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Comparative bioavailability of synthetic vitamin C and Nutra-C (calcium ascorbate) in Korean healthy volunteers

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Abstract: The purpose of this study was to compare the relative bioavailability of synthetic Vitamin C and Nutra-C® (calcium ascorbate) using a randomized parallel pharmacokinetics study design. Under fasting conditions, 20 healthy volunteers were randomly allocated to receive a single oral dose (500 mg of ascorbic acid) of either synthetic Vitamin C or Nutra-C®. Fasting blood was collected pre-dose and 1, 2, 3, 4, 7 and 10 hr post-dose. The ascorbic acid content of human serum was determined using HPLC with ultraviolet detection. The fasting serum ascorbic acid concentrations of synthetic Vitamin C and Nutra-C® were 6.734 ± 2.09 ng/mL (n = 10) and 7.542 ± 2.96 ng/mL (n = 10), respectively. The bioavailability of Nutra-C® was significantly greater (128 %, $p < 0.05$) than that of the synthetic Vitamin C.

Key words: Vitamin C, Nutra-C, Bioavailability, Human serum

1. Introduction

Vitamin C, also known as *L*-ascorbic acid, is a water-soluble vitamin that is naturally present in some foods, added to others, and available as a dietary supplement. Because humans lack the enzymes to synthesize vitamin C endogenously, it is necessary to intake this nutrient through dietary sources.[1] Vitamin C is a crucial component of biosynthesis of collagen, *L*-carnitine, and numerous neurotransmitters and is involved in certain protein metabolsim.[1,2] In addition, Vitamin C is known to have anti-cancer effects, as it impedes the production of carcinogenic nitrosamines in the acidic environment of the stomach, supported by the correlation between high vitamin C intake and low rate of stomach cancer.[3,4] Vitamin C is absorbed into intestinal cells of animals via several different mechanisms. Vitamin C uptake is mediated by either Na^+-dependent active transporters – transporting both the oxidized ascorbic acid (dehydroascorbic acid) and the reduced form (L-ascorbic acid) – or facilitated-diffusion glucose transporters – in the oxidized form only. These absorption mechanisms on cell membranes regulate Vitamin C concentration in tissues and the plasma.[5] The consumption of 2000 mg of Vitamin C per day is considered safe, but

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studies indicate that uptake declines to 50 % at oral intakes above 1000 mg per day.[5,12] The unabsorbed Vitamin C molecules, before being excreted in the urine, benefits the body by decreasing nitrosamine production, reducing the risk of stomach cancer, increasing non-heme iron uptake, and improving body status.[4-7] As pharmacokinetic studies conclude that Vitamin C relative bioavailability peaks at 200 mg dose, ingestion of several smaller doses each day is preferable to a single large dose.[13,14] Other studies examined the effect of Vitamin C tablet formulations on its relative bioavailability and demonstrated that slow-release formulations resulted in the highest bioavailability.[15-17]

There are several known factors that influence the uptake of Vitamin C, such as iron and dietary fibers. Studies support that iron merely has effect on Vitamin C intake in cultured intestinal cells but not on bioavailability during human intervention studies.[8-10] Likewise, it is uncertain whether specific fibers, such as hemicellulose and pectin, determine the intake and excretion of Vitamin C.[11]

The composition of Vitamin C tablets also influence the relative bioavailability. Sodium and calcium ascorbic acid, also known as Ester-C, was taken in at a faster rate and excreted at a lower rate and observed superior anti-scorbutic activity than ascorbic acid in tested animals,[18,19] nevertheless, a significant difference in bioavailability between Ester-C and Vitamin C in humans has yet to be found.[20] Despite the uncertainties, Ester-C, which contains calcium ascorbic acid, DHA, and calcium threonate, reduces gastronomical symptoms in individuals with acid-sensitivity.[18,21] The addition of calcium into Vitamin C pills reduces side effects of taking Vitamin C by itself – depletion of calcium stores in the body and chronic digestive difficulties in sensitive stomachs – as calcium ions neutralize the acidic properties of ascorbic acid in the stomach, resulting in no significant increase in pH.[21] Furthermore, calcium is known as an essential nutrient for proper health, serving as the building block of strong bones and teeth and improving the immune system. Nutra-C®, another form of calcium ascorbic acid, is not only a pH neutral product, manufactured via a proprietary, water-based process, but also contains Vitamin C metabolites to faciliate intercellular transport of Vitamin C molecules for numerous health benefits.

