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Determination of the Phospholipid Composition of Bovine Muscle by High Performance Liquid Chromatography with Emphasis on the Choline and Ethanolamine Plasmalogens

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ABSTRACT

Bovine muscle phospholipids contain large proportions of plasmalogens. A method for the separation of the plasmalogens from the corresponding diacyl compounds by high performance liquid chromatography (HPLC) has not been available although HPLC has many other advantages for lipid separations. The separation of phospholipid classes by HPLC was modified in order to separate hydrolysis products of plasmalogens from other phospholipids. The choline and ethanolamine glycerophospholipids were collected from the first HPLC separation, hydrolyzed at room temperature with 50 mM HCl in chloroform–methanol–water (50:47·5:2·5, by vol), and rechromatographed. In bovine semimembranosus *muscle, the content of each phospholipid determined by phosphorus assay was 38·2, 21·4, 10·6, 17·9, 4·3, 2·4, and 5·2% for acid-stable choline glycerophospholipids, choline plasmalogens, acid-stable ethanolamine glycerophospholipids, ethanolamine plasmalogens, inositol glycerophospholipids, serine glycerophospholipids, and sphingomyelin, respectively. This HPLC method efficiently separated the phospholipid species, especially the lyso compounds corresponding to plasmalogens. The gradient of the solvent system composed of hexane-2-propanol (3:2 by vol), A, and hexane-2-propanol (3:2 by vol) with 5·5% water, B, was adjusted. For the gradient elution for the phospholipid class separation, the initial solvent ratio was 50% B, and B was increased to 100% at 10·5 min and decreased to 50% at 22 min, whereas, for resolution of lyso*

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compounds from diacyl-glycero-3-phosphocholine and diacyl-glycero-3-phosphoethanolamine, the initial solvent ratio was 50% B, then 100% B at 17 min, and 50% B at 38 min. With HPLC separation of phospholipid classes and mild acid hydrolysis of choline and ethanolamine glycerophospholipids prior to rechromatography, it is possible to recover quantitatively all of the phospholipid components for any desired assay or determination of composition.

Abbreviations: ChoGpl, choline glycerophospholipids; EtnGpl, ethanolamine glycerophospholipids; InsGpl, inositol glycerophospholipids; SerGpl, serine glycerophospholipids; Cer*P*Cho, sphingomyelin; PlsCho, choline plasmalogen (plasmenylcholine); PlsEtn, ethanolamine plasmalogen (plasmenylethanolamine); GPC, glycero-3-phosphocholine; GPE, glycero-3-phosphoethanolamine; HPLC, high performance liquid chromatography.

INTRODUCTION

Ether phospholipids are important components of many vertebrate tissues. Appreciable quantities of plasmalogens are found in brain, heart, and other muscle tissues of mammals (Rapport & Norton, 1962; Klenk & Debuch, 1963; Horrocks, 1972), but bovine *semimembranosus* muscle has not been included in those studies. Because plasmalogens differ from the corresponding diacyl types by having their hydrocarbon chains attached to glycerol through a dehydrated hemiacetal linkage, the fatty acid composition of the plasmalogens should be determined separately from that of the diacyl phospholipids of the same class (Sun & Horrocks, 1969; Horrocks & Sun, 1972; Horrocks & Sharma, 1982).

The two-dimensional thin layer chromatographic method presently used for the separation of plasmalogens and diacyl compounds derived from ChoGpl and EtnGpl has the disadvantages of low resolution, variable separations due to variations in humidity, and the difficulty in removing lipids from the silica gel. Only the separation of PlsEtn from diacyl-GPE by HPLC has been reported (Dugan *et al.*, 1986), although a number of methods for the separation of phospholipids are available (Jungalwala *et al.*, 1976; Geurts van Kessel *et al.*, 1977; Kiuchi *et al.*, 1977; Gross & Sobel, 1980; Hsieh *et al.*, 1981; Hanson *et al.*, 1981; Chen & Kou, 1982; Kaduce *et al.*, 1983; Chan *et al.*, 1983; Yandrasitz *et al.*, 1983; Chen & Kou, 1984). For the present study, a new method utilizing HPLC was developed for the separation of both choline and ethanolamine plasmalogens in the form of their lyso derivatives from the corresponding diacyl Gpl.

MATERIALS AND METHODS

A beef round steak (USDA choice grade) from a steer fed with grain was obtained from the Kroger Meat Laboratory (Columbus, Ohio) at 1 day post mortem. After removal of the external fat the *semimembranosus* muscle from the steak was homogenized with a Waring blender, and the lipids were extracted by the method of Folch *et al.* (1957).

