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Determination of the phospholipid content of human milk, cow's milk and various infant formulas

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Summary: The phospholipid (PL) content of human milk, cow's milk, and various infant formulas was determined by recently developed high performance liquid chromatography (6). As the examinations promised, the content of phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholin (PC), and sphingomyelin (SP) was not changed by homogenization and pasteurization of cow's milk. Levels of phosphatidylglycerol (PG) were below the detection limit. Furthermore it has been proved that human milk and cow's milk are more or less identical in PL content.

Some of the PL in human milk varies during the course of pregnancy and postpartum. PI, PC, and SP content in the prepartum mammarial secretion lies above the average content of mature human milk after delivery. Before the contractions start, all the PL examined show a more or less considerable decrease. PC drops to 30 % of the value at the beginning of the examination six weeks before delivery. PG contents are very low throughout the whole period. Contrary to the others, PC content recovers three weeks after delivery, which may be the result of the endogenous surfactant replacement system.

To compare PL content with human milk and cow's milk, 13 different infant formulas have been examined. There are considerable differences to be found in and among adapted milk, partially adapted milk, and special formulas. None of the PL examined could be found in all the infant formulas, where PG content was usually low, except in some Milupa formulas. PE and PI were not to be found in some special formulas. Most of the formulas contain high amounts of SP, in some cases higher than the amount of PC. To a certain extent infant formulas contain a considerably greater amount of other PL concentrations than human milk and cow's milk. In most of the formulas examined the PL content is generally so high, that it can be used as a source of PL for the newborn.

Zusammenfassung: Mit Hilfe einer speziell hierfür entwickelten (6) Hochdruckflüssigkeitschromatographie-Methode (HPLC) wurde der Phospholipidgehalt (PL) von Frauenmilch, Kuhmilch und verschiedenen Säuglingsnahrungen bestimmt. Die Untersuchungen zeigen, daß der Gehalt an Phosphatidylinositol (PI), Phosphatidyläthanolamin (PE), Phosphatidylcholin (PC) und Sphingomyelin (SP) durch Homogenisieren und Pasteurisieren von Kuhmilch nicht verändert wird. Der Gehalt an Phosphatidylglycerol (PG) liegt hier, wie bei der Frauenmilch, unter der Nachweisgrenze. Auch bei den anderen untersuchten PL konnten zwischen Frauen- und Kuhmilch keine nennenswerten Unterschiede festgestellt werden.

Der Gehalt einiger PL verändert sich im Verlauf der Spätschwangerschaft und der Post-partum-Periode charakteristisch. So liegt der Gehalt an PI, PC und SP in der Vormilch deutlich über dem Gehalt reifer Frauenmilch. Bereits vor Beginn der

Wehen zeigen alle PL einen mehr oder weniger scharfen Abfall. So verliert Vormilch vor der Geburt 70 % des Gehaltes, den PL-Proben sechs Wochen vor der Geburt zeigen. Die PG-Gehalte sind allgemein in der gesamten Beobachtungsperiode sehr niedrig. Im Gegensatz zu den anderen Parametern erholt sich der PC-Gehalt drei Wochen nach der Geburt wieder, was als Ausdruck eines endogenen Surfactant-Regelmechanismus betrachtet werden kann.

Weiterhin wurden 13 verschiedene Säuglingsnahrungen im Vergleich mit Kuhmilch und Frauenmilch untersucht. Dabei wurden teilweise erhebliche Unterschiede zwischen adaptierter Milch, teilweise adaptierter Milch und Spezialnahrungen, aber auch innerhalb dieser Gruppen, gefunden. Keines der bestimmten PL konnte in allen Säuglingsnahrungen nachgewiesen werden. Mit Ausnahme einiger Milupa-Säuglingsnahrungen war der PG-Gehalt allgemein niedrig. PE und PI wurden nur in einigen Spezialnahrungen nicht gefunden. Die meisten Säuglingsnahrungen enthalten hohe Gehalte an SP, die teilweise höher als der Gehalt an PC liegen.

Allgemein kann gesagt werden, daß die untersuchten Säuglingsnahrungen z.T. wesentlich andere Phospholipidmuster als Frauen- und Kuhmilch aufweisen und daß zwischen einzelnen Säuglingsnahrungen erhebliche Unterschiede in der Zusammensetzung bestehen. In den meisten der untersuchten Nahrungen ist der Phospholipidgehalt so hoch, daß er durchaus als Phospholipidquelle für das Neugeborene genutzt werden kann.

