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Vitamin C and Glycohemoglobin

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Three groups of 10 age- and sex-matched nondiabetic volunteers took 0, 750, or 1500 mg of vitamin C each day for 12 weeks. Glycohemoglobin (GHb) was measured by HPLC, electrophoresis, affinity chromatography, and immunoassay at baseline (−4 weeks and −1 day), during supplementation (6 weeks and 12 weeks), and after supplementation ended (6 and 12 weeks). Plasma vitamin C increased twofold during supplementation but, in contrast with the results of Davie et al. (Diabetes 1992; 41:167–73), there were no between-group differences in GHb, glucose, and fructosamine concentrations. Fructosamine may have increased with storage time. The net effects of vitamin C on absolute GHb at 12 weeks vs −1 day (and at 12 weeks vs 12 weeks after) in %GHb amounted to: HPLC −0.035 (−0.050); electrophoresis +0.005 (+0.035); affinity chromatography −0.070 (+0.015); and immunoassay −0.110 (+0.025). We conclude that supplementation of nondiabetics with 750 or 1500 mg of vitamin C daily for 12 weeks does not cause interference in GHb determinations by HPLC, electrophoresis, affinity chromatography, or immunoassay, and does not reduce in vivo Hb glycation.

Indexing Terms: *variation, source of/intermethod comparison/ascorbate/glucose/fructosamine*

The glycohemoglobin (GHb) percentage of total hemoglobin in blood reflects the blood glucose concentrations of the previous 2–3 months and is therefore widely accepted as a valuable indicator for long-term control of diabetes (*1*). We recently reported a study on the influence of hemoglobin (Hb) derivatives (carbamylated Hb, acetylated Hb, those in stored blood, and GHb–Schiff base) on GHb results (*2*). Davie et al. (*3*) described the influence of a Hb derivative produced from the reaction of Hb with vitamin C. In their study of 12 nondiabetic subjects they found that daily supplementation with 1 g of vitamin C for 3 months decreased GHb determined by affinity chromatography from 6.18% to 5.05%, but increased HbA_1 determined by electrophoresis from 6.17% to 7.16%; 2 months after supplementation, both GHb and HbA_1 returned to baseline values. They concluded that vitamin C supplementation has both analytical and in vivo effects on GHb results. The analytical effect was attributed to the reaction of Hb with vitamin C or vitamin C metabolites, leading to formation of some Hb derivative with charge properties similar to HbA_1. Analogous to carbamylated Hb, acetylated Hb, and GHb–Schiff base, "ascorbinated Hb" might interfere in methods for quantifying HbA_{1c} that are based on differences in Hb charge (i.e., electrophoresis and HPLC). The in vivo effect might result from competitive inhibition of Hb glycation by vitamin C, causing genuinely lower GHb when specific methods (i.e., affinity chromatography and immunoassay) are used.

The study of Davie et al. (*3*) prompted us to examine the influence of vitamin C supplementation on GHb. Three groups of 10 age- and sex-matched nondiabetic volunteers took 0, 750, or 1500 mg of vitamin C each day for 12 weeks. We used four basically different methods—electrophoresis, HPLC, affinity chromatography, and immunoassay—to measure GHb at baseline, during this supplementation period, and after supplementation.

Materials and Methods

Subjects, study design, and blood sampling. Thirty nondiabetic Caucasian volunteers (mean ± SD fasting plasma glucose 5.0 ± 0.4 mmol/L; range 3.7–5.9) were divided into three equal groups of age- and sex-matched subjects. Subjects in two groups were daily supplemented with 3 × 250 mg (six women, four men; mean age 34 years, range 23–49) and 3 × 500 mg (seven women, three men; mean age 34 years, range 22–46) of vitamin C (Pharbita bv, Zaandam, The Netherlands), respectively, for 12 weeks. The doses were equally divided for intake at breakfast, lunch, and dinner. The third group (seven women, three men; mean age 35 years, range 26–49) was not supplemented. Subjects in the control group were asked to refrain from vitamin C supplementation during the entire study period. Those in the vitamin C-supplemented groups were requested to abstain from the vitamin for the 4 weeks preceeding the study period and in the 12 weeks after the supplementation period.

