Micronutrient Concentrations in Paired Skin and Plasma of Patients with Actinic Keratoses: Effect of Prolonged Retinol Supplementation[1]

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Abstract

Much of our knowledge about the relationship between micronutrients and cancer comes from studies in which plasma (serum) micronutrient levels have been correlated with cancer incidence; however, the relationship between the concentrations of micronutrients in the plasma and in the target tissues has not been established. Ninety-three subjects (62 males and 31 females ages 42–86, median age 69) with actinic keratoses were recruited for investigation of this relationship. The subjects were randomly assigned and received placebo or retinol (25,000 IU/day) intervention for 48 to 65 months as part of a skin cancer chemoprevention trial. Shortly before the end of the trial, three fasting plasma samples and one skin biopsy were obtained from each subject. The concentrations of lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene, cis-β-carotene, retinol, retinyl palmitate, α-tocopherol and γ-tocopherol in the plasma and skin were simultaneously measured using HPLC. The profiles of the eleven micronutrients in the plasma and skin were similar. Lycopene, β-carotene and α-tocopherol were the predominant micronutrients in both plasma and skin, but the ratio of retinyl palmitate to retinol was much greater in the skin than plasma. The three fasting plasma concentrations from the same subject during a one-month period were very consistent; however, the between-person variations were very large. The retinol supplementation caused a significant increase in the plasma concentrations of retinol, retinyl palmitate, lutein and α-tocopherol, especially retinyl palmitate as well as the skin concentrations of retinol and retinyl palmitate. The correlations for all the micronutrients in the paired plasma and skin were highly significant (most Spearman correlation coefficients > 0.615, p = 0.0001). However, in retinol and retinyl palmitate, the good correlations were mainly due to the retinol supplementation.

Introduction

Epidemiological and experimental data point to the anticancer effect of several carotenoids, retinoids, and tocopherols (1–12); however, the concentration of these micronutrients in the target tissues is largely unknown. Much of our knowledge about the relationship between micronutrients and cancer comes from studies in which plasma or serum micronutrient levels have been correlated with cancer incidence. Although blood micronutrient levels are presumed to reflect their tissue concentrations, human data in this area are limited, due in part to a lack of appropriate analytical procedures. Since the micronutrients may exert their influence on cancer through actions occurring in the target tissues (13), the validity of using blood concentrations to assess the micronutrient status of target tissues needs to be established.

To gain information on tissue micronutrient concentration and its relationship to the plasma level, paired skin and plasma samples from 93 patients with actinic keratoses were obtained. These subjects were randomly assigned and received placebo or retinol (25,000 IU/day) intervention for 48 to 65 months as part of an on-going study on chemoprevention of skin cancer by retinol. The paired skin and plasma samples were analyzed for various carotenoids, retinoids, and tocopherols using our newly developed nonsaponification method (14, 15). The micronutrients being analyzed include 7 carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene, and *cis*-β-carotene), 2 retinoids (retinol and retinyl palmitate), and 2 tocopherols (α-tocopherol and γ-tocopherol).[3]

Materials and Methods

Human Subjects. Ninety-three subjects who participated in a National Cancer Institute-sponsored chemoprevention trial of skin cancer by retinol at the Arizona Cancer Center (principal investigator, Dr. Thomas Moon) were recruited for this study. A demographic description of the study subjects is shown in Table 1. There were 62 males and 31 females of Caucasian background, between the ages of 42 and 86 years; the median and mean ages

Received 5/15/92.

[1] Supported in part by USPHS Grants CA51477 and CA27502 from the National Cancer Institute, Bethesda, MD, and a grant from Amoco Technology Company, Naperville, IL.

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[3] This procedure cannot separate γ-tocopherol and β-tocopherol; thus, the γ-tocopherol values reported in this paper may contain some β-tocopherol.

