Journal of Chromatography, 413 (1987) 79-89
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3384

DETERMINATION OF TOCOPHEROL AND TOCOPHEROL ACETATE CONCENTRATIONS IN HUMAN FECES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 29th, 1986; revised manuscript received August 6th, 1986)

SUMMARY

A method of sample clarification and high-performance liquid chromatography (HPLC) specifically developed to permit simple and rapid determination of vitamin E (α-tocopherol, E) and vitamin E acetate (EA) in feces is reported. Retinol acetate (RA) was used as an internal standard. The vitamins of interest were extracted from an aqueous stool suspension into an organic phase (ethyl acetate-butanol), which was injected directly onto the reversed-phase HPLC system. An isocratic mobile phase of methanol-water (97:3) was employed, with ultraviolet detection at 275 and 285 nm (to permit simultaneous monitoring and absorbance ratio determination). Recoveries of exogenous RA, E, and EA from stool suspensions (relative to water) were 99.0 ± 7.0, 100.9 ± 7.0, and $101.2 \pm 13.3\%$, respectively ($n=10$). The organic matrix could be stored at $-35\,^{\circ}C$ overnight with no change in E or EA results. Sensitivities for E and EA were 80 and 102 $\mu g/g$ of stool, respectively. Each analysis required nine min. The within-day coefficients of variation were 2.9, 3.6, and 3.0% ($n=7$) for RA, E, and EA, respectively. Neither E nor EA were detected in baseline fecal samples from fourteen subjects, but both were present in high but varied concentrations after four weeks supplementation with oral *d,l*-EA. E but not EA was present in blood samples drawn during periods of oral supplementation with EA. There was poor correlation between fecal levels of E and EA, and the increase in serum levels of E. This method permits rapid, selective, and precise determination of E and EA in human fecal samples.

INTRODUCTION

Carcinoma of the colon is the most common visceral cancer in the U.S.A., resulting in more than 50 000 deaths annually [1]. Environmental factors, with special reference to regional dietary habits, may play an important role [2]. The presence of mutagenic substances in the feces of high-risk populations in the U.S.A. and Canada has been identified [3,4]. Dietary factors may affect the actual amount of carcinogen intake or somehow affect the formation of mutagenic sub-

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stances in vivo [5,6]. One group of substances which have such mutagenic properties have been identified and labelled "fecapentaenes" and are thought to be the by-products of bacterial metabolism in human feces [7–9].

The inhibition of chemical carcinogens has been studied in various animal tumor models utilizing a broad range of compounds. The use of various antioxidants to inhibit carcinogenesis has been based upon the concept that they will exert a scavenging effect, thereby protecting cells from attack [10]. Selenium, α-tocopherol, ascorbic acid, and other normal dietary antioxidant constituents with antioxidant activity have generated much interest because of their minimal toxicity and potential role in "naturally" occurring geographic differences in specific organ cancer rates [2]. Multiple studies using animal models have demonstrated that tocopherols may have a protective effect against nitrosamine- or dimethylhydrazine-induced colonic tumors [11–14], although conflicting results have also been reported [15].

In light of the potential relationship between fecal mutagenicity and colorectal neoplasms and the efficacy of tocopherol in preventing tumor induction in some animal models, several issues require further study as colon cancer chemoprevention studies with either oral α-tocopherol (E) or α-tocopherol acetate (EA) are attempted in human subjects. First, since fecal concentrations of E rather than serum E concentrations may be critical for its effects upon colonic mucosa, a sensitive and specific analytical method for quantifying E in fecal material is necessary.

Second, oral supplementation with tocopherol is usually in the form of EA rather than E, since EA is more resistant to oxidation by exposure to the atmosphere, heat, light, etc. [16]. However, EA is not active as an antioxidant itself; E, in the form of the free alcohol, is the active form of the vitamin. In humans, EA is hydrolyzed in the intestinal lumen to E, which can then be absorbed [16]. Even when EA is ingested orally, no measurable EA is found in serum [17]. Therefore, an assay for fecal tocopherol concentration should be able to separate and quantify both E and EA. A Medline search covering the last five years revealed one article which described an assay for fecal total tocopherol content [18], but no method which could separate and quantify both E and EA.

