Stability of Fat-Soluble and Water-Soluble Vitamins in Artificially Prepared, Vitamin-Enriched, Lyophilized Serum

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Vitamin-enriched, lyophilized serum (VES) was prepared for an inter-laboratory study to compare vitamin assays. The VES contained water-soluble vitamins (vitamin B1, vitamin B12, vitamin C, and folate), fat-soluble vitamins (vitamin A and vitamin E), and cholesterol. We performed stability studies and determined vitamin concentrations and total cholesterol in VES stored at -20°C for 12 months. Our recovery of the water-soluble vitamins in reconstituted VES was 70–142%, but we recovered only 33–45% of the fat-soluble vitamins. Physico-chemical properties, such as specific gravity and viscosity of the reconstituted VES did not affect manual or automated measurements of these vitamins. Vial-to-vial

differences found for the VES were the same as the within-day analytical variations. There was no evidence of degradation of vitamin A, vitamin B1, vitamin B12, vitamin C, folate, and cholesterol over 12 months in VES stored at -20°C . Following deproteinization, vitamin C concentration was found to be lower than when not deproteinized. Vitamin E was less stable in VES, however, and the degradation during 12 months was lower than the between-day analytical variation of the assay. Our VES is the first preparation of lyophilized control serum that contains water-soluble and fat-soluble vitamins. *J. Clin. Lab. Anal.* 18:240–246, 2004.

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Key words: vitamins; reference value; lyophilized serum; standard reference materials 968c (SRM 968c)

INTRODUCTION

Certain vitamins are essential micronutrients for the normal metabolic functioning of the body. Most essential vitamins must be obtained from the diet, because humans do not synthesize them. At the present time, nutritional status for vitamins is assessed by measuring their concentrations in blood. The recommended dietary allowance (RDA) of vitamins, defined for Americans and Canadians (1), is based on the amounts of intake needed for maintenance of serum or blood vitamin concentrations above a lower reference limit. This applies to vitamin B6, folate, vitamin B12, pantothenic acid, biotin, vitamin C, and vitamin E. In establishing the RDA for a Japanese population, a similar approach was used for recommended doses of

vitamin D, vitamin E, vitamin B6, folate, vitamin B12, and vitamin C (2).

However, assay values for most vitamins are currently method-dependent, and each laboratory has to establish its own reference limits using its own selective procedures for measuring vitamins (3). Thus, we concluded that the present RDA cannot be definitive until the

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Received 18 November 2003; Accepted 12 March 2004

DOI 10.1002/jcla.20030

Published online in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Vitamins and cholesterol concentrations observed in VES after liquid-frozen serum pool from healthy volunteers was enriched with six vitamins*

Constituent	Original concentration	Added concentration	Expected concentration	Found concentration	Amount recovered in VES (%)
Vitamin A ($\mu\text{g/L}$)	442	1477	1919	865	45
Vitamin E (mg/L)	4.14	21.96	26.10	8.55	33
Vitamin B ₁ (ng/mL)	2.9	107.0	109.9	99.9	91
Vitamin B ₁₂ (pg/mL)	510	760	1270	1174	92
Vitamin C (mg/L)	0.4	28.5	28.9	20.2	70
Folate (ng/mL)	3.84	5.34	9.18	13.08	142
Total cholesterol (mg/L)	1360	0	1360	1277	94

*Enriched % was calculated by the following formula: $= 100 \times (\text{found concentration}) / (\text{original concentration} + \text{added concentration})$. Vitamin C concentration was determined by the enzymatic method.

lower reference limit independent of the assay method is determined. We also thought that assay values could be standardized with the use of control serum. However, control serum does not usually have vitamins. Therefore, we prepared vitamin-enriched lyophilized serum (VES) to compare vitamin assay values coming from different laboratories. The aim of the present study was to investigate the 12-month stability of both water-soluble (vitamin B₁, vitamin B₁₂, vitamin C, and folate) and fat-soluble vitamins (vitamin A and vitamin E) in VES.

