Enzymatic Sample Hydrolysis and HPLC in a Study of Phylloquinone Concentration in Human Milk

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Phylloquinone (vitamin K) is essential to prevent hemorrhagic diseases in newborns. We were interested in determining the concentration of phylloquinone in human milk and in elucidating the factors that influence that concentration. Human milk contains lipid constituents encapsulated with phylloquinone in the fat globules. To eliminate the lipids, we combined sonication and an enzymatic treatment with lipase to prevent degradation by trastic hydrolysis. Despite the low phylloquinone concentrations and the procedure’s complexity (lipase treatment, wo HPLC steps, and an on-line thermoinduced postcolumn reduction followed by fluorescence detection), within-day and day-to-day CVs of 5.2% and 5.8% were obtained (n = 8, \( \bar{x} = 1.86 \) µg/L, and n = 7, \( \bar{x} = 1.74 \) µg/L, respectively). The mean concentration of phylloquinone in 126 samples was 1.15 ± 0.82 µg/L (\( \bar{x} \pm SD \)); no correlation was observed between the vitamin concentration and the postpartum date of collection. There was a positive correlation between phylloquinone concentration and phospholipid and cholesterol content (\( r = 0.5578 \) and 0.6020, respectively).

Additional Keyphrases: postcolumn reaction · fluorescence detection · lipase treatment · vitamin K

The results of their bioassay in the early 1940s for phylloquinone in human milk suggested to Dam et al. 1) that an insufficient supply of phylloquinone from maternal milk can lead to the classical hemorrhagic disease of newborns. Other studies supported this hypothesis by demonstrating that the incidence of this syndrome is much higher in infants who are exclusively breast-fed (2, 3). This observation was even more pronounced for babies who developed a late-onset form between the third and eighth weeks postpartum in which intracranial bleeding is common (4–6). Even before the later studies, the American Academy of Pediatrics recommended that all newborns be administered 0.5–1.0 mg of vitamin K (7). The latest observations also triggered an increased interest in the concentration of phylloquinone in human milk as well as in the factors that might influence the presence of the vitamin in breast milk.

Human milk contains a large amount of lipids in fat globules that are encased in protein–phospholipid membranes (8). Consequently, the quantitation of the fat-soluble phylloquinone is an analytical challenge, and the removal of the lipids is a crucial step in the determination of phylloquinone in human milk.

It is clear that the chick bioassay offered only a rough estimate of the phylloquinone content and suffered from interferences. However, with the advent of high-performance liquid chromatography (HPLC) several more sensitive and more selective procedures for the measurement of phylloquinone in milk have been reported (9). In addition to a final measurement by HPLC on two different materials (silica and reversed-phase packing), most of these procedures include chromatographic purification of the crude extract (10–14).

A totally different approach consisted of the enzymatic hydrolysis of triglycerides in the milk, followed by extraction and chromatographic measurement. However, there are two major points for criticism of these techniques. First, sometimes a large amount of a concentrated sodium hydroxide solution (10 mol/L) was added to the hydrolyzed sample to eliminate the fatty acids liberated in the extraction with n-hexane by keeping them in the water layer (15–17). Such treatment should be avoided because phylloquinone is unstable under these drastic alkaline conditions (18). Other factors, such as matrix interference and the phylloquinone content of the mother’s food, can still result in higher concentrations of phylloquinone than we find. Second, is the well-documented existence of fat globules that contain milk lipids. These globules are encapsulated by membranes that inhibit direct lipase activity on the triglycerides (19, 20). However, in none of the above-mentioned work was a special treatment incorporated to disrupt the membrane (15–17). One research group used ultrasonic treatment to increase the extraction recovery of phylloquinone from human milk (21). However, no enzymatic hydrolysis of the triglycerides was incorporated, and the result was an oily residue left after extraction with n-hexane.

We combined an ultrasonic treatment of the milk sample with enzymatic hydrolysis of the triglycerides. After extraction under much milder conditions, phylloquinone was determined in 126 human milk samples by an HPLC procedure that includes on-line thermoinduced postcolumn reduction and fluorescence detection (22, 23).

We also investigated the influence of other factors on phylloquinone concentration in human milk. These include the time of lactation and the lipid composition (cholesterol, triglycerides, and phospholipids). Human milk composition can change drastically in one person and there are large differences among individuals, as a function of time postpartum (e.g., differences in the

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composition of colostrum, transition milk, and mature milk; 14, 24).