Table 1 Demographic description of study subjects

	n	%
Age distribution[a]		
42-55	9	10
56-65	18	20
66-75	49	54
76-86	14	16
Gender		
Male	62	67
Female	31	33
Treatment		
Placebo	41	44
Retinol	52	56

[a] Mean and median ages were 67.6 and 69 years, respectively.

were 69 and 67.6 years, respectively. The subjects were first diagnosed with actinic keratoses 5 or 6 years earlier and had no history of other diseases. Shortly after diagnosis, they were randomized to receive either placebo (n = 41) or 25,000 IU of retinol/day (n = 52) since that time. The placebo and the retinol intervention lasted between 48 and 65 months. Dietary intakes (except retinol) were not restricted. Two to three months prior to the termination of the trial, each subject provided fasting blood samples, three times over a 1-month period, at 7- to 10-day intervals. The subjects also provided skin biopsies once during the same month. All the subjects signed an informed consent form which was approved by the Human Subjects Committee of the University of Arizona.

Sample Collection. Approximately 7 ml of blood were drawn from each subject after an overnight fast by venipuncture into a foil-wrapped, green-top (heparinized) tube and immediately placed on ice. A total of 279 blood samples were collected; they were centrifuged under refrigeration within 2 h of collection. Plasma was saved and stored in 0.25-ml aliquots at −80°C until analysis.

Skin biopsies, one from each subject, were taken from the upper thigh area. Prior to removal, the skin was cleaned with a betadine preparation, and the area was anesthetized with 1% xylocaine. A piece of skin (about 1 inch x 1/4 inch in size) and the subcutaneous fat directly underneath were removed. Stitches were used to close the incision, and remained in place for approximately 7 to 10 days. The biopsies were rinsed with phosphate-buffered saline, and any visible subcutaneous fat was removed with a scalpel. The remaining samples were then weighed, and about 100 mg/biopsy were stored in a 17 x 100 mm polypropylene test tube (Falcon 2059; Becton Dickson, Lincoln Park, NJ) at −80°C until analysis. All the cleaning and weighing were carried out under dim red light.

Homogenization and Extraction. The skin biopsies were homogenized as described (14). Essentially, 1 to 2 mg of BHT,[4] 560 μl of phosphate-buffered saline, and 70 μl of a collagenase solution (50 mg/ml) were added to 100 mg of skin biopsy in the storage tube. After mixing, the samples were incubated at 37°C for 1 h and then homogenized using a polytron homogenizer (model PT 10/35; Brinkmann Instruments, Westbury, NY). Next, 70 μl of a protease solution (20 mg/ml) were added to the whole homogenate. The samples were mixed and then incubated at 37°C for another 30 min.

The extractions of micronutrients for HPLC analysis were carried out as described (14). Briefly, after the second incubation, 250 μl of a sodium dodecyl sulfate-ethanol-BHT solution were added to 0.25 ml of the homogenate. After vortex mixing, the samples were extracted twice with 500 μl of hexane containing 0.1% BHT. When the concentration of the micronutrients was too low to be accurately determined, more aliquots of the digested homogenates were extracted, and all of the hexane layers were combined and dried. The dried extracts were stored at −20°C for analysis within 2 days. The recovery of spiked standard lycopene, β-carotene, retinol, retinyl palmitate, and α-tocopherol in this method was found to be 85 to 98% (14).

The plasma did not require homogenization. Aliquots of frozen plasma, 0.25 ml/tube, were allowed to thaw, were mixed with 250 μl of the sodium dodecyl sulfate-ethanol-BHT solution, and were extracted twice with 500 μl of hexane as described (15).

HPLC Analysis. HPLC analysis was carried out as previously described (15). The analysis was performed using a Waters 600E multisolvent delivery system, a Waters 715 Ultra-WISP autoinjector, and a Hewlett-Packard 1040M photodiode array detector with a chem station. Two Novapak C_{18} columns (4 μm, 300 x 3.9 mm; Waters Associates, Milford, MA) connected in series preceded by a guard column (70 x 2.1 mm; Waters Associates) packed with CO:Pell octadecyl silane (Alltech, Deerfield, IL) were used for analysis.

