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Simultaneous determination of amounts of major phospholipid classes and their fatty acid composition in erythrocyte membranes using high-performance liquid chromatography and gas chromatography

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ABSTRACT

A method for the simultaneous determination of amounts of major phospholipid classes and their fatty acid composition in erythrocyte membranes is described. The method consists in extraction of phospholipids from erythrocyte membranes, separation of phospholipid classes by high-performance liquid chromatography, methylation of phospholipids and determination of phospholipid-bound fatty acids by capillary gas chromatography. The amounts of phospholipid classes are calculated from the total weight of phospholipid-bound fatty acids and their average molecular weights. The method was applied to erythrocytes from rats. The results show that the method is reproducible and is useful for the determination of amounts of phospholipid classes and their fatty acid composition in small blood samples.

INTRODUCTION

Phospholipids are important constituents of all biological membranes. Both the nature of the polar head groups and the acyl chains influence the physical and chemical properties of membranes [1,2]. Especially in erythrocytes, membrane parameters such as shape, deformability, permeability and osmotic fragility are determined by membrane phospholipids [3–6]. The most important phospholipid classes in erythrocyte membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM), which contain a variety of aliphatic chains that vary in length and number of double bonds [7–10]. For their determination erythrocyte membrane phospholipids are extracted with various solvents or binary solvent mixtures. The main phospholipid classes are usually separated by one- and two-dimensional thin-layer chromatography (TLC), quantified by measurement of inorganic phosphate and their fatty acid composition is determined by capillary gas chromatography (GC) after methylation [11–14]. Although TLC is used routinely for the separation of phospholipid classes, it has some disadvantages. The amount of lipid that can be applied to the plate is small and oxidation of polyunsaturated fatty acids during plate development and drying and losses of phospholipids during scraping off can occur [15,16]. Moreover, quantification of phospholipid classes by phosphorimetry is not very exact as only the number of moles of phospholipid and not the molecular weight of the individual phospholipid classes depending on their fatty acid composition is considered.

The aim of this work was to develop a method for the determination of amounts of individual phospholipid classes and their fatty acid composition in erythrocyte membranes which should overcome the disadvantages of the TLC method. For this purpose phospholipid classes extracted from erythrocyte membranes were separated by high-performance

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liquid chromatography (HPLC) and the amounts of total bound fatty acids of individual phospholipid classes were determined by capillary GC. Amounts of individual phospholipid classes were then calculated from the sum of their bound fatty acids.

EXPERIMENTAL

Chemicals, standards and blood samples

Phospholipid standards [PC, lyso-PC and lyso-PE from egg yolk; PE (diacyl), PE (plasmalogen), PS and SM from bovine brain; PI from bovine liver; cardiolipin from bovine heart) were purchased from Sigma (Taufkirchen, Germany). The purity of all phospholipid standards was at least 98%.

Fatty acid standards (with a chain length between 8 and 24 carbon atoms) were obtained from Sigma, Roth (Karlsruhe, Germany) and Fluka (Buchs, Switzerland) in the highest purity available. Methylation of standard fatty acids for GC was carried out using boron trifluoride–methanol reagent (Fluka, purissimum) in accordance with Morrison and Smith [17]. All other chemicals were purchased from Merck (Darmstadt, Germany). Blood samples were taken from adult female Sprague–Dawley rats.

Apparatus

Separation of phospholipid classes was carried out on a Merck–Hitachi (Darmstadt, Germany) HPLC system consisting of a gradient pump (L-6200), a diode array (L-3000), a 25 cm × 0.4 cm I. D. Si 60 (5-μm) cartridge (LiChroCART, Merck), an integrator (D-2000) and a fraction collector (Model 201; Gilson, Villiers-le-Bel, France).

Analysis of fatty acid methyl esters (FAMEs) was performed on a Sichromat 2 gas chromatograph (Siemens, Karlsruhe, Germany) equipped with a programmed-temperature vaporizer (PTV), a CP-Sil 88 wall-coated open-tubular (WCOT) fused-silica column (50 m × 0.25 mm I.D., film thickness 0.2 μm) (Chrompack, Middelburg, Netherlands), a flame ionization detector and an integrator (D-2500, Merck).