The analytical method for detemination of Vitamin C were Classic (conventional) techniques : volumetric methods—titration with an oxidant solution such as dichlorophenol indophenol,[22,23] potassium iodate,[24] or bromate,[25] sensors and biosensors.[26-28] Hyphenated instruments consisting of flow injection analysis,[29-32] high performance liquid chromatography[33-35] or capillary electrophoresis[36-39] instruments and a detector are mostly utilized for the determination of Vitamic C. Liquid chromatography is a successful method for vitamin C determination when selectivity and specificity are concerned.[40-42]

The objective of the present study was to determine the relative bioavailability of synthetic Vitamin C and Nutra-C® in Korean healthy volunteers.

2. Materials and Methods

2.1. Study design

This study was approved by the Human Subjects Committee of the Chungnam National University Hospital. This was a single-dose, randomized, open-label, parallel-group pharmacokinetic study. Healthy volunteers subjects aged 19–35 years were randomly assigned to either one of two sample groups (n = 10/group) to receive ascorbic acid : synthetic Vitamin C (as ascorbic acid 500 mg) in one group and Nutra-C® (as ascorbic acid 500 mg) in the other.

2.2. Chemicals and materials

HPLC-grade solvents (Merck & Co., Inc., USA) were purchased from Sigma-Aldrich Chemicals (Korea): acetic acid (99.4 %), formic acid (> 98 %), and metaphosphoric acid (Sigma-Aldrich, ACS reagent). Water, used in HPLC, was purified with the Milli-Q water purification system (Millipore, MA, USA). Nutra-C® tablet (Celltrion Pharm., Inc., Korea) contains a non-acidic vitamin C powder - a tan, free-flowing powder with a slight caramel odor - 9.0 ± 0.5 % calcium as calcium ascorbate dehydrate

and no more than 8 % moisture by weight, resulting in a pH neutral product. Other ingredients in Nutra-C® tablet were manufactured by Novotech (Ventura, CA, USA). Nutra-C® is a unique combination of vitamin C and calcium, manufactured by a patented, water-based process, neutralizing the acid and enabling those who are sensitive to the acidic nature of ascorbic acid to tolerate vitamin C. Synthetic Vitamin C (DSM Co., UK), used as a reference, contains 100 % vitamin C (ascorbic acid).

2.3. Instrumentation and analysis conditions

The ACQUITY UPLC system (Waters Corp., MA, USA) equipped with a binary solvent delivery system, an autosampler and a TUV detector was developed for analysis of ascorbic acid in human serum matrix. The mobile phase solution was pumped into a Gemini C18 110A column (Phenomenex) (4.6 × 150 mm, 3 μm) in isocratic mode was maintained at 0.8 mL/min flow rate. The mobile phase was isocratic system consisting of 0.1 % formic acid. The injection volume was 10 μL and acquisition time (2.0 min) was equilibrated with mobile phase flowing through the system. The auto sampler temperature was 5 ± 10ºC. Chromatograms were monitored at 245 nm. The calibrated curve and quality control samples were injected into the liquid chromatographic system. The chromatograms were obtained. Accordingly, the peak area was determined for each concentrations of ascorbic acid. Calibration curve of ascorbic acid was constructed by plotting peak area versus applied concentrations of ascorbic acid by using the linear calibration function fit curve.

2.4. Sample preparation

Standards from an ascorbic acid stock solution (1 mg/L) in the range of 1.0–100 μg/mL were prepared by dilution with 200 μL of 0.1 % metaphosphoric acid was added to a 200 μL of human serum. Acid addition was used to maintain the stability of ascorbic acid.[22] The respective samples were vortex mixed at 10 seconds, and centrifuged at 13,000 rpm for 5 min. After centrifugation, 10 μL of supernatant was used for UPLC analysis.