We describe a high-performance liquid chromatographic (HPLC) assay for both E and EA in human feces, based upon our prior assay for E in human serum [17]. In addition, we report the fecal concentrations of both E and EA in fourteen subjects at baseline and again after they had ingested either 400 or 800 mg of EA daily over a four-week period. Finally, we demonstrate a lack of correlation between changes in serum E concentrations and fecal concentrations of E and EA.

EXPERIMENTAL

Reagents

All chemicals were the highest grade commercially available; all solvents were HPLC grade. Water was house distilled and then passed through a Milli-Q purification system (Millipore, Milford, MA, U.S.A.). D-α-tocopherol (oil) was

obtained from Kodak (Rochester, NY, U.S.A.). D-α-tocopherol acetate and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, U.S.A.). Butanol-1 and ethyl acetate were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). Methanol was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Clinical samples

Stool samples were collected over three-day periods by each subject at baseline and again after four weeks of daily EA supplementation with oral EA. Specimens were stored frozen until brought to the central lab, then thawed sufficiently to allow approximately 1 g of stool to be removed and placed in a tared 50-ml polypropylene screw-capped centrifuge tube. Samples were frozen at −75°C until analysis. The fecal samples were analyzed for E and EA content in a blinded fashion; the laboratory did not know prior to analysis whether the subjects had been randomized to receive placebo, low-dose EA, or high-dose EA.

Fasting 8:00 a.m. serum samples werer obtained at baseline and after four weeks of EA supplementation. Blood was collected in a red-top serum Vacutainer tube (Becton-Dickinson, Rutherford, NJ, U.S.A.). After separation, serum was transferred to polypropylene freezer tubes and stored at −75°C until analysis. All human subjects had given informed consent to a protocol previously approved by our Institutional Review Board.

Stock solutions

A stock solution of E was prepared by dissolving 100 mg of E oil in 10 ml of acetonitrile (approximate concentration 10 mg/ml). E was stored at 4°C in its oil form and at 4°C under nitrogen when in solution. EA was prepared by dissolving 400 mg crystalline EA in 20 ml of acetonitrile (20 mg/ml). In its crystalline form EA was stored at 4°C in a dessicator and in solution it was stored at 4°C under nitrogen. Retinol acetate (RA) stock solution was prepared by dissolving 1 g of crystalline RA in 20 ml of acetonitrile (50 mg/ml). A further dilution of RA in acetonitrile (3 mg/ml) was prepared and used for adding the internal standard to stool suspensions. All stock solutions were filtered through Nylon 66 0.2-μm disposable filter units (Rainin Instruments, Woburn, MA, U.S.A.) after preparation to remove insoluble material.

An aliquot of each stock solution was diluted each day in acetonitrile in order to measure exact vitamin concentrations. The extinction coefficients ($E^{1\%}_{1\text{cm}}$ in ethanol) and wavelengths used were 1850 and 325 nm for RA [19], 75.8 and 292 nm for E [20], and 43.6 and 285 nm for EA [20]. We had previously determined that UV absorption for all compounds was identical (to within 0.5%) in ethanol and acetonitrile.

Sample clarification

To approximately 1 g of stool (wet weight) was added sufficient water to make up to 16 g total weight (approximately 16 ml total volume). Eight 3-mm glass beads (Fisher Scientific, Fairlawn, NJ, U.S.A.) were added to assist in preparing the aqueous stool suspension. The sample was then vortexed for 1 min. Diluted

RA stock solution (3 mg/ml, 100 μl) and blank acetonitrile (900 μl) were added, the sample was again vortexed and then placed on an automatic shaker for 10 min. The tube was removed from the shaker and 8 ml of ethyl acetate–butanol (1:1) was added. The tube was vortexed for 15 s and placed on the shaker again for 10 min. The sample was then centrifuged at 1500 *g* for 10 min. Approximately 5 ml of the top organic layer was removed using a pasteur pipet and placed in a 0.2-μm Nylon 66 disposable centrifugal filter unit (Rainin). The filter unit was centrifuged for 5 min at 1500 *g*. A volume of 10 μl of this organic matrix was then injected directly onto the liquid chromatograph.

