Vitamin C Activity of 2-*O*- α -D-Glucopyranosyl-L-Ascorbic Acid in Guinea Pigs

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Summary The vitamin C activity of 2-*O*- α -D-glucopyranosyl-L-ascorbic acid (AA-2G), which is one of chemically stable derivatives of L-ascorbic acid (AsA), in guinea pigs was investigated. Male guinea pigs were divided into 9 groups and fed AsA-deficient diet for 24 days with the following supplement: AA-2G- or AsA-supplemented groups were orally supplemented with 0.96, 1.92, 9.6 and 192 AA-2G mg/animal/day or equimolar amounts of AsA (0.5, 1, 5 and 100 mg/animal/day, respectively); AsA-deficient group received neither of them. The body weight gain, serum alkaline phosphatase activity, and the concentration of AsA and AA-2G in the liver, adrenals and urine of the guinea pigs were measured at the end of the experimental period. The AA-2G-supplemented guinea pigs showed similar body weight gain to the animals supplemented with equimolar amount of AsA. Serum alkaline phosphatase activity in both AA-2G- and AsA-supplemented groups was significantly higher than that of AsA-deficient group. But there was no significant difference between the groups supplemented with AA-2G and the equimolar amount of AsA. AA-2G-supplemented guinea pigs showed no apparent symptoms of scurvy. In AA-2G-supplemented groups, AA-2G was not detected in the liver, adrenals and urine, but AsA was found and the AsA concentration increased with increasing AA-2G dosage. The AsA concentration in the tissues of each AA-2G-supplemented groups was higher than that of AsA-deficient group, which was similar to that of the groups supplemented with equimolar amount of AsA.

These results showed that AA-2G has the same vitamin C activity as AsA on a molar basis for the orally supplemented guinea pigs.

Key Words L-ascorbic acid, 2-*O*- α -D-glucopyranosyl-L-ascorbic acid,

vitamin C activity, guinea pigs

L-Ascorbic acid (AsA), known as vitamin C, has various physiological and pharmacological functions. Humans, monkeys and guinea pigs cannot synthesize AsA due to lack of hepatic L-gulonolactone oxidase, and must take this essential nutrient from foods. AsA is very unstable on thermal and oxidative degradation in aqueous solution. Thus the development of stable and easily synthesized AsA-derivatives having the same vitamin C activity as AsA has been desired. There are several reports about stable AsA-derivatives such as ether- and ester-derivatives; however, some of these derivatives have no vitamin C activity. For example, L-ascorbic acid 2-sulfate (AA-2S) which was found in the tissues of rats (1) and in urine of humans (2), rats and trouts (1), is very stable, but it has been reported to have no antiscorbutic activity in guinea pigs and rhesus monkeys (3-8).

Recently, Yamamoto *et al.* reported a new AsA derivative, 2-O- α -glucopyranosyl-L-ascorbic acid (AA-2G) synthesized through transglucosylation with mammalian and rice seed α -glucosidase (9-11), and cyclodextrin glucanotransferase from *Bacillus stearothermophilus* (12,13). In contrast to AsA, AA-2G is characterized by its high stability toward thermal and oxidative degradation in aqueous solutions and its nonreducibility (10,11,14). In addition, Yamamoto *et al.* have confirmed that AA-2G is able to stimulate the collagen synthesis in cultured human skin fibroblasts (15). However, the bioavailability of AA-2G *in vivo* such as optimum intake to prevent scurvy, the intestinal absorption, metabolism and retention mechanism of tissues have not been defined yet. Therefore, the present study was designed to examine the vitamin C activity of AA-2G in guinea pigs by supplementing graded doses of AA-2G with comparison of AsA. Based on the adequate AsA doses (5 mg/day) to keep the guinea pigs healthy (16,17), suboptimal (0.5 and 1 mg AsA/day), optimal (5 mg AsA/day) and excessive (100 mg AsA/day) doses were chosen for low, normal and high level, respectively, for guinea pigs. These graded levels of AA-2G and their equimolar amounts of AsA were orally supplemented to guinea pigs fed AsA-deficient diet and their body weight gain, serum alkaline phosphatase activity which is considered to be closely related with vitamin C level in the body, concentration of AsA and AA-2G in tissues and urine were measured to evaluate the vitamin C activity of AA-2G.

