¹ and 8.2×10⁻¹, coefficient of variance: 6.1%, 7.8%, 3.9% and 3.2%, respectively).

The normal-phase gradient-HPLC system [16] used in this study seemed to be the most competent method for the separation of phospholipid classes. Although the normal-phase HPLC methods with the isocratic elution of
Fig. 1. HPLC patterns of equine erythrocyte phospholipids simultaneously detected at 206 nm (top) and 233 nm (bottom). PI; phosphatidylinositol, PS; phosphatidylserine, PE; phosphatidylethanolamine, PC; phosphatidylcholine, SPM; sphingomyelin.

Fig. 2. HPLC patterns of equine muscular phospholipids simultaneously detected at 206 nm (top) and 233 nm (bottom). PE; phosphatidylethanolamine, PC; phosphatidylcholine.

The separation of phospholipid classes, it was highly difficult to resolve the PI from the solvent front [4, 6, 9, 12]. In addition, the acid solvents would degrade the phospholipids, especially the alkenyl ether phospholipids (plasmalogens) [12] which were contained in the tissue with high electrical activity such as nerves and muscles [1].

The selective detection of the peroxidized phospholipids used in the HPLC methods was based on the ultraviolet (UV) absorbance of conjugated dienes [20, 22], the electrochemical properties of hydroperoxides [8], the post-column iornithocyanate reaction with hydroperoxides [14], or the post-column chemiluminescent reaction between hydroperoxides and a peroxidase [7, 13, 21]. The UV detection seemed to be the most available method which was accompanied by neither troublesome handling for the post-column reagents nor highly expensive equipment like the electrochemical- or chemiluminescence-detector.

The absorption at 233 nm and 203–210 nm was reflected by the conjugated dienes indicating peroxidized phospholipids and the nonspecific double bonds indicating phospholipids, respectively [20, 22]. In the present study, the absorbance of eluates was simultaneously monitored at 233 nm and 206 nm with 2 UV-detectors, and the ratio of

| Table 1. Retention time of each phospholipid class and the reproducibility |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|
|                            | 206 nm      |             | 233 nm      |             |
|                            | RT(min)a    | CV(%)b      | RT(min)     | CV(%)b      |
| Phosphatidylinositol       | 21.17       | 0.69        | 21.10       | 0.76        |
| Phosphatidylserine         | 23.98       | 1.22        | 24.04       | 1.69        |
| Phosphatidylethanolamine   | 27.24       | 0.90        | 27.29       | 1.04        |
| Phosphatidylcholine        | 31.09       | 0.75        | 31.56       | 0.84        |

a) Retention time.
b) Coefficient of variance (n=21).
the peak areas (A233 nm/A206 nm) of each phospholipid class was calculated as an index of the peroxidation levels. Our results revealed high reproducibilities in the samples of skeletal muscle and erythrocytes.

On the other hand, it is notable that the present HPLC system with UV-detectors cannot discriminate the phospholipid hydroxides from the corresponding hydroperoxides, since the phospholipid hydroperoxides and the hydroxides, both of which have the conjugated dienes, give almost the same retention times as that for corresponding unoxidized classes in the normal-phase HPLC [19]. However, adequately prepared biological samples would contain few phospholipid hydroxides, because the phospholipid hydroperoxides in the biological membrane were hardly reduced into their hydroxides by the cytosolic glutathione peroxidase [17].

It can thus be concluded that the present HPLC method is a convenient tool for the selective determination of the peroxidized phospholipids. This method will make it possible to estimate the oxidative changes of phospholipids in small amounts of biological samples.

REFERENCES