MATERIALS AND METHODS

Materials

Vitamin A (all-trans-retinol, catalog no. 594-10621), vitamin B₁ (thiamine hydrochloride, catalog no. 203-00851), vitamin B₁₂ (cyanocobalamin, catalog no. 226-00343), vitamin C (L-ascorbic acid, catalog no. 012-04802), vitamin E (DL- α -tocopherol, catalog no. 209-01791), and folate (pteroylglutamic acid, catalog no. 062-01801) were all purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. A frozen serum pool from healthy volunteers was purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. The pool contained 442 $\mu\text{g/L}$ retinol, 4.14 mg/L α -tocopherol, 2.9 ng/mL thiamine, 510 pg/mL vitamin B₁₂, 0.4 mg/L ascorbic acid, and 3.84 ng/mL folate (Table 1). The pool was negative for human immunodeficiency virus (HIV 1, HIV 2) antibody, HBs antigen, and ATL and HCV antibodies.

Preparation of VES

Retinol (5,906 mg/L) and α -tocopherol (87,859 mg/L) were dissolved in ethanol. Thiamine hydrochloride (428 mg/L), cyanocobalamin (152 mg/L), L-ascorbic acid (114,000 mg/L), and pteroylglutamic acid (214 mg/L) were dissolved in physiological saline. Aliquots of the

above solutions of vitamin A (0.325 mL), vitamin E (0.325 mL), vitamin B₁ (0.325 mL), vitamin B₁₂ (0.0065 mL), and folate (0.0325 mL), in this order, were added to 1,300 mL of the pooled serum. It was gently mixed at 6–10°C in the dark for 30 min, and then vitamin C solution (0.325 mL) was added. After mixing for 5–10 min at 6–10°C in the dark, the mixture was filtered through a 0.45- μm filter. Then 2.0 mL aliquots of this mixture were dispensed into opaque brown 5-mL sterilized glass vials and lyophilized. These VES were stored at –80°C for 5 months and delivered to each laboratory packed in dry ice. No preservatives were added to the VES.

Vitamin Analysis

VES was reconstituted with 2.0 mL of distilled water 15–30 min prior to the analysis. Vitamin A and vitamin E were measured by reversed-phase high-performance liquid chromatography (HPLC) with fluorescent detection after extraction with hexane (4,5). Vitamin B₁ was measured with a precolumn derivatized HPLC method after oxidation of thiamine with mercuric bichloride in sodium hydroxide (6,7). Vitamin C (ascorbic acid, here) and dehydroascorbic acid were measured by an HPLC method with UV detection after the VES was deproteinized with 10% metaphosphoric acid (8). Vitamin C was also measured enzymatically using ascorbate oxidase without deproteinization (9). Ascorbate oxidase reacts only with ascorbic acid, not with dehydroascorbic acid. Vitamin B₁₂ and folate were measured by competitive protein binding assay with an Elecsys 2010 (Roche Diagnostics GmbH, Mannheim, Germany). The total cholesterol concentration was measured enzymatically with a Hitachi 7600 automated analyzer (Hitachi High-Technology Corporation, Tokyo, Japan). VES was also measured by two other laboratories. In one, vitamin A and vitamin E were measured by the same HPLC method and a normal-phase HPLC with UV detection

TABLE 2. Mean values and vial-to-vial differences of vitamins and cholesterol found between 20 vials of VES

Constituent	Median (range)	Mean (SD)	Vial-to-vial CV, % ^a	Within-day precision CV of analysis
Vitamin A (μg/L)	862 (841–909)	865 (17)	2.0	2.2
Vitamin E (mg/L)	8.56 (8.31–8.75)	8.55 (0.15)	1.8	0.4
Vitamin B ₁ (ng/mL)	99.8 (93.3–106.1)	99.2 (2.6)	2.6	3.5
Vitamin B ₁₂ (pg/mL)	1,173 (1,122–1,214)	1175 (25)	2.1	2.2
Vitamin C, HPLC (mg/L)	21.9 (21.3–22.8)	21.9 (0.4)	1.8	4.5
Vitamin C, enzymatic (mg/L)	20.4 (19.0–21.0)	20.2 (0.6)	3.0	2.0
Folate (ng/mL)	13.13 (12.65–13.91)	13.08 (0.37)	2.8	3.2
Total cholesterol (mg/L)	1,275 (1,260–1,290)	1277 (12)	0.9	0.7