Key words: cow's milk, human milk, high performance liquid chromatography (HPLC), infant formulas, phospholipids

Schlüsselwörter: Frauenmilch, Hochdruck-Flüssigkeitschromatographie (HPLC), Kuhmilch, Phospholipide, Säuglingsnahrungen

Introduction

Among other factors lung surfactant phospholipids (PL) play an important role in the development of the fetal lung and in the adaptation to breathing via the lung at birth, due to their surface activity. When the fetal lung is unable to function properly due to lack of phospholipids after delivery, this leads to the momentous respiratory distress syndrome (RDS). Despite extensive therapeutic measures for prevention, and where possible prenatal attempts at therapy, it is still the main cause of death in premature newborns.

For lung function not only is the main component lecithin (phosphatidylcholin = PC) of importance, but also other phospholipids such as phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). Therefore, a highly specific and sensitive method of analysis is necessary when assessing lung maturity. We used high performance liquid chromatography (HPLC) for determining the phospholipids in amniotic fluid (4). A variation of this method which we recently developed (6) is also suitable for the determination of phospholipids in other biological media such as gastric and tracheal secretion, alveolar lavages and in various kinds of milk and in infant formulas. Up to now the physiological value of phospholipids in milk has not been clear. The importance of phospholipids in human milk for lung maturation of the newborn remains unknown as well.

It was the aim of this study to answer the following questions:

a) Is the phospholipid content of cow's milk changed by homogenization and pasteurization?
b) Are there differences between the phospholipid content of human milk and cow's milk?
c) Do changes take place in the phospholipid content of human milk during pregnancy and postpartum?
d) Do infant formulas have a different phospholipid pattern than human milk or cow's milk?
e) Are there notable differences in the phospholipid content between the various infant formulas?

There are very few answers to be found in the literature to these questions. This is probably because it has only recently become possible to make precise investigations of the phospholipid content of milk at all, due to the previous lack of suitable specific methods. A few observations to question c can be taken from a study by Bitman et al. (1). They examined the fat composition of the prepartum mammarial secretion (PMS) of five women and compared it to the composition of colostrum and mature human milk (MHM). The whole fat content of the milk lay between 1 g/100 ml in the first phase, starting 42 days before delivery and rose to levels of up to 3–4 g/100 ml in colostrum and MHM. Both before delivery (93 %) and after delivery (97 %) the main part of the fats consisted of triglycerides. In looking for the membrane active components it was found that PMS contained higher proportions of PL (3.2 g/100 ml), cholesterol (2.3 g/100 ml), and cholesterol esters (1.1 g/100 ml) than MHM. After delivery all three components decreased of the phospholipids for example from 3.2 g/100 ml to 0.65 g/100 ml. Harzer et al. (3) present data for 550 human milk samples (collected through the first five weeks after delivery) and their contents of triglycerides, cholesterol, PL, proteins and the trace elements copper, zinc and magnesium. They asked, "what about the high levels of cholesterol and living cells? Are they of any physiological benefit?" As cholesterol and PL form the membranes of secreted fat globes and therefore derive from the apical membrane of secretory cells, the question of physiological benefit of PL content should be raised too.

We must find out whether the secretory cells in the mamma play a role in the neonatal adaptation as it does in the lung pool (5). In addition we had to clarify whether the obligatory surfactant proteins in mammarial material are similar to those of pulmonary surfactant (8).

Material and Methods

Milk samples were stored at 4 °C and examined shortly thereafter. Deep freezing is not recommended as it may lead to protein coagulation which may complicate the further treatment of the samples. Before extracting the material according to our method (6), samples should be homogenized in order to reverse any decomposition that may have occurred during storage. 200 to 500 mg of milk are required per examination. In order to enable the calculation to be made using an internal standard, defined amounts of lysolecithin (LL) are added to the milk before the extraction is executed. After the HPLC separation the compounds are determined quantitatively by computer, so volumina errors have no influence on the precision