Materials and Methods

Apparatus

A first HPLC system used for the cleanup of the extracts consisted of an LC2-XP dual-piston pump (Fye Unicam, Cambridge, UK), a 20 × 0.46 cm (i.d.) column packed with RSL1 5-μm particles (RSL, Eke, Belgium), and a Fye Unicam LC3-UV variable-wavelength detector set at 254 nm.

Final quantitative analysis was performed on a second HPLC equipped with a Model 2150 dual-piston pump (LKB, Bromma, Sweden), a 15 × 0.32 cm (i.d.) reversed-phase column (RoSIL, C18, H4, 5 μm, also from RSL), and a Model LS-4 fluorescence detector with double monochromator (Perkin-Elmer, Norwalk, CT). Injections were made through a Model CV-6-UHPS-N60 injection valve (Valco, Houston, TX), and chromatograms were recorded on a Model PM 8251 single-pen recorder (Philips, Eindhoven, The Netherlands).

Chemicals and Reagents

We used porcine pancreas lipase Type II (EC 3.1.1.3; Sigma Chemicals, St. Louis, MO) and a 200 g/L solution of albumin (Nichiyaku, Nihon Seiyaku Company, Japan). All other chemicals used in the sample preparation came from Merck (Darmstadt, FRG). Solvents used for extraction and chromatography were HPLC grade (Fisher Scientific Co., Fair Lawn, NJ). Phylloquinone (2-methyl-4-phytyl-1,4-naphthoquinone) as well as the internal standard, a structural analog of phylloquinone that contained one extra isoprene unit in the side chain, were donated by Hoffmann-La Roche (Basel, Switzerland). The reducing reagent, tetramethylethanol octahydridotriborate, (CH3)4NB3H8, was from Alfa Products (Danvers, MA). Lipid analyses (phospholipids, triglycerides, cholesterol) were done with a Cobas-Bio centrifugal analyzer (Roche, Basel, Switzerland) enzymatic tests from Boehringer (Mannheim; FRG; kits 691 844, 290 319, and 701 882 for phospholipids, triglycerides, and cholesterol, respectively).

Milk Sampling

The phylloquinone concentration and the lipid composition of breast milk during the course of lactation were evaluated by analyzing milk samples obtained by manual expression during a single feeding, but at different days of lactation and at all nursing times throughout the day, ad libitum. In addition, care was taken not to collect milk at the very beginning or end of the feeding. Samples (8 mL) were obtained starting from the day of birth up to 82 days postpartum. Milk samples were collected away from direct sunlight and were frozen immediately at −18 °C until analysis.

Enzymatic Hydrolysis and Extraction

Before the pipetting, milk samples were kept for ≥30 min at 25 °C to ensure homogeneity. To a 500-μL aliquot of human milk we added 2.7 ng of the internal standard (50 μL of an ethanolic solution). We also added 10 μL of a 200 g/L albumin solution and 200 μL of an aqueous solution of sodium taurocholate (0.05 mol/L), calcium chloride (0.1 mol/L), and sodium chloride (0.1f mol/L) before ultrasonic treatment for 2 min in a 150 W ultrasonic disintegrator, equipped with a titanium probe (MSE Scientific Instruments, Sussex, UK). This treatment was performed at short intervals (30 s treatment, 30 s waiting period) while the sample was kept at low temperature by placing the vial in ice water. Directly after sonication we added 1.2 mL of 0.2 mol/L Tris buffer (pH 7.7) that contained 4.08 mg of crude lipase powder. During the next 45 min, the samples were kept at 37 °C and mixed at 100 strokes/min to allow optimal lipase activity.

To the reaction mixture we added 4 mL of ethanol, 2 mL of water, and 200 μL of NH4OH (50 g/L) before we extracted the phylloquinone with 7.5 mL of n-hexane by vortex-mixing for 2 min.

Chromatography

Purification of the crude extract was performed on the adsorption column eluted with n-hexane and diisopropyl ether (98.5:1.5, by vol) at a flow rate of 0.86 ml/min. We collected the fraction of the column effluent that contained both the phylloquinone and the internal standard, and we evaporated this to dryness. The residue was redissolved in 75 μL of a mixture of methanol and ethylacetate (96:4, by vol), and a 50-μL sample was injected onto the reversed-phase system. The column was eluted with the same methanol–ethylacetate mixture, which now contained the reducing reagent, (CH3)4NB3H8 (2.25 g/L). Phylloquinone and the internal standard were separated by an on-line thermodinduced postcolumn reaction in a knitted coil, and the fluorescence was monitored at 430 nm (λex 325 nm).