^aCV, % was calculated by the following formula:
 $= 100 \times (\text{SD})/(\text{mean})$

TABLE 3. Stability of vitamins and cholesterol in VES for 12 months*

Constituent	Storage duration					Between-day precision CV of analysis
	Before (20 vials)	1–4 months	5–8 months	9–12 months	1–12 months (CV of 12 months)	
Vitamin A (μg/L)	865 ± 17	866 ± 10	863 ± 12	848 ± 16	859 ± 15 (1.7)	4.2
Vitamin E (mg/L)	8.55 ± 0.15	8.24 ± 0.15 ^b	8.31 ± 0.11 ^b	8.38 ± 0.23	8.31 ± 0.17 ^a (2.0)	2.2
Vitamin B ₁ (ng/mL)	99.2 ± 2.6	98.6 ± 1.2	99.2 ± 2.6	97.4 ± 3.7	98.5 ± 2.4 (2.4)	5.0
Vitamin B ₁₂ (pg/mL)	1175 ± 25	1169 ± 24	1192 ± 37	1174 ± 39	1178 ± 32 (2.7)	5.5
Vitamin C, HPLC (mg/L)	21.9 ± 0.4	20.4 ± 0.1 ^c	20.0 ± 0.4 ^a	18.5 ± 0.2 ^a	20.2 ± 1.2 ^a (6.2)	3.6
Vitamin C, enzymatic (mg/L)	20.2 ± 0.6	20.3 ± 0.4	20.3 ± 0.2	20.6 ± 0.3	20.4 ± 0.3 (1.6)	4.3
Dehydroascorbic acid (mg/L)	0.10 ± 0.16	0.11 ± 0.09	0.20 ± 0.11	0.40 ± 0.07 ^b	0.24 ± 0.15 ^a (62.5)	63.6
Folate (ng/mL)	13.08 ± 0.37	13.01 ± 0.07	13.38 ± 0.36	13.44 ± 0.97	13.24 ± 0.57 (4.3)	5.6
Total cholesterol (mg/L)	1277 ± 12	1285 ± 11	1261 ± 28	1266 ± 12	1271 ± 20 (1.6)	1.7

*VES was stored in freezer at –20°C before reconstitution. Values in stored VES were compared with those values obtained at the start of the experiment. All values are mean ± SD.

^a $P < 0.001$.

^b $P < 0.01$.

^c $P < 0.05$.

(10). Vitamin B₁ was also measured using a precolumn HPLC after oxidation with cyanogen bromide, and a postcolumn HPLC and potassium ferricyanide as an oxidation reagent (6). In the other laboratory, vitamin C was measured by the same HPLC method. Vitamin B₁₂ and folate were measured using an Immulite (Diagnostics Products, Los Angeles, CA) and Advia Centaur (Bayer Diagnostics, Tarrytown, NY).

The coefficients of variation (CV) of the within- and between-day analytical precisions are shown in Tables 2 and 3. However, because the analytical stability of these methods during the study period was unclear, assay values of vitamin A, vitamin E, vitamin B₁, and vitamin C were corrected with the use of solutions of retinol, α -tocopherol, thiamine, and ascorbic acid. Their concentrations were certified by molar absorptivity at 325, 292, 248, and 245 nm, respectively (11–13), while assay values of vitamin B₁₂ and folate with the Elecsys 2010 were

corrected with use of the manufacturer's control serum (Elecsys PreciControl Universal).