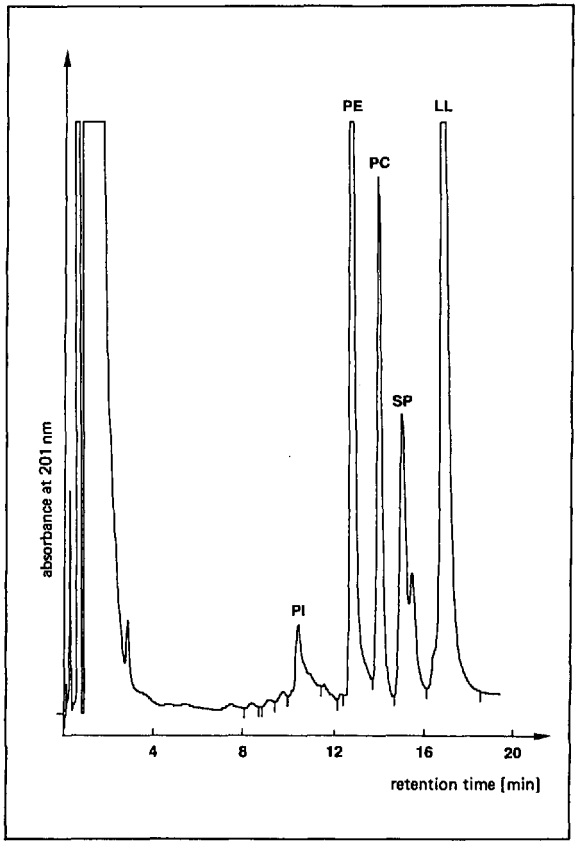

Fig. 1. HPLC-chromatogram of cow's milk before homogenization and pasteurization (untreated milk).

of the method. If measurable amounts of LL are present in the samples, which is the case in some infant formulas, then the less exact manual calculation according to the 100 % method has to be used in the quantitative calculation of the HPLC chromatograms (6). The HPLC method for the determination of PL in amniotic fluid which we described first (4), does not appear to be suitable for milk because a second extraction is not possible on account of the high fat content of the milk. Therefore the levels obtained for PE and PI are too low.

Results

Influence of homogenization and pasteurization on the phospholipid content of cow's milk

Figures 1 and 2 show HPLC chromatograms of cow's milk before and after homogenization and pasteurization. There is practically no difference in the chromatograms. The levels measured for every five samples of

untreated milk and homogenized and pasteurized unskimmed milk examined can be seen in Table 1. As the table shows, the levels of PG content of both samples lie below the detection limit. PI, PE, PC and SP values are within the margin of error conditional to the method. In this way it is assured that there is no reduction of phospholipids in the cow's milk caused by homogenization and pasteurization.

Comparative examinations of human milk and cow's milk

Figure 3 shows a typical chromatogram of mature human milk five days after delivery. The missing separation of the SP peak in Figure 3 is insignificant. It occurs according to the age and package thickness of the columns and has no analytical importance. PG is present in such small amounts in both cow's and human milk that its presence cannot be proven.

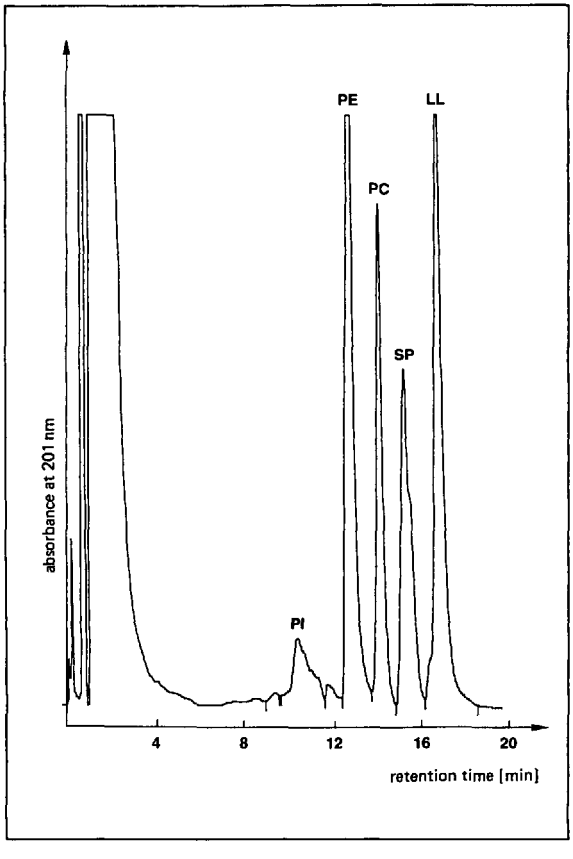

Fig. 2. HPLC-chromatogram of homogenized and pasteurized cow's milk (unskimmed milk).