Preparation of erythrocyte membranes and extraction of phospholipids

Blood from rats was collected in glass tubes containing heparin to prevent clotting. The fresh blood was centrifuged (1100 *g*, 10 min) and plasma and buffy coat were removed by suction. The red cells were washed three times with isotonic saline by centrifugation (1100 *g*, 10 min) and resuspension. The washed cells were haemolysed by adding 5 ml of distilled water per millilitre of cells and freezing. The red cell membranes were then washed three times according to Hanahan and Ekholm [18] using Tris buffer (pH 7.6). After washing, liquid was drained from the packed membranes by placing the centrifuge tubes upside down. A 1-ml volume of distilled water per gram of packed membranes was added and the membranes were suspended ("membrane solution"). Extraction of phospholipids was carried out with isopropanol by a modification of the method of Peuchant *et al.* [10]. To 1 g of membrane solution 20 ml of isopropanol [containing butylated hydroxytoluene (BHT) as an antioxidant] were added. This mixture was sonicated with a Branson Sonifier for 1 min and was then left to stand for 4 h at room temperature. After extraction the mixture was filtered and the residue was washed twice with 10 ml of isopropanol. The filtrate was evaporated to dryness and the residue consisting of lipids was dissolved in chloroform and diluted to 5 ml with chloroform. This lipid extract was filtered using an HPLC (45-μm) filter and was then ready for injection into the HPLC system.

Separation of phospholipid classes by HPLC

For separation of phospholipid classes a modification of the method of Seewald and Eichinger [19] based on the silmultaneous use of a pH gradient and a polarity gradient was used. The mobile phase

TABLE I

SOLVENT GRADIENTS AND FLOW-RATES USED FOR SEPARATION OF PHOSPHOLIPID CLASSES

Time (min)	A (%)	B (%)	C (%)	Flow-rate (ml/min)
0.0	100	0	0	1.5
5.0	100	0	0	1.5
5.1	0	100	0	1.5
15.0	0	100	0	1.5
15.1	0	100	0	1.0
35.0	0	0	100	1.5
50.0	0	0	100	1.5

was composed of solvent A (acetonitrile), solvent B [acetonitrile–85% phosphoric acid (99.8:0.2)] and solvent C [(methanol–85% phosphoric acid 99.8:0.2)]. The solvent gradients and the flow-rates used are shown in Table I.

A 50-μl portion of the lipid extract was injected manually into the HPLC system. Separation was carried out at room temperature. The eluted phospholipid classes were detected at 205 nm and collected with a fraction collector for further analysis. To each tube containing the collected eluent with an individual phospholipid class a solution containing a known amount of methyl heptadecanoate (as internal standard for GC determination of FAMEs) and BHT (as antioxidant) was added.

Methylation of phospholipid classes

The individual phospholipid classes were evaporated from the mobile phase under vacuum at room temperature. The residues of the phosphoglyceride classes (PC, PE, PS, PE–plasmalogen) were resuspended in 0.5 ml of chloroform and then 4 ml of 0.5 *M* methanolic sodium methoxide solution were added. This mixture was stirred at room temperature for 1.5 h. For extraction of FAMEs 2 ml of water and 2 ml of hexane were added. The mixture was stirred for a further 10 min, then the hexane phase containing the FAMEs was transferred into a vial for analysis. The extraction was repeated by adding a further 2 ml of hexane. The hexane phases were collected and the solvent was evaporated under vacuum at room temperature. The FAMEs were dissolved in a small volume (50 μl) of hexane and were then ready for injection into the GC system. This methylation procedure gave conversions of fatty acids from standard phosphoglyceride classes into FAMEs between 97.1 and 103.4% [20].

The residue of sphingomyelin was dissolved in 1 ml of chloroform, transferred into a tube provided with a PTFE-lined screw cap, the solvent was evaporated again and 2 ml of methanolic boron trifluo-

