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High-performance liquid chromatography with evaporative light-scattering detection for the determination of phospholipid classes in human milk, infant formulas and phospholipid sources of long-chain polyunsaturated fatty acids ☆

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Abstract

We developed and validated a new high-performance liquid chromatographic method for the separation of phospholipid classes in human milk, infant formulas and phospholipidic sources of long-chain polyunsaturated fatty acids (LC-PUFAs) used in paediatric nutrition. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin were separated in less than 25 min using an Extrasil silica column (150×4.0 mm I.D., 3-μm particle size) by isocratic elution with a mixture of isopropanol–hexane–water. Phospholipids were determined by an evaporative light-scattering detector. Several chromatographic conditions were assayed to optimise the method, whose suitability is shown by the detection limits, linearity ranges and precision rates obtained. The main advantages of the proposed method are its speed and the direct determination of the main phospholipids present in human milk, infant formulas and the phospholipid sources of LC-PUFAs used in paediatric nutrition.

Keywords: Milk; Infant formulas; Food analysis; Phospholipids; Lipids; Fatty acids; Choline

1. Introduction

Human milk is regarded as the most appropriate source of food for a healthy infant during the first 4–6 months of life [1]. Like most infant formulas, it contains more than 98% of milk fat in the form of triglycerides (TGs) [2]. These are within small-emulsified globules surrounded by a structural membrane made of phospholipids (PLs) [3]. PLs do not only contribute to the physical stability of fat milk, but also perform a key nutritional function. Is well known that sphingomyelin (SM) and phosphatidylcholine (PC), which are the main PLs in human milk, provide a source of choline to the neonate [4–7].

Choline is involved in several biological pro-

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cesses. This amino alcohol is a major constituent of membranes. It is critical in brain development of both fetuses and infants. Several studies have suggested that choline supplementation alters hippocampal function in rats. Choline is also essential for the formation of the neurotransmitter acetylcholine. Furthermore, it has been reported to regulate cellular signal transduction. Moreover, it provides a source of labile methyl groups in intermediate metabolism [4–14].

Maternal reserves of choline are depleted in pregnancy and lactation [13], suggesting that neonates require especially large amounts. Thus, it is an essential nutrient for the preterm infant whose hepatic pathway for choline biosynthesis is not yet completely functional [14]. For this reason, the American Academy of Paediatrics (AAP) has recommended that infant formulas should contain at least 7 mg of choline/100 kcal [4] (1 cal=4.184 J). Choline is ingested mainly in the form of PC and SM rather than as a free base [4,5,7].

Further research on the composition of human milk and infant formulas may improve our understanding of the infant nutritional requirements and, thus, of the most suitable feeding practices.

Several analytical methods have been described for the determination and quantification of PL classes in human milk fat: thin-layer chromatography [15], column chromatography [16], and high-performance liquid chromatography (HPLC). Polarity differences have been exploited in normal-phase (NP) chromatography to obtain the PL profile of milk fat [17–19].

Various detection methods have been used in the analysis of PL separation by HPLC. Evaporative light-scattering detection (ELSD) affords advantages over ultraviolet detection, refractive index and flame ionisation in that it does not exist baseline drift. ELSD is not sensitive to the flow-rate of the solvent and quantifies any solute less volatile than the solvent.

The aim of this study was to develop a method for the determination of PLs in human milk, infant formulas and phospholipidic sources of long-chain polyunsaturated fatty acids (LC-PUFAs) by NP-HPLC with ELSD and thus to improve the design of new infant formulas to incorporate PLs resembling those of human milk.

2. Experimental

2.1. Materials

HPLC-grade *n*-hexane, HPLC-grade isopropanol and HPLC-grade methanol were purchased from SDS (Peypin, France). Milli-Q water was obtained with a Milli-Q Plus ultra-pure water system from Millipore (Milford, MA, USA). All other reagents were of analytical grade. Dichloromethane and absolute methanol were purchased from Panreac (Barcelona, Spain). Absolute *n*-hexane and absolute isopropanol were from Merck (Darmstadt, Germany). PLs standards were of analytical grade (purity greater than 99%) including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM) were purchased from Sigma (St. Louis, MO, USA).

