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Note

Rapid method for the determination of the phospholipid subclass distribution in human breast milk samples

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Phospholipids are distributed ubiquitously in living matter, including nutrients [1—4]. Their correct analysis is therefore of great interest to clinical, biochemical and nutritional researchers.

Separation of the subclasses sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) is commonly achieved by thin-layer chromatography (TLC) [5—7] or by liquid column chromatography (LC) [8—12]. These techniques allow good separation; however, quantitation of the subclasses remains a problem, especially in the case of the TLC, which is the method chosen when only small sample amounts are available [13]. Appropriate high-performance liquid chromatographic (HPLC) techniques are still in the process of being developed and, in addition, expensive laboratory equipment is needed.

In a recent publication from our laboratory a TLC method for the analysis of the phospholipid subclass distribution in milk was described. Good separation could be obtained by using solvent systems of different polarity, and quantitation was achieved by phosphorus assay [14, 15]. Although this method gives good results, it is somewhat inconvenient for the analysis of large series of samples because it is a time-consuming process. We have therefore developed a new method for the quantitation of the subclass distribution, which is based on reflectance measurement of properly sprayed TLC plates.

EXPERIMENTAL

Materials
Lipid standards were obtained from Sigma, St. Louis, MO, U.S.A. Nano-Sil 20 TLC plates (20 × 20 cm) came from Macherey-Nagel, Düren, G.F.R. All

chemicals and organic solvents in use were of analytical grade and were purchased from E. Merck, Darmstadt, G.F.R. Human milk samples were frozen immediately after sampling and stored at $-30°C$.

Instrumentation

Reflectance measurement was performed on a dual-wavelength chromatogram scanner, Model CS-910 from Shimadzu, Kyoto, Japan. Spots were measured in the dual-wavelength mode -- sample beam at 600 nm and reference beam at 450 nm — when molybdatophosphoric acid was used as spraying reagent. Plates sprayed with hydroxylamine—ferric chloride or ammonium heptamolybdate—perchloric acid were measured in the same mode at appropriate wavelengths (605 and 700 nm, 600 and 700 nm, respectively). Slit size was set to 10 mm (height), 0.5 mm (width). The linearizer was adjusted as described in the manual of the CS-910. The scanner was connected to a Sigma 10 data processor (Perkin-Elmer, Überlingen, G.F.R.). Quantitation was achieved by normalisation of the peak areas.

Methods

Preparation of lipid extracts from human milk, TLC and determination of phospholipids by phosphorus assay were carried out as described previously [14, 15]. For quantitative TLC scanning, samples (20 μl of lipid extract, or standard, containing a total of 1—10 μg of phospholipids) were applied as spots on the TLC plates.

Triglyceride and fatty acid standards (1--50 μg per spot) were chromatographed on Nano-Sil 20 plates using hexane—diethyl ether—methanol—acetic acid (70:20:10:1, v/v) in a first run (8 cm) and hexane-diethyl ether—chloroform—acetic acid (70:20:10:1, v/v) in a second run (17 cm).

The fatty acid composition of the phospholipids was determined as described elsewhere [16].

Molybdatophosphoric acid was used in ethanolic solution (20%, w/v) [17], and plates were kept at 80°C for exactly 10 min after spraying with this reagent. Hydoxylamine—ferric chloride and ammonium heptamolybdate—perchloric acid reagents were prepared and applied according to the description of Krebs et al. [17]. They were used within 1 h after preparation.

RESULTS

Three lipid-staining reagents — hydroxylamine—ferric chloride (HF), ammonium heptamolybdate—perchloric acid (AM) and molybdatophosphoric acid (MP) — were studied as to their usage for quantitative reflectance measurements of TLC-separated phospholipids. Only on using MP did an intensive color develop when milk samples containing about a total of 1 μg of phospholipid were subjected to chromatography. Much larger amounts (20 μg of total phospholipids per spot) had to be applied to obtain a comparable color intensity when using the other reagents. This, however, led to overloaded TLC plates, due to the high triglyceride concentration in milk, and therefore the separation of phospholipid subclasses was significantly impaired.