Statistics

The concentrations of vitamins and total cholesterol in the VES stored for 12- month or 4-month intervals were compared with those values obtained at the start of the study. The values before and after storage were analyzed using Student's *t*-test, and differences of $P < 0.05$ were considered to be statistically significant.

RESULTS

Physicochemical Properties

We measured the specific gravity of the VES with an Erma SU-202 Clinical Refractometer (Erma Inc., Tokyo, Japan), and found it to be 1.042 ± 0.000 g/cm³

(mean $\pm$ SD of three vials) at 25°C. We determined the viscosity of VES with a digital viscometer, DVM-E (Tokyo Keiki Co., Ltd., Tokyo, Japan), and it was 2.42 and 2.51 mPa at 20°C. The pH of VES was 8.0 ± 0.0 (mean $\pm$ SD of three vials) at 27°C. The optical density of VES was measured at 710 nm with the Shimadzu CL-770 Clinical Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The turbidity of VES was 0.321 ± 0.012 (10 mm light path, mean $\pm$ SD of three vials). Our VES present in a human serum matrix had a higher specific gravity and viscosity than the values recommended in the Japanese Society of Clinical Chemistry (JSCC) Enzyme Reference Materials (ERM). For the JSCC material, specific gravity of 10.20–1.035 g/cm³ and viscosity of 1.40–1.70 mPa, equal to natural serum, were required (14). These differences had no effect on the assays. The VES was clear, and had a bilirubin concentration of 7 μ mol/L as recommended by the U.S. National Committee for Clinical Laboratory Standards (NCCLS, C37-A) (15).

Recovery of Added Vitamins

We investigated the enrichment of VES with added vitamins (Table 1). The serum concentrations of vitamin B1, vitamin B12, and vitamin C were increased by the addition of these vitamins; the fractional enrichment was 70–92% of the expected increase. Here, the expected values were set to about twice the concentration of the upper reference limit for each vitamin present in serum or blood. For vitamin A and vitamin E, the recovered amounts from the VES were less than the expected quantity. For folate, we recovered 142% of the expected amount. Serum folate is mainly composed of 5-methyltetrahydrofolic acid. We found that pteroylglutamic acid added to VES reacted at nearly twice the rate of 5-methyltetrahydrofolic acid with the Elecsys 2010, which explains the increased recoveries of folate.

Effects of Dilution Volume

To examine whether the different concentration levels were prepared from VES, we reconstituted the VES with 4.0 mL of distilled water instead of 2.0 mL of distilled water, and then assayed them for all the vitamins and cholesterol. Except for vitamin B12 and folate, the observed values in the reconstituted VES were the expected values; we found the values in VES to be one-half those when reconstituted with 2.0 mL of distilled water. The bias between the two values was 11 μ g/L for vitamin A, 0.01 mg/L for vitamin E, 0.6 ng/mL for vitamin B1, 0.1 mg/L for vitamin C (enzymatic), and 0 mg/L for total cholesterol. The values found for the 4-mL dilutions were always lower than the expected values. The bias was 53 pg/mL for vitamin B12 and 1.2

ng/mL for folate. These biases observed with the Elecsys could be reduced with the use of the manufacturer's diluent, Elecsys Diluent Universal.

Vial-to-Vial Differences

To examine the vial-to-vial differences of VES, 20 vials were reconstituted with 2.0 mL of distilled water, and the concentrations of the vitamins and cholesterol were determined (Table 2). The vial-to-vial CVs were nearly equal to the within-day analytical CVs of the methods.

Stability After Reconstitution

We reconstituted VES with 2.0 mL of distilled water and stored it at 4°C in the dark for 5 hr. Except for vitamin C, all vitamins and cholesterol were stable for at least 5 hr. The concentration of vitamin C decreased from 5% to 10% of the original concentration after 1 and 3 hr of storage (Fig. 1).

