# Vitamin A and Carotenoids in Human Milk[†]

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The relationship of dietary carotenoid consumption and the concentration of retinol and several carotenoids in mature human milk was investigated. Forty-two lactating mothers of infants from 0.5 to 7 months participated in this study. All measurements of milk retinol and carotenoids were determined by HPLC. Mean retinol concentration in milk was $57 \pm 25$ $\mu$g/100 g. Milk retinol concentration of mothers who took vitamin supplements, 60 $\mu$g/100 g, was not significantly different from those who did not, 49 $\mu$g/100 g ($p < 0.05$). Duration of lactation produced no significant differences in retinol and $\beta$-carotene concentrations of milk. Correlation coefficients between milk and estimated dietary intake of retinol, $\alpha$-carotene, $\beta$-carotene, lycopene, and lutein were 0.15, 0.52, 0.42, 0.03, and 0.25, respectively.

## INTRODUCTION

The carotenoids are a relatively inefficient source of vitamin A for man, yet $\beta$-carotene from plants represents a major source of dietary vitamin A (Krause and Mahan, 1979). In addition to their role as provitamin A, carotenoids might also have an anticancer effect. Several epidemiological studies have appeared within the past 10 years which show that retinoids are effective in inhibiting carcinogenesis (Peto et al., 1981; Anonymous, 1980; Wolf, 1982; Shekelle et al., 1981; Anonymous, 1982). Vitamin A is well-known to be important in the general growth and differentiation of epithelial tissues. In photosynthetic organisms, carotenoid pigments serve as protective agents against photosensitized oxidation (Krinsky, 1977, 1978, 1979; Anderson et al., 1974).

Animals, including humans, cannot generally synthesize carotenoids but can convert it to vitamin A (Krause and Mahan, 1979), primarily in the intestinal mucosa (Smith and Goodman, 1976). Man is capable of absorbing small but significant amounts of unchanged dietary carotenoids into the lymph (Goodman et al., 1966), and these may be converted to vitamin A in the liver. Carotenoids absorbed as such from the intestine contribute a yellow color to the blood and milk (Krause and Mahan, 1979) and may be deposited in various organs (Simpson and Chichester, 1981).

In well-nourished women, the level of vitamin A in mature milk is relatively constant (Wallingford and Underwood, 1986). This is because uptake of vitamin A by the mammary gland is dependent upon the maternal blood concentration, which is under relatively tight control. The vitamin A content of breast milk from mothers with diets marginal in vitamin A is low and can be raised by increased dietary supply (Wallingford and Underwood, 1986). The blood retinol complex remains fairly constant as long as there are small reserves of vitamin A present in the liver

(Lui and Roels, 1980). Even though the blood retinol complex is fairly constant, the concentration of lipoprotein-associated retinyl esters will increase in proportion to the vitamin intake, and transfer of this material to the milk will also contribute to the total (Valquist and Nelson, 1979).

A major problem in nutrition regarding vitamin A and provitamin A is that of analysis. Recently, reversed-phase high-performance liquid chromatography (HPLC) was applied to retinol determination (Bui-Nguyen and Blanc, 1980) and to the separation of various carotenoids (Zakaria et al., 1979; Broich et al., 1983).

The present study was therefore conducted to investigate the extent to which dietary carotenoid intake can be correlated with human milk levels of retinol and carotenoids, using HPLC for analysis of retinol and carotenoids.

## MATERIALS AND METHODS

**Milk Collection.** Fifty-four mothers from Rhode Island and Massachusetts volunteered to participate in this study. Mothers with infants aged 0.5–7 months comprised the study group. Mothers were instructed to collect the first morning milk for up to 3 consecutive days. An equal volume of milk was expressed from both breasts by hand or electric pump before and immediately after the feedings. For each mother, the milk collected over the 1–3 consecutive days was pooled to give one sample per mother. Samples were then stored at $-20$ °C until analysis. Milk samples were collected between March and August.