Heparin-anticoagulated blood (8 mL) and EDTA-anticoagulated blood (8 mL) were collected in the fasting state at baseline (−4 weeks and −1 day), during supplementation (6 weeks and 12 weeks), and at 6 and 12 weeks after discontinuation of supplementation. Blood samples during vitamin C supplementation were taken 1 h after the subjects ingested 250 or 500 mg of vitamin C in the fasting state. All samples were immediately put in melting ice. The participants

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gave informed consent. The study was approved by the hospital's medical ethical committee and was in accordance with the Helsinki declaration of 1975, as revised in 1983.

Sample processing and analyses. EDTA-anticoagulated blood was stored at 4°C for measurement of GHb within 2 days. Heparin-anticoagulated blood was centrifuged for 10 min at 1500*g* and 4°C. Heparin-plasma glucose was measured without delay, and 1 mL of heparin-plasma was stored at −30°C for analysis for fructosamine. To assay vitamin C, we added 1 mL of heparin-plasma to 4 mL of 50 g/L trichloroacetic acid. The mixture was stored for 20 min at 4°C in the dark and centrifuged for 10 min at 1500*g* and 4°C. The supernate was stored at −30°C. Fructosamine and vitamin C were determined 2–4 weeks after study completion, implying that storage time ranged from 2–28 weeks.

Analytical methods. GHb was measured in duplicate with the following four methods according to the manufacturer's instructions: HPLC (Diamat; Bio-Rad Labs., Hercules, CA); electrophoresis (Diatrac; Beckman Instruments, Brea, CA); affinity chromatography (Glycotest II; Pierce, Rockford, IL) and immunoassay (Tina-quant; Boehringer Mannheim, Mannheim, Germany). All GHb methods were calibrated with calibrators (*4*) that are used in the Dutch external quality assurance program of the SKZL ("Foundation for Quality Assurance in Clinical Chemical Laboratories"). Fasting plasma glucose was determined by the hexokinase method with a Beckman Synchrom CX-7. Plasma fructosamine was measured with the nitroblue tetrazolium reaction (second-generation fructosamine, calibrated on glycated polylysine; NBT-test combination; Boehringer Mannheim) on a Technicon RA 1000 (Miles Technicon, Tarrytown, NY). Plasma vitamin C was determined by HPLC with fluorometric detection, according to Speek et al. (*5*).

Net effect of vitamin C on GHb. The net effect of vitamin C supplementation on GHb was calculated by comparing: (*a*) GHb after 12 weeks' supplementation with GHb at baseline (−1 day), and (*b*) GHb after 12 weeks' supplementation with GHb 12 weeks after discontinuation (washout), both corrected for the corresponding GHb changes in the control group. Results were calculated as [(A + B)/2 − C] − [(D + E)/2 − F], in which:

A = Mean GHb after 12 weeks for group that took 750 mg of vitamin C daily,
B = Mean GHb after 12 weeks for group that took 1500 mg of vitamin C daily,
C = Mean GHb after 12 weeks for control group,
D = Mean GHb at −1 day (or after 12 weeks of washout) for the group that took 750 mg of vitamin C per day,
E = Mean GHb at −1 day (or after 12 weeks of washout) for the group that took 1500 mg of vitamin C per day,
F = Mean GHb at −1 day (or after 12 weeks of washout) for the control group.

Data evaluation and statistics. Means of duplicate assays were used for evaluating GHb results. Multivariate analysis of variance (MANOVA, SPSS program; SPSS Benelus, Gorinchem, The Netherlands) was used to investigate between-group differences in GHb, glucose, fructosamine, and vitamin C; $P < 0.05$ was considered significant. The Wilcoxon rank test (*6*) was used to evaluate differences between analyte concentrations at different sampling times; $P < 0.05$ was considered significant.

