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Improved method for rapid determination of vitamin A in small samples of breast milk by high-performance liquid chromatography

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Abstract

Employing a short, potassium hydroxide (KOH) saponification (25 min) and rapid extraction with *n*-hexane–toluene (1:1) and a <4-min retention time, a convenient, rapid, accurate and precise determination of vitamin A in breast milk was developed. The recovery rate was 102.3±2.5% and the small relative standard deviation (intra-assay 1.9%, inter-assay 2.3%) resulted in high precision. The amount of breast milk needed is very low (1 ml) which makes the method suitable for the use in free field studies with large sample sizes. The simple extraction and separation steps allow a high throughput screening under routine laboratory conditions.

Keywords: Milk; Vitamins; Retinol

1. Introduction

For decades, vitamin A deficiency has been recognized as a major public health problem in many parts of the world. Due to the high homeostatic control of retinol in the circulation, it is only when clinical symptoms like night blindness or xerophthalmia occur that a drop in the vitamin A content can be measured, thus allowing a meaningful analysis to be made. That means, that subclinical vitamin A deficiency of an individual cannot be assessed – or is very difficult to determine – using serum analysis for retinol. In comparison, earlier studies indicate that the amount of vitamin A in breast milk is directly related to the maternal dietary intake and less homeostatically controlled [1]. Therefore, one appropriate method for assessing the vitamin A status of lactating women, one which is less invasive and convenient to handle, is the collection and analysis of breast milk for retinol [2]. In addition, breast milk is seen to be an important – and often the only – source of vitamin A for the infant [1,2]. As a result, vitamin A in breast milk contributes not only as an appropriate indicator for measuring the prevailing vitamin nutriture or the impact of vitamin A interventions on women, but it also provides information about infants' intake and risk of vitamin A deficiency [2]. In developing countries sampling of blood is often culturally unacceptable; analysis of milk for vitamin A could present a viable alternative approach.

Vitamin A, which is strongly bound in the fat globules of human milk, is present primarily as retinyl esters [3]. As these fat globules are very stable, extreme conditions of saponification must be applied to assure the complete release of vitamin A

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from the linkage. In this process, the retinyl esters are hydrolyzed so that vitamin A is quantified as retinol using high-performance liquid chromatography (HPLC) after extraction.

Former studies [4], dealing with methods for determination of vitamin A in breast milk, emphasize the requirement of long saponification time for releasing all vitamin A, complicate extraction procedures, or both along with multiple evaporation steps [5–8]. To overcome these problems, we developed a method to measure vitamin A in breast milk using isocratic HPLC with: (1) a short saponification time, and (2) a simple extraction.

2. Experimental

2.1. Chemicals and samples

Pooled breast milk from four healthy German women in various stages of lactation (2 to 6 months) was used for pilot tests. The milk was collected after feeding the child, pooled and stored at −20°C. The calibration was done by using breast milk from only one healthy German woman (second month of lactation). During 7 days about 100 ml breast milk was collected and pooled.

All chemicals used were of analytical grade. All-*trans*-retinol (purity: 92%) and retinyl palmitate (purity: 98%) were obtained from Hoffmann–La Roche (Basel, Switzerland), potassium hydroxide and toluene were obtained from Merck (Darmstadt, Germany), ethanol from Roth (Karlsruhe, Germany), BHT (2,4-di-*tert*.-butyl hydroxytoluene) from Sigma (Deisenhofen, Germany) and liquid nitrogen from Stickstoffwerke Friedrichshafen (Friedrichshafen, Germany).

2.2. HPLC

The HPLC system used consisted of a HPLC-pump T414 and an autosampler 465 from Kontron (Neufahrn, Germany) and a Grom-Sil-CN-2PR column, 250×4.6 mm I.D., 3 μm from Grom (Herrenberg, Germany). As mobile phase *n*-hexane–2-propanol (98:2, v/v) was used at a flow-rate of 2 ml/min. Retinol was quantified using a fluorescence detector (emission wavelength: 480 nm; excitation wavelength: 325 nm) from Waters (Eschborn, Germany) (Fig. 1).

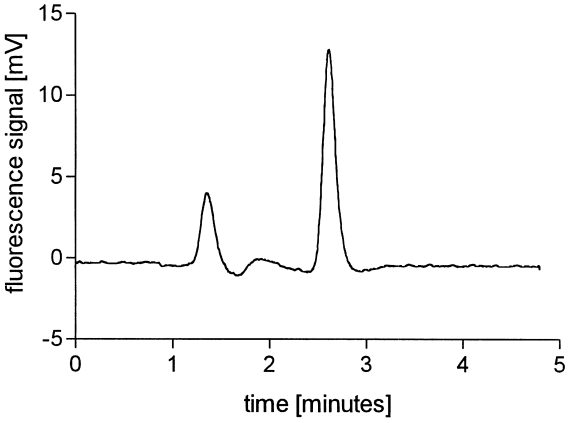

Fig. 1. HPLC chromatogram of mature human milk using a fluorescence detector. The conditions were as described in the Experimental section.