Table 1. Comparison of the phospholipid content of untreated cow's milk and homogenized and pasteurized unskimmed milk.

Type of milk	Number of samples	Mean values of phospholipid content (mg/100 ml)				
		PG	PI	PE	PC	SP
Untreated milk	5	nd	1.99	4.17	3.31	5.53
SD			0.14	0.14	0.17	0.15
SD (%)			7.0	3.3	5.2	2.7
Unskimmed milk homogen. + past.	5	nd	1.49	4.50	3.45	7.39
SD			0.38	0.17	0.13	0.18
SD (%)			5.4	3.7	3.6	2.4

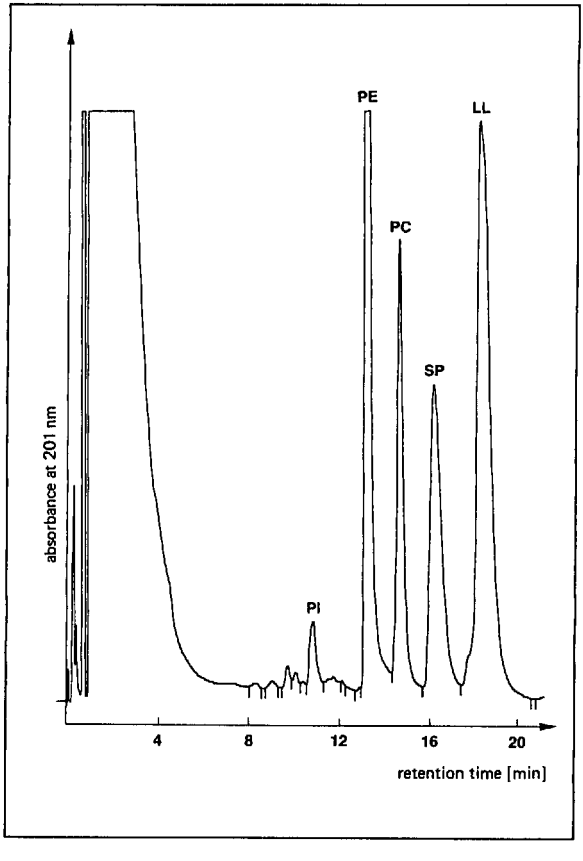

Fig. 3. HPLC-chromatogram of mature human milk.

Observation of the course of the phospholipid content of human milk

In two single examinations the course of the concentration of the most important phospholipids was examined before and after delivery in human milk within a period of six weeks before until eight weeks after delivery in patient A and four weeks before until three weeks after delivery in patient B. Fortunately we found two motivated patients who were willing to compress milk free of contamination from each breast almost every day. In both cases it was possible even long before delivery to obtain more than 0.2 ml secretion per sample which is the minimum amount needed for the analytical examination.

At the beginning the samples from each side of the breast were analyzed separately and differences were made between the first milk and milk withdrawn later. As in the analytical examinations no notable differences emerged in excess of the method's precision; this separation was discontinued during the course of the measurements. In Figs. 4–7 the courses for both patients are given separately. In spite of individual fluctuations of the contents, characteristic parallels are to be seen: In both cases the content of PG lies below the detection limits. Beyond this, a clear fall of the PL one to two weeks *before* delivery – not *during* delivery (!) to levels which partially lie more than 50 % below the highest levels in PMS was characteristic too. During the course of the breast-feeding period a slight increase