The HPLC instrument was equipped with two pumps (Model 100, Altex Scientific Company, Berkeley, CA), an injection system with a 20 μl injection loop (Model 500, Altex), a column block heater (Jones Chromatography Co., Columbus, OH), and a variable wavelength detector (LC-75, Perkin-Elmer, Norwalk, CT) set at 205 nm with full scale absorbance of 0·64. A

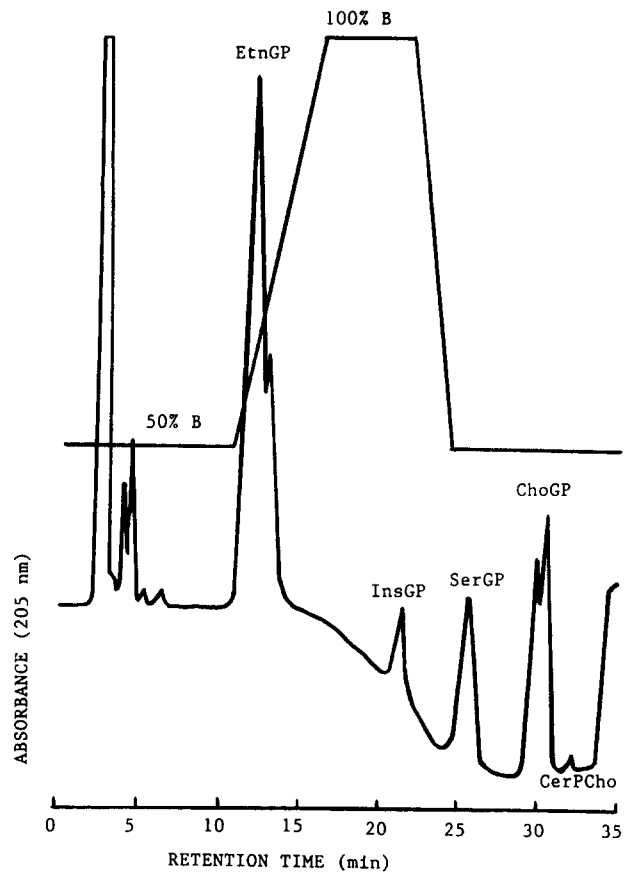

Fig. 1. HPLC separation of the phospholipid classes from bovine *semimembranosus* muscle. Column, Zorbax Sil.; mobile phase, hexane–isopropanol (3:2 by vol) for solvent A, hexane–isopropanol (3:2 by vol) with 5·5% water for solvent B; flow rate, 1·5 ml/min; gradient elution, 50 to 100% B (duration 5 min) at 10·5 min and 100 to 50% B (duration 3 min) at 22 min; detection, 205 nm with full-scale absorbance of 0·64.

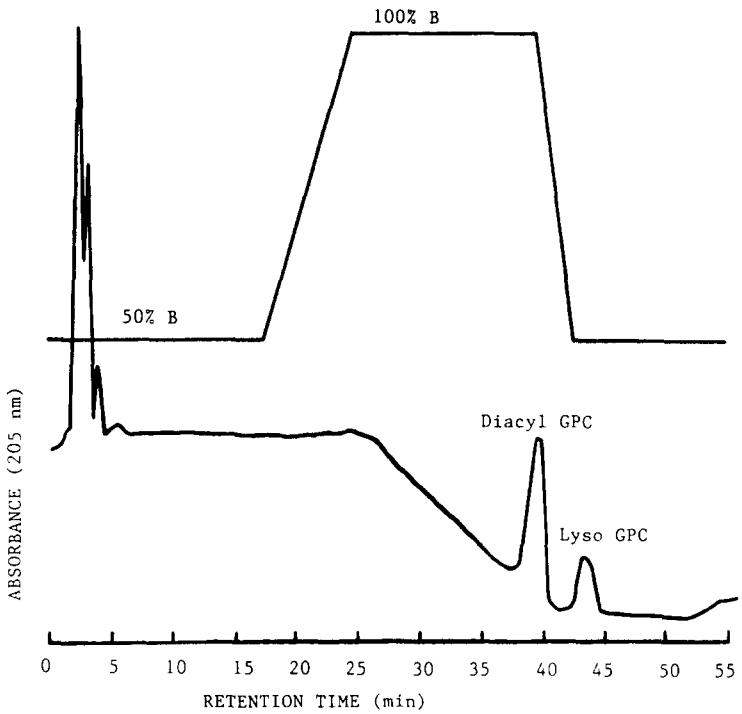

Fig. 2. HPLC separation of the diacyl and monoacyl GPC obtained by acid hydrolysis of the ChoGpl fraction from bovine *semimembranosus* muscle. Column, Zorbax Sil.; mobile phase, hexane–isopropanol (3:2 by vol) for solvent A, hexane–isopropanol (3:2 by vol) with 5·5% water for solvent B; flow rate, 1·5 ml/min; gradient elution, 50 to 100% B (duration 7 min) at 17 min and 100 to 50% B (duration 3 min) at 38 min; detection, 205 nm in UV with full-scale of absorbance of 0·64.

microprocessor (Model 420, Altex) was used to control the pumps and recorder (Model BD-40, Kipp and Zonen, Delft, Holland).