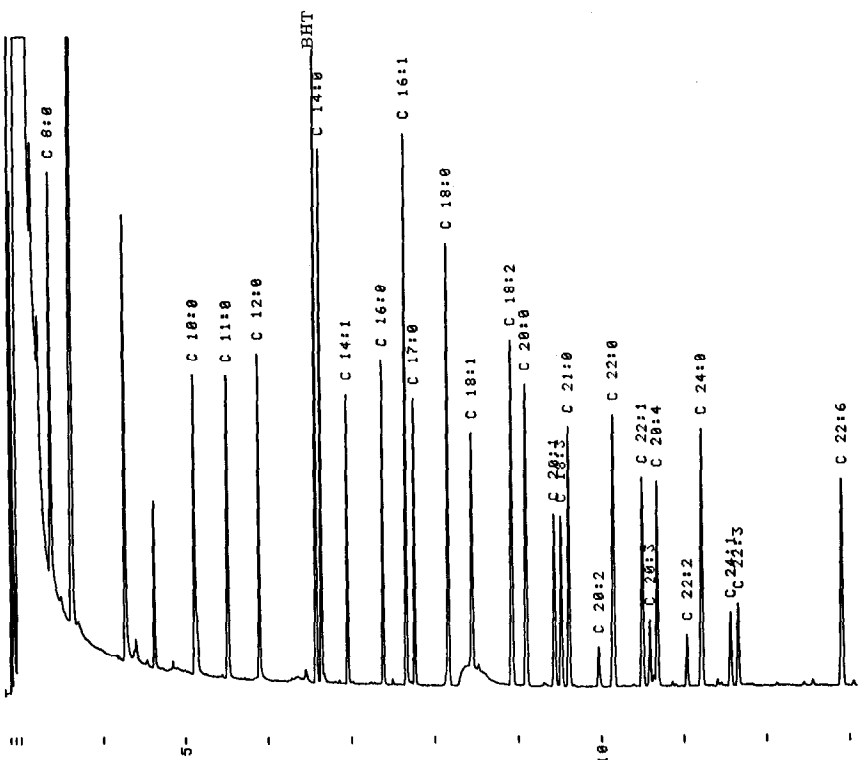

Fig. 1. Separation of standard FAMEs using PTV split injection. Splitting ratio, 1:2; amounts of FAMEs injected into the injector chamber, 0.8–7 ng. Time scale in minutes.

ride solution (140 g/l) and a small volume (10 μl) of a solution containing BHT (6 mg/ml) were added. The tube was closed and heated for 15 h at 90°C. This very long period of heating was necessary as it has been shown [20,21] that for complete methylation of sphingomyelin with boron trifluoride–methanol reagent very long periods are required. Thus in our studies [20] the recoveries of FAMEs from a sphingomyelin standard were 35.5, 60.4, 82.7 and 99.1% for periods of 1, 2, 6 and 15 h, respectively. FAMEs were extracted by adding twice each time 0.75 ml of hexane and water. The hexane phases were collected, the solvent was evaporated and the FAMEs were dissolved in a small volume of hexane.

Gas chromatographic analysis of FAMEs

A 2-μl portion of each FAME extract was injected manually into the GC system using a PTV. The PTV programme was 25°C held for 1 min after injection, increased at 800°C/min to 300°C, 300°C held for 10 min, then the PTV was cooled. The oven temperature was 50°C held for 1 min, increased at 30°C/min to 160°C and at 15°C/min to 200°C, 200°C held for 1.5 min, increased at 10°C/min to 225°C, 225°C held for 15 min. Hydrogen was used as the carrier gas at a flow-rate of 2.0 ml/min; the splitting ratio was 1:2. FAMEs were calculated using methyl heptadecanoate ester as internal standard. For more details, see ref. 22. A typical separation of standard FAMEs with chain lengths between 8 and 24 carbon atoms is shown in Fig. 1.

Calculation of amounts of individual phospholipid classes

Amounts of individual phospholipid classes were calculated as proposed by Seewald and Eichinger [19]. The amount of each individual fatty acid of each phospholipid class was determined by GC. The total fatty acid weight of each phospholipid class was calculated as the sum of the amounts of the individual fatty acids. Using the relative amount of each fatty acid and its moleculare weight, an average molecular weight of the fatty acids of each phospholipid class can be calculated. The molecular weight of the phospholipid can be calculated as the sum of the molecular weight of the phospholipid core and the average molecular weight of bound fatty acids. The number of moles of fatty acids can be calculated as the ratio between total amount of fatty acids and the average molecular weight of the fatty acids bound to each phospholipid class. For PC, PE and PS, the number of moles of phospholipid in the injected extract is half that of the number of their bound fatty acids, and for lyso-phospholipids and sphingomyelin the number of moles of phospholipids and their bound fatty acids is identical. From the number of moles of each phospholipid class and its average molecular weight, the amount in micrograms of each phospholipid class contained in the extract injected into the HPLC system can be calculated.