2.5. Method validation procedures

The purpose of the method validation was to evaluate the method in terms of the parameters, such as linearity response, sensitivity, selectivity, precision and accuracy, stabilities and recovery. The calibration curve standards spiking solutions were prepared by adding 200 μL of ascorbic acid stock solution (1.0 mg/mL) with diluents (MPA: metaphosphoric acid, 0.1 g/mL) in different vials vortex for 10 seconds. The calibration curve standards were prepared by spiking the respective calibration curve standards spiking solutions in screened blank serum in different vials to obtain final concentrations of 1, 5, 10, 20, 50 and 100 μg/mL of ascorbic acid. The quality control spiking solutions were prepared by adding 200 μL of ascorbic acid stock solution (1.0 mg/mL) with diluents (MPA, 0.1 g/mL) in different vials and mixing with vortex for 10 seconds. The quality control samples were prepared by spiking the respective quality control spiking solutions in screened blank serum in different vials to obtain LLOQ (lower limit of quantification) QC (quality control), LQC (liquid quality control), MQC (medium quality control), HQC (high quality control), ULOQ (upper limit of quantification) QC of concentrations of 1, 3, 50 and 100 μg/mL of ascorbic acid, respectively. The LLOQ QC and ULLOQ QC solutions were prepared only for method validation and screening of blank serum samples. The working spiking solutions were prepared for long-term solution stability and short-term solution stability by adding ascorbic acid stock solution (1.0 mg/mL) and diluents (MPA, 0.1 g/mL) in different vials and mixing with vortex for 10 seconds.

2.6. Statistical analysis

The pharmacokinetic parameters of ascorbic acid were estimated by non-compartmental analysis using Phoenix WinNonlin® software version 6.1 (Pharsight Corporation, Sunnyvale, CA, USA). Descriptive statistics involved calculation of least square geometric mean for C_{max}, AUC_{0-10} and AUC_{0-inf}. Analysis of variance was carried out for least square means, the difference between the adjusted formulation means

and the standard errors associated with the difference.

3. Results and Discussion

3.1. Participant characteristics

At screening, the participants' mean ± SD age was 22.5 ± 4.3 years, weight was 53.3 ± 4.8 kg, height was 162.6 ± 5.4 cm. The participants selected for this study had "healthy" fasting serum ascorbic acid concentrations, their mean ± SD at screening being 6.734 ± 2.09 ng/mL (n = 10), 7.542 ± 2.96 ng/mL (n = 10) for synthetic Vitamin C and Nutra-C® groups, respectively. This method was specifically designed prevent any potential confounding effects of preferential tissue uptake in individuals with suboptimal ascorbic acid status at baseline, possibly affecting the comparative ascorbic acid levels observed in serum.

3.2. Method performance

A representative HPLC chromatogram obtained from a human serum is shown in *Fig.* 1. Sensitivity i.e. the lowest limit of reliable quantification was set at the concentration of the LLOQ (1 μg/mL) from human serum (*Fig.* 1). Linearity was achieved in serum over the concentration ranges of 1.0 – 100 μg/mL for ascorbic acid. Calibration curve parameters (including slope and intercept of the calibration function $y = ax + b$) showed high reproducibility with $r^2 > 0.999$. The assay LLOQ was determined to be 1.0 μg/mL for Ascorbic acid. Intra-batch precision ranged from 0.47 to 1.95 % and accuracy ranged from 92.68 to 98.63 % respectively. Inter-batch precision ranged from 1.77 to 4.71 % and accuracy ranged from 95.20 to 100.70 % respectively. The accuracy and precision results of the method, presented in