The phospholipid classes were separated by a modification (Dugan *et al.*, 1986) of the method of Chan *et al.* (1983) using a 4·6 mm × 25 cm Zorbax Sil column. The mobile phases were hexane-2-propanol (3:2 by vol) for Solvent A, and hexane-2-propanol (3:2 by vol) with 5·5% water for solvent E. The column was stored in hexane-2-propanol (3:2 by vol), and equilibrated with the solvent system until a stable baseline was obtained. For the gradient elution, the initial solvent ratio was 50% B (50% A) at a flow rate of 1·5 ml/min. The % B was increased to 100% (duration 5 min) at 10·5 min and decreased to 50% B (duration 3 min) at 22 min (Fig. 1). The identity of each peak was established by injecting individual phospholipid standards.

After collecting each class of phospholipid, quantitation was by phosphorus assay (Rouser *et al.*, 1970). Each phospholipid class had a single spot by thin layer chromatography.

Duplicate phospholipid separations were made for the collection of the

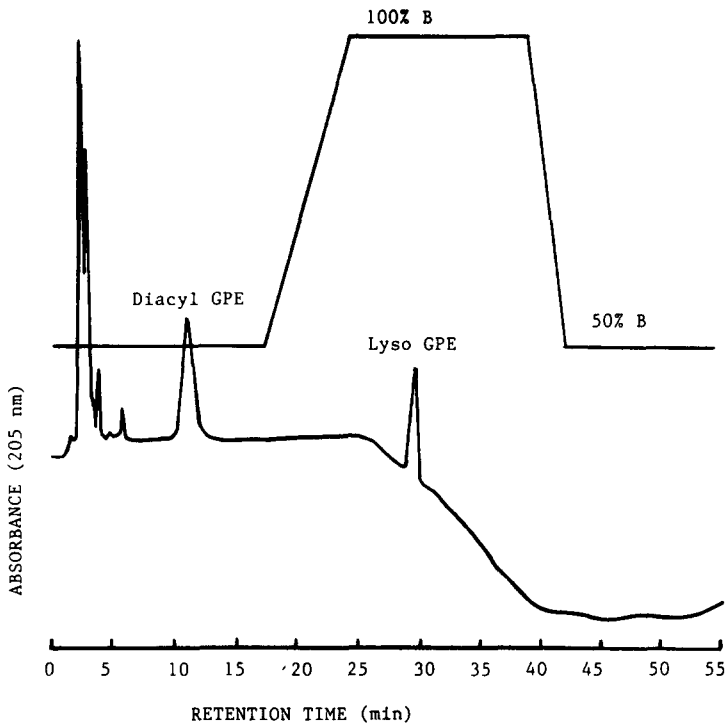

Fig. 3. HPLC separation of the diacyl and monoacyl GPE obtained by acid hydrolysis of the EtnGpl fraction from bovine *semimembranosus* muscle. The conditions were the same as described in Fig. 2.

ChoGpl and EtnGpl. The alkenyl groups of the choline and ethanolamine plasmalogens were cleaved with hydrochloric acid by a modification of the method of Renkonen (1966). The sample was taken to dryness with N_2 and dissolved in 1·5 ml $CHCl_3$. Then 1·5 ml of 0·1M HCl in 95% CH_3OH was added, the tube was flushed with N_2 and capped, vortexed, and incubated at room temperature for 40 min. The sample was then rechromatographed to separate the lyso derivatives corresponding to the plasmalogens in the ChoGpl and EtnGpl from the corresponding diacyl compounds as described above. For gradient elution, the proportion of B was increased to 100% (duration 7 min) at 17 min and decreased to 50% (duration 3 min) at 38 min. The contents of diacyl-GPC, 2-acyl-GPC, diacyl-GPE, and 2-acyl-GPE were determined by phosphorus assay of the separated compounds (Figs 2 and 3).

HPLC grade organic solvents were purchased from EM Science (Gibbstown, NJ). Distilled water was purified with a Milli-Q system plus an Organex-Q cartridge (Millipore Co., Bedford, MA). Aqueous and organic solutions were filtered with 0·22 μm GS (Millipore Co., Bedford, MA) and degassed before use.