Daily standard curve

To 16 ml of water were added sufficient volumes of the three stock solutions to produce aqueous solutions having the following approximate concentrations of RA, E, and EA: low standard (20, 50, and 70 μg/ml); medium standard (20, 200, and 280 μg/ml); and high standard (20, 350, and 490 μg/ml). After addition of these acetonitrile solutions, sufficient blank acetonitrile was added such that the total volume of acetonitrile added was 1.0 ml. These three aqueous solutions were then extracted as usual and two daily standard curves (E/RA peak-height ratio versus known E concentration and EA/RA peak-height ratio versus known EA concentration) were prepared. Daily standard curves were accepted only if $r > 0.99$. The ranges of the standard curves for E (50–350 μg/ml) and EA (70–490 μg/ml) were established after preliminary studies of human fecal samples. The corresponding concentrations of E and EA in the fecal samples themselves were sixteen-fold greater: 600–5600 and 1120–7840 μg/g, respectively.

HPLC system

The HPLC system consisted of a Waters 510 dual-piston pump (Waters Assoc., Milford, Mass., U.S.A.), an SSI 0.5-μm in-line filter (Rainin), a Rheodyne 7125 injector with a 500-μl loop (Rainin), a Brownlee C_{18} precolumn (30×4.6 mm) packed with 5-μm spherical material (Rainin), a Waters μBondapack C_{18} column (300×3.9 mm) packed with 10-μm irregular RP-18 material, a Waters 490 multiwavelength detector (Waters Assoc.), and three strip-chart recorders (Houston Instruments, Austin, TX, U.S.A.). The mobile phase consisted of methanol–water (97:3) and was vacuum-filtered through a 0.45-μm Nylon 66 filter prior to use and then degassed under vacuum. Flow-rate was 2.5 ml/min at ambient temperature generating a back-pressure of approximately 100 bar (1500 p.s.i.). Peak identification was confirmed in all samples by comparison of retention times (within 0.05 min) with those of known standards and by comparison of the absorbance ratio for each peak of interest to absorbance ratios of known E and EA peaks. Detector sensitivity was set at 0.04 a.u.f.s. and detection was carried out at 285 and 275 nm. The absorbance ratio was calculated as absorbance at 285 nm divided by the absorbance at 275 nm.

E concentrations in serum were measured using a previously described HPLC assay [18]. We had previously confirmed that no EA is detectable in serum or plasma, even when oral supplementation with EA is provided.

Calculations

Concentrations of E and EA in each aqueous suspension were calculated from the standard curves, and expressed as μg vitamin per ml suspension. This value was re-expressed as μg vitamin per g of stool (wet weight). When necessary, differences between group means were explored using one-way analysis of variance; the Student-Neuman-Keuls test was applied as needed [21].

Special extraction procedures

Occasionally, the above extraction procedure was modified in order to perform a particular experiment. In order to quantitate recoveries of added RA, E, and EA from stool suspensions from different subjects, duplicate samples of water and aqueous stool suspensions from 10 subjects were obtained. For each pair, one sample was spiked with RA (20 μg/ml), E (70 μg/ml), and EA (260 μg/ml) and analyzed. The second sample from each subject was also analyzed, but without adding RA, E, and EA. In this way, net recoveries from stool suspensions could be determined.

In order to test stability of the extraction matrix during storage, stool samples from eight subjects were prepared and extracted in duplicate, immediately chromatographed and concentrations of E and EA calculated. After chromatography, the organic matrices were stored at $-35\,^{\circ}C$ overnight. The next day the organic matrices were warmed to room temperature and re-chromatographed (using a new standard curve prepared that day). Results of E and EA determinations on the second day were expressed as a percentage of results obtained the first day.

Finally, the effect of butylated hydroxytoluene upon the recoveries of RA, E and EA were measured by "spiking" duplicate stool suspensions from six subjects with RA. For each subject, one sample had 100 μl blank ethanol added, while the second suspension had an equal volume of a concentrated ethanolic solution of BHT (330 mg/ml) added, such that the final concentration of BHT in the aqueous suspension was 2.06 mg/ml.

Human subjects and drug administration

Normal volunteers were randomized to receive placebo, EA 400 mg per day or EA 800 mg per day. Serum and stool samples were collected at baseline and again after four weeks of oral ingestion of placebo or EA capsules. Serum and stool samples were analyzed in a blinded fashion. However, this paper reports the results of the first fourteen subjects who were in fact receiving low- or high-dose EA supplementation (code broken after analysis). All capsules were supplied by BASF (Wyandotte, MI, U.S.A.). The gelatin shell was made of gelatin, glycerin, water, methyl paraben, and propyl paraben. The contents included either *d,l*-EA (400 or 800 mg, $>96\%$ purity) in soybean oil, or soybean oil (for placebo capsules).