MATERIALS AND METHODS

Animals and diets. Male Hartley guinea pigs, weighing about 170 g, were obtained from Saitama Animal Research Co. They were housed individually in cage with wire mesh bottoms in a room where the temperature and humidity were maintained at $22 \pm 2^\circ\text{C}$ and $55 \pm 5\%$, respectively, and in a 12-h light-dark cycle. During the experimental period, the animals were fed an AsA-deficient diet (Table 1). Water and diet were provided *ad libitum*, and their body weights were

Table 1. Composition of the AsA-deficient diet.

Constituents	Amount (%)
Casein	20
Sucrose	12
Corn	20
Soybean oil	5
Soybean meal	5
Wheat bran	20
Alfalfa meal	10
Vitamin mixture (AsA-free)* ¹	1
Mineral mixture* ²	7

AsA content determined by Fujita's method (21) was 0.95 mg/100 g diet. *¹ Vitamin mixture contains the following (mg/100 g diet): vitamin A (500,000 IU/g), 2.0; vitamin D₃ (500,000 IU/g), 0.4; vitamin B₁, 0.2; vitamin B₆, 0.4; vitamin B₁₂ (0.1%), 1.0; vitamin K₃ (50%), 0.6; DL- α -tocopherol, 8.0; riboflavin, 0.5; nicotinic acid, 2.0; DL-Ca-pantothenate, 4.0; ρ -aminobenzoic acid, 10.0; inositol, 10.0; folic acid, 0.4; biotin (2%), 1.5; choline chloride, 80.0; sucrose, 879.0. *² Mineral mixture contains the following (mg/100 g diet): NaCl, 200.0; CaCO₃, 1650.0; MgSO₄, 750.0; Na₂PO₄·H₂O, 375.32; K₂HPO₄, 450.0; CaH₄(PO₄)₂·H₂O 1,000.0; Fe(C₆H₅O₇)·5H₂O, 127.8; Ca-lactate, 650.0; Ca(IO₃)₂, 0.03; MnSO₄·4H₂O, 8.2; KCl, 1150.0; sucrose, 638.65.

measured daily.

At the beginning of the experiment, these animals were randomly divided into nine groups and were orally supplemented with AA-2G or AsA in the following doses: AA-2G-supplemented groups supplemented with 0.96, 1.92, 9.6 and 192 mg AA-2G/animal/day (groups G-0.5, G-1, G-5 and G-100, respectively), AsA-supplemented groups supplemented with 0.5, 1, 5 and 100 mg AsA/animal/day (groups A-0.5, A-1, A-5 and A-100, respectively), AsA-deficient group (A-0) received neither of them. AA-2G and AsA were dissolved in water immediately before use and orally supplemented every 9:00 A.M. for 24 days.

On the day before the guinea pigs were killed, urine was collected in foiled flask containing 10 ml of 20% metaphosphoric acid for 24 h.

On the 24th day, they were killed under Nembutal anesthesia at around 9:00 A.M. After blood was collected in glass tube, the whole body was perfused with ice-cold 0.9% (W/V) NaCl. The liver and adrenals were removed and homogenized with 4% metaphosphoric acid and centrifuged at $10,000 \times g$ for 5 min. The supernatants were used for the measurement of AsA and AA-2G, and stored at -30°C until analysis.

Assay of serum alkaline phosphatase activity. It was reported that, when the guinea pigs were in the biological vitamin C-deficient state (although no clinical symptoms of scurvy can be observed), the serum alkaline phosphatase activity was lower than that of the normal guinea pigs (18). Furthermore, this activity tended to decrease with the depletion of AsA (19). The serum alkaline phosphatase activity might reflect the vitamin C level in the guinea pigs. Therefore, we

Fig. 1. HPLC profiles of AsA and AA-2G. A: standard AsA and AA-2G. B: adrenals extract. C: adrenals extract added AA-2G.

measured this activity in order to clarify the vitamin C activity of AA-2G in guinea pigs.