Fig. 1. Stability of vitamins and total cholesterol in reconstituted VES. Values are the percent of the original concentrations. Reconstituted VES was stored in a refrigerator (4°C) for 5 hr prior to analysis. Open triangles, vitamin A; open circles, vitamin B1; solid circles, vitamin B12; solid lozenges, vitamin C (by enzymatic); open squares, vitamin E; solid triangles, folate; solid squares, total cholesterol.

Long-Term Stability Study

Two of the VES vials stored in the freezer at -20°C were reconstituted monthly, and the vitamins and cholesterol were determined every month during 1 year of storage (Figs. 2–4). Vitamin A, vitamin B1, vitamin B12, vitamin C (enzymatic method), folate, and cholesterol were stable for at least 12 months. When we compared the means of observed values every 4 months, we found that the values of vitamin C (HPLC method) and vitamin E decreased significantly during storage (Table 3). Decreases in these two antioxidant vitamins as compared to their original concentrations would therefore arise within a month after start of storage. Except for vitamin C, the CVs of all of these vitamin and cholesterol assays obtained during 12 months were smaller than the between-day analytical CVs.

Fig. 2. Stability of vitamin A, vitamin E, and total cholesterol in VES. Two vials of VES stored at -20°C were reconstituted every month for the assay of vitamins and cholesterol. Solid and open circles represent the single analysis of two vials. The solid and dashed lines indicate mean $\pm 95\%$ confidence levels obtained on 20 vials at the start of the storage.

Fig. 3. Stability of vitamin B1, vitamin B12, and folate in VES. Solid and open circles represent the single analysis of two vials. The solid and dashed lines indicate mean $\pm 95\%$ confidence levels obtained on 20 vials at the start of the storage. Folate was measured at 13 months and was plotted on this figure.

Interlaboratory Differences

Analytical values of vitamins in VES were compared in three laboratories that used different procedures for most vitamins (Table 4). The analytical bias found for the three laboratories for all tests were larger than the vial-to-vial differences (i.e., mean $\times 2\text{CV}/100$) from any one of the laboratories. The CVs of vial-to-vial differences are from Table 2.

DISCUSSION

Assay-independent reference values for the vitamins in serum or plasma are needed not only in evaluations of vitamin deficiency, but also in the nutritional assessment of vitamins. We prepared VES to compare the interlaboratory differences. To accomplish this, Standard Reference Materials 968c (SRM 968c) purchased from the National Institute of Standards and Technology (NIST) could be also used as a control serum. A disadvantage of the NIST material is that it contains

Fig. 4. Stability of vitamin C determined by the enzymatic and HPLC methods. As vitamin C degrades, dehydroascorbic acid forms (middle graph). Solid and open circles represent the single analysis of two vials. The solid and dashed lines indicate mean $\pm$ 95% confidence levels obtained on 20 vials at the start of the storage.

only fat-soluble vitamins (retinol, retinyl esters, α -tocopherol, and carotenes) and cholesterol (16). The VES we prepared contained four water-soluble vitamins (vitamin B₁, vitamin B₁₂, vitamin C, and folate) in addition to fat-soluble vitamins and cholesterol. Cholesterol was used to correct α -tocopherol values, because vitamin E secreted from hepatocytes is bound to serum lipids (17).

Results of vial-to-vial differences showed that VES had excellent uniformity for all vitamins including cholesterol. Water-soluble vitamins added to VES gave a recovery of 70–142% after lyophilization. By comparison, the recovery of fat-soluble vitamins (i.e., retinol and α -tocopherol) was 33–45% of the added concentration. In the preparation of VES, a very small quantity (4.04 mg) of retinol was dissolved with small volumes (0.684 mL) of ethanol. For other vitamins, more than 10 mg of vitamins were weighed and/or dissolved in more than 10 mL of physiological saline. Because α -tocopherol was purchased as an oily liquid, the weighing of α -tocopherol was inaccurate. In future studies, concentrations of vitamin A and vitamin E should be checked by measuring the absorbance at 325 and 292 nm, respectively, before adding these solutions to pooled serum.