RESULTS

Chromatographic and clarification conditions

The three peaks of interest eluted in less than 8 min; injections could be made every 9 min. There was nearly baseline separation of RA, E, and EA from all

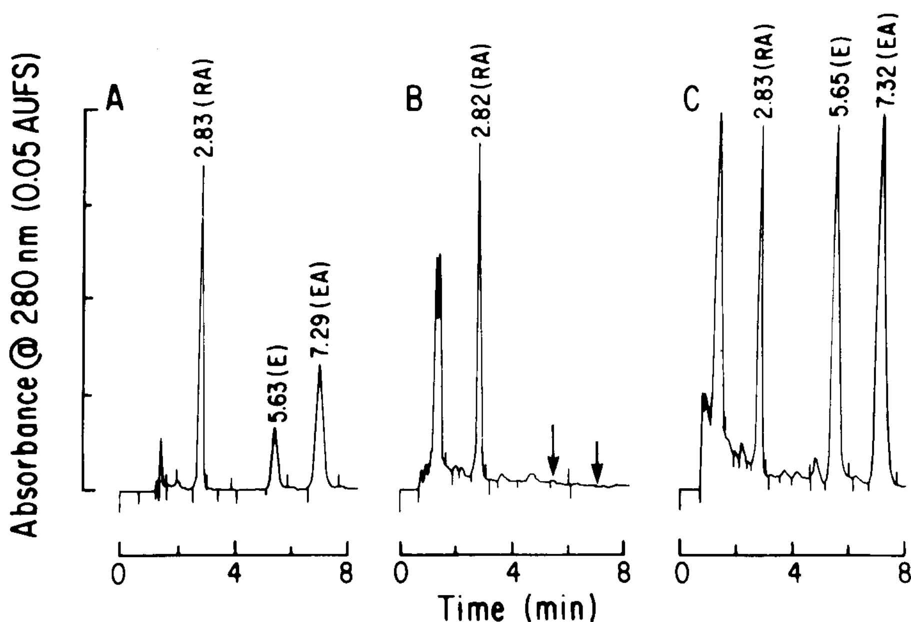

Fig. 1. Chromatograms of an aqueous standard solution and two aqueous stool suspensions after extraction. UV absorption was measured at 280 nm (0.05 a.u.f.s.) and recorded on a recorder-integrator. (A) Aqueous standard solution with exogenous RA, E, and EA peaks. (B) Baseline stool sample with exogenous RA peak (internal standard); two arrows indicate retention times for E and EA. (C) Stool sample after four weeks oral supplementation with EA, demonstrating exogenous RA peak (internal standard) and endogenous E and EA peaks.

surrounding peaks (see Fig. 1). Net recoveries of exogenous RA, E, and EA from the aqueous stool suspensions were 99.0 ± 7.0% (n=4), 100.9 ± 7.0% (n=10), and 101.2 ± 13.3% (n=10), respectively, relative to recoveries from "spiked" water (differences not significant).

Sensitivity and precision

Conservative limits of detection for E and EA in stool suspensions were 5 and 7 μg/ml, respectively (peak size > five times baseline noise). These limits of sensitivity correspond to concentrations of E and EA of 80 and 102 μg per g of stool, respectively. The within-day precision (coefficient of variation, C.V.) for RA, E, and EA was 2.9, 3.6, and 3.0%, respectively, based upon extractions of seven aliquots of the same stool suspension.

Stability of E and EA in the organic matrix

Compared to the results obtained on day 1, the results obtained for E and EA on day 2 were 97.8 ± 4.8 and 100.9 ± 13.3%, respectively. These differences were not statistically significant.

Use of an antioxidant during extraction

Recoveries of RA, E, and EA in the presence of BHT, relative to recoveries in the absence of BHT, were 90.9 ± 8.7, 98.4 ± 6.5, and 98.3 ± 6.5%. These non-sig-

TABLE I

CONCENTRATIONS OF E AND EA IN STOOL SAMPLES OBTAINED DURING THE EA SUPPLEMENTATION COLLECTION PERIOD

When more than one stool sample was assayed, the mean values for E and EA were listed. Also tabulated are the dose of EA received, total stool tocopherol concentration in E equivalents (Total), E as a percentage of total stool tocopherol concentration (% E), serum E concentration during baseline (Base) and supplementation (Drug) periods, and net change (Change) in serum E concentration.