All mothers were asked to fill out investigator-designed food-frequency forms (Table I) designed to estimate the carotene consumption during the 2 months previous to milk collection. Of the 54 mothers surveyed, 42 food-frequency forms were filled out correctly and were analyzed by computer using the U.S. Department of Agriculture (USDA) Data Bank at the University of Massachusetts.

Values obtained from the USDA Analysis were expressed as total vitamin A. To estimate the dietary value for individual vitamin A components, food items on each dietary record were regrouped and records reanalyzed by using the National Cancer Institute (NCI) computer program. This program, which was not available at the time of the data collection, estimates both the total vitamin A content of the diet as well as vitamin A components. Calorie and vitamin A values from the two programs were correlated to determine whether it would be acceptable to utilize the more detailed dietary estimates from the NCI program (Table II). Because of the high correlations between the two analyses, the NCI values were used in this study. Both sets of dietary values should be viewed as rough estimates, however, because the initial data collection used an untested questionnaire and dietary information was regrouped to match the

**Table I. Foods Included on the Diet Questionnaire[a]**

| | | | |
|---|---|---|---|
| tomato, fresh | apple | margarine | tomato sauce |
| apricot | butter | tomato soup | banana |
| milk, whole | tomato ketchup | cantaloup | milk, 2% |
| carrots | cherry | milk, 1% | chili |
| lettuce, iceburg | fruit cocktail | milk, skim | cheese |
| milk, low lactose | peach pepper, green | lettuce, leaf | grapefruit |
| beans, green | cottage cheese | cabbage, green | tangerine |
| peas, green | watermelon | yogurt, lowfat | broccoli |
| papaya | yogurt | spinach | prune |
| ice cream | asparagus | tomato/vege-table juice | sherbet/ice milk |
| avocado | orange juice | cereals, plain | pepper, red |
| grapefruit juice | liver milk, whole | squash, winter cereals, foritified | other juices |
| pumpkin | squash, summer | sweet potato | tofu |
| corn | collard greens | orange | grapes |

[a] Subjects were asked to list the serving size of each food and whether it was consumed rarely, 1 time/week, 2-4 times/week, once/day, 2-3 times/day, or never.

**Table II. Comparison of Diet Records Analysis**

| | HHHQ[a,d] | USDA[b,d] | correlation |
|---|---|---|---|
| calories | 1 290 ± 414 | 1 635 ± 623 | 0.9091[c] |
| vitamin A (IU) | 11 328 ± 4 819 | 20 935 ± 10 179 | 0.7172[c] |

[a] Health Habits and History Questionaire, National Cancer Institute. [b] U.S. Department of Agriculture Handbook 8 tapes, University of Massachusetts. [c] Significantly correlated ($p < 0.001$). [d] Data are presented as means ± standard deviation, $N = 42$.

format of the NCI program. Breast milk and diet correlations were performed for retinol, $\alpha$-carotene, $\beta$-carotene, lycopene, and lutein. Use of a standardized and tested food-frequency questionnaire is recommended. The NCI program, which estimates both total vitamin A and its components, is appropriate for future research.

**Liquid Chromatography.** The HPLC instrumentation consisted of a Waters Model 6000A solvent delivery system (Waters Associates, Milford, MA), a Waters U6K injector system, and a Perkin-Elmer LC-85 spectrophotometric detector (Norwalk, CT). Chromatographic peaks were recorded on a Hewlett-Packard 3390A integrator. A 7-$\mu$m Zorbax ODS stainless steel column (25 × 0.46 cm) was purchased from Du Pont (Wilmington, DE) and used in the course of this study. The chromatographic mobile phases consisted of a mixture of HPLC grade acetonitrile (ACN), dichloromethane (DCM), and methanol (MeOH) (Fisher Scientific, Medford, MA) (7:2:1). All solvents were filtered through a <0.45-$\mu$m membrane filter (Gelman, Ann Arbor, MI). Acetone and petroleum ether (PE) were freshly distilled before use.