2.2. Liquid chromatography

The chromatographic equipment consisted of a Hewlett-Packard (Waldbronm, Germany) Model 1050 pump system, a Waters 717 Plus Autosampler (Milford, MA, USA), a ELSD detector ACS (Macclesfield, UK) Model 750/14 and a HP 3365 series II Chemstation, which acquired data from the ELSD detector.

The analytical column used was an Extrasil silica (150×4.0 mm I.D., 3-μm particle size) with a precolumn (2×4.0 mm) from Tracer Analitica (Teknokroma, Barcelona, Spain).

2.3. Samples

Three samples were analysed: a mature human milk sample, collected by manual expression, heated rapidly to inactivate lipases [20] and stored at −20 °C until analysis; an infant formula supplemented with LC-PUFAs, which provides around 10% of its fat from egg phospholipids, and Ovothin® 120 (Lucas Meyer, Hamburg, Germany), which is used as source of LC-PUFAs (mainly arachidonic acid and docosahexaenoic acid) in some infant formulas. Ovothin® is a natural, highly viscous mixture of egg PLs and egg oil that contains around

30% of PLs, mainly phosphatidylcholine and phosphatidylethanolamine.

2.4. Sample preparation

2.4.1. Human milk

A total of 1.5 ml of mature human milk stored in a 16×125-mm test tube covered with PTFE-lined caps was extracted with 6 ml of absolute *n*-hexane, 2 ml of absolute methanol and 1 ml of absolute isopropanol. The mixture was shaken mechanically for 1 min and centrifuged at 1500 *g* for 2 min at 20 °C. As much of the upper hexane fraction as possible was removed. The hydroalcoholic layer, containing the polar lipids, was separated and evaporated by removing the solvent under a gentle stream of nitrogen.

The extract was redissolved in 2 ml of HPLC-grade methanol. The tube was shaken for 1 min and the mixture was centrifuged at 1500 *g* for 2 min at 20 °C. Finally, the supernatant was passed through a 0.45-μm filter. The samples were stored at −20 °C. The solution time before injection into the HPLC may require up to 3 h [21].

2.4.2. Infant formula

A total of 0.5 g of infant formula was exactly weighed in 16×125-mm test tubes. We added 6 ml of dichloromethane-absolute methanol (2:1, v/v). After mechanical shaking for 1 min, the mixture was centrifuged at 1500 *g* for 2 min at 20 °C. Finally, the organic phase was separated and evaporated under nitrogen stream. Polar lipids contained in fat were dissolved in 2 ml of HPLC-grade methanol. The tube was shaken for 1 min and the mixture was centrifuged at 3000 rpm for 2 min at 20 °C. Finally, the supernatant was passed through a 0.45-μm filter. The samples were stored at −20 °C until injection into the HPLC.

2.4.3. Ovothin®

A total of 0.2 g of Ovothin® was weighed in 16×125-mm test tubes. Polar lipids contained in the sample were extracted and dissolved as described above (in Section 2.4.2).

2.5. Chromatographic conditions

PLs were separated by chromatography with isocratic elution with isopropanol–hexane–water (55:37.2:7.8, v/v/v) in less than 25 min. The flow-rate of the eluent was 1 ml/min and the column temperature was 35 °C. The volume of sample injected was 20 μl. The temperature of the detector was 85 °C and the gas flow (from air compressor) was 10 l/min. Compounds were identified by comparing the retention times of the sample peaks with those of the phospholipid class standards run earlier. Quantification was carried out by external standardization.

3. Results and discussion

3.1. Dissolution of PLs

First, we would like to highlight the differences in the PL content of the three samples analysed. Mature human milk is a dynamic system that contains around 0.002% (w/v) of PL [3]. The infant formula used, which includes egg phospholipids, consists of around 0.36% (w/w) of PL. Finally, Ovothin® is a mixture that contains around 30% (w/w) of PLs. The lipid fraction of the three samples contains mainly TGs.

Several experiments were carried out to optimise the dissolution of PLs present in the infant formula fat and Ovothin®. It is well known that fats containing mainly saturated fatty acids such as these can only be completely dissolved with chlorinated solvents [18,22]. However, given the low concentration of PLs in the samples, the direct injection of this fat leads to the masking of peaks corresponding to PE, PI and PS into the peak corresponding to neutral lipids and free fatty acids (peaks 1 and 2 in all three chromatograms). Moreover, chlorinated solvents are evaporated rapidly, which significantly increases the concentration of the analytes. In addition, we observed that these solvents did not completely dissolve the most polar PL standards, such as PC and SM.