To separate the 11 micronutrients of interest, a gradient elution at a flow rate of 1.3 ml/min was used (15). The total run time, including reequilibration, was 47 min. The HPLC effluent was monitored at 300 nm (tocopherols), 325 nm (retinoids), and 452 nm (carotenoids). Information regarding the analytical standards and the validity of the homogenization, micronutrient extraction, and HPLC procedures has been reported (14, 15).

Statistical Analysis. A SAS statistical program was used for all of the statistical analyses. Since the size was small and since many of the micronutrient distributions were highly skewed, the Wilcoxon rank-sum test was used to compare the concentration difference between the placebo and the retinol intervention group. Spearman correlation coefficients were used to evaluate the relationship of the micronutrients in the paired plasma and skin samples.

Results and Discussion

The primary purposes of this study were to determine: (*a*) whether the prolonged retinol intervention/supplementation would increase the concentration of retinol and retinyl palmitate in the plasma, and most importantly in the skin (target tissue) of patients with actinic keratoses; and (*b*) whether the concentration of the 11 micronutrients in the plasma and skin were correlated. Since the concentration of the micronutrients in the plasma may fluctuate with recent dietary intake, three plasma samples after an overnight fast were collected from each individual over a 1-month period to assess how consistent the

[4] The abbreviations used are: BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography.

Table 2 Mean concentrations of the designated micronutrients in three fasting plasma samples collected over a 1-month period and coefficients of variation of the three measurements

Micronutrients	Mean concentrations ± SD (ng/ml)			Coefficients of variation of the 3 means (%)
	PL-1	PL-2	PL-3	
Placebo group (n = 41)				
Lutein	143 ± 48	119 ± 51	116 ± 48	12.2
Zeaxanthin	26 ± 9	21 ± 9	21 ± 7	12.6
β-Cryptoxanthin	119 ± 68	104 ± 67	101 ± 59	8.9
Lycopene	348 ± 167	340 ± 195	312 ± 149	5.7
α-Carotene	63 ± 45	58 ± 40	57 ± 37	5.4
β-Carotene	268 ± 260	241 ± 225	234 ± 219	7.2
cis-β-Carotene	17 ± 17	16 ± 14	16 ± 15	3.6
Retinol	589 ± 144	562 ± 154	557 ± 127	3.0
Retinyl palmitate	31 ± 16	26 ± 11	27 ± 17	9.4
α-Tocopherol	12,990 ± 3,947	12,296 ± 3,431	12,688 ± 4,182	2.7
γ-Tocopherol	2,111 ± 1,329	2,040 ± 1,285	2,243 ± 1,299	4.8
Retinol intervention group (n = 52)				
Lutein	119 ± 54	101 ± 43	94 ± 40	12.2
Zeaxanthin	22 ± 11	20 ± 9	20 ± 9	5.5
β-Cryptoxanthin	130 ± 68	110 ± 64	113 ± 59	9.1
Lycopene	322 ± 148	286 ± 124	291 ± 150	6.5
α-Carotene	60 ± 40	54 ± 39	53 ± 42	6.8
β-Carotene	305 ± 296	267 ± 279	271 ± 282	7.4
cis-β-Carotene	21 ± 21	19 ± 19	20 ± 21	5.0
Retinol	653 ± 122	642 ± 109	638 ± 111	1.2
Retinyl palmitate	145 ± 176	115 ± 90	113 ± 109	14.5
α-Tocopherol	15,617 ± 7,049	14,456 ± 5,406	14,754 ± 5,281	4.0
γ-Tocopherol	2,100 ± 1,426	2,073 ± 1,207	2,176 ± 1,316	2.5

three fasting plasma concentrations of the micronutrients were. As shown in Table 2, the mean concentrations of the 11 micronutrients in the three fasting plasma samples were very close, although dietary (except retinol) intake was not restricted. The coefficients of variation of the three measurements, expressed as SD/mean × 100%, for all the micronutrients in the placebo group ranged from 2.7% to 12.6%, indicating consistency. Thus, it appears that it was appropriate to use the fasting plasma concentration to study its relationship to the skin micronutrient concentration. The skin concentration probably reflects the chronic nutritional status of the micronutrients in the target tissue.