Subject	Dose (mg per day)	E (μg/g)	EA (μg/g)	Total (μg/g)	% E	Base (μg/ml)	Drug (μg/ml)	Change (μg/ml)
A	400	810	4650	4995	16.2	9.73	20.15	10.42
B	400	2281	289	2544	89.7	10.87	19.61	8.74
C	400	618	1461	1948	31.7	11.41	22.67	11.26
D	800	3914	6464	9796	40.0	9.65	21.65	12.00
E	800	593	4120	4342	13.7	12.14	19.55	7.41
F	800	3817	5986	9264	41.2	12.88	17.99	5.11
G	800	1363	2265	3424	39.8	18.51	31.95	13.44
H	800	2081	5491	7078	29.4	12.46	19.33	6.87
I	800	850	1842	2526	33.7	15.08	21.22	6.14
J	800	3285	4833	7683	42.8	14.99	26.65	11.66
K	800	2678	3347	5724	46.8	13.82	19.95	6.13
L	800	2593	4445	6638	39.1	15.10	30.28	15.18
M	800	2969	8086	10327	28.7	13.33	19.90	6.57
N	800	1781	3417	4890	36.4	10.88	15.85	4.97
Mean		2117	4050	5799	37.8	12.85		8.99
S.D.		1155	2128	2770	17.7	2.41		3.30
C.V. (%)		54.6	52.5	47.8				

nificant trends indicated that recoveries decreased slightly in the presence of BHT, especially for RA.

Levels of E and EA in feces

In the baseline samples, the amount of E detected in stool based upon peak-height analysis was non-detectable (less than 80 μg/g) in eight subjects. In six subjects, a peak with the appropriate retention time suggested E concentrations of 113–470 μg/g. However, when the absorbance ratios of these six peaks were measured at 285 and 275 nm, it was clear that none of these six peaks represented E. Injections of pure E had absorbance ratios of 1.80 ± 0.03; these six peaks all demonstrated absorbance ratios of less than 1.32. Thus, none of the fourteen subjects had detectable concentrations of E in baseline stool samples. In addition, none of the fourteen subjects had a detectable EA peak in the baseline stool sample (less than 102 μg/g).

All fourteen subjects had large peaks at retention times identical to E and EA after four weeks of ER supplementation (see Table I). In all fourteen samples, the absorbance ratios for all peaks identified as E by retention time were 1.80 ± 0.03; peaks identified as EA by retention time had absorbance ratios of 1.11 ± 0.00, identical to the absorbance ratio seen when pure EA was injected. The concentrations of E and EA in stool samples after oral EA supplementation

varied widely from subject to subject, as did the total tocopherol equivalent (equal to E + 0.91 × EA) and the percentage of total tocopherol equivalent present in the form of E itself.

In ten subjects, extractions were prepared from two or three different stool samples obtained during the three-day collection period. The concentration of E and EA in the different samples from the same subject varied considerably over the three-day period. However, the average coefficients of variation of E and EA within these ten subjects (17.2 and 23.5%) were considerably less than the coefficients of variation of mean E and EA levels between subjects (54.6 and 52.5%).

Serum E concentrations

The serum E concentrations in the fourteen subjects at baseline and again four weeks later, are listed in Table I. Baseline E levels varied considerably, but all were within the generally accepted "normal" range (5–25 μg/ml). The mean level was 12.85 μg/ml. After supplementation, each subject's E level increased by an average of 8.99 μg/ml (range, 4.97–15.18 μg/ml).

DISCUSSION

The particular analytical column was selected because we had previously used it in our serum and plasma determinations of retinol and E with excellent separation of retinol, E, and EA, but with sufficiently short retention times to permit analysis of many samples each day [17]. The composition of the mobile phase was altered to decrease retention time because we were not interested in quantifying retinol in feces. RA was used as an internal standard because it was commercially available in pure form, did not interfere with other peaks of interest and had excellent UV absorption at wavelengths used to assay E and EA.

We demonstrated that recoveries of RA, E and EA from stool suspensions were similar to recoveries from water. This was expected, based upon similar findings concerning net recoveries of exogenous retinol, E, and EA from serum [17]. While we did not measure absolute recoveries in this study, prior studies have demonstrated that extraction of exogenous retinol, RA, E, and EA from spiked aqueous samples was greater than 98.8% [17,22].