Results

Figure 1 shows the mean plasma concentrations of vitamin C in the groups that took 750 and 1500 mg of vitamin C per day. The data are expressed as percentages of the corresponding means of the control group. The vitamin C concentrations of the two vitamin C-supplemented groups were not significantly different at baseline and at the end of the study. The mean relative plasma concentrations of vitamin C doubled during supplementation at both dosages and returned to baseline thereafter.

For the various GHb methods, mean calibrated (and mean uncalibrated) GHb percentages for the 60 blood samples collected from the 10 control subjects at six sampling points were (%GHb): HPLC 4.71 (5.26); electrophoresis 5.13 (3.46); affinity chromatography 4.78 (3.83); and immunoassay 4.81 (4.78). To assess the effect of calibration on analytical drift, we calculated the CV for mean GHb percentages of the 10 control subjects at six sampling points. With (and without) calibration, the CVs (%) amounted to: HPLC 1.3 (1.8); electrophoresis 3.5 (5.9); affinity chromatography 1.9 (3.1); and immunoassay 1.7 (4.7). Thus, calibration

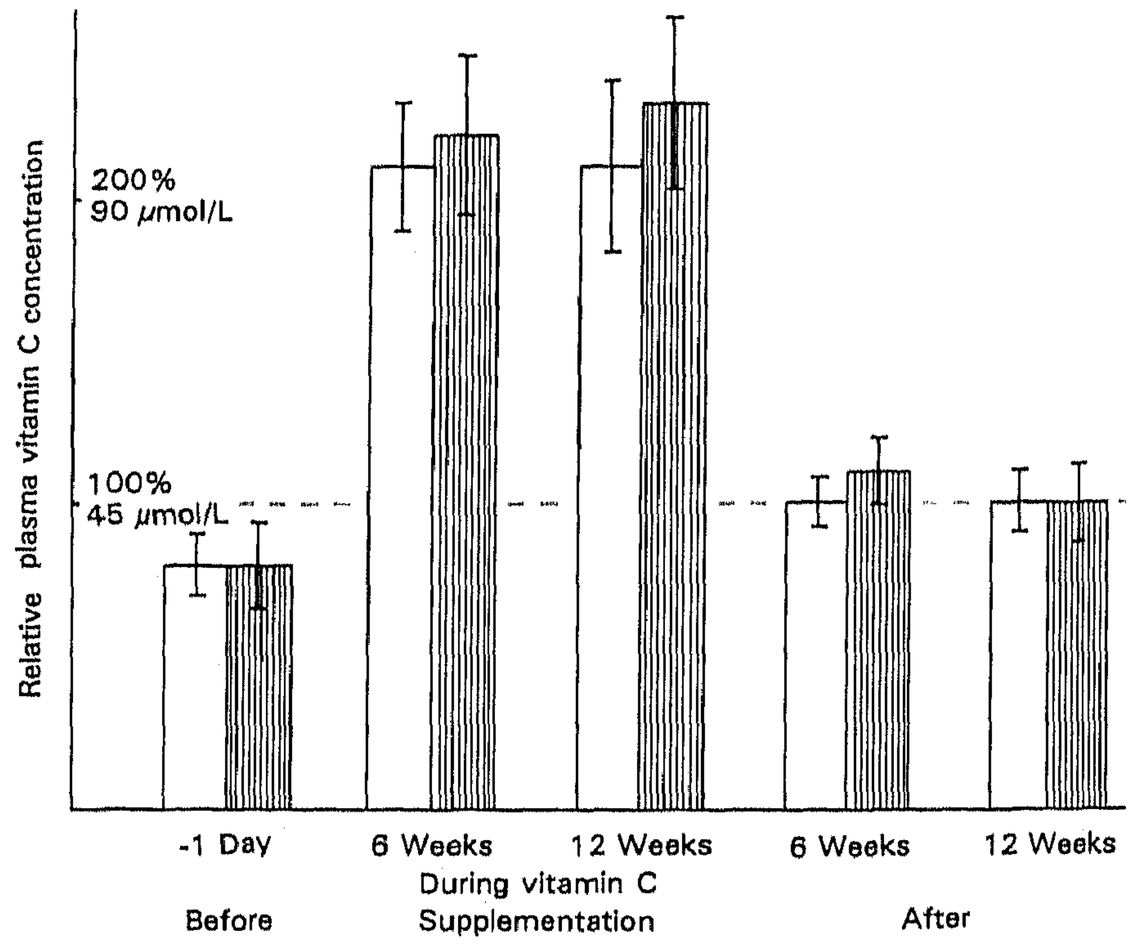

Fig. 1. Relative plasma vitamin C concentrations before, during, and after supplementation with 750 (□) or 1500 (▥) mg of vitamin C daily. Concentrations are relative to the control group (100%) at each of the indicated sampling points. *Bars* indicate SEM.