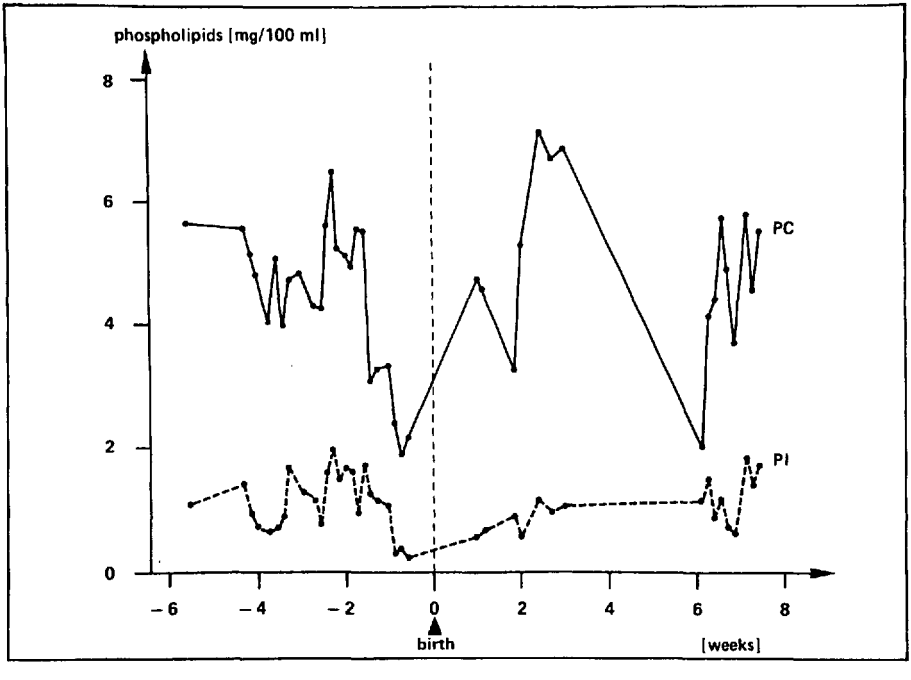

Fig. 4. PC and PI content of mammarial secretion of patient A before and after delivery.

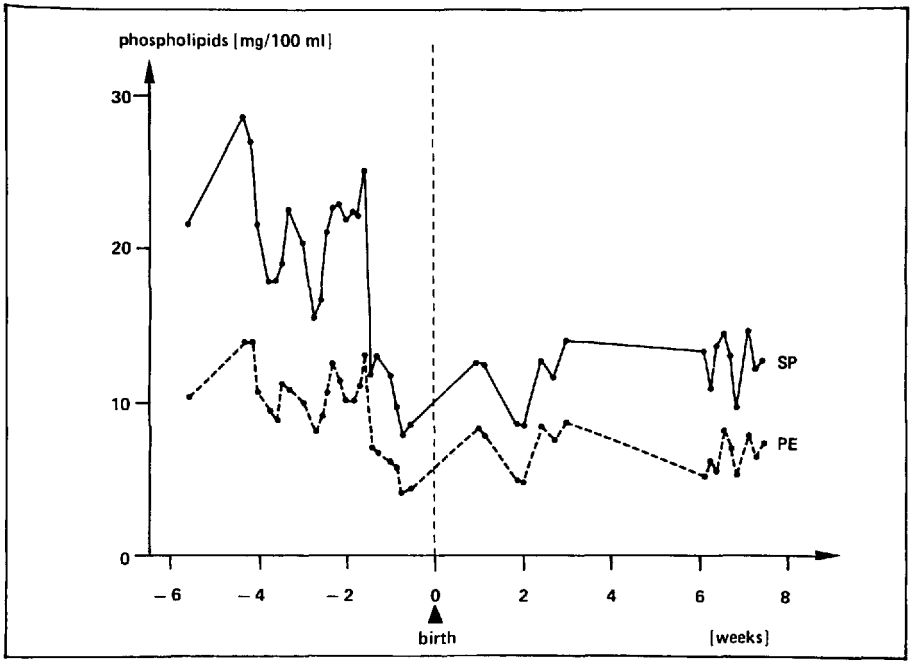

Fig. 5. PE and SP content of mammarial secretion of patient A before and after delivery.

in PC and PI (Fig. 4, 6) was observed. However the highest levels of PMS were only achieved again in patient A (Fig. 4). In addition to the small content of PG the equally lower content of PI in contrast to amniotic fluid is remarkable. Before delivery the levels lie at about 1.5 mg/100 ml and after delivery at about 1.0 mg/100 ml (Fig. 4, 6). The content of PE is considerably higher. In patient A it lies before delivery around 11 mg/100 ml (Fig. 5). In patient B it even exceeds 30 mg/100 ml (Fig. 7). During the period of breast feeding the values still amount to 7–10 mg/100 ml. PC shows a similar profile on a lowered basis (Fig. 4, 6). The concentration course of SP is of particular interest because it is very similar for both patients. The original levels lie in the range of 20–30 mg/100 ml and in comparison to amniotic fluid are extremely high (Fig. 5, 7). Even after a sharp fall during the delivery the levels at about 10 mg/100 ml are clearly higher than in amniotic fluid and, contrary to amniotic fluid, lie within the range of the levels for PE.