Because of the presence in baseline stool samples of small peaks with retention times similar to E, we used a multichannel variable-wavelength detector to monitor UV absorption on all stool samples. For serum samples, fixed-wavelength monitoring at 280 nm with peak-area integration was satisfactory, since there were no peaks interfering with E in serum [18]. In fact, stool samples with high levels of E and EA could be monitored using a fixed-wavelength UV detector at 280 nm with no difficulty. The more expensive and more complicated multichannel variable-wavelength detector which we used was necessary only when monitoring stool samples at baseline.

Our same-day precision was similar to that obtained with assays of E or retinol in serum [17,20,22]. Sensitivity could have been increased, but operating our detector at higher sensitivity required 32 min for each run, since many smaller later-eluting peaks were found.

We found it more convenient to perform the sample clarification steps on one day and perform the actual chromatography of all samples on the next day. This appeared to be acceptable, since E and EA levels determined by analysis of samples stored in the freezer overnight did not differ from those same samples analyzed immediately after sample extraction. This stability of the organic matrix thus allowed the sequence of sample extraction and subsequent chromatography to be conveniently interrupted.

The prolonged shaking required to prepare a proper stool suspension could have exposed the E and EA present in the suspension to possible oxidation. In theory, this would be more of a problem with E than with EA, which is considerably more resistant to such destruction [18]. Such oxidative losses during sample extractions have been noted for other compounds such as retinol and β-carotene [23,24]. Thus, our finding that the inclusion of high concentrations of an antioxidant such as BHT in the extraction step did not increase vitamin recovery is reassuring. Since our extraction procedure does not expose the sample to heating during evaporation [20], nor to strong acids to precipitate protein [24], this finding was not surprising.

Our observations concerning fecal E and EA concentrations during baseline and oral supplementation periods require further comment. As expected, fecal concentrations of E and EA during baseline periods were undetectable. However, oral supplementation with EA produced high concentrations of both E and EA in the feces. Subjects varied considerably in their fecal concentrations of E and EA, their total tocopherol equivalents, and the percent of total tocopherol equivalent represented by E. If tocopherol proves to play a chemopreventive role in the colon, it is possible that the luminal (fecal) concentrations of E may be more important than serum E levels. In addition, it is likely that if any tocopherol demonstrates such a chemopreventive effect, it will be E (free alcohol) rather than EA (acetate ester). This large between-subject variability in fecal E concentration may have important implications for further studies employing oral supplementation with EA.

The large within-subject variation in stool levels of E and EA perhaps reflected different stool consistencies, or samples obtained at different times relative to the oral dosing regimen for each patient. It was reassuring to find that this within-subject variability was still much less than between-subject variability in the same parameters. In the future, we intend to combine all stool samples from each subject obtained over the three-day collection period, and mix the samples before obtaining our 1-g sample for analysis. We hope that in this way, our fecal E and EA measurements will represent more of an "average" value for the entire three-day collection period.

Finally, the baseline serum E levels, and increases in serum E levels seen in our fourteen subjects after one month of EA ingestion, were similar to values reported by others following ingestion of tocopherol analogues [25,26]. The net change in serum E concentration correlated poorly with the fecal total tocopherol equivalents in the feces ($r=-0.076$) and with the percentage E in the stool ($r=0.047$). In addition, the percentage E in the stool correlated poorly with the total tocopherol equivalents ($r=-0.164$). Thus, we saw little relationship

between the fecal concentrations of E, EA, or total tocopherol equivalents and the changes in serum E concentration.

This observation may be useful in future cancer chemoprevention studies, especially those related to the colonic mucosa, since all fourteen subjects demonstrated increases in serum E levels (4.97–5.18 μg/ml), while the same subjects demonstrated varied concentrations of all fecal tocopherol parameters. Whether these changes in either the serum or fecal concentration of tocopherol prove to be important in chemoprevention of colonic polyps or colonic adenocarcinoma remains to be seen.

ACKNOWLEDGEMENTS

We wish to thank Sandra Nann and Laurie Hildebrandt for their expert technical assistance. This work was supported in part by Grants CA 37287 and CA 35860 from the National Cancer Institute, National Institutes of Health, DHHS.

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