**Analytical Procedure.** Retinyl esters were hydrolyzed to retinol by saponification, and the total retinol concentration was measured. Sixteen grams of milk was saponified directly with 24 mL of KOH solution in methanol. Fifteen percent (w/v) KOH in methanol was prepared for saponification. The alkaline mixtures were heated on a water bath (75 °C) for 25 min. After cooling, the alkaline mixtures were added to 16 mL of petroleum ether in a separatory funnel. When two phases appeared, the lower aqueous phase was drained off and extracted 4 times with 16 mL of PE. The ethereal solutions were then combined in a separatory funnel and washed free from alkali by repeated additions of water until the alkali was removed as determined by pH paper. The aqueous layer was discarded. The solvent was evaporated, and the residue was dissolved in 2 mL of chromatographic solvent system. The solution was filtered through a <0.45-$\mu$m Gelman membrane filter, and 50 $\mu$L of solution was injected into the HPLC for analysis.

**Standards and Standard Curves.** Crystalline retinol and $\beta$-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). $\alpha$-Carotene, lycopene, and lutein were extracted from carrot, tomato, and spinach tissues, respectively. $\beta$-Carotene, $\alpha$-carotene, and lycopene standards were separated and purified several times by use of a magnesium oxide column and were then crystallized (Davies, 1976). The lutein standard was separated and purified several times by sucrose and magnesium col-

umns (Yamamoto et al., 1962) and crystallized (Davies, 1976). The retinol standard was purified by alumina column chromatography (Bieri et al., 1979). For quantitation, retinol was dissolved in absolute methanol, and the concentration was measured with a spectrophotometer at 325 nm. The extinction coefficient of *all-trans*-retinol in absolute alcohol was assumed to be 1850 (Arroyave et al., 1982). Methanol was evaporated in a rotary evaporator, and the residue was reconstituted in the chromatographic solvent system. After filtering, 50 $\mu$L of the resulting retinol solution was injected into the HPLC, and the peak area was recorded by an integrator. The detection wavelength for all carotenoids was 450 nm. A linear relationship was obtained between each standard concentration and peak area.

**Data Analysis.** The results are presented as mean ± SD. Correlation coefficients were calculated and statistical significance was determined by use of $t$-test (Winer, 1971; Zar, 1974).

## RESULTS

Mothers with infants aged 0.5-7 months were included in this study. There was a fairly uniform distribution of infant ages over this period. In this study, colostrum and transitional milk were excluded because of their high vitamin A concentration (Ajans, et al., 1965). After 15 days postpartum, the milk is considered mature (Harzer et al., 1983) and the vitamin A concentration becomes steady (Thomas et al., 1983).

Retention time of retinol was about 4.6 min with a flow rate of 1 mL/min. For milk carotenoids, the first peak, with a retention time of 2.8 min, was lutein. Retention times of $\beta$-carotene, $\alpha$-carotene, and lycopene were about 12.8, 11.9, and 7.9 min, respectively. Peak identification was based on the retention time, compared with standards, cochromatography with standards, and wavelength scans. The retinol and carotenoid concentrations of human milk are shown in Table III. Mean retinol concentration was 57 $\mu$g/100 g of milk. The retinol concentrations of milk ranged from 19 to 148 $\mu$g/100 g. The high standard deviation indicated a large variation between samples from different mothers. According to biochemical criteria (Arroyave et al., 1982), a milk retinol concentration of 50 $\mu$g/100 mL indicated that the mother is well-nourished, while 20 $\mu$g/100 mL represents adequate status. Twenty-five of 42 (60%) samples contained more than 50 $\mu$g/ of retinol/100 g of milk. Only one sample contained less than 20 $\mu$g of retinol (19 $\mu$g), a level indicating that the mother was in slightly low vitamin A status. The mean $\beta$-carotene concentration was 4.6 $\mu$g/100 g of milk, ranging from 2.6 to 10.6 $\mu$g. The mean $\alpha$-carotene, lycopene, and lutein concentration in milk were 3.2, 3.8, and 11.5 $\mu$g/100 g, respectively.