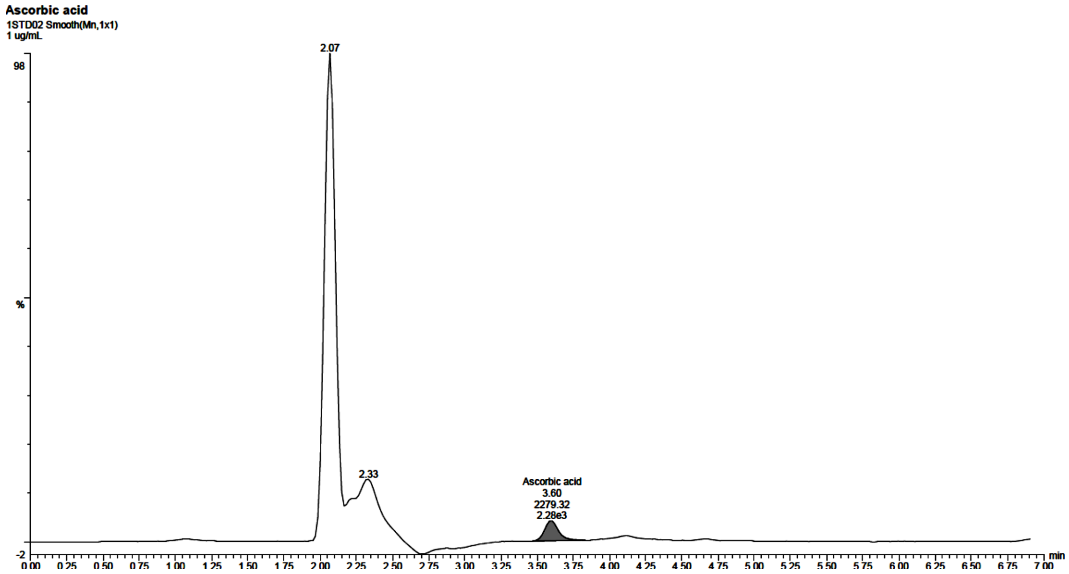

Fig. 1. The HPLC chromatogram of extracted from human serum spiked ascorbic acid at LLOQ (1 μg/mL).

Table 1. Intra-batch accuracy and precision of ascorbic acid in human serum

Batch	Nominal conc. (μg/mL)	Measured concentration (μg/mL)					Mean conc. (μg/mL)	CV (%)	Accuracy (%)
		No. 1	No. 2	No. 3	No. 4	No. 5			
Within run	1	0.942	0.946	0.918	0.918	0.910	0.927	1.74	92.68
	3	2.794	2.791	2.813	2.788	2.816	2.800	0.47	93.35
	50	49.932	49.230	48.865	47.615	47.923	48.713	1.95	97.43
	100	100.563	99.743	97.268	99.205	96.395	98.635	1.77	98.63

Table 2. Inter-batch accuracy and precision of ascorbic acid in human serum

Batch	Nominal conc. (μg/mL)	Measured concentration (μg/mL)			Mean conc. (μg/mL)	CV (%)	Accuracy (%)
		Day 1	Day 2	Day 3			
Between run	1	0.927	0.996	1.014	0.979	4.71	97.90
	3	2.800	2.899	2.868	2.856	1.77	95.20
	50	48.713	48.899	51.468	49.693	3.10	99.39
	100	98.635	102.271	101.207	100.704	1.86	100.70

Table 3. Absolute recoveries of ascorbic acid in human serum

No.	Nominal conc. (μg/mL)	Mean area	CV (%)	Recovery (%)
Post-extraction spiked	3	4306	0.63	
	50	54737	3.20	
	100	113711	4.57	
Pre-extraction spiked	0	4320	0.31	100.31
	0	55478	1.07	101.35
	0	110270	1.71	96.97

Table 1 and 2, were acceptable. The recovery results of the method, presented in *Table* 3, were based on a comparison between HPLC/UV response from un-extracted serum and that of extracted serum blank. The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations; QL(quality low), QM(quality medium), and QH(quality high), with un-extracted standards. The mean recovery for 3, 50,100 μg/mL were 100.31, 101.35 and 96.97 % respectively.

3.3. Stability

Working solution of ascorbic acid was stable after approximately 20 hrs at room temperature. The % change for LQC and HQC were 0.62 and 0.20 respectively. The stability samples of LQC and HQC was found to be stable for approximately 20 hrs in autosampler (at 10 ℃). The % change for LQC and HQC were 0.5 and 0.5 respectively. The freeze and thaw stability samples each of QL and QH were found to be stable in human serum after three freeze and thaw cycles (at -70 ± 5 ℃). The % change for LQC and HQC were 0.15 and 0.32 respectively. The long-term stability results of the ascorbic acid in serum concentration are given in *Table* 4. The % ascorbic acid change found for LQC and HQC were 0.55 and 0.36 respectively. The % mean long term working solution stability for ascorbic acid in serum concentration were in the range of 99.47 to 101.59 % and % CV was within 15.00 %. The result suggest that ascorbic acid was stable in human serum at -70 ± 5 °C for at least 11 days and expected to be more stable at -70 ± 5 °C.

