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Short Communication

Serum concentrations of retinol, *d*-α-tocopherol and β-carotene: effects of storage at −70°C for five years

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ABSTRACT

To investigate the effects of prolonged storage of serum samples at −70°C on concentrations of micronutrients, we measured concentrations of retinol, *d*-α-tocopherol, and β-carotene in serum samples drawn in 1986. We compared values we measured in 1991 to values we obtained in 1986, using the same analytical methods. The relative concentrations obtained in 1991 (mean ± S.D.) were: retinol 99.7 + 12.6% (n = 23), *d*-α-tocopherol 100.7 ± 6.4% (n = 19), and β-carotene 103.4 ± 13.7% (n = 28). Using these techniques of sample preparation and high-performance liquid chromatographic analysis, we found that the effects of storage of serum at −70°C for five years appear insignificant in a small population of patients. However, we did identify clinically important changes in concentration (>20% difference) in several individual subjects.

INTRODUCTION

Available epidemiologic and biomedical data indicate that retinol (RET), *d*-α-tocopherol (TOC), and β-carotene (CAR) may serve as cancer-preventive agents [1–3]. Several large-scale observational and interventional studies have been conducted to examine the efficacy of these agents in patients at high cancer risk. Many of these studies have involved determination of serum or plasma concentrations of RET, TOC, and CAR in selected populations, and several have reported an inverse correlation between the micronutrient of interest and the risk of developing cancer.

In many of the observational studies, serum samples are analyzed years after they are drawn. While some analytes in serum are stable for up to ten years at −70°C [4], limited information is available concerning the effects of storage at subzero temperatures on the degradation of RET, TOC, and CAR in human serum or plasma samples. One study [5] involving HPLC analysis of human serum samples stored at −20°C for two

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to six years demonstrated that RET and CAR begin to degrade during the extraction step immediately after adding ethanol to the serum, possibly due to free radical oxidation of the polyunsaturated vitamins. Such degradation was found in 74 of the 180 serum samples studied. The loss of RET was reportedly eliminated by addition of ascorbic acid [0.1% (w/v) or 1 mg/ml] to the ethanol before it was used in the pre-assay extraction [5,6]. Other researchers concur with this recommendation [7].

Previous studies involving carotenoid extraction from human serum and plasma have demonstrated that total carotenoids in plasma are not stable at −20°C for six months or ten years [8,9] and that serum CAR substantially degrades after storage at −74°C for ten years [2]. Preliminary data indicate that CAR is stable in plasma for six to twenty-two months when stored at −35°C or −70°C, although storage at −7°C leads to marked loss [1].

We have previously reported analytical methods for measuring RET, TOC, and CAR in human serum or plasma samples that utilized reversed-phase HPLC with ultraviolet detection [10,11]. Using these analytical methods for measuring concentrations of RET, TOC, and CAR in serum, we examined the effects of storage at −70°C for five years on the degradation of RET, TOC, and CAR in serum samples drawn in 1986. The patients were previously selected for participation in a large multicenter prospective clinical trial to determine whether vitamin C, TOC, and CAR (in various combinations) were effective in preventing recurrent colonic polyps. We selected serum samples that were originally obtained in 1986 and analyzed in our laboratory in November 1986. We then re-analyzed these samples in November 1991, using the same analytical techniques, after the samples had been stored at −70°C for five years. Our findings suggest that storage at −70°C for five years has insignificant effects on RET, TOC, and CAR in serum samples for a population of patients. However, clinically important changes (>20% difference) were observed for several individual subjects.

EXPERIMENTAL

Blood samples

All blood samples were drawn in 1986 as part of a large multicenter prospective clinical trial. This clinical trial was approved by the local Review Board at each study site, and was conducted in accord with the Helsinki Declaration of 1975 as revised in 1983. The samples were drawn directly into royal blue-topped Vacutainer sample tubes (Becton-Dickinson, Rutherford, NJ, USA). After centrifugation at 1500 *g* for 10 min and separation, the serum was transferred to polypropylene freezer tubes (Sarstedt, Princeton, NJ, USA) and stored at −70°C until needed. All tubes were handled under 25-W incandescent bulbs only and were protected from sunlight and fluorescent light to prevent photoisomerization or oxidation of agents [1]. On the day of analysis, serum samples were thawed slowly to room temperature and vortex-mixed to resuspend any lipids.

Chemicals

All chemicals were the highest grade commercially available; all solvents were HPLC grade. Detailed description of sources of all chemicals used is provided in refs. 10 and 11.