Serum alkaline phosphatase activity was measured by using Alkaline phosphatase B-test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to a modified method of Bessey *et al.* (20). The values were expressed in international units (IU).

Assay for AsA and AA-2G in tissues and urine. Total amount of AsA excreted in 24 h urine was determined by 2,4-dinitrophenylhydrazine method (22, 23).

Determination of both AsA and AA-2G in the liver and adrenals and AA-2G in urine were carried out using high-performance liquid chromatography (HPLC) with a modified condition of Kitada *et al.* (24).

The system used was liquid chromatograph (Model LC-5A Shimadzu) equipped with a UV spectrometric detector set at 250 nm and a Inertsil ODS-2 column (250 × 4.6 mm i.d.; GL Sciences Inc., Tokyo Japan). The mobile phase consisted of 20 mM potassium phosphate buffer (pH 3.5) and methanol (80:20 v/v) containing 0.03 mM EDTA and 5 mM tetra-*n*-amylammonium bromide, and flow rate was 0.5 ml/min. Under these conditions, AsA and AA-2G were analyzed simultaneously and retention times were 8.32 and 13.48 min, respectively (Fig. 1A). Figure 1B

shows typical HPLC profile of the AsA extracted from adrenals and Fig. 1C shows HPLC profile of the adrenals extract to which AA-2G was added. In these conditions, there were no interfering peaks on the chromatograms, and the detection limit of both AsA and AA-2G was 1 pmol.

Statistical analysis. The significant difference between the means of two groups was statistically analyzed by Student's *t*-test or the Cochran-Cox test, depending on whether the variance was equal or different, respectively.

RESULTS

Body weight gain

Body weight gains for 23 days are shown in Fig. 2. Body weight of AsA-supplemented groups increased continuously during the experimental period. The AsA-deficient guinea pigs (group A-0) stopped gaining weight from day 15, while there was a continued increase in body weight of AA-2G-supplemented groups similar to those of AsA-supplemented groups. At the end of the experimental period, subcutaneous hemorrhage was observed by autopsy in AsA-deficient group, but none of this phenomenon was observed in either AsA- or AA-2G-supplemented groups.

Alkaline phosphatase activity

Serum alkaline phosphatase activity of experimental groups is shown in Table 2. Alkaline phosphatase activity of guinea pigs supplemented with 5 mg AsA/day

Fig. 2. Body weight gains of guinea pigs supplemented with AsA and AA-2G. $\diamond$, group A-0.5, 0.5 mg AsA-supplemented group; $\blacklozenge$, group G-0.5, 0.96 mg AA-2G-supplemented group; $\triangle$, group A-1, 1 mg AsA-supplemented group; $\blacktriangle$, group G-1, 1.92 mg AA-2G-supplemented group; $\circ$, group A-5, 5 mg AsA-supplemented group; $\bullet$, group G-5, 9.6 mg AA-2G-supplemented group; $\square$, group A-100, 100 mg AsA-supplemented group; $\blacksquare$, group G-100, 192 mg AA-2G-supplemented group; $\times$, group A-0, AsA-deficient group.

Table 2. Serum alkaline phosphatase activity of guinea pigs supplemented with AA-2G or AsA.

Group ^{*1}	Alkaline phosphatase activity (mIU/ml)
A-0	53.4±29.4 ^a
A-0.5	145.6±22.7 ^b
G-0.5	142.3±11.9 ^b
A-1	156.8±12.2 ^b
G-1	166.2±15.0 ^b
A-5	167.8±11.1 ^b
G-5	168.5±24.6 ^b
A-100	166.2±10.2 ^b
G-100	178.2±18.0 ^b

Values are mean±SEM for 5 guinea pigs except group A-5 ($n=15$). Means within the same column not sharing a common superscript letter are significantly different ($p<0.05$). One unit of alkaline phosphatase activity is defined as the activity of enzyme that 1 μ mol of *p*-nitrophenol liberated from substrate per 1 min at 37°C.

^{*1} See Fig. 2 for group identification.