reduced intermethod variation and bias due to analytical drift.

Table 1 shows the mean (SD) concentrations for calibrated GHb, fasting plasma glucose, and fructosamine as measured at baseline (−4 weeks, −1 day), during vitamin C supplementation (at 6 and 12 weeks), and after vitamin C supplementation (6 and 12 weeks after stopping the dosing). MANOVA showed no between-group differences for indicated analytes. The Wilcoxon rank test showed no changes between GHb and glucose concentrations at baseline (i.e., −4 weeks and −1 day) and after 12 weeks of supplementation, or between 12 weeks of supplementation and 12 weeks of washout. The mean fructosamine concentrations decreased from baseline (−1 day) to the study's end (12 weeks after discontinuation) by 21 mmol/L ($P = 0.01$) for the control group, 8 mmol/L ($P = 0.05$) for the group that took 750 mg/day, and 21 mmol/L ($P = 0.01$) for the group that took 1500 mg/day.

Table 2 shows the net effects of vitamin C concentration on GHb measurements. Comparison of GHb after 12 weeks of vitamin C supplementation with the baseline values (−1 day), after 12 weeks of supplementation, and after 12 weeks of discontinuation caused insignificant changes in GHb percentages (from −0.11% to 0.04%).

Discussion

In studying the effect of vitamin C supplementation on GHb percentages of nondiabetic subjects, vitamin C doses of 0 (n = 10), 3 × 250 (n = 10), and 3 × 500 (n = 10) mg/day were taken during 12 weeks. GHb was determined by HPLC, electrophoresis, affinity chromatography, and immunoassay. We used calibrated GHb percentages to maximize the comparability of GHb data collected during the 28-week study period. By this approach we found no effect of vitamin C on GHb as measured by each of the indicated methods.

Mean calibrated GHb percentages (Table 1) were close to the mean of 5% that was used in the Diabetes Control and Complications Trial study (*7*). Although there were no changes in concentrations of GHb and fasting plasma glucose, fructosamine decreased. Because fructosamine was measured within 2–4 weeks after study completion, we consider calibration drift to be improbable. Lyophilized samples stored at 4°C exhibit increasing fructosamine concentrations (unpublished). The external quality assurance program prompted us to first dialyze serum before lyophilization. We did not expect ongoing glycation in plasma frozen at −30°C, but the results presented may indicate that this assumption is incorrect. Ongoing glycation in frozen plasma was previously described (*8, 9*). Because the phenomenon affected samples from both the control group and the vitamin C-supplemented groups, it did not cause between-group differences as analyzed by MANOVA.

Our results contrast with those of Davie et al. (*3*), who reported an absolute decrease of 1.13% GHb by affinity chromatography and a 0.99% increase by electrophoresis after 12 weeks of supplementing nondiabetics with 2 × 500 mg of vitamin C per day. We observed an increase of about 100% in plasma vitamin C concentrations, whereas Davie et al. (*3*) reported an increase of ~50%. Perhaps the moment of blood collection accounts for this variability. The time interval since the last vitamin C intake was 1 h in our study vs 12–15 h in the study of Davie et al. The major differ-

Table 1. Concentrations of glycohemoglobin, fasting glucose, and fructosamine before, during, and after supplementation with 0, 750, or 1500 mg of vitamin C daily for 12 weeks.