Determination of retinol and d-α-tocopherol

Our serum clarification procedure employed extraction of analytes of interest from denatured serum into an organic matrix, as previously described [11]. HPLC was employed using an isocratic mobile phase, an ODS reversed-phase column, and UV peak detection at 280 nm as previously described [11]. RET and TOC values measured in November 1991 (with and without addition of ascorbic acid during extraction) were compared to baseline values obtained in 1986 (defined as 100%). Results of relative concentrations were expressed as mean ± S.D. The significance of differences between the means (samples measured in 1986; samples measured in 1991 with ascorbic acid; samples measured in 1991 without ascorbic acid) was analyzed using the ANOVA option in the Macintosh computer program Stat-

view 512+ (Abacus Concepts, Berkeley, CA, USA). We formally evaluated each result for the presence of outliers [12].

Two curves were generated correlating the 1986 RET (or TOC) values (abscissa) and the 1991 RET (or TOC) values (ordinate). Best-fit linear regression lines were calculated using the method of least squares. The correlation coefficient (r^2) was calculated for each curve.

Determination of β-carotene

Our serum clarification procedure, employing extraction of CAR from denatured serum into an organic matrix, was previously described [10]. HPLC utilized an isocratic mobile phase, an ODS reversed-phase column, and UV detection at 436 nm [10].

CAR values measured in 1991 were compared to values obtained in 1986 using a paired t-test, contained in the software package Statview 512+ (Abacus Concepts).

RESULTS

Retinol

Concentrations of RET obtained in 1991 were compared to values obtained in 1986 (defined as 100%) for a total of 23 blood samples extracted with and without addition of ascorbic acid. Because of limited amounts of serum available, all serum samples were assayed once in 1986; all but four were assayed once in 1991; four serum samples were measured three or four times in 1991 to confirm changes in serum RET concentrations. In these four cases, the mean of all 1991 measurements was used. RET concentrations measured in 1991 with the addition of ascorbic acid (94.2 ± 11.7%) were slightly (and barely significantly, $p = 0.03$) less than RET concentrations measured in 1991 without use of ascorbic acid (99.7 ± 12.6%), and RET concentrations measured in 1986 without use of ascorbic acid (100%). There was no significant treatment effect within subjects for samples analyzed in 1986 and 1991 without use of ascorbic acid ($p = 0.90$). Further analysis of the data includes only serum samples analyzed in 1986 and 1991 without addition of ascorbic acid.

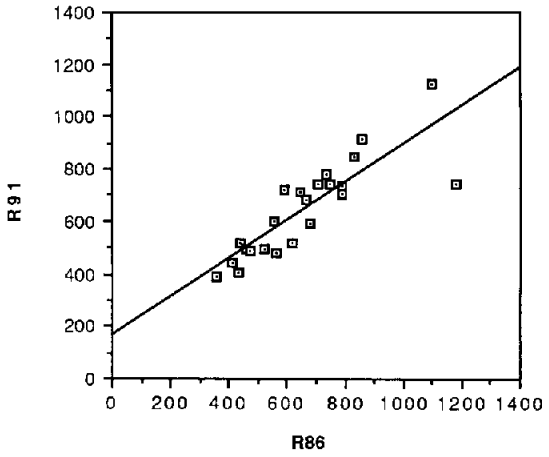

Fig. 1. Relation of serum retinol measured in 1991 (R91) to values measured in 1986 (R86) for 23 samples ($y = 0.731x + 163.42$; $r^2 = 0.721$).

Serum samples measured from two subjects in 1991 had a >20% difference in relative RET concentration compared to the 1986 mean value (62.6%, 121.4%); however, neither of these samples was formally identified as a possible outlier. The 1991 RET values were correlated with the 1986 values as shown in Fig. 1 for a total of twenty-three samples. A correlation coefficient (r^2) of 0.721 was obtained. If one eliminated the single subject whose 1991 RET concentration was only 62.6% as great as the 1986 concentration, then