Thirty-nine mothers studied were taking vitamin pills regularly, while 15 took no regular vitamin supplement. Average milk retinol concentration of mothers taking supplements was not significantly different, 60 $\mu$g/100 g (standard deviation of 27 $\mu$g/100 g) from that of mothers who did not take vitamin pills, 49 $\mu$g/100 g (standard deviation of 19 $\mu$g/100 g).

Milk retinol concentration with duration of lactation is presented in Table IV. The mean retinol concentration from the mothers with infants aged 0.5-1 month was the highest (72 $\mu$g/100 g). Retinol concentration decreased thereafter. The lowest milk retinol concentration was obtained at 3-4 months (36 $\mu$g/100 g). However, the number of samples in each age group was small, and differences between groups were not statistically significant ($p > 0.05$). Distribution of $\beta$-carotene in milk according to duration of lactation is also presented in Table IV. Little variation of milk $\beta$-carotene concentration was observed.

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**Table III. Comparison of Retinol and Carotenoid Concentrations in Breast Milk and Dietary Estimation**

|  | retinol | β-carotene | α-carotene | lycopene | lutein |
|---|---|---|---|---|---|
| breast milk |  |  |  |  |  |
| mean,[a] | 57 μg/100 g | 4.6 μg/100 g | 3.2 μg/100 g | 3.8 μg/100 g | 11.5 μg/100 g |
| SD | 25 μg/100 g | 1.6 μg/100 g | 0.9 μg/100 g | 2.1 μg/100 g | 3.4 μg/100 g |
| dietary estimate[b] |  |  |  |  |  |
| mean | 2588 RE/day[c] | 3247 μg/day | 651 μg/day | 389 μg/day | 3106 μg/day |
| SD | 1124 RE/day[c] | 2093 μg/day | 393 μg/day | 213 μg/day | 2611 μg/day |
| correlation | +0.1463 | +0.4174[d] | +0.5216[d] | +0.0279 | +0.2487 |

[a] $N = 54$. [b] $N = 42$. [c] Calculated as 6 μg of β-carotene = 1 RE, 12 μg of α-carotene = 1 RE, and 1 μg retinol = 1 RE. [d] Significantly correlated ($p < 0.01$), $N = 42$.

**Table IV. Distribution of Retinol and β-Carotene Concentration in Milk according to Duration of Lactation**

|  | duration of lactation, months | | | | | | |
|---|---|---|---|---|---|---|---|
|  | 0.5–1 (N = 8) | 1–2 (N = 6) | 2–3 (N = 14) | 3–4 (N = 5) | 4–5 (N = 8) | 5–6 (N = 5) | 6–7 (N = 8) |
| retinol |  |  |  |  |  |  |  |
| mean, μg/100 g | 72 | 65 | 59 | 36 | 58 | 38 | 54 |
| SD | 37 | 9 | 18 | 15 | 32 | 17 | 25 |
| β-carotene |  |  |  |  |  |  |  |
| mean, μg/100 g | 4.6 | 4.6 | 4.7 | 4.4 | 4.9 | 3.4 | 5.1 |
| SD | 2.6 | 1.4 | 1.4 | 1.4 | 1.7 | 0.7 | 1.5 |

The amount of vitamin A and carotenoids in the diet is shown in Table III; the correlation of carotenoids between milk and estimated dietary intake is also presented in Table III. The correlation coefficient for milk α-carotene and dietary α-carotene was the highest, 0.52, followed by β-carotene at 0.42. Correlations between milk and dietary values for lutein, retinol, and lycopene are 0.25, 0.15, and 0.03, respectively. Both milk α-carotene and β-carotene correlated significantly with their respective dietary components ($p \leq 0.01$). The analytical recovery of retinol and β-carotene of milk was determined by adding known amounts of retinol and β-carotene to bovine milk and included the alkaline hydrolysis of the milk. Retinol recovery ranged from 91% to 94%, with an average of 93%. β-Carotene recovery ranged between 91% and 102%, with an average of 97%.