Table 3 shows the concentration ranges and the group mean concentrations of the designated micronutrients in the plasma of the placebo and retinol interven-

Table 3 Concentration ranges and group mean concentrations of the designated micronutrients in the plasma of the placebo and retinol intervention groups

Micronutrients	Concentration ranges (ng/ml)	Group means ± SD (ng/ml)	P
Placebo group (n = 41)			
Lutein	53-243	126.3 ± 46.6	
Zeaxanthin	8-42	22.7 ± 7.6	
β-Cryptoxanthin	21-155	107.9 ± 62.9	
Lycopene	55-740	333.3 ± 161.6	
α-Carotene	12-199	59.4 ± 40.1	
β-Carotene	35-1,492	247.7 ± 233.6	
cis-β-Carotene	3-98	17.0 ± 15.6	
Retinol	249-931	569.3 ± 135.5	
Retinyl palmitate	14-84	27.9 ± 13.2	
α-Tocopherol	7,382-23,316	12,658 ± 3,629	
γ-Tocopherol	267-5,628	2,131 ± 1,239	
Retinol intervention group (n = 52)			
Lutein	33-204	104.8 ± 44.1	0.0395[a]
Zeaxanthin	8-50	20.7 ± 9.3	0.1369
β-Cryptoxanthin	20-259	117.9 ± 62.2	0.3611
Lycopene	69-785	299.7 ± 131.7	0.4670
α-Carotene	10-246	56.5 ± 39.4	0.7746
β-Carotene	40-1,778	280.6 ± 284.1	0.8134
cis-β-Carotene	3-125	19.9 ± 20.4	0.8252
Retinol	461-908	644.5 ± 104.5	0.0010[a]
Retinyl palmitate	36-776	124.0 ± 109.3	0.0001[a]
α-Tocopherol	7,253-3,6152	14,942 ± 5,625	0.0258[a]
γ-Tocopherol	518-5,871	2,116 ± 1,239	0.7984

[a] Significantly different from the placebo group by Wilcoxon rank-sum test.

Table 4 Concentration ranges and group mean concentrations of the designated micronutrients in the skin biopsies of the placebo and retinol intervention groups

Micronutrients	Concentration range (ng/g wet weight)	Group means ± SD (ng/g wet weight)	*p*
Placebo group (n = 26)			
Lutein	8–34	17.0 ± 6.6	
Zeaxanthin	1–6	3.1 ± 1.0	
β-Cryptoxanthin	2–25	11.6 ± 6.4	
Lycopene	12–258	102.2 ± 57.4	
α-Carotene	3–31	13.2 ± 7.1	
β-Carotene	18–89	48.1 ± 23.3	
cis-β-Carotene	3–21	9.9 ± 5.0	
Retinol	6–81	25.7 ± 15.6	
Retinyl palmitate	5–43	16.2 ± 7.6	
α-Tocopherol	2,722–35,549	6,542 ± 6,605	
γ-Tocopherol	452–3,746	1,428 ± 738	
Retinol intervention group (n = 36)			
Lutein	7–44	16.3 ± 8.2	0.2944
Zeaxanthin	1–10	3.8 ± 1.9	0.2449
β-Cryptoxanthin	3–49	15.7 ± 10.3	0.1557
Lycopene	24–333	105.7 ± 55.0	0.7809
α-Carotene	3–29	14.2 ± 7.0	0.5302
β-Carotene	15–374	65.9 ± 63.7	0.3960
cis-β-Carotene	2–66	13.2 ± 11.2	0.3215
Retinol	18–98	37.4 ± 16.5	0.0010[a]
Retinyl palmitate	12–72	34.9 ± 14.4	0.0001[a]
α-Tocopherol	2,197–33,627	8,689 ± 7,712	0.2508
γ-Tocopherol	553–3,046	1,419 ± 627	0.9886

[a] Significantly different from the placebo group by Wilcoxon rank-sum test.