Phospholipid content of various infant formulas

There is a great variety of infant formulas on the market. In order to get a more complete impression of the PL content of various infant formulas, a possible representative selection had to be made. As Table 2 shows, samples from the following groups of infant formulas were examined:
1) adapted infant formulas (AF);

2) partially adapted infant formulas (PF);
3) special formulas (SF).

Table 2 gives examples of 13 products available commercially. Remarkable differences were found between the adapted formulas. Aponti Pre (No. 1) in contrast to all the other examples in this group contains resonable amounts of PG and has a high PC content, making this formula very different from Hippon A (No. 2) and Pre Humana (No. 3). None of those formulas contains LL.

Considerable variations in the content of phospholipids were to be found in the partially adapted formulas Aponti 1 (No. 4) and Hippon 1 (No. 5). In the first, all the PL including PG and LL are present in high concentrations; Hippon 1 appears to be very low in PL and contains no measurable amounts of PG and LL.

Bessau Reisschleim (No. 7) and Humana Hydrolysat (No. 9) enriched with trace elements appeared to be particularly worthy of mention in the group of special formulas. These formulas are the only ones not containing

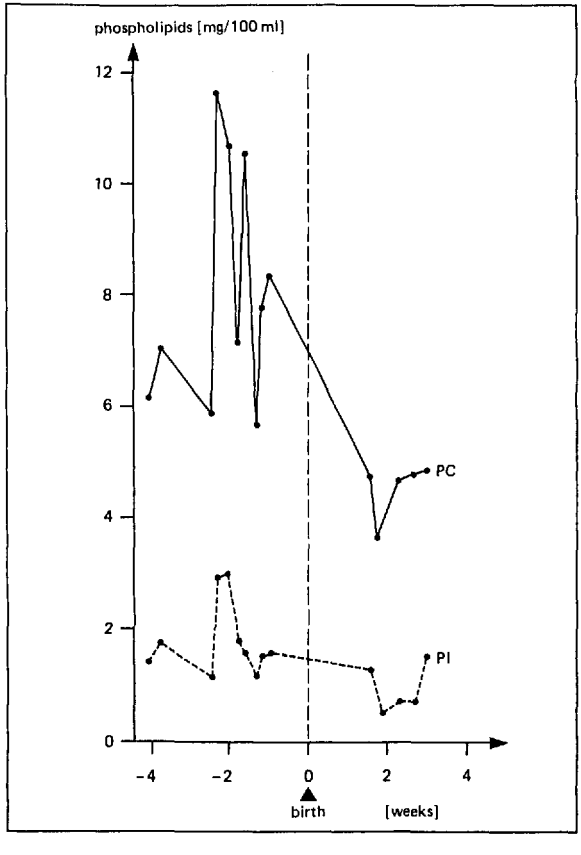

Fig. 6. PC and PI content of mammarial secretion of patient B before and after delivery.

Table 2. Phosopholipid contend of different infant formulas. AF = adapted formula; TF = partially adapted formula; SF = special formula.

No	Type of formula	Name of formula	Phospholipid content (mg/100 g)					
			PG	PI	PE	PC	SP	LL
1	AF	Aponti Pre	1.5	5.0	7.0	11.7	7.1	–
2	AF	Hippon A	–	1.5	3.7	4.2	7.4	–
3	AF	Pre Humana	–	1.9	3.4	3.7	6.7	–
4	PF	Aponti 1	0.5	1.0	2.5	4.4	1.6	1.9
5	PF	Hippon 1	–	0.6	1.5	1.8	2.7	–
6	SF	Beba HA	2.8	10.8	1.7	9.4	13.0	4.9
7	SF	Bessau Reis-schleim	0.2	–	–	–	0.8	6.1
8	SF	Humana Heil-nahrung	0.3	0.9	0.1	2.9	2.0	2.2
9	SF	Humana Hydrolysat	0.7	–	0.1	–	1.9	4.0
10	SF	Milupa Heil-nahrung	4.8	17.5	15.7	40.4	3.6	5.1
11	SF	Milupa Pregomin	7.9	11.6	0.7	10.0	11.4	17.1
12	SF	Milupa Prematil	–	1.0	2.5	3.3	6.2	–
13	SF	Milupa Som	6.7	32.4	5.6	62.3	–	0.6