Most reports on methods for SM determination

Table 1
Recovery rates (%) in infant formula

Compound	Initial content (μg)	Level 1 (μg added)	Recovery (%)	Level 2 (μg added)	Recovery (%)	Average recovery rates
Phosphatidylethanolamine	10.93	10	108.1±0.42	25	107.5±0.74	107.8±0.42
Phosphatidylinositol	6.88	7	91.3±1.62	20	92.4±1.56	91.85±0.77
Phosphatidylserine	3.15	3	98.9±1.73	10	100.8±2.10	99.85±1.34
Phosphatidylcholine	30.80	30	94.6±1.29	50	96.8±0.47	95.70±1.55
Sphingomyelin	4.90	5	103.1±2.01	15	102.8±1.86	102.95±0.21

use high percentages of methanol to dissolve the sample and the PL standards [23–28]. For this reason, we aimed to solubilize samples and standards in methanol. All PL standards were totally solubilized, whereas samples were partially solubilized, since a precipitate containing apolar lipids (mainly saturated short-chain TGs and cholesterol) was formed. However, recovery assays revealed that none of phospholipids was lost in precipitate. Recovery was tested by the standard addition procedure using two addition levels for each compound. Three determinations were performed for each addition level. Results are summarised in Table 1.

To avoid the injection of precipitates into the HPLC system, which may interfere with the analysis and affect the column, a previous centrifugation step before HPLC-filtering was added.

The separation of polar lipids from human milk was performed by a single extraction using methanol and isopropanol. The redissolution of PLs in methanol showed no formation of precipitate, since TGs and cholesterol were recovered mainly in the discarded hexane phase. Recovery assays revealed that none of PLs was lost in hexane phase (Table 2).

3.2. Mobile phase

Various mobile phases have been used in PL analysis by NP-HPLC with ELSD for the separation of complex mixtures like those found in several samples [23–33]. Hexane, isopropanol and water mixtures are widely used in the separation of PLs. Nevertheless, in most cases a gradient is required to achieve high resolution, which may modify the retention times of analytes owing to the low stability of normal-phase columns when compared with reversed-phase columns [34]. Yoon et al. [33] reported an isocratic NP-HPLC method to separate PC and SM from natural samples. They found that the best resolution was obtained with a mobile phase composed of isopropanol–hexane–water (55:36:9, v/v/v). The method showed short analysis time (less than 7 min). When we applied this method good resolution was obtained when standards were injected, but not with samples, since the peaks corresponding to neutral lipids and PE were not fully separated. Therefore, the mobile phase was modified for better resolution: water content was reduced, which increased retention times and lowered column

Table 2
Recovery rates (%) in human milk

Compound	Initial content (μg)	Level 1 (μg added)	Recovery (%)	Level 2 (μg added)	Recovery (%)	Average recovery rates
Phosphatidylethanolamine	2.93	10	89.1±0.24	25	91.4±2.70	90.25±1.62
Phosphatidylinositol	0.74	3	92.3±1.39	10	93.6±1.97	92.95±0.91
Phosphatidylserine	1.05	3	98.9±1.73	10	100.1±1.05	99.5±0.84
Phosphatidylcholine	3.41	10	101.6±1.47	25	99.8±2.55	100.7±1.27
Sphingomyelin	3.12	10	104.2±1.29	25	107.9±1.00	106.05±2.61

pressures. Finally, we achieved total separation of PE and neutral lipids using isopropanol–hexane–water (55:37.2:7.8, v/v/v) as mobile phase. The resulting chromatogram was relatively simple (Fig. 1), showing a positive identification of the compounds in less than 25 min. Neutral lipids (e.g., triglycerides, diglycerides and cholesterol) eluted very early (within 2 min) (Fig. 1, peaks 1 and 2).

The stability of column was studied by injecting a solution of 5% (w/v) of standard PLs over the course of 1 day. Results showed minimal changes in retention time (Table 3).