A more detailed investigation of the observed color reactions was done by

using different fatty acid, triglyceride, and phospholipid standards. When 50 μg of lipid standard were applied, AM reacted only with lipids containing phosphorus, and MP only with unsaturated lipids, while HF reacted with all lipids under investigation. Therefore, because unsaturation of the fatty acids derived from the phospholipids of different biological materials might differ, AM and HF would seem to be more appropriate than MP for phospholipid quantitation. However, the better colour development makes MP the only one really suitable for this purpose. This caused us to investigate the colour reaction of MP with fatty acid standards of different degrees of unsaturation and chain length. These standards were applied as a spot and developed in the solvent systems for free fatty acids as described under Experimental. All unsaturated fatty acids had identical R_F values and, as mentioned above, only they reacted with MP. As a result, the degree of unsaturation and the chain length could be shown to have no influence on the color density.

For the analysis of the phospholipid subclass distribution in milk, samples were also applied as a spot onto the TLC sheets and separated as described elsewhere [14]. By analyzing the TLC sheets with the TLC scanner it could be demonstrated that signals are linear over the range 0.3—3 μg of a single phospholipid subclass or 1—10 μg of total phospholipids applied. This fairly wide range could only be obtained when the linearizer of the CS-910 was properly set. The lower limit for the phospholipid subclass quantitation was therefore 5 mg of total phospholipids per 100 ml of milk. Without linearization, linearity was only obtained for 0.5—1.5 μg of a single phospholipid subclass applied.

As SPH and lyso-phospholipids have only one fatty acid per molecule, data obtained for these subclasses have to be multiplied by a factor of 2 to compensate for the resulting differences in colour development.

Fig. 1 shows that the signal for PE compared to that for PC is high, although these two phospholipid subclasses occur in almost equivalent amounts in human milk [18]. This discrepancy can be explained as a factor of the different R_F values [19—21]. We could demonstrate by using reference mixtures of PC and PE having the same degree of unsaturation, that PE gives at least a 30% larger peak area than PC. The area for PI and PE obtained from milk samples were therefore corrected by multiplication with a response factor, which could be obtained by division of the PC area by the PE area (standards with the same degree of unsaturation). This factor has to be calculated for each TLC plate analyzed. Table I summarizes the relative response factors (RRF) for individual phospholipid subclasses as these have been used for the processing of raw data.

Fivefold determinations of the phospholipid subclass distribution in three milk samples gave variances ranging from 2.1% to 3.2% for SPH, PC and PE, and from 4.3% to 5.5% for PS and PI. These variances are much smaller than those observed when using the phosphorus assay [15]. Many analyses of a single milk sample over a six-month period showed a very good reproducibility with a variance not exceeding the values described above.

A reflectance chromatogram of the phospholipid subclasses derived from human milk sprayed with MP is shown in Fig. 1. It demonstrates that all subclasses are resolved well.
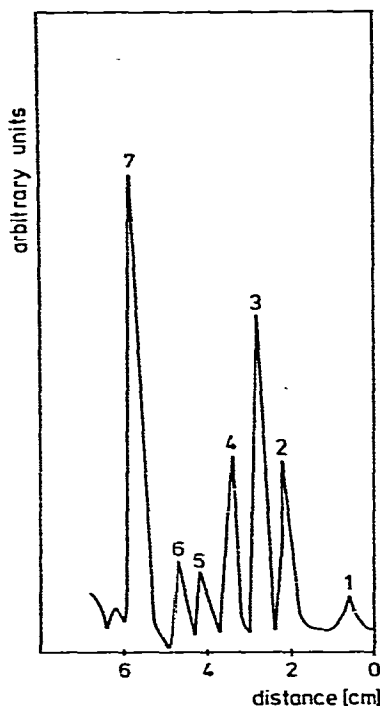
Fig. 1. Reflectance chromatogram of TLC-separated human milk phospholipids stained with MP. 1 = application spot, 2 = SPH, 3 = PC, 4 = PS, 5 = LPE, 6 = PI, 7 = PE. Details under Experimental.