any lecithin (PC) and no PI and are also very low on the other PL examined with the exception of LL. On the other hand, Milupa Heil-nahrung (No. 10), Milupa Pregomin (No. 11), and Milupa Som (No. 13) were shown to have very high quantities of PI and PC. Formula No. 11 contains an usually high amount of LL. With the exception of Milupa Prematil (No. 12), all the Milupa formulas examined showed a high PL content. In Fig. 8 a HLPC chromatogram of Aponti Pre (No. 1) is given as an example of formula chromatograms. Similarities with mature amniotic fluid can clearly be seen.

Discussion

The PC content of cow's milk is not changed by homogenization and pasteurization, so it is possible to establish that this procedure does not influence the nutritional value of cow's milk as far as the PL content is concerned. Furthermore it is interesting that human milk and cow's milk are nearly identical in their PL content. If the nutritional importance of PL for feeding newborns should be proven, then it can be assumed that cow's milk would be an adequate substitute.

It is also of interest that the content of some of the phospholipids in human milk clearly varies during the course of pregnancy. The compari-

son of Figs. 4–7 illustrates a similar course of PL content in mammarial secretion of the two patients. In both cases the PE, PC, and SP content in the PMS lies clearly above the average content after delivery. It is also characteristic for both cases that even one to two weeks before delivery, before the contractions start, all the PL examined showed a significant decrease.

Through this decrease the content of PC drops to 30 % of the values found at the beginning of our examinations. It is not clear why the PL start to lessen in the PMS at this time, but it recovers later in MHM. This phenomenon could offer interesting aspects for the improvement of lung maturity.

It is also a startling fact that parallel examinations of our milk samples regarding the content of trace elements by Brätter et al. (2), show that trace elements like iron, zinc, copper, and magnesium demonstrate a similar concentration behavior during the course of the pregnancy and after delivery. The same milk samples that were examined here were used and this cannot be a coincidence. Further, it is worth mentioning that during the whole examination period, PG does not play an important role. An increase during later pregnancy, something like a sign of lung maturity, cannot be proved in human milk. Contrary to the other parameters, the PC

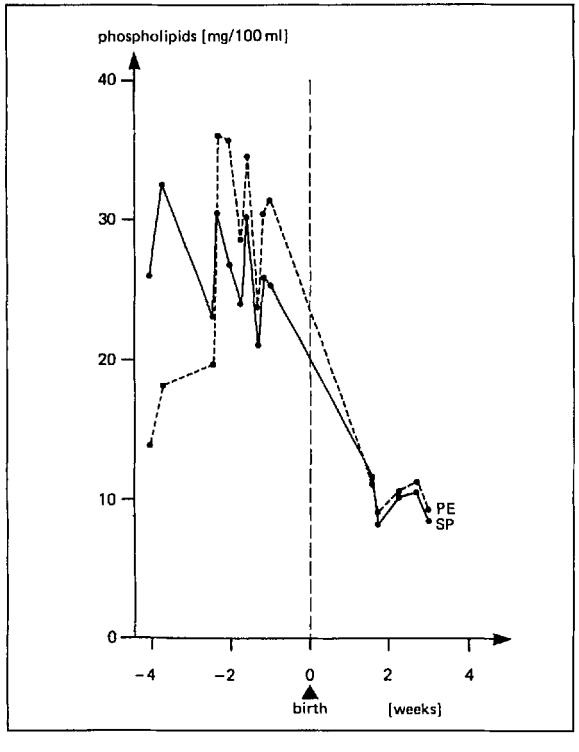

Fig. 7. PE and SP content of mammarial secretion of patient B before and after delivery.

content starts to rise about three weeks after delivery and then remains on a constant level. This behavior raises the following questions:

Do lecithin losses caused by delivery recover in this period? Is this picture the expression of a regulation mechanism to adjust the loss of lecithin during delivery?