3.4. Ascorbic acid uptake into human serum

Serum ascorbic acid levels, following ingestion of either 500 mg synthetic Vitamin C tablets or Nutra-C® are shown in *Fig.* 2. A statistically significant increase in serum ascorbic acid was observed as early as 1 h post intervention ($P = 0.008$). There was a significant increase in serum ascorbic acid from two hours post intervention ($P < 0.001$) and also a significant difference between the two interventions, with enhanced ascorbic acid observed in the Nutra-C® ($P = 0.016$, *Fig.* 2). This difference was confirmed with ascorbic acid area under the time-concentration curves (*Table* 5). The validated method has been

Table 4. Matrix stability of ascorbic acid under different conditions

Time	Nominal conc. (μg/mL)	Measured conc. (μg/mL)	Mean conc. (μg/mL)	Accuracy (%)	CV (%)
Short-term Stability[a]	3	2.930 2.964 2.936	2.943	98.11	0.62
	100	102.289 102.357 101.975	102.207	102.21	0.20
Autosampler Stability[b]	3	2.998 2.969 2.978	2.982	99.39	0.50
	100	102.259 101.782 101.235	101.759	101.76	0.50
Freeze-thaw Stability[c]	3	2.825 2.827 2.819	2.824	94.12	0.15
	100	98.677 99.092 98.483	98.751	98.75	0.32
Long-term Stability[d]	3	3.003 2.976 2.973	2.984	99.47	0.55
	100	101.360 102.007 101.406	101.591	101.59	0.36

[a]Short-term Stability (at room temperature) for 20 hr in polypropylene tube.
[b]Autosampler stability (at below 10ºC) for 20 hr in polypropylene tube.
[c]Three freeze-thaw cycles (3 F/T).
[d]Long-term Stability (at below -70ºC) for 11 days in polypropylene tube.

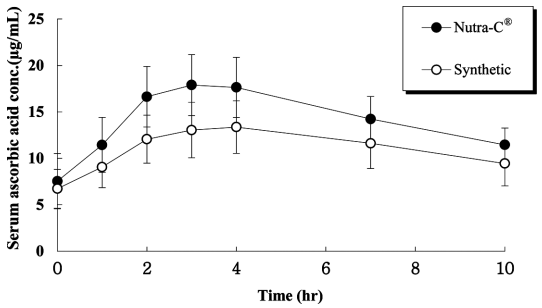

Fig. 2. Change in human serum ascorbic acid uptake following ingestion of 500 mg synthetic Vitamin C (○) or Nutra-C® (●). Data represent mean ± SEM (n = 10). Baseline plasma ascorbic acid concentrations were 6.734 ± 2.09 μg/mL and 7.542 ± 2.96 μg/mL for the synthetic Vitamin C and Nutra-C® groups, respectively.

Table 5. PK parameter for Nutra-C® and Synthetic ascorbic acid in human serum

PK Parameter	Nutra-C®	Synthetic
AUC(last) (μg·h/mL)	144.92 ± 25.48	113.28 ± 24.85
AUC(inf) (μg·h/mL)	319.46 ± 63.54	291.99 ± 98.73
C_{max} (μg/mL)	18.05 ± 3.22	13.62 ± 2.94
T_{max} (hr)	3.30 ± 0.67	3.60 ± 0.70
CL(inf)/F	1.30 ± 0.27	1.61 ± 0.85
Vz(terminal)/F	18.98 ± 3.76	26.16 ± 6.82
Half time(hr)	10.57 ± 3.43	12.59 ± 3.86

successfully applied to investigate the bioavailability in healthy human volunteers after a single dose of 500 mg synthetic Vitamin C and Nutra-C®. As a

result, when evaluating the pharmacokinetics after administration of the developed material in synthetic Vitamin C and Nutra-C® in the healthy volunteers, it was confirmed that the pharmacokinetics of the difference. C_{max} as compared to the control group, the test groups ware increased 1.34 times, compared with the control group, the test group it was confirmed that the AUC_t 1.28 times increase. The bioavailability of the Nutra-C® was significantly greater (128 %, $p < 0.05$) than that of the synthetic Vitamin C. Administration compared to the control material was developed with a neutral vitamin C was confirmed that there is no difference in stability.

4. Conclusions

This study was undertaken to compare the bioavailability of synthetic ascorbic acid and a neutralized calcium ascorbate. The validated method has been successfully applied to investigate the bioavailability in healthy human volunteers after a single dose of 500 mg synthetic ascorbic acid and a neutralized calcium ascorbate. The proposed method is suitable for pharmacokinetics and bioequivalence study to determine Ascorbic acid in human serum. The bioavailability of the neutralized calcium ascorbate was significantly greater (128 %, $p < 0.05$) than that of the synthetic ascorbic acid.

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