2.3. Preparation of samples

2.3.1. Saponification

The entire pool of human milk was homogenized by sonification for 2 min in a water bath sonicator (Bandelin Electronic, Berlin, Germany) and afterwards divided into 500-μl breast milk samples. These samples were again stored at −20°C. For analyzing vitamin A the 500-μl breast milk sample was placed together with 1 ml of ethanol (containing 20 mg/ml BHT) in a screw top polypropylene tube (15 ml Falcon). A 500-μl volume of KOH (12.5 mol/l) was added and oxygen was displaced by flushing with nitrogen. The samples were kept in darkness for 25 min at 80°C whilst being slightly agitated. After cooling down to room temperature retinol was extracted as described below.

2.3.2. Extraction

A 1-ml volume of solvent (*n*-hexane–toluene, 1:1) was added to the saponified milk and vigorously mixed by vortexing for 1 min. Subsequently, the mixture was centrifuged for 3 min at 4500 *g*. A 200-μl volume of the organic phase was directly transferred to a HPLC vial and injected.

2.4. Preparation of standards

2.4.1. External calibration

A stock solution was prepared by dissolving retinol in *n*-hexane. The absorption was measured by photometry at a wavelength of 325 nm against *n*-hexane and diluted with *n*-hexane until a value of approx. 0.5 was achieved. The concentration was calculated according to Lambert–Beer's law. Six different volumes of the diluted solution were dried by evaporation under nitrogen and re-dissolved in 200 μl *n*-hexane–toluene (1:1) resulting in concentrations between 0.58 and 1.53 μmol/l (0.58, 0.77, 0.96, 1.15, 1.34 and 1.53 μmol/l). These solutions were used directly for HPLC.

2.4.2. Internal calibration (method of standard addition)

Retinyl palmitate was dissolved in ethanol and the absorption was measured. To be in the same range as the external calibration, different concentrations (0, 0.118, 0.237, 0.355, 0.473 and 0.591 μmol/l) of this solution were added to milk samples and treated as described above.

2.5. Statistical evaluation

To take the sample preparation into consideration all the statistical evaluations were done by means of the internal calibration curve.

2.5.1. Precision

The precision was tested by examining the homogeneity of variances. For this the variances of samples with the highest amount of added retinyl palmitate were compared with variances of samples without added ester using the *F*-test. The relative standard deviation (RSDs) for the intra- and the inter-assays were calculated analyzing the same milk samples six times on the first day and once on each of the following 10 days. The dependence over time was also tested by comparing the standard deviation within a batch with the standard deviation between batches using the *F*-test.

2.5.2. Linearity

The linearity of the established method was tested by determining the residuals of the calibration.

2.5.3. Recovery

In order to determine the recovery rate, the external calibration and the calibration using the method of standard addition were compared. For this purpose, the measured absorption of each value of the standard addition method was inserted into the equation of the external calibration curve and the *x* values were calculated. Plotting these values against the added concentrations of retinyl palmitate to the milk samples provided the recovery curve. The slope of this curve yields information about proportional systematic variations, and is equivalent to a recovery rate. The *y*-intercept provides information about constant systematic variations, and therefore provides quantitative answers about vitamin A levels in the analyzed breast milk.

3. Results and discussion

3.1. Preparation of samples

In the current trial, it was found that using a saponification temperature of 80°C and 500 μl KOH (12.5 mol/l) total saponification could be achieved after 25 min. The high temperature did not harm the retinol due to protection from the added BHT. In comparison with former studies done by de Pee and co-workers [6,9], Canfield and co-workers [4,5], and Stoltzfus et al. [8], all saponifying the esters for 16 h at 25°C, the increase of the temperature results in shorter reaction time without any loss of retinol [2].

To optimize the extraction, two solvent systems were compared. Using an *n*-hexane–toluene (1:1) mixture, the yield was significantly increased ($P < 0.05$) (Table 1) compared to pure *n*-hexane. To achieve the same yield with pure *n*-hexane repeated extraction steps are necessary. Consequently, steps such as total separation of the organic layers, evaporation and redissolving are unavoidable. Using *n*-hexane–toluene (1:1) these steps can be bypassed and a part of the organic layer from a single extraction can be injected directly into the HPLC system.

The use of a straight-phase method in this trial means no evaporation steps are necessary. Using the traditional reversed-phase chromatography, as was done in aforementioned studies [2,5,7,10], a polar

Table 1
Comparison of two different extraction solvents: *n*-hexane and *n*-hexane–toluene (1:1)

Solvent	Mean (peak area of the fluorescence signal), $n=4$	SD	RSD (%)
Pure *n*-hexane	1.69	0.115	6.9
n-Hexane–toluene (1:1)	2.26	0.045	1.9

mobile phase is required, for which retinol has to be evaporated and re-dissolved.