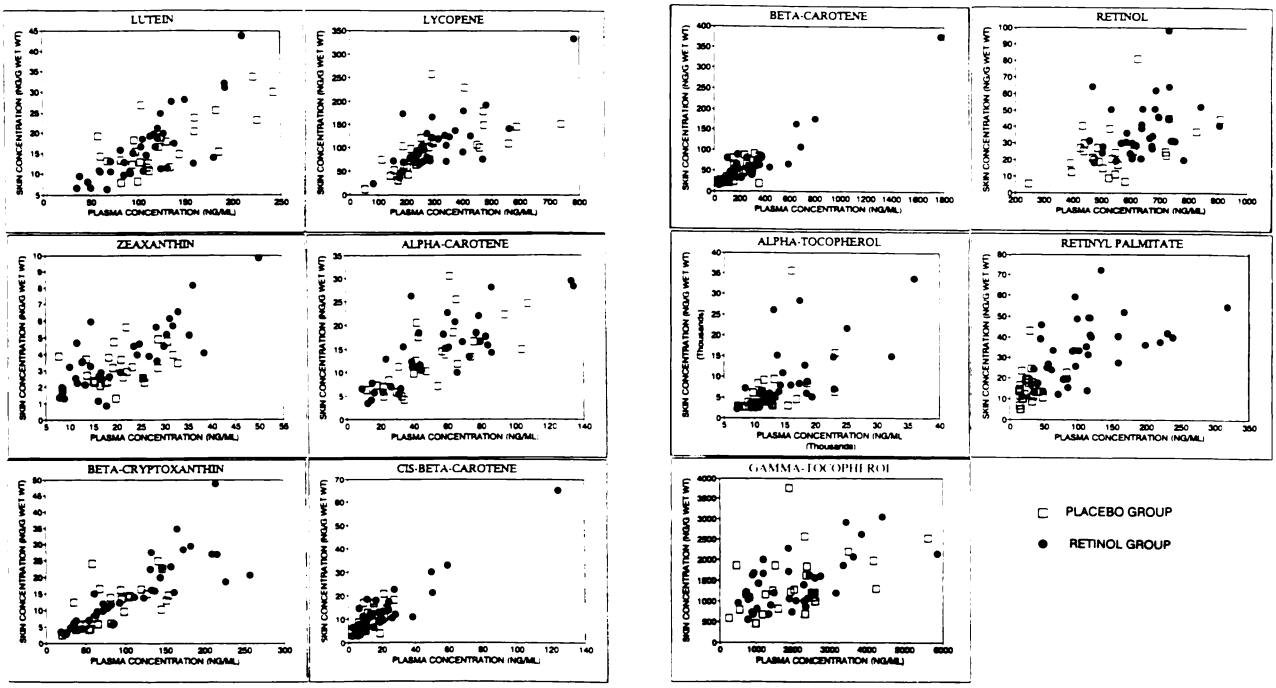

Fig. 1. Plots of plasma concentration (average of three fasting concentrations) *versus* skin concentration for each micronutrient in 62 paired plasma and skin samples.

tion groups. The data indicate that the concentration ranges (*i.e.*, between-person variations) were very large in both placebo and intervention groups. The extent of the variations differed between micronutrients. Other investigators have also reported such large variations for β-carotene, retinol, and α-tocopherol in plasma (16–19).