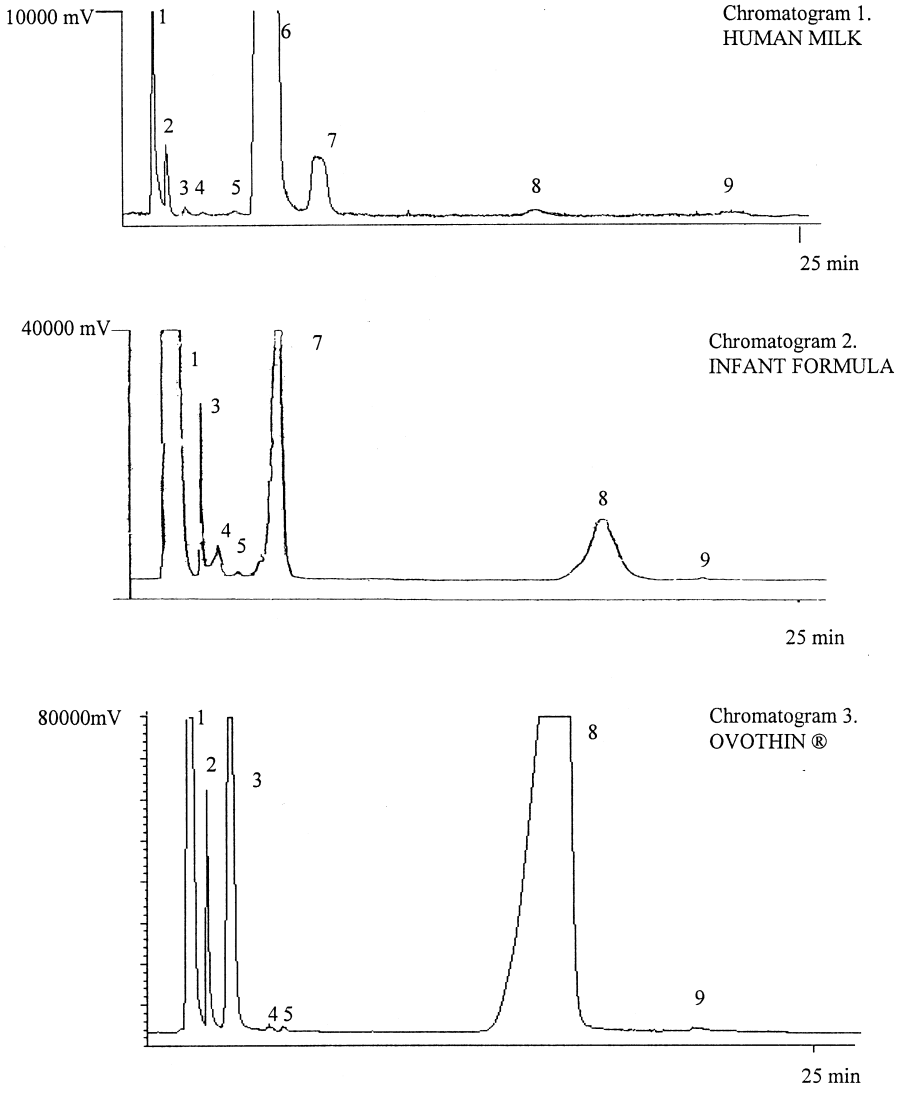

Fig. 1. HPLC–ELSD chromatograms of human milk PLs (1), infant formula PLs (2) and Ovothin® (3). HPLC–ELSD conditions: isocratic elution with isopropanol–hexane–water (55:37.2:7.8, v/v/v). The flow-rate of the eluent was 1 ml/min and the column temperature was 35 °C. The temperature of the detector was 85 °C and the gas flow (from air compressor) was 10 l/min. Peak identification: (1,2) neutral lipids; (3) phosphatidylethanolamine; (4) phosphatidylinositol; (5) phosphatidylserine; (6,7) unidentified compounds; (8) phosphatidylcholine; (9) sphingomyelin.

Table 3
Retention time repeatability of standards

Compound	Retention time (min)	RSD (%)
Phosphatidylethanolamine	2.593±0.003	0.136
Phosphatidylinositol	3.951±0.009	0.250
Phosphatidylserine	4.208±0.056	1.344
Phosphatidylcholine	16.222±0.183	1.133
Sphingomyelin	22.880±0.209	0.914

3.3. Temperature optimisation

To determine the effect of temperature, the analyses were performed with the column thermostated at a range of temperatures, from 25 to 45 °C at 5 °C intervals. Temperature enhanced the solubility of PLs in the mobile phase. In contrast, higher column temperatures resulted in lowered selectivity. Solutions of 5% (w/v) of standard PLs were injected in triplicate at each of the above temperatures. The best resolution was obtained at 35 °C.