Quality control

The reproducibility of the method was studied by applying the whole method (Washing of cells, extraction and separation of phospholipids and GC analysis of FAMEs) to five aliquots of pooled blood (5 ml each). The recovery of phospholipids was checked by using a phospholipid standard solution containing PC, PE and SM. A 25-ml volume of this

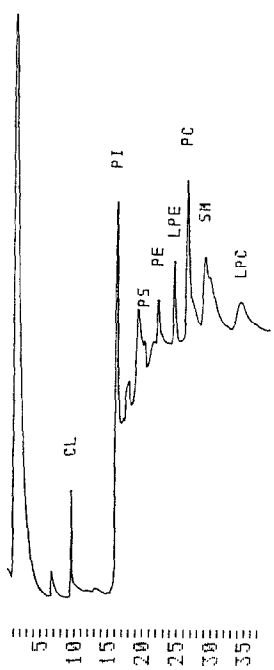

Fig. 2. Separation of standard phospholipid classes by HPLC as described under Experimental. The amounts of phospholipids applied to the column were 20 μg for PE, 25 μg for cardiolipin (CL), PI, PS, lyso-PE and lyso-PC and 50 μg for PC and SM. Time scale in minutes.

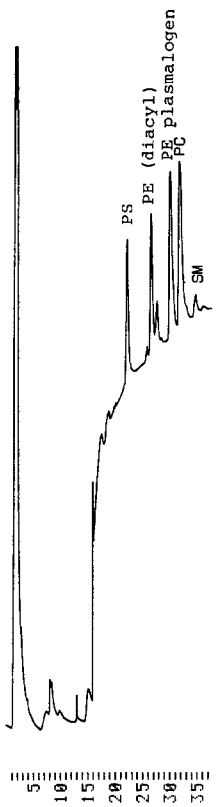

Fig. 3. Separation of rat erythrocyte membrane phospholipids by HPLC as described under Experimental. Peaks represent phospholipids contained in 40 μl of packed erythrocytes. Time scale in minutes.

solution (containing 3.12 mg of PC, 5.43 mg of PE and 3.21 mg of SM) was treated in the same way as the lipid extract was treated in the method described above. The standard solution was evaporated to dryness and phospholipids were dissolved in chloroform. The solution was diluted up to 5 ml with chloroform and 50 μl of it were injected into the HPLC system. The subsequent analytical steps were identical with those described above. Phospholipid recoveries were calculated as the ratio between the calculated amount of each phospholipid class and the known amount of each phospholipid class injected into the HPLC system.

RESULTS

The chromatographic separation of standard phospholipid classes [PC, PE, PS, phosphatidylinositol (PI), lyso-PC, lyso-PE, cardiolipin and SM] and phospholipid classes from rat erythrocyte membranes are shown in Figs. 2 and 3. The chromatogram of rat erythrocyte membranes contains peaks of PS, PE, lyso-PE, PC and SM. However, the lyso-PE peak respresents the plasmalogen fraction of PE as the use of acidic mobile phases leads to hydrolysis of the labile enol ether binding [23–25].

Fig. 4 shows typical separations of FAMEs from rat erythrocyte PC, PE, PE–plasmalogen, PS and SM. The fatty acid composition of these phospholipid classes is given in Table II. Amounts of phospholipid classes per millilitre of cells as calculated by their total bound fatty acids are given in Table III. The relative standard deviations (n = 5) were 2.7% for PC, 3.1% for PE (diacyl), 6.3% for PE (plasmalogen), 5.4% for PS, 1.0% for SM and 0.1% for the sum of these phospholipid classes.

The recoveries of phospholipid standards were 98.9 ± 0.8% for PE (diacyl), 101.8 ± 4.2% for PC and 96.3 ± 0.1% for SM, indicating that there were no losses of phospholipid between extraction and fatty acid analysis.

DISCUSSION

In the analysis of phospholipids, TLC is the most widely used separation technique. Although the separation of major phospholipid classes can be easily achieved, this method has disadvantages such as possible losses of phospholipids during scraping off and oxidation of polyunsaturated fatty acids during plate development and drying. Another problem is the quantification of the separated phospholipids. Phosphorimetry, which is the most commonly used technique for the quantification of phospholipids, does not give exact results because different molecular weights of phospholipids depending on their fatty acid composition are not considered. Using HPLC for separation of phospholipid classes, the disadvantages of the TLC method can be avoided. However, phospholipids cannot be quantified directly by UV absorption measurement as the molar absorption coefficients of phospholipids are dependent on their fatty acid composition [26,27]. Therefore, phospholipid classes separated by HPLC must be collected and quantified in a further analytical step.