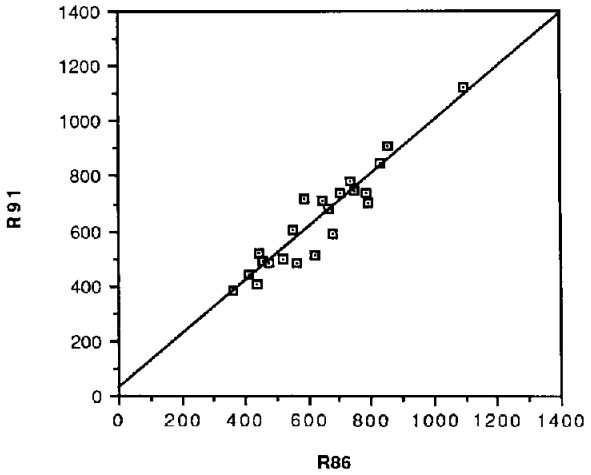

Fig. 2. Relation of serum retinol measured in 1991 (R91) to values measured in 1986 (R86) for 22 samples ($y = 0.967x + 26.285$; $r^2 = 0.895$).

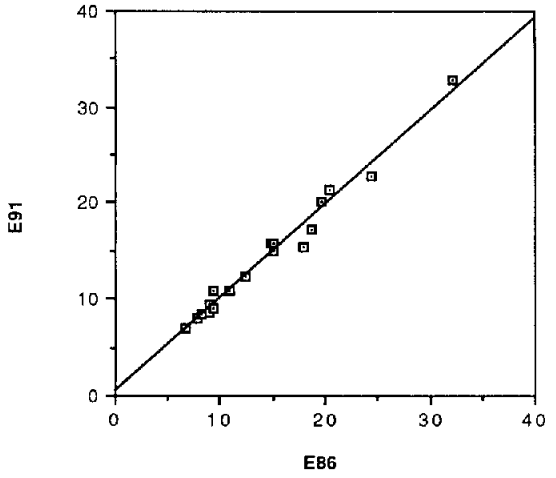

Fig. 3. Relation of serum α-tocopherol measured in 1991 (E91) to values measured in 1986 (E86) for 19 samples ($y = 0.974x + 0.375$; $r^2 = 0.978$).

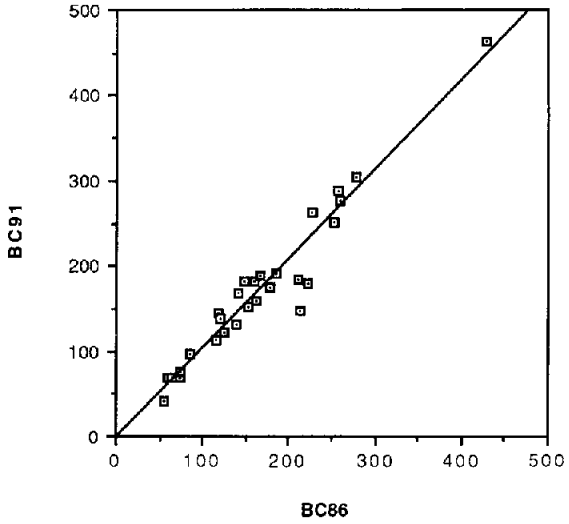

Fig. 4. Relation of serum β-carotene measured in 1991 (BC91) to values measured in 1986 (BC86) for 28 samples ($y = 1.045x - 1.819$; $r^2 = 0.932$).

one obtains the regression shown in Fig. 2, with a correlation coefficient of 0.895.

α-Tocopherol

Concentrations of TOC obtained in 1991 were compared with baseline values obtained in 1986 (defined as 100%) for a total of nineteen serum samples extracted with and without addition of ascorbic acid. All serum samples were analyzed once in 1986; twelve were analyzed once in 1991; seven were analyzed two or three times to 1991 to confirm 1991 values. There were no siginificant treatment effects within subjects identified using ANOVA ($p = 0.61$). Group means were similar for samples measured in 1986 (100%), samples measured in 1991 without ascorbic acid (100.6 ± 6.4%), and samples measured in 1991 with ascorbic acid (99.1 ± 7.4%). Therefore, further analysis of the data includes only serum samples measured in 1986 and 1991 without addition of ascorbic acid.

All serum samples measured in 1991 had relative TOC differences within 15% of the 1986 mean value; none was identified as a possible outlier. The 1991 TOC values were related to the 1986 values as shown in Fig. 3 for a total of nineteen samples. A correlation coefficient of 0.978 was obtained.

β-Carotene

Concentrations of CAR obtained in 1991 were compared with values obtained in 1986 (defined as 100%) for a total of twenty-eight serum samples. All serum samples were analyzed once in 1986; all but one sample were analyzed once in 1991. Samples measured in 1991 (103.5 ± 13.9%) did not differ significantly from those measured in 1986 (100%; $p = 0.20$).