TABLE I

RELATIVE RESPONSE (RRF) FOR REFLECTANCE MEASUREMENT OF TLC SEPARATED PHOSPHOLIPID SUBCLASSES

Plates were sprayed with MP. Details under Results.

Phospholipid subclass	RRF
Lyso-PC	2.000
SPH	2.000
PC	1.000
Lyso-PS	2.000
PS	1.000
Lyso-PE	2.000
PI	0.700*
PE	0.700*

*RRF values were obtained by dividing the PC area by the PE area from a standard mixture containing equivalent amounts of these phospholipids.

Table II shows the comparison of the phospholipid distribution in nine human milk samples determined by reflectance measurement and phosphorus assay. The analyzed milk samples were donated by several mothers at different stages of lactation.

TABLE II

MOLAR DISTRIBUTION OF PHOSPHOLIPID SUBCLASSES IN HUMAN BREAST MILK SAMPLES OBTAINED FROM DIFFERENT MOTHERS

Data, expressed as percentage total phospholipid, are the average of two separate analyses.

Sample	Method*	SPH	PC	PE
1	a	27.0	33.9	31.7
	b	25.5	32.9	33.5
2	a	31.6	17.2	27.8
	b	31.8	19.4	29.3
3	a	29.3	21.6	28.1
	b	31.3	24.7	27.5
4	a	30.0	32.3	34.0
	b	29.1	31.5	33.0
5	a	26.9	26.7	25.7
	b	28.9	22.4	27.5
6	a	25.0	20.5	35.7
	b	23.1	18.8	35.3
7	a	28.1	23.0	35.6
	b	29.0	23.6	33.7
8	a	25.7	23.1	32.3
	b	28.0	25.5	34.5
9	a	27.5	23.5	31.1
	b	22.7	23.0	35.9

*a = phosphorus assay; b = reflectance measurement.

DISCUSSION

The quantitative determination of the phospholipid subclass distribution in human breast milk based on reflectance measurement as described herein compares well with the determination by phosphorus assay [14]. In order to show the validity of the new method, we analyzed a total of nine different human breast milk samples, which were obtained from several mothers at different stages of lactation. Their total phospholipid content, subclass distribution and the fatty acid composition therein was therefore expected to show rather significant variations [16]. As seen from Table II, the data obtained by the two different methods are comparable, even though the variations of the subclass composition between the samples are considerable. This can only be explained by the fact that, despite the changes in the fatty acid composition of total phospholipids during lactation, the total unsaturated fatty acids therein remains relatively constant [16].

Due to the phosphorus threshold of the TLC plates used, data for SPH, PC and PE obtained by phosphorus assay showed considerable variations in multiple analyses of the same sample (variance: SPH $\approx$ 13%, PC $\approx$ 10%, PE $\approx$ 10%) [15].

The reproducibility of data from reflectance measurements was better. To obtain such reproducible values, however, some care had to be taken. Spotting, developing and spraying had to be performed without any delay between these

particular steps. Furthermore, chromatographic conditions, e.g. chromatographic tank saturation, had to be considered. The final phospholipid determination (phosphorus assay or reflectance measurement) could, however, be done later. The colour of the MP-sprayed plates was stable for three days when the plates were stored in the dark. During this period no changes of the subclass distribution could be observed upon repeated scanning.

Taking into account the results presented above, the described method should also be useful for the determination of the phospholipid subclass distributions of biological material other than human breast milk. Of course, appropriate response factors would have to be evaluated to process the raw data. For serial analysis, necessary in many biological studies, these additional experiments may take only a small part of the time that would be needed for performing a phosphorus assay for all samples under investigation.

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