Analyte and method[a]	Vitamin C, mg/day	Mean (SD) conc[b] 4 weeks before	1 day before	6 weeks of vitamin C	12 weeks of vitamin C	6 weeks after	12 weeks after
GHb, %	0	4.81 (0.28)	4.77 (0.29)	4.77 (0.38)	4.62 (0.36)	4.66 (0.36)	4.61 (0.32)
HPLC	750	4.92 (0.53)	4.86 (0.51)	4.79 (0.44)	4.70 (0.43)	4.68 (0.49)	4.69 (0.48)
	1500	4.83 (0.21)	4.81 (0.22)	4.80 (0.25)	4.60 (0.21)	4.60 (0.21)	4.69 (0.19)
GHb, %	0	4.97 (0.62)	5.23 (0.69)	5.32 (0.53)	5.00 (0.62)	4.82 (0.53)	5.37 (0.43)
Electrophoresis	750	5.32 (1.00)	5.13 (0.70)	5.12 (0.50)	4.87 (0.51)	5.30 (0.98)	4.99 (0.70)
	1500	4.93 (0.62)	4.94 (0.69)	5.29 (0.50)	4.75 (0.51)	4.99 (0.85)	5.30 (0.73)
GHb, %	0	4.68 (0.32)	4.55 (0.21)	4.84 (0.37)	4.71 (0.45)	4.69 (0.45)	4.72 (0.32)
Affinity chrom.	750	4.78 (0.51)	4.65 (0.61)	4.76 (0.36)	4.81 (0.47)	4.82 (0.46)	4.72 (0.33)
	1500	4.82 (0.20)	4.79 (0.33)	4.80 (0.38)	4.81 (0.28)	4.69 (0.38)	4.89 (0.27)
GHb, %	0	4.74 (0.27)	4.84 (0.25)	4.72 (0.30)	4.68 (0.31)	4.59 (0.37)	4.73 (0.35)
Immunoassay	750	5.08 (0.49)	5.04 (0.37)	4.62 (0.60)	4.59 (0.49)	4.59 (0.39)	4.76 (0.34)
	1500	4.81 (0.18)	4.88 (0.23)	4.71 (0.19)	4.79 (0.20)	4.67 (0.32)	4.67 (0.20)
Glucose, mmol/L	0	5.07 (0.21)	5.13 (0.39)	5.19 (0.36)	5.31 (0.80)	4.90 (0.43)	5.34 (0.16)
	750	4.87 (0.55)	4.89 (0.35)	5.31 (0.49)	5.33 (0.37)	5.04 (0.39)	5.62 (0.51)
	1500	5.06 (0.30)	5.09 (0.32)	5.32 (0.42)	5.25 (0.42)	5.12 (0.31)	5.16 (0.36)
Fructosamine, μmol/L	0	n.m.	244 (14)	231 (23)	232 (16)	228 (20)	223 (12)
	750	n.m.	235 (13)	244 (18)	240 (12)	229 (17)	227 (13)
	1500	n.m.	238 (16)	239 (16)	231 (14)	223 (17)	217 (21)

[a] HPLC, Diamat (Bio-Rad); electrophoresis, Diatrac (Beckman); affinity chromatography, Glycotest II (Pierce); immunoassay, Tina-quant (Boehringer Mannheim).
[b] Results for duplicate samples from 10 subjects per group.

Table 2. Net effect of vitamin C supplementation on glycohemoglobin.[a]

Method	Absolute GHb difference, %, between results after 12 weeks of supplementation and results	
	1 day before	12 weeks after
HPLC	−0.035	−0.050
Electrophoresis	0.005	0.035
Affinity chromatography	−0.070	0.015
Immunoassay	−0.110	0.025

[a] Data are for the combined group of subjects who took 750 and 1500 mg of vitamin C per day after correction for the corresponding GHb difference of the control group.

ence with the study of Davie et al. was inclusion of a control group and GHb calibration. The use of a control group and calibration reduce analytical GHb drifts, whereas calibration reduces intermethod variation.

We conclude that supplementation of nondiabetics with 750 or 1500 mg of vitamin C daily for 12 weeks does not cause interference in GHb determinations by HPLC, electrophoresis, affinity chromatography, or immunoassay and does not reduce in vivo Hb glycation.

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