Assay Performance

For calibration, 0.5-mL portions of a human milk pool were supplemented with phylloquinone to yield concentrations of 0.75, 1.13, 1.98, and 3.75 μg/L. The standard curve was obtained by plotting peak-height ratios (phylloquinone to internal standard) vs the amounts added. This curve intercepted the y-axis because of the endogenous phylloquinone in the human milk pool. After subtraction of the intercept, the concentrations of the unknowns were determined from the new curve.

Within-day reproducibility was evaluated by analyzing eight 0.5-mL samples of a human milk pool in one run. Day-to-day reproducibility was tested by analyzing 0.5-mL portions of two different pools on different days, over a period of five weeks.

Total recovery was evaluated by analyzing calibration samples. After the whole analytical procedure, the peak heights of phylloquinone and of the internal standard obtained for these calibration samples were compared with the peak heights obtained for a similar amount of phylloquinone and of the internal standard injected directly on the reversed-phase system.
Stability during Extraction

To check the eventual degradation during the extraction, we added to seven 0.5-mL aliquots of ethanol, 3.5 mg of phylloquinone, 1.2 mL of buffer (pH 7.7), and 200 μL of NH₄OH (50 g/L). After various contact times, the mixtures were extracted with n-hexane. The internal standard was added and the peak-height ratios were calculated before injection.

Results and Discussion

Effect of Enzymatic Hydrolysis

The major impediment to the quantitation of phylloquinone in human milk was the large quantity of lipids. To avoid drastic hydrolysis procedures and time-consuming column chromatographic procedures, we preferred the much milder treatment by lipase. Because it is well known that the membrane that encapsulates the triglycerides can inhibit lipase activity, we incorporated ultrasonic treatment. After the membrane was disrupted, the triglycerides were easily accessible for subsequent hydrolysis.

For the enzymatic treatment several variables had to be optimized, such as temperature (37°C), time (45 min), and pH (7.7). Albumin was added to bind the liberated fatty acids and to prevent them from attaching to the substrate surface. Furthermore, the albumin protected the enzyme against surface denaturation and enhanced lipolytic activity. There was no loss in recovery of phylloquinone as a result of eventual binding to the albumin. The denaturation step in the extraction procedure clearly liberated the phylloquinone from albumin. Sodium chloride also inhibited denaturation of the enzyme, whereas Ca²⁺ and sodium taurocholate enhanced enzymatic activity (20, 25).

Representative chromatograms obtained from the adsorption system and from the reversed-phase column are shown in Figures 1A and 1B and in Figures 2A–F. In addition, the effect of lipase treatment and sonication can be followed by the disappearance of interfering peaks in these chromatograms.

The effect of lipase treatment can be seen in the chromatograms obtained on the adsorption column. Omitting lipase treatment or sonication (Figure 1A) resulted in a high peak that eluted exactly in the collection window. In Figure 1B (sonication and the addition of 4 mg of lipase) this peak is much smaller. Addition of more lipase (6 mg) did not result in a total removal of the peak. The effect of lipase treatment was even more clear in the chromatograms obtained on the reversed-phase column combined with the fluorescence detection (Figures 2A–F). A too-small amount of lipase added or the omission of the sonication always resulted in an interfering peak eluting close to the phylloquinone peak. Only the combination of sonication and the addition of 4 mg of crude lipase resulted in a complete elimination of the interfering peak and allowed quantitative measurement. The lipase treatment also drastically prolonged column life because fewer lipid constituents are injected under these conditions.
Table 1. Results of the Analysis of 126 Human Milk Samples

<table>
<thead>
<tr>
<th></th>
<th>Phospholipids, mg/L</th>
<th>Triglycerides, mg/L</th>
<th>Cholesterol, mg/L</th>
<th>Vitamin K, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>880</td>
<td>9880</td>
<td>260</td>
<td>1.15</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>580</td>
<td>3230</td>
<td>160</td>
<td>0.82</td>
</tr>
<tr>
<td>Median</td>
<td>870</td>
<td>10 710</td>
<td>230</td>
<td>0.98</td>
</tr>
<tr>
<td>Lowest concentration</td>
<td>20</td>
<td>1870</td>
<td>20</td>
<td>0.09</td>
</tr>
<tr>
<td>Highest concentration</td>
<td>2580</td>
<td>18 800</td>
<td>1120</td>
<td>3.98</td>
</tr>
</tbody>
</table>

Fig. 3. Frequency histogram for milk samples (N, number of samples) as a function of the time of collection postpartum (D, days postpartum)