The retinol supplementation caused a significant increase in the plasma concentration of retinol, a chemopreventive agent, and its fatty acid ester, retinyl palmitate. The magnitude of the increase was much greater for retinyl palmitate than for retinol. The net increases were 344% and 13%, respectively. The mean concentration of lutein

Table 5 Spearman correlation coefficients of skin concentration *versus* three plasma concentrations and the average of the three plasma concentrations

Micronutrients	Spearman correlation coefficients			
	PL-1	PL-2	PL-3	Average of concentrations
Combined (n = 62)				
Lutein	0.7024	0.5865	0.7015	0.6931
Zeaxanthin	0.6018	0.5931	0.5419	0.6164
β-Cryptoxanthin	0.8573	0.8197	0.8029	0.8349
Lycopene	0.7063	0.6743	0.6536	0.7446
α-Carotene	0.7707	0.7453	0.7340	0.7612
β-Carotene	0.7661	0.7458	0.7223	0.7552
cis-β-Carotene	0.7586	0.7395	0.6990	0.7385
Retinol	0.5180	0.3865[a]	0.5476	0.5082
Retinyl palmitate	0.7062	0.7611	0.6703	0.7315
α-Tocopherol	0.7129	0.6544	0.6421	0.7088
γ-Tocopherol	0.5266	0.4682	0.4829	0.4958
Placebo group (n = 26)				
Retinol	0.4304	0.2301	0.3149	0.3327
	(0.0282)[b]	(0.2582)	(0.1172)	(0.0968)
Retinyl palmitate	0.3170	0.2660	0.3191	0.2988
	(0.1146)	(0.2068)	(0.1121)	(0.1382)
Retinol intervention group (n = 36)				
Retinol	0.2888	0.1910	0.4688	0.3787
	(0.0875)	(0.2646)	(0.0039)	(0.0227)
Retinyl palmitate	0.5137	0.6757	0.4349	0.6027
	(0.0014)	(0.0001)	(0.0080)	(0.0001)

[a] P was 0.0019; all other P values for the combined groups were 0.0001.
[b] P in parentheses.

appeared to be lower (104.8 *versus* 126.3) and the mean concentration of α-tocopherol appeared to be higher (14,942 *versus* 12,658) in the intervention group than in the placebo group. The reason for such changes is not apparent.

The concentration ranges and the group mean concentrations of the 11 micronutrients measured in 62 skin biopsies are summarized in Table 4. The other 31 skin biopsies had been analyzed earlier using a different method, *i.e.*, saponification with KOH-methanol prior to homogenization.[5] Thus, the data are not included in the table. As seen in other tissues and organs (20–23), the concentration range of each micronutrient in the skin was also very large. Between the placebo and the intervention group, only the concentrations of retinol and retinyl palmitate were significantly different. The retinol supplementation caused the two retinoids, especially retinyl palmitate, to increase. The net increases were 14.6% and 115.4%, respectively. The outcome of the chemoprevention trial on skin cancer by retinol will be able to address the issue of whether the increase in the concentrations of retinol and retinyl palmitate in the target tissue, *i.e.* skin, have any clinical significance.

The concentrations of the 11 micronutrients in human skin have not been reported by others. Thus, comparisons of data between laboratories are not possible at this time. Nevertheless, two groups have reported the concentration of carotene in the epidermis and dermis of human skin; their values differed by more than 10-fold (24, 25). The reason for such a large disparity is not apparent, since both studies used KOH-methanol saponified skin samples for the analysis. Vahlquist *et al.* (25) have also reported the concentration of retinol in epidermis and dermis, which was about 10-fold higher than the concentrations reported in Table 4. Thus, it is apparent that any comparison of data between laboratories is very difficult, unless a standardized procedure is used.