Knowledge of the stability of vitamins in the VES is essential in judging the shelf-life of these vitamins in lyophilized serum. Stability results for VES stored at -20°C showed that vitamin A, vitamin B₁, vitamin B₁₂, vitamin C (by enzymatic), folate, and total cholesterol were stable for up to 12 months. Vitamin C (by HPLC) and vitamin E tended to deteriorate during storage. We suspect that ascorbic acid in VES was made fragile by acidic deproteinization, and thus easily converted to 2,3-diketo-L-gluonic acid via

TABLE 4. Inter-laboratory differences in three laboratories evaluated by VES

Constituent	Lowest value	Middle value	Highest value	Mean $\pm$ SD of three laboratories (CV, %)	CV of vial-to-vial difference
Vitamin A ($\mu\text{g/L}$) ^a	865 (reversed phase HPLC)	875 (reversed phase HPLC)	902 (normal phase HPLC)	881 $\pm$ 19 (2.2)	2.0
Vitamin E (mg/L) ^a	7.70 (reversed phase HPLC)	8.55 (reversed phase HPLC)	10.60 (normal phase HPLC)	8.95 $\pm$ 1.49 (16.6)	1.8
Vitamin B ₁ (ng/mL) ^b	78.0 (potassium ferricyanide)	82.9 (cyanogen bromide)	99.2 (mercuric chloride)	86.7 $\pm$ 11.1 (12.8)	2.6
Vitamin B ₁₂ (pg/mL) ^c	1,138 (Centaur)	1,148 (Immulate)	1,260 (Elecys)	1182 $\pm$ 68 (5.8)	2.1
Vitamin C (mg/L) ^d	19.2 (HPLC)	20.2 (enzymatic)	22.0 (HPLC)	20.5 $\pm$ 1.4 (6.9)	3.0
Folate (ng/mL) ^a	11.10 (Immulate)	11.63 (Centaur)	14.27 (Elecys)	12.33 $\pm$ 1.70 (13.8)	2.9

^aVitamin A and vitamin E: reversed phase HPLC with fluorescent detection and normal phase HPLC with UV detection.

^bVitamin B₁ was oxidized with potassium ferricyanide, mercuric bichloride, or cyanogen bromide in sodium hydroxide.

^cVitamin B₁₂ and folate by three automated analyzers: Bayer Advia Centaur, Diagnostics Products Immulate, and Roche Elecys.

^dVitamin C by HPLC and enzymatic methods.

dehydroascorbic acid when VES was determined by HPLC after deproteinization with metaphosphoric acid. Dhariwal et al. (18) reported that 2,3-diketo-L-gulonic acid and dehydroascorbic acid were formed during treatment of serum with acidic deproteinization. Actually, dehydroascorbic acid determined by HPLC was found to be significantly increased after storage for 12 months (Fig. 4). In future studies, we think that the addition of some reductant or stabilizer to VES will be needed to make stable preparations, and that vitamin C should be measured without deproteinization.

We used the values obtained from the three laboratories that analyzed VES to estimate their analytical differences. As shown in Table 4, we found substantial inter-laboratory differences for all the vitamins. Clearly, the values were not standardized whether the same method was used or not. For example, with the same HPLC method minor modifications were observed on columns purchased from different manufacturers and in constituents of the eluent. Moreover, in the analysis of vitamin B12 and folate with three different automated analyzers (Elecsys 2010, Immulite, and Advia Centaur), characteristics of the vitamin B12 (or folate)-binding protein and/or the sort of calibration material would affect the results (19).

The biases of the assay values would affect reference values for the concentrations of blood or serum (plasma) vitamin concentrations as established by different procedures. In a planned study, we would like to assign reference values based, as determined, on the best analyzer available and the best estimates of the true values by definitive or reference methods. Our VES could serve as a reliable reference material in judging inter-laboratory differences and their effect on reference values.

ACKNOWLEDGEMENT

We thank Dr. John A Lott, Professor of Pathology, Ohio State University Medical Center, Columbus OH, for his helpful suggestions on this manuscript.

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