(group A-5) was about 168 mIU/ml, and there was no significant difference among the AsA-supplemented groups. The activity of AsA-deficient guinea pigs (group A-0) was 1/3 that of normal group (group A-5), while those of AA-2G-supplemented groups were higher than that of group A-0 and almost the same as that of group A-5.

Concentration of AsA and AA-2G in tissues and urine

Tables 3 and 4 show the concentration of AsA and AA-2G in the liver and adrenals of experimental groups. In AA-2G-supplemented groups, AA-2G was not found in the liver and adrenals, even when 192 mg AA-2G was supplemented (group G-100), although AsA was found in those tissues.

In AsA-supplemented groups, concentration of AsA in the liver and adrenals increased with increasing AsA dosage, and the same tendency was observed in AA-2G-supplemented groups. In the low (group G-0.5 and G-1) and normal (group G-5) AA-2G-supplemented groups, the AsA concentrations in the liver and adrenals were lower than the groups supplemented with the equimolar amount of AsA. However, in the case where excessive dose of AA-2G was supplemented to the guinea pigs (group G-100), the AsA concentrations in both the liver and adrenals seemed to be higher than in the guinea pigs supplemented with the equimolar amount of AsA (group A-100). Although differences were noticed on the AsA concentration in tissues among the AsA- and AA-2G-supplemented groups, the difference was not significant except in groups A-5 and G-5, where significant difference was observed at $p<0.05$ level.

Table 5 shows the amount of total AsA and AA-2G in urine of experimental

Table 3. Concentration of AsA and AA-2G in liver of guinea pigs supplemented with AA-2G or AsA.

Group* ¹	AsA	AA-2G
	nmol/g tissue wet wt.	nmol/g tissue wet wt.
A-0	trace	—
A-0.5	9.37±6.71 ^a	—
G-0.5	9.09±2.25 ^a	N.D.
A-1	36.33±10.27 ^{a,b}	—
G-1	11.26±3.17 ^a	N.D.
A-5	121.70±13.76 ^{c,d}	—
G-5	79.69±23.18 ^{b,c}	N.D.
A-100	310.62±38.20 ^d	—
G-100	355.37±39.45 ^d	N.D.

Values are mean±SEM for 5 guinea pigs except group A-5 ($n=15$). Means within the same column not sharing a common superscript letter are significantly different ($p<0.05$). N.D., not detected. *¹ See Fig. 2 for group identification.

Table 4. Concentration of AsA and AA-2G in adrenals of guinea pigs supplemented with AA-2G and AsA.

Group* ¹	AsA	AA-2G
	μmol/g tissue wet wt.	μmol/g tissue wet wt.
A-0	0.13±0.03 ^a	—
A-0.5	0.29±0.05 ^{b,c}	—
G-0.5	0.22±0.02 ^b	N.D.
A-1	0.56±0.16 ^{a,b,d}	—
G-1	0.43±0.05 ^{c,d}	N.D.
A-5	1.79±0.10 ^e	—
G-5	1.21±0.21 ^f	N.D.
A-100	2.75±0.21 ^{g,h}	—
G-100	3.13±0.59 ^{e,h}	N.D.

Values are mean±SEM for 5 guinea pigs except group A-5 ($n=15$). Means within the same column not sharing a common superscript letter are significantly different ($p<0.05$). N.D., not detected.

*¹ See Fig. 2 for group identification.

groups. In AA-2G-supplemented groups, AsA was found in urine, but AA-2G was not. The amount of total AsA in urine of AA-2G groups increased with increasing AA-2G dosage and was similar to that of equimolar amount of AsA-supplemented groups, except of group G-5.

DISCUSSION

It is reported that serum alkaline phosphatase activity decreased with decre-

Table 5. Amount of total AsA and AA-2G in urine of guinea pigs supplemented with AsA or AA-2G.