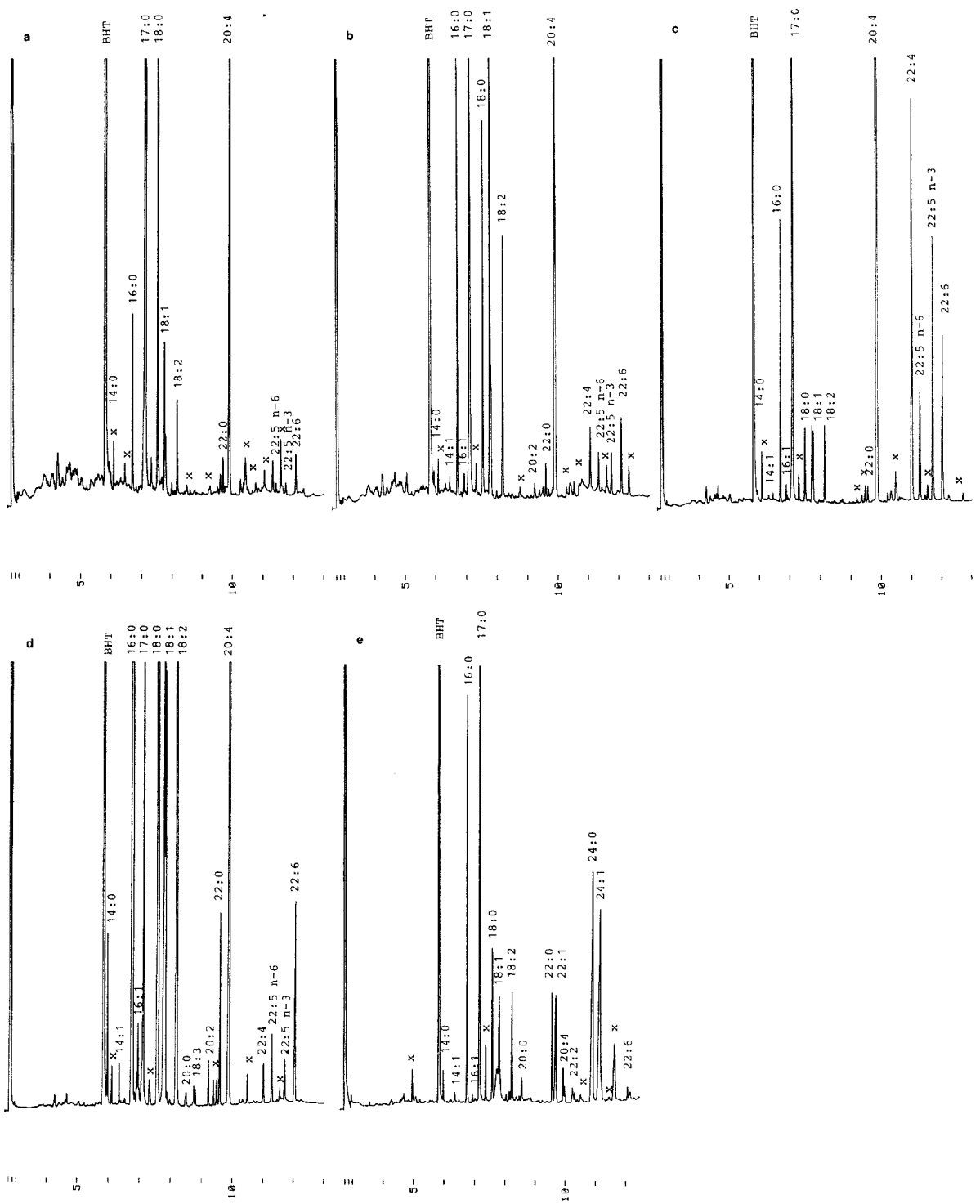

Fig. 4. GC separation of FAMEs from rat erythrocyte membrane phospholipid classes. (a) Phosphatidylserine; (b) phosphatidylethanolamine (diacyl); (c) phosphatidylethanolamine (plasmalogen); (d) phosphatidylcholine; (e) sphingomyelin. 17:0 = Internal standard representing *ca.* 4 ng of substance; × = unidentified peaks. Time scale in minutes.