The precision of the method was determined by repeated analysis of pooled milk samples. The coefficients of variation of retinol, β-carotene, and α-carotene were 5%. The coefficients of variation of lycopene and lutein were 8% and 4%, respectively.

DISCUSSION

As the total lipid content of human milk varies in the course of suckling as well as diurnally (Hall, 1979), retinol and carotenoid contents in human milk may vary. Therefore, the proper sampling of milk is very important (Gaull et al., 1982). Complete emptying of both breasts is the best way of obtaining milk samples for analysis of lipid or lipid-soluble substances, but this is impractical in field studies. In the present study, an equal volume of fore and hind milk was obtained from both breasts to compensate for changes in fat content in the course of a feeding. The first morning milk sample was used to avoid problems with diurnal variation in lipid content.

There are numerous methods suggested for the separation of carotenoids from foodstuffs and plasma. These methods may or may not result in complete resolution of the carotenoids mixture into purified components that can be quantified by spectrophotometric analysis (Simpson and Chichester, 1981). The HPLC method, now available, allows better separation, more rapid analysis, and less destructive conditions than the other methods so far developed. In this study, α-carotene, β-carotene, lycopene, and lutein were separated from other carotenoids by HPLC.

The carotenoid concentration of milk in this study was low compared with other studies. β-Carotene concentration of breast milk in Ethiopian mothers with infants aged 0.5–6.5 months ranged from 23.9 to 28.1 μg/100 mL, while in Swedish mothers the range was from 16.3 to 20.8 μg/100 mL (Gebre-Medhin et al., 1979). In this study, β-carotene concentration ranged from 2.6 to 10.6 μg/100 g at the same infant age. There are several possible causes of these differences. Gebre-Medhin et al. (1979) did not differentiate the individual forms of carotene. Any compound that absorbed light at 450 nm was considered β-carotene. Since other carotenes and xanthophylls also absorb light at 450 nm, the tendency would be to probably overestimate the β-carotene content. The sampling time was also different. In the present study, the first morning milk was obtained, while Gebre-Medhin et al. (1979) collected milk throughout the morning. In this study, retinol and the carotenoids were extracted at the same time. If the extraction was incomplete, retinol as well as carotenoids concentration would have been low. However, the retinol concentration in milk of almost all mothers studied was within the normal range, and the results of recovery tests indicated that a 93% recovery was obtained. The carotene could have been lost during storage. All milk samples were stored at −20 °C for less than 5 months. According to Arroyave et al. (1982) milk samples can be kept safely at −20 °C for up to 6 months. To assess the effects of storage, several samples were analyzed on the day that the samples were obtained. The values were in the same range as the stored samples.

To estimate carotenoid consumption, a simplified food-frequency form was filled out. This form imposed less of a burden on respondents than do most conventional methods for assessing dietary intake (Stuff et al., 1983). Mothers were asked to estimate the intake of carotene-containing foods for the previous 2 months. The turnover rate of carotenoids in milk is unknown; however, a 2-month period was chosen because Hume and Krebs (1949) reported that plasma carotenoids fell rapidly with carotenoid-deficient diets and reached a minimum stable level after an 8-week period. Use of a standardized and tested food-frequency questionnaire is recommended. The NCI program, which estimates both total vitamin A and its components, is appropriate for future research.

α- and β-carotene correlations between milk and diet (0.52, 0.42) were positive, although the relationship was

only moderate. There was little or no correlation between the remaining vitamin A components in the diet and those found in milk. This limited relationship could be due to the high level of vitamin A consumed by participants in this study (11 328 IU). The vitamin A content of breast milk is relatively constant in well-nourished mothers (Wallingford and Underwood, 1986). Thus, it is not surprising to see a limited relationship in this study. Both β-carotene and retinol from vitamin supplements, which would increase dietary intake, were found not to significantly raise the milk retinol levels.

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