In addition to this endogenous surfactant replacement system for surfactant recycling, synthesis, and secretion, exogenic sources for complementary application should be developed and tested (7). As genes for the key surfactant proteins have already been cloned, such postnatal external replacement therapy seems to become possible in the future. At this point it should be mentioned that the comparison between human milk and infant formulas on the one hand and amniotic fluid on the other hand seems to be quite reasonable: as milk does for the newborn, amniotic fluid acts as a source of nutritive substances for the fetus.

Whereas in the phospholipids discussed up to now the concentration was comparable to amniotic fluid, this was not the case as far as sphingo-

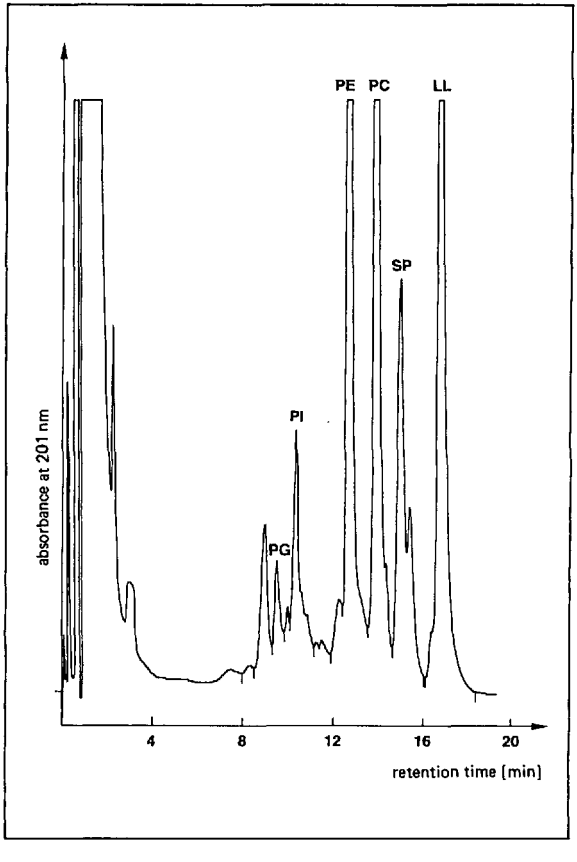

Fig. 8. HPLC-chromatogram of infant formula Aponti Pre (No. 1).

myelin is concerned. Its concentrations lie much higher in human milk than those of the other phospholipids and also clearly higher than in amniotic fluid. It would be interesting to check the importance of this fact for infant formula production.

Our results correlate with the findings of Harzer et al. (3), who determined the total amount of PL at 30–40 mg/100 ml, but did not investigate the different fractions of PL. A second finding published by Bitman et al. (1) is confirmed by our results too: there is a significant drop in PL values during delivery, starting one to two weeks before delivery.

The results of the examinations of 13 different infant formulas are listed in Table 2. As already mentioned, there are considerable differences to be found in the three groups examined: adapted milk, partially adapted milk, and special formulas. But also within the groups considerable differences were found by which we can assume that the various firms use different ingredients for the production of infant formulas. None of the PL examined could be found in all infant formulas. Normally the PG contents were low, except for Milupa formulas 10, 11 and 13 which had considerably higher PG contents. PI was not traceable in special formulas 7 and 9. PE was not to be found in special formula 7, which – like formula 9 – occupies an exceptional position since it does not contain any of the main phospholipids.

With the exception of special formulas 7 and 9, all the products exhibit a relatively high content of lecithin (PC). Bearing in mind that lecithin, in addition to its function for lung maturation of the fetus, is also of great importance for nutritional physiology in general; therefore, this fact is not surprising. On the other hand it is more surprising that some of the formulas contain more SP than PC. When considering lysolecithin (LL), formula 11 (Milupa Pregomin) is remarkable, since it has an extremely high LL content of 17.1 mg/100 ml.

In conclusion we can state that to a certain extent infant formulas contain considerably different amounts of other phospholipid concentrations than human milk and cow's milk. There are enormous differences in the composition between the infant formulas. In most of the formulas examined the phospholipid content is generally so high, that – provided the realization is possible – it can be used as a source of phospholipids for the newborn. In addition to giving results, this publication also includes some questions and speculations. Further examinations are necessary for explanations.

Acknowledgement

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