TABLE II

FATTY ACID COMPOSITION (mol%) OF PHOSPHOLIPID CLASSES FROM RAT ERYTHROCYTE MEMBRANES

Values represent means ± S.D. for five analyses of a pooled blood sample.

Fatty acid	Phospholipid class					
	PS	PE (diacyl)	PE (plasmalogen)	PC	SM	Total
14:0	–[a]	–	–	1.01 ± 0.02	1.34 ± 0.05	0.70 ± 0.02
16:0	7.21 ± 0.53	13.67 ± 0.42	5.24 ± 0.13	39.13 ± 0.66	23.43 ± 0.94	27.25 ± 0.60
16:1	–	0.42 ± 0.03	–	0.64 ± 0.01	–	0.40 ± 0.01
18:0	25.15 ± 0.68	9.53 ± 0.16	1.83 ± 0.08	17.96 ± 0.50	8.40 ± 0.53	14.55 ± 0.39
18:1	8.97 ± 0.32	22.48 ± 0.62	3.23 ± 0.26	10.03 ± 0.89	7.39 ± 0.58	9.72 ± 0.48
18:2	4.56 ± 0.08	8.82 ± 0.07	1.73 ± 0.06	12.16 ± 0.30	6.94 ± 0.20	9.07 ± 0.20
20:0	–	–	–	0.17 ± 0.00	1.29 ± 0.04	0.21 ± 0.00
20:2	–	0.65 ± 0.03	–	0.34 ± 0.00	–	0.25 ± 0.00
20:4	47.48 ± 1.76	35.21 ± 0.67	54.43 ± 0.61	14.19 ± 0.63	1.79 ± 0.07	24.16 ± 0.61
22:0	–	0.81 ± 0.06	–	1.04 ± 0.02	4.90 ± 0.21	1.12 ± 0.01
22:1	–	–	–	–	5.93 ± 0.35	0.54 ± 0.03
22:4	1.36 ± 0.09	2.29 ± 0.05	14.32 ± 0.16	0.35 ± 0.01	–	2.72 ± 0.14
22:5 $n-6$	1.97 ± 0.16	1.37 ± 0.03	3.85 ± 0.17	0.64 ± 0.01	–	1.26 ± 0.04
22:5 $n-3$	0.70 ± 0.02	1.60 ± 0.10	9.43 ± 0.48	0.37 ± 0.02	–	1.87 ± 0.14
22:6	2.60 ± 0.12	3.15 ± 0.09	5.94 ± 0.07	1.97 ± 0.10	0.88 ± 0.03	2.64 ± 0.07
24:0	–	–	–	–	18.25 ± 0.98	1.68 ± 0.08
24:1	–	–	–	–	19.46 ± 0.81	1.79 ± 0.05

[a] Dashes indicate that the fatty acid exists only in trace amounts.

Seewald and Eichinger [19] proposed a method for quantification of phospholipids by determination of the total amounts of phospholipid bound fatty acids. This method offers the advantage that in a single analysis both the amount of individual phospholipid classes and their fatty acid composition can be determined. In contrast, most other methods require two analyses for the same purpose. A disadvantage of this method for calculation is that the exact determination of phospholipid amounts depends on the determination of all bound fatty acids. In practice, however, when small sample amounts are used it is hardly possible to identify and quantify fatty acids which are present in very small amounts ("minor fatty acids").

TABLE III

AMOUNTS OF PHOSPHOLIPID CLASSES IN RAT ERYTHROCYTE MEMBRANES

Values represent means ± S.D. for five analyses of a pooled blood sample.

Phospholipid	Amount	
	μg/ml of cells	%
PS	294 ± 16	8.2
PE (diacyl)	253 ± 8	7.1
PE (plasmalogen)[a]	878 ± 55	24.5
PC	1717 ± 47	47.9
SM	443 ± 4	12.3
Sum	3584 ± 4	100.0

[a] For calculation of the amount of PE–plasmalogen it was assumed that the aldehyde in the α-position has an average chain length of 17.5 carbon atoms.

