METHODS

Complete Separation of Phospholipids from Human Heart Combining Two HPLC Methods

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The separation of phospholipid classes from human heart was achieved in two steps by high performance liquid chromatography (HPLC) using a silica column with an ultraviolet spectromonitor at 206 nm. A complete partitioning of phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PI), phosphatidylserines (PS), cardiolipins (CL), lysophosphatidylcholines (LPC) and sphingomyelins (Sph) was obtained for further analysis.
Lipids 21, 239-240 (1986).

The separation of phospholipid classes traditionally uses thin layer or column chromatography (1). Many HPLC methods (2) have been developed that offer advantages over other chromatographic methods. However, the separations were not easy because of the complexity and diversity of phospholipid structures as well as their limits of detection (absorption in the 203-210 nm region). Many applications of separation came from the techniques of Hax et al. (3) and Geurst Van Kessel et al. (4), which allowed the separation of all phospholipid classes except PC and Sph, or from the work of Jungalwala et al. (5), which allowed the separation of PC and Sph but not other classes.

Our aim was to separate all of the phospholipid classes of human or rat heart (CL, PC, PE, PI, PS, LPC and Sph). Moreover, we wished to obtain these fractions as pure as possible and in sufficient quantity (milligrams) for further analysis or for preparation of membrane models.

The preliminary assays with the existing methods (6-9) were less satisfactory than the combined methods of Hax et al. (3) and Jungalwala et al. (5). Particularly mobile phases containing acidic solvents which can alter plasmalogens (7,10) were avoided. The present paper shows the results of a complete separation of phospholipid classes from human heart.

EXPERIMENTAL PROCEDURES

Materials. The HPLC was a Varian model Vista 54 with a Pye Unicam detector, model PU4020. The fraction collector was a 401 Gilson model.

The HPLC columns were packed in our laboratory according to the procedure of Coq et al. (11). Organic solvents were of HPLC grade and used as such after degassing. Distilled water was purified on an RP-8 column (E. Merck, Darmstadt, West Germany). Standards of phospholipids were obtained from Sigma Chemical Co. (St. Louis, Missouri).

Methods. Samples of human heart (about 1 g) were obtained from the Surgical Clinic at the Dijon University Hospital. They were removed in the course of an open-heart surgery and stored immediately in a chloroform/methanol mixture (2:1, v/v). The total lipids were extracted according to the method of Folch et al. (12) and the phospholipids were separated from the nonphosphorus lipids in silica cartridges (13). The phospholipids were dissolved in 2-propanol/hexane (8:6, v/v). The analyses were done at room temperature.

The first separation followed the procedure of Hax et al. (3). A column with a similar length (25 cm) was used but with a bigger internal diameter (7.5 mm) packed with Lichrosorb Si60, 5 μm (Merck). This modification compelled us to modify the gradient. The elution mixtures of 2-propanol/hexane/water varied from 54:41:5 (v/v/v) to 52:39:9 (v/v/v) in 10 min instead of 5 min plateau and 20 min gradient. The flow rate was only double (2 ml/min) in order to avoid excessive head pressure (100 atm).

The second separation was a modification of the procedure of Jungalwala et al. (5). We used a shorter (25 cm) column of larger internal diameter (4.8 mm) packed with Lichrosorb Si60, 10 μm (Merck).

The quantity injected (3 or 4 mg) and the ratio of the PC and Sph compelled us to modify the mixture of acetonitrile/methanol/water to 71:21:8 (v/v/v) instead of 65:21:14 (v/v/v). The flow rate was tripled (3 ml/min). The detector in the two analyses was set at 206 nm with a sensitivity of 1.28 absorbance unit full scale (AUFS). The collected fractions were evaporated to dryness at 42 C in a rotary vacuum apparatus and kept in chloroform/methanol (2:1, v/v) until analyzed.

RESULTS AND DISCUSSION

Figure 1 shows the results obtained after the first separation. The CL, PE, PI, PS and LPC were well separated. PC and Sph were collected together. Ethanolamine and choline plasmalogens are eluted with PE and PC respectively. Peaks were identified by comparing their retention times to those of phospholipids standards. The purity of the collected fractions was tested by thin layer chromatography (TLC) (14). As shown by Hax et al. (3) and Geurst Van Kessel et al. (4), a part of Sph was in the PC peak. This first analysis lasted 60 min (45 min for complete elution and 15 min for column equilibration). The maximum injected quantity was 7 mg of phospholipids, according to the column characteristics. We verified with a phosphorus assay (15) that no phospholipids remained on the column. The column was used for more than 200 runs without loss of reproducibility and allowed us to automate the collecting of the different fractions.