The profiles of carotenoids, retinoids, and tocopherols in the plasma (Table 3) and skin (Table 4) were very similar, except that the skin contained a relatively higher amount of retinyl palmitate in relation to retinol. The three major antioxidants (lycopene, β-carotene, and α-tocopherol) were present in relatively large amounts in both plasma and skin. In fact, lycopene, which has not been investigated as extensively as β-carotene, was present in a greater amount than β-carotene in the plasma and skin, as well as other tissues of most human subjects studied.[6] In view of the potential role of lycopene in cancer prevention (6–9) and its abundance in the target tissues, this carotenoid also deserves investigation.

The plots of plasma concentration (average of the three fasting plasma concentrations) *versus* skin concentration for each micronutrient in 62 paired plasma and skin samples are shown in Fig. 1, and the Spearman correlation coefficients of plasma concentration *versus* skin concentration using the three individual fasting plasma concentrations as well as the average of the three concentrations are shown in Table 5. All the data from the placebo and intervention groups were combined because the sample size was small and because the concentrations of most micronutrients (except retinyl palmitate) did not differ greatly between the two groups. The micronutrients in all of the paired plasma and skin

[5] Saponification of skin biopsies with KOH-methanol caused substantial destruction of micronutrients, especially retinyl palmitate and α-tocopherol (14).

[6] Peng *et al.*, unpublished data.

were highly correlated ($P = 0.0001$), except in one case (retinol in PL-2) in which $P = 0.0019$. Thus, it is possible to use one set of the fasting plasma concentrations to correlate with the skin concentration; however, using the average of the three plasma concentrations produced a more uniform result. The correlations were generally better for the seven carotenoids, α-tocopherol, and retinyl palmitate. Retinol and γ-tocopherol had weaker correlations. The weaker correlation of retinol was not unexpected, because plasma retinol concentration was under a strict homeostatic control, while skin retinol concentration probably was not.

Table 5 also shows the Spearman correlation coefficients of retinol and retinyl palmitate in the two respective groups. In the placebo group, neither retinol nor retinyl palmitate in the paired plasma and skin samples was correlated; the use of the average of the three fasting plasma concentrations did not improve the correlation. Since significant correlations of the two retinoids in the paired plasma and skin samples were observed when all the data from the placebo and intervention groups were combined, it appears that the retinol supplementation was the key to the good correlations of retinol and retinyl palmitate observed. In the retinol intervention group, the correlation was much better for retinyl palmitate than for retinol. The difference was due to the differential response of the two retinoid concentrations to the retinol supplementation which caused a much greater increase in the concentration as well as between-person variation of retinyl palmitate than retinol in both plasma and skin (Tables 3 and 4).

In conclusion, the retinol supplementation caused a significant increase in the concentration of retinol and retinyl palmitate in the plasma and skin (target tissue) of subjects with actinic keratoses. In addition, significant correlations were observed between the concentrations of the 11 micronutrients in the paired plasma and skin of the subjects, 56% of whom had received retinol supplementation. The good correlations suggest that the plasma micronutrient concentrations can be used to estimate the micronutrient status of the skin; however, in the case of retinol and retinyl palmitate, the correlation would have been much weaker if the subjects receiving the retinol supplementation were excluded. Under the experimental conditions, the fasting plasma concentrations of the 11 micronutrients were very consistent. Thus, using fasting plasma concentrations in the correlation study was appropriate.

Parker (20, 21) has reported good correlations between the concentrations of carotenoids in plasma and adipose tissue. Our preliminary data have also indicated good correlations between the concentrations of carotenoids and α-tocopherol in paired plasma and buccal mucosal cell samples. Although the subjects in the present study had actinic keratoses, they were essentially normal and healthy. Thus, the data generated in this study may be used as baseline reference values for future epidemiological and chemoprevention trials in older subjects.

Acknowledgments

The authors would like to thank Dr. Kevin Welch of the Department of Dermatology, University of Arizona, for his assistance in obtaining skin biopsies. The authors also would like to thank Dr. David S. Alberts for his enthusiastic support.

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