Group* ¹	AsA	AA-2G
	$\mu\text{mol/day}$	$\mu\text{mol/day}$
A-0	trace	—
A-0.5	$0.89 \pm 0.21^{\text{a,b,c}}$	—
G-0.5	$0.41 \pm 0.12^{\text{a}}$	N.D.
A-1	$1.08 \pm 0.54^{\text{a,d}}$	—
G-1	$0.71 \pm 0.38^{\text{a,e}}$	N.D.
A-5	$1.46 \pm 0.37^{\text{b,d,e}}$	—
G-5	$0.51 \pm 0.16^{\text{a}}$	N.D.
A-100	$2.09 \pm 0.35^{\text{d,f}}$	—
G-100	$1.69 \pm 0.31^{\text{c,d,e}}$	N.D.

Values are mean $\pm$ SEM for 5 guinea pigs except group A-5 ($n=15$). Means within the same column not sharing a common superscript letter are significantly different ($p < 0.05$). N.D., not detected. *¹ See Fig. 2 for identification.

asing vitamin C level in the tissues (18, 19). In this experiment, when graded doses of AA-2G were supplemented to guinea pigs, serum alkaline phosphatase activity was higher than that of AsA-deficient guinea pigs and was similar to that of 5 mg AsA-supplemented group (A-5, normal group). Also the body weight of guinea pigs in all AA-2G supplemented groups increased continuously, as in normal AsA-supplemented ones, although AsA-deficient animals stopped gaining weight from day 15 after supplementation. The results showed AA-2G might have the same effect on serum alkaline phosphatase activity and body weight gain as AsA.

Moreover, in all AA-2G-supplemented guinea pigs, AsA was found in the liver, adrenals and urine, but AA-2G was not, and their AsA concentration increased with increasing AA-2G dosage and was almost the same as that of guinea pigs which were supplemented with equimolar amount of AsA. These results suggested that AA-2G has the same vitamin C activity as AsA in guinea pigs.

Recently, Yamamoto *et al.* have demonstrated (25, 26) that AA-2G is hydrolyzed by α -glucosidase which exists in several tissues of guinea pigs, and, *in vitro*, the enzyme activity of intestinal homogenates was higher than that of any other tissues homogenates. Therefore, orally supplemented AA-2G might be hydrolyzed to AsA and glucose by α -glucosidase which exist in brush border of small intestine, and if AA-2G is absorbed as it is in its original form in small intestine, it might also be hydrolyzed easily by α -glucosidase which exists in tissues. Even in guinea pigs supplemented with the highest dose of AA-2G (192 mg of AA-2G; equivalent to 100 mg AsA) AA-2G was not detected in tissues and urine, but AsA was detected. Therefore, we might be able to say that AA-2G could show vitamin C activity as AsA only after hydrolysis to AsA by α -glucosidase.

Another AsA-derivative, L-ascorbic acid-2-O-phosphate (AA-2P), is also a stable form of AsA and has vitamin C activity as AsA in guinea pigs (7, 8), and this

vitamin C activity is explained by enzymatic hydrolysis (phosphatase) to AsA. However, AA-2P is expensive and it is not practical. Recently, Tanaka *et al.* (12) established a simple enzymatic synthesis method of AA-2G by using *Bacillus stearothermophilus* cyclodextrin glucanotransferase (CGTase). This enzymatic method for AA-2G formation is more applicable than chemical methods being applied for the synthesis of other stable AsA-derivatives (27–30) in terms of reaction steps, regiospecificity and cost.

In this study, it was clarified that AA-2G has the same vitamin C activity as AsA on a molar basis for orally supplemented guinea pigs. Therefore, it is hoped that this fact would be useful information as basic data for AsA supplement from the nutritional point of view.

CONCLUSION

In order to evaluate the vitamin C activity of 2-*O*- α -D-glucopyranosyl-L-ascorbic acid (AA-2G), various doses of AA-2G and their equimolar amounts of AsA were supplemented to guinea pigs.

All AA-2G-supplemented guinea pigs' body weight increased continuously and their serum alkaline phosphatase activity was also similar to that of 5 mg AsA-supplemented animals. AsA, but not AA-2G, was found in the liver, adrenals and urine of all AA-2G-supplemented guinea pigs, and the amount of AsA increased with increasing AA-2G dosage which was similar to that of AsA in guinea pigs supplemented with equimolar amount of AsA.

From our results, it was shown that AA-2G has the same vitamin C activity as AsA in guinea pigs.

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