A total of three serum samples measured in 1991 were found to have a >20% difference in relative CAR concentration compared to the 1986 mean values (69.2%, 73.2%, 123.8%): none of these samples was identified as a possible outlier. The 1991 CAR values were related to the 1986 CAR values as shown in Fig. 4. A correlation coefficient of 0.932 was obtained.

DISCUSSION

Our data indicate that for this small population of subjects, the effects of serum storage at −70°C for five years on RET, TOC, and CAR

concentrations were not clinically (or statistically) significant. While the addition of ascorbic acid to serum samples prior to extraction had no significant effect on TOC concentrations, this addition appeared to produce a slight but detectable decrease in measured RET concentrations.

Our findings of analyte stability over years during storage at −70°C are consistent with previous results reported by our group and others. One group, using a different extraction procedure that employed saponification, found that storage of serum samples for even six months at −20°C led to loss of at least 15% of CAR, while storage at −70°C maintained the CAR concentration for at least one year [8]. We found in our earlier report, using the same CAR assay that we used in this paper, that CAR was stable in plasma for up to two years when stored at −35°C or −75°C, but not when stored at −7°C [1]. Driskell's group [5,6] used a different assay for RET, and found that some sera exhibited loss of RET when stored at −20°C for two to six years; this loss could be prevented by the addition of ascorbic acid during the extraction procedure. Our extraction conditions are considerably different from those described by Driskell's group [5,6], and we stored our sera at −70°C.

Interestingly, we were unable to demonstrate any advantage to adding ascorbic acid to serum samples prior to use of our extraction method. In fact, the addition of ascorbic acid may have caused a slight decrease in recovery of RET. Presumably, the addition of ascorbic acid to samples prior to analysis can have beneficial effects, no effects, or minimally destructive effects depending on the analyte of interest, length and conditions of storage, extraction scheme, etc.

As reported by Driskell's group [5,6], we also found that sera from several individuals showed clinically important changes in serum RET and CAR values (>20% difference) over time. While none of these individuals were identified as statistical outliers, differences of >20% would be clinically important. Sources for these differences over time could include loss or destruction of the analyte of interest, loss of serum water via lyophilization, error in the initial measurement in 1986, error in the measurement in 1991, or simple between-day variation in the assay itself. Serum from different individuals could vary in their stability in the freezer, possibly because of variable amounts of "serum antioxidant activity" which could protect polyene compounds from free radical oxidation [5]. Loss of serum water was unlikely, since our freezers routinely have ice on the interior which would make lyophilization less likely; also, mean micronutrient concentrations did not increase over time for any analyte.

Since most serum samples were measured only once, laboratory error is possible. Duplicate analyses would be preferable, but our serum volumes were limited. In the few patients in whom larger changes were observed, we did reanalyze serum samples in 1991 several times; these additional analyses did not suggest the presence of laboratory error (at least in 1991). Finally, all such assays will have measurable between-day variation in the results obtained from identical samples. It is likely that between-day variation would be greatest when assays are separated by five years; papers usually report between-day precision with assays done on consecutive days. Our assays for RET, TOC, and CAR have reported between-day precision of the order of 5 10% (coefficient of variation), and thus would be unlikely to account for the larger declines in concentration observed in the sera from several subjects.

Of the three analyses, the measurement of TOC exhibited the least variation, while those for RET and CAR exhibited more variation. The reason for this is not readily apparent, but may reflect greater inherent stability in tocopherol compounds.

The observed differences in the relative concentrations of RET, TOC, and CAR for this population may be due to chance alone (a Type I error). Further, larger-scale studies involving greater numbers of serum samples and similar methods of sample preparation and HPLC analysis are needed to provide more conclusive results regarding the stability of RET, TOC, and CAR during long-term storage at −70°C. It is likely that stability may be dependent upon storage temperature, length of storage, and specific meth-

ods used for sample extraction and clarification.

The finding that RET, TOC, and CAR serum concentrations are stable in most subjects despite storage of serum for five years at −70°C is particularly relevant to large observational studies, in which sera are analyzed after years of storage. One of the strengths of this study is that we used methods of sample preparation and HPLC analysis that have been previously determined to provide sensitive and precise determinations of RET, TOC, and CAR in relatively small volumes of human blood (250 or 500 μl). These observations may be particularly useful for additional large observational or interventional studies aimed at determining the possible role of RET, TOC, and CAR in patients with high cancer risk.

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