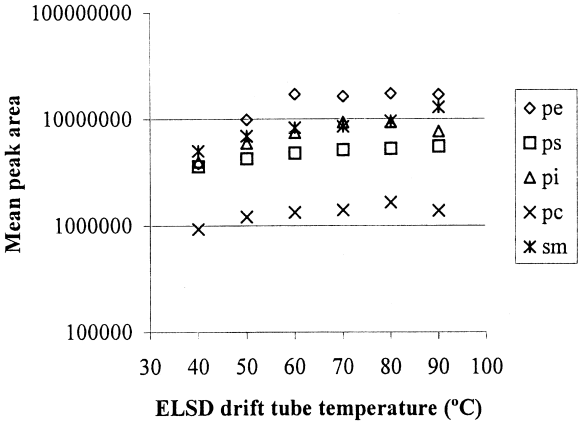

Fig. 2. Effect of drift-tube temperature on detector response. pe, phosphatidylethanolamine; ps, phosphatidylserine; pi, phosphatidylinositol; pc, phosphatidylcholine; sm, sphingomyelin.

The evaporation temperature was found to be the most critical parameter for the detector. Solutions of 2% (w/v) of PL standards were injected at a range of drift tube temperatures, from 40 to 90 °C at 10 °C intervals. The optimum temperature was between 80 and 90 °C (Fig. 2). Above this temperature, resolution was poor, especially for PI. Solutions were then injected at a range from 80 °C to 90 °C at 1 °C intervals. Finally, the selected temperature was 85 °C.

3.4. HPLC–ELSD method validation

The following parameters were determined: linearity, reproducibility, detection and quantification limits. Several authors have established that the ELSD response is linear for a broad range of concentrations [23,35,36]. Nevertheless, other authors have reported that the response of the mass detector to increasing amounts of solute injected is non-linear [37–39] i.e., the response (y) is proportional to the amount injected (x) raised to a power ($y = bx^a$). The exponent, a, is closely linked to the nebulizer shape (pressure and evaporator conditions in the evaporator) [38].

The calibration curves were prepared with standard solutions of each compound at levels similar to those contained in the samples analysed (see ranges in Table 4). Each PL standard calibration curve was prepared in triplicate, and each sample was injected in duplicate. Power relationships were detected with coefficients of determination of 0.99 in the range studied. Table 4 shows the results by power curve

Table 4
Results from the treatment of experimental data points of PLs determined by power curve fitting; estimated equations in the linearity study of ELSD

Compound	Range (μg/20 μl)	Power curve ($y = bx^a$)		
		b	a	r^{2a}
Phosphatidylethanolamine	6–48	2453.9	1.6980	0.9975
Phosphatidylinositol	1–8	872.81	1.8818	0.9998
Phosphatidylserine	0.3–15	2501.7	1.5581	0.9957
Phosphatidylcholine	7.5–45	20494	1.2587	0.9988
Sphingomyelin	7.5–60	515.74	1.9202	0.9978

[a] r^2 = coefficient of determination.

Table 5
Results of sensitivity study of ELSD

Compound	DL[a] (ng)	QL[b] (ng)
Phosphatidylethanolamine	17	34
Phosphatidylinositol	57	108
Phosphatidylserine	76	164
Phosphatidylcholine	9	29
Sphingomyelin	50	93

[a] Detection limit (ng).
[b] Quantification limit (ng).

fitting from plot of PL peak areas versus amounts injected.

The inter-laboratory precision (reproducibility) was measured by calculating the relative standard deviation (RSD) of eight replicates of the samples on various days, which were between 2.19 and 5.39. The reproducibility was consistent with the acceptable precision proposed by Horwitz [40] for analyte concentrations of the order of μg/ml.

The detection limit (DL) and the quantification limit (QL) were obtained following the USP criteria [41]. Sensitivity data are summarised in Table 5. DL and QL for most of the standards were estimated to be in the low nanogram range, according to results found in bibliography [23,26].

Therefore, the method proposed is sensitive and precise. Its simplicity, absence of baseline drift and short analysis time make it suitable for the routine determination of phospholipid classes in infant formulas and phospholipid materials used in paediatric nutrition. It may thus be helpful to industry to improve the incorporation of phospholipids sources resembling those in human milk structural and nutritional properties.

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