Figure 2 shows the separation of PC and Sph. These two classes were well separated (TLC assay) and identified with standards. There was no retention of

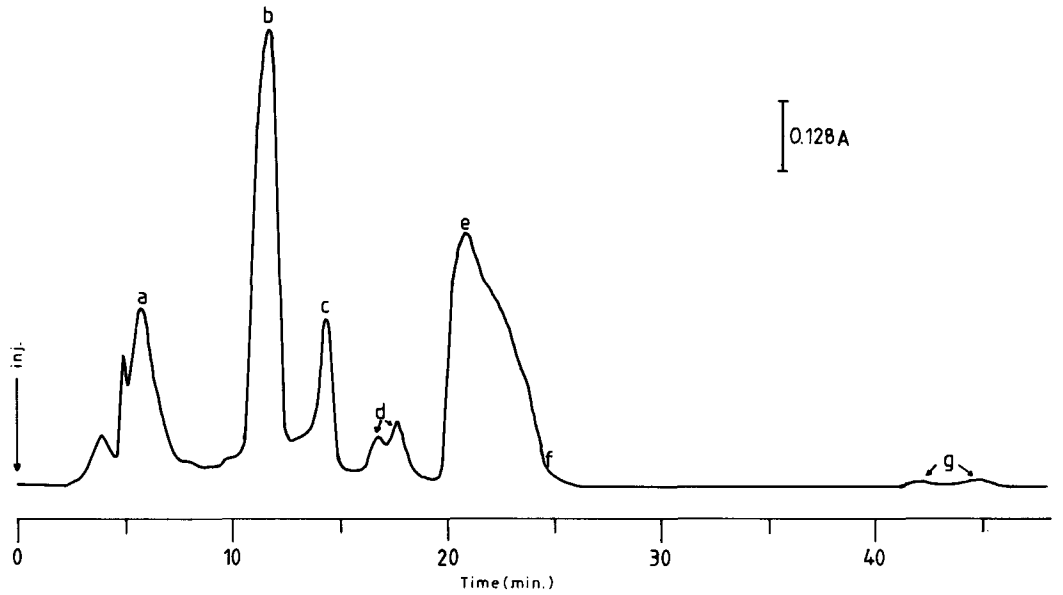

FIG. 1. Separation of human heart phospholipids. Solvent: 2-propanol/hexane/water (54:41:5 to 52:32:9, v/v/v in 10 min); flow rate: 2 ml/min; column: Lichrosorb Si60, 5 μm; UV detection at 206 nm; sensitivity: 1.28 absorbance unit full scale (AUFS). a: Cardiolipin, b: phosphatidylethanolamine, c: phosphatidylinositol, d: phosphatidylserine, e: phosphatidylcholine, f: sphingomyelin, g: lysophosphatidylcholine.

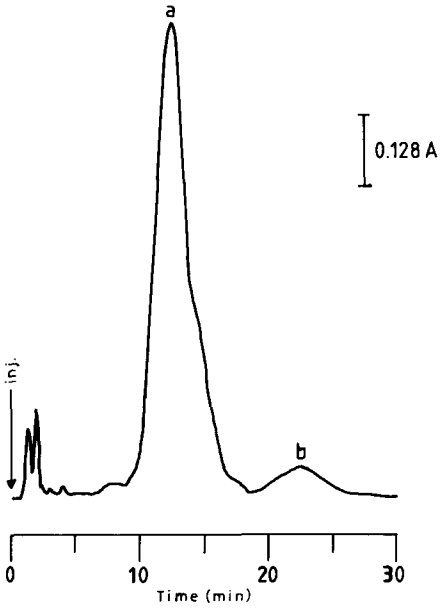

FIG. 2. Separation of the phosphatidylcholine (a) and sphingomyelin (b) fraction from human heart phospholipids. Solvent: acetonitrile/methanol/water (71:21:8, v/v/v); flow rate: 3 ml/min; column: Lichrosorb Si60, 10 μm; UV detection at 206 nm; sensitivity: 1.28 absorbance unit full scale (AUFS).

phospholipids on the column. The analysis took 30 min.

We could similarly separate phospholipids from rat heart, kidney and testes. For rat liver phospholipids, it was necessary to modify the relative proportions of acetonitrile/methanol/water in the second step to 72:22:6 (v/v/v) instead of 69:21:8 (v/v/v) for a complete separation of PC and Sph.

The quantity injected (7 mg) produced sufficiently pure classes and quantity of minor classes like PS, PI, Sph and LPC. With the automated collections of fractions, the time of analysis was 90 min for a complete separation, but the evaporating time was rather long, partly because of the water contained in the mobile phase but mainly because of the number of fractions.

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