In this work, the method of Seewald and Eichinger [19] originally used for the determination of phospholipids from muscle was adapted to erythrocyte membranes from small blood samples. There are many differences between pig muscle and rat erythrocyte membranes. First, in contrast to pig muscle, in most instances rat erythrocytes are available only in small amounts. Second, erythrocyte membranes contain smaller amounts of phospholipids than muscle. Third, phospholipid from erythrocyte membranes contain a greater variety of fatty acids (some of which exist in very small amounts, "minor fatty acids") than phospholipids of pig

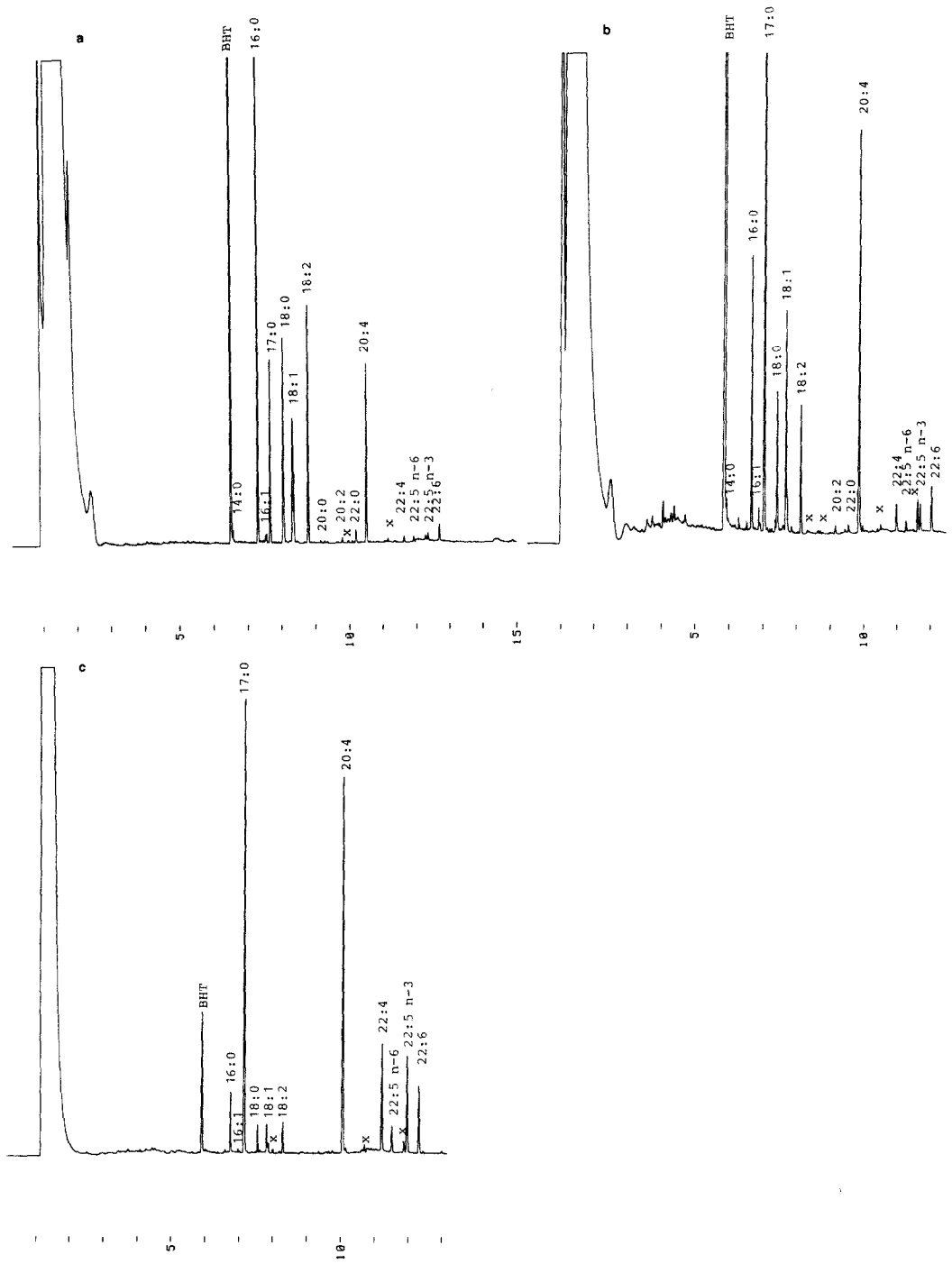

Fig. 5. Chromagrams of FAMEs from the rat erythrocyte membrane phospholipid classes. (a) PC; (b) PE (diacyl); (c) PE (plasmalogen). The results indicate that peak areas of FAMEs which were not identified (×) represent only 1–3% of the peak areas of total FAMEs. 17:0 = Internal standard. Time scale in minutes.

muscle. Owing to these differences, the determination of erythrocyte membrane phospholipids requires a higher sensitivity than the determination of phospholipids from pig muscle.