Assay Performance

Calibration samples were made to cover concentrations to 4 µg/L. Linearity, however, can be obtained for much higher concentrations (100 µg/L). In a typical experiment the calibration equation was \( y = 0.4238x + 0.2507, r = 0.9992 \). The significant intercept of this equation was attributable to the endogenous phylloquinone present in the pool (0.59 µg/L). It was possible to analyze as little as 0.5 mL of human milk, and quantitation was possible down to 80 ng/L; the detection limit was estimated at 35 ng/L. A within-day reproducibility (CV) of 5.2% was obtained at 1.86 µg/L for eight replicate analyses of 0.5-mL portions of a pool of human milk. The day-to-day precision (CV) over 5 weeks was 5.8% (n = 7) for 1.74 µg/L and 7.3% for 1.32 µg/L (n = 8). Total recovery over the whole analytical procedure yielded 62.5%.

In view of the complexity both of the matrix and of the analytical procedure (enzymatic treatment, two chromatographic systems, and postcolumn reaction), linearity, within-day reproducibility, and day-to-day reproducibility are quite acceptable. The 62.5% recovery seems low, but incomplete transfer of the n-hexane layer (maximum 7.0 of 7.5 mL) and the partial injection of the redissolved residue (100/120 µL) already represent a loss of >20%.

Compound Stability

There was no remarkable difference between the degradation with and without contact with NH₄OH even over a period of 30 min.

Phylloquinone and Lipid Constituents

The results of the analyses of phylloquinone and of the lipids in the human milk samples are presented in Table 1. The mean concentration of 1.15 µg of phylloquinone per liter of human milk (1.15 ± 0.82 µg/L, \( \bar{x} \pm SD, n = 126 \)) is in close correlation with other data (9–11, 13) but substantially lower than the results reported by some groups (26, 27). Figure 3 is a frequency histogram by sampling day. Most of the milk samples were obtained during the first week postpartum because it was easier to have the mothers express the milk while they were in the hospital. There was no correlation observed between phylloquinone concentration and the day of collection during the 80-day period of

Fig. 4. Phylloquinone vs collection day postpartum (A) and cholesterol (B) in human milk

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Table 2. Circadian Variation of Phylloquinone in Human Milk

<table>
<thead>
<tr>
<th>Volunteer no.</th>
<th>Day postpartum</th>
<th>Time of collection</th>
<th>Vitamin K, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>0200</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>0.76</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>0100</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>1.95</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>0200</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>1.08</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1200</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>1.40</td>
</tr>
<tr>
<td>5</td>
<td>77</td>
<td>1400</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2400</td>
<td>0.91</td>
</tr>
<tr>
<td>6</td>
<td>79</td>
<td>0900</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1900</td>
<td>1.93</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>0200</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0800</td>
<td>0.71</td>
</tr>
<tr>
<td>8</td>
<td>81</td>
<td>0300</td>
<td>2.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>3.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1400</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2300</td>
<td>0.67</td>
</tr>
<tr>
<td>9</td>
<td>82</td>
<td>0800</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1300</td>
<td>1.52</td>
</tr>
</tbody>
</table>

lactation ($r = 0.0457$, Figure 4A). Similar observations also were described recently by Greer et al. (28). However, in both studies samples were obtained ad libitum, and other factors (meal composition and time of collection after a meal) probably influence the concentration to a much greater degree and mask the eventual effects of the number of days postpartum. On the other hand, a positive correlation was observed between the phylloquinone concentration and the phospholipid ($r = 0.5578$) and cholesterol content ($r = 0.6020$) of the milk (Figure 4B). No correlation was observed between phylloquinone and triglyceride content ($r = 0.1489$).

In a separate experiment we obtained from nine mothers two or four milk samples on the same day. For this study, it was important that the sampling method was exactly the same during that day (11). Some results indicate a large variation in phylloquinone concentration within one day (Table 2), corresponding with similar observations (24) for cholesterol and phospholipids. The concentration often was higher in the evening sample. This can be correlated with the appearance in the milk of the phylloquinone absorbed from the noon meal. In addition to the time of sampling after a meal (10), the composition of the meal itself is an important influence on the concentration of phylloquinone in human milk. However, this was difficult to evaluate from our results because the exact composition and weight of the food were not known (28).

It is difficult to state that colostrum contains less or more phylloquinone than does mature milk, because the factors of meal composition and time of sampling can hardly be kept constant over the whole lactation period. Although extremely low phylloquinone concentrations in human milk were not encountered in our study, the human concentration was still below the concentrations reported for cow milk (10). Consequently, for breast-fed babies the input of phylloquinone is clearly lower than it is for babies who are fed cow milk. Disease-related causes or effects were not considered in our study.

References


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