RESULTS

The phospholipids from bovine *semimembranosus* muscle were separated with baseline resolution. The order of elution was EtnGpl, InsGpl, SerGpl, ChoGpl, and Cer*P*Cho with the solvent system of hexane-2-propanol-water and a silica column as found previously by Chen *et al.* (1983) and Dugan *et al.* (1986). The small peaks eluted before EtnGpl included triacylglycerols, cholesterol, and free fatty acids. All phospholipid components were eluted

TABLE 1
Composition of Phospholipids in Bovine *Semimembranosus* Muscle

Phospholipids	*% total P*
ChoGpl	38·2 ± 0·3
PlsCho	21·4 ± 0·1
EtnGpl	10·6 ± 0·2
PlsEtn	17·9 ± 0·2
InsGpl	4·3 ± 0·1
SerGpl	2·4 ± 0·2
Cer*P*Cho	5·2 ± 0·3

The means ± SD are from three replicate determinations. Phospholipids were separated from lipid extracts of beef round steak by HPLC as shown in Figs 1, 2 and 3, and quantitated by phosphorus assays of each collected fraction. Diphosphatidylglycerol was not collected.

within 35 min. An efficient resolution of the ChoGpl fraction into diacyl-GPC and 2-acyl-GPC from the hydrolysis of choline plasmalogens was achieved with a different gradient system (see 'Materials and Methods') of the same mobile phases utilized for the phospholipid classes. The same gradient elution was used for the separation of 2-acyl-GPE from diacyl-GPE. The retention times for diacyl-GPE and 2-acyl-GPE were 11 and 29 min.

The most abundant phospholipid class in the bovine *semimembranosus* muscle was ChoGpl which represented 59·6% of the total phospholipids, while EtnGpl accounted for 28·5% (Table 1). The other phospholipids, such as InsGpl, SerGpl, and Cer*P*Cho, were present in much lower amounts. The choline and ethanolamine plasmalogens from the bovine muscle accounted for 21·4% and 17·9%, respectively. The recovery of total phosphorus from the HPLC separation was 99·4%.

DISCUSSION

With the method of Dugan *et al.* (1986), baseline resolution of the five principal phospholipid classes was achieved. Hexane-2-propanol (3:2) was used as the eluting solvent with water for adjustment of polarity. Gradient elution was very effective for the separation of phospholipids that vary in polarity and charge. To quantify each phospholipid fraction from HPLC, phosphorus assay was necessary because the absorption at 205 nm is due primarily to double bonds in the phospholipids.

Previous HPLC methods are not suitable for the separation of all classes and lyso compounds. The method of Jungalwala *et al.* (1976) has a poor resolution of EtnGpl, SerGpl, and InsGpl. Gross & Sobel (1980) separated ChoGpl, 2-acyl-GPC, 2-acyl-GPE, and Cer*P*Cho but not EtnGpl and SerGpl. Hanson *et al.* (1981) employed a silica-based anion exchange column and a gradient elution to resolve ChoGpl, 2-acyl-GPC, EtnGpl, and Cer*P*Cho, but had difficulty with the resolution of SerGpl and InsGpl. Geurts van Kessel *et al.* (1977) separated Cer*P*Cho from ChoGpl only partially with different proportions of the same solvents that we have used. The method of Chen & Kou (1982) includes an acidic solvent mixture which causes plasmalogen hydrolysis. In fact, an alternative method for the assay of the proportion of plasmalogens in a glycerophospholid could be the rechromatography of the isolated glycerophospholipid with an acidic solvent with recovery of the acid-stable and acid-labile (lysoglycerophospholipid) fractions (Kawasaki *et al.*, 1986).

The proportion of choline plasmalogen in the *semimembranosus* muscle (21·4%) is considerably higher than that reported for ox *longissimus* muscle (13·6%) by Davenport (1964). The latter method was subsequently found to give low results for plasmalogens due to incomplete alkaline hydrolysis (Horrocks, 1972). Lower proportions of PlsCho and PlsEtn are found in rodent muscle (Okano *et al.*, 1980) than in bovine muscle.

Simon & Rouser (1969) analyzed phospholipids of skeletal muscles in several vertebrate and invertebrate species and found rather different distributions according to species. Their results for the phospholipid composition of skeletal muscle from bovine neck are somewhat different from the composition of the *semimembranosus* muscle found in this investigation. Simon and Rouser did not separate plasmalogens and reported a much lower proportion of choline glycerophospholipids. The HPLC method developed in this study contributed to efficient separation of each phospholipid species, including the lyso compounds derived from plasmalogens. Current HPLC methods should be used to reinvestigate the composition of the phospholipids in different muscle types in a number of different species.

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