In the proposed method a high sensitivity was achieved by the following means. The phospholipid and FAME extracts were evaporated and dissolved in a small volume of solvent. Hence sufficient amounts of phospholipids and FAMEs could be injected into HPLC and GC systems. Moreover, for GC injection a very small splitting ratio was used. A further possibility for increasing the sensitivity is to repeat the HPLC separation of phospholipid classes and collect phospholipid classes from more than one HPLC separation. This is useful for very small blood samples (less than 1 ml). Preliminary studies have shown that there is a linear correlation (r = 0.99) between the amount of fatty acids measured and the number of succesive collections of separated phospholipid classes.

The fourth difference between erythrocyte membranes and muscle is the different behaviour of the sample during extraction. Whereas lipids from muscle can be extracted with the widely used Folch extraction [chloroform–methanol (2:1)], this method cannot be applied for extraction of lipids from erythrocyte membranes without problems, as erythrocyte membranes aggregate after addition of the chloroform–methanol mixture, making it difficult to resuspend them [28–30]. However, in another study we found that isopropanol is a good solvent for the extraction of phospholipids from erythrocyte membranes [30]. Another difference is that erythrocyte membranes are to be isolated by some preparative steps such as washing and haemolysis of cells and washing of membranes whereas muscle can be extracted in its original form.

In this work, methylation of phosphoglycerides was carried out at room temperature using methanolic sodium methoxide. Hence a one-vial procedure could be used. Phospholipids contained in the lipid extract were separated by HPLC. The eluent containing separated phospholipids was collected in 10-ml centrifuge tubes and the solvent was evaporated under vacuum at room temperature. Then methanolic sodium methoxide was added and, after methylation, hexane and water were added for extraction of FAMEs. Thus losses of phospholipids during transfer from one vial to another could be completely avoided. This could be seen in the recovery data for phophoglycerid standards, which were close to 100% (98.9% for PE, 101.8% for PC).

Using the described method, the amounts of the major phospholipid classes PC, PE, PS and SM (which represent approximately 95% of total phospholipids in erythrocyte membranes [31]) and their fatty acid compositions can be determined.

In contrast to diacyl phospholipids, plasmalogens contain one "fatty aldehyde". Using the method described, which is based on fatty acid analysis, it is not possible to determine the exact molecular weight of plasmalogens. Therefore, for calculation of the amounts of PE–plasmalogen it was assumed that the aldehyde contained in the molecule has an average chain length of 17.5 carbon atoms, as data from Farquhar [32] showed that 15.9% of aldehyde molecules in PE–plasmalogen from human erythrocytes contained sixteen carbon atoms, 11.3% contained seventeen carbon atoms and 56.2% contained eighteen carbon atoms. Using this assumption also the amounts of PE–plasmalogen contained in erythrocyte membranes can be calculated. This might be interesting, as PE–plasmalogen, which represents 78% of the total PE in rat erythrocyte membranes (Table III), contains a large portion of long-chain polyunsaturated fatty acids ($C_{20:4}$, $C_{22:4}$, $C_{22:5}$ and $C_{22:6}$) and therefore might be of physiological significance.

The method described shows good reproducibility. The fatty acids determined in the present analyses represent 97–99% of the total phospholipid-bound fatty acids as compared with fatty acid composition data from other investigators [7,8,33]. Therefore, the error in the calculation of amounts of phospholipids caused by bound fatty acids which could not be detected or identified is small. This can also be seen from Fig. 5, illustrating areas of peaks identified and not identified. Moreover, the results for the amounts of phospholipids in rat erythrocyte membranes and also those of the fatty acid composition of individual phospholipid classes are in good agreement with those of other investigators [13,14,33–38]. The method can be applied to small blood samples (less then 1 ml) without loss of precision if the sensitivity of the method is optimized by the means discussed above. Hence the method is useful for blood samples obtained from small laboratory animals such as mice and rats. The method

gives simultaneous results for amounts of the major phospholipid classes and their fatty acid composition in erythrocyte membranes.

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