With these three methodological alterations, time and effort in the analysis of vitamin A in breast milk can be considerably reduced.

3.2. Statistical evaluation

The slope of the external calibration (sensitivity) curve was calculated to be 1.95±0.03 area l/μmol and a correlation coefficient of 0.999 was obtained. This calibration curve was used for the quantification of retinol in the milk samples. Using the method of standard addition a second calibration curve was determined. The calculated slope of 1.99±0.05 area l/μmol showed a very good correlation coefficient of 0.998. This calibration curve was used for preparing the recovery curve (Fig. 2) and determining the recovery rate as described above.

Using the modified saponification conditions (80°C, 25 min) and extraction (*n*-hexane–toluene, 1:1) described above, a recovery rate of 102.3±2.5% was obtained. The standard deviations of the slope (equivalent to the standard deviation of the recovery rate) and the *y*-intercept (supplying information about the matrix effects) were 0.025 μmol/l and 0.009 μmol/l, respectively (Table 2).

Table 2
Characteristics of the recovery curve[a]

Slope±SD	1.023±0.0258
y-intercept±SD	1.245±0.009 μmol/l
SD recovery curve	0.0125 μmol/l

[a] $y = 1.245\text{Ext.} + 1.023 \cdot \frac{\text{Ext.}}{\mu\text{mol/l}} \cdot x$

In Fig. 3 the residuals of the calibration curve are plotted against the concentrations of added retinyl palmitate. As the residuals demonstrated a Gauss distribution, the calibration curve can be considered to be linear. The homogeneity of variances was found by comparing standard deviations from samples without additional retinyl palmitate (SD=0.057) and samples with additional retinyl palmitate (SD=0.069) using the *F*-test ($\alpha=0.01$).

The RSD for the intra-assay was calculated to be 1.9% and for the inter-assay as 2.3% (Table 3). Comparing the standard deviation within a batch (SD=0.045) with the standard deviation between batches (SD=0.05) no significant differences could be found (*F*-test, $\alpha=0.05$). As a result, precision

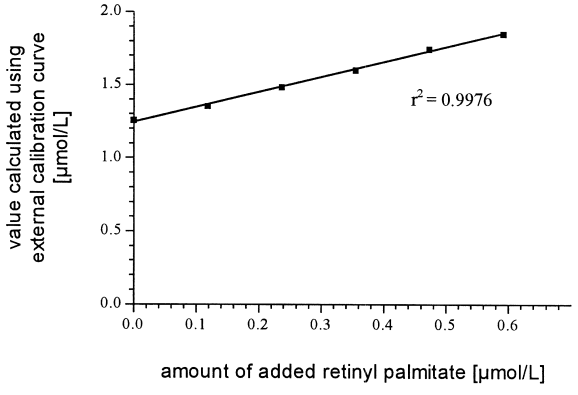

Fig. 2. Recovery curve: the concentrations of added retinyl palmitate (*x* value) are plotted against the concentrations calculated using the external calibration curve* (*y* values).

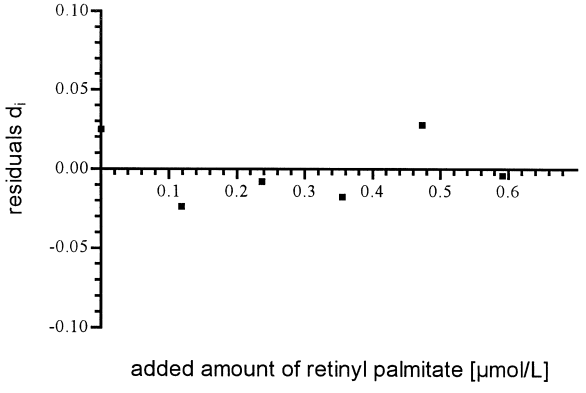

Fig. 3. Plot of residuals calculated from the calibration curve using the method of standard addition against added amount of retinyl palmitate.

Table 3
Determination of the intra- and inter-assay relative standard deviations

	Mean (peak area of the fluorescence signal)	SD	RSD (%)
Intra-assay[a]	2.26	0.045	1.9
Inter-assay[b]	2.18	0.05	2.3

[a] $n=10$.
[b] $n=6$.

over time is ensured. The detection limit is 30 nmol/l.

4. Conclusions

This paper describes a simple and rapid method for the measurement of retinol in breast milk. The higher temperature allows for a shorter saponification time, whereas the solvent mixture is decisive in reducing the number and effort of extractions. Only a single extraction step is necessary, and the organic layer can be injected directly into the HPLC system without any evaporation steps. This method requires only small amounts of breast milk, and is therefore suitable for use under field conditions. The high precision and reliability of this method guarantee its applicability in routine analyses of breast milk from surveys and intervention studies in developing country populations.

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