Effect of Various Halide Salts on the Incompatibility of Cyanocobalamin and Ascorbic Acid in Aqueous Solution

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Combination of cyanocobalamin (VB12) and ascorbic acid (VC) has been widely seen in pharmaceutical products and dietary supplements. However, VB12 has been reported that its behavior in stability in aqueous solution is quite different when VC is mixed. In the present study, we examined the stabilities of these vitamins in acetate buffer (pH 4.8) using high performance liquid chromatography. Degradation of VB12 was not observed in the absence of VC in the buffer. However, when VC was mixed in the VB12 solution, VB12 concentrations decreased in accordance with VC degradation. VB12 and VC degradations were inhibited by adding sodium halides to acetate buffer at pH 4.8. These stabilization effects were also observed in the range from pH 3.5 to 5.3 and by adding potassium, magnesium, and calcium halides. Furthermore, our data demonstrated that increases in the halide anion concentrations and atomic number ($Cl^- < Br^- < I^-$) were proportionally associated with better stabilities of both VB12 and VC. Therefore, choosing an appropriate condition with a certain halide salt may be useful for stabilizing pharmaceutical products and dietary supplements when VB12 and VC are combined in solution.

Key words halide salt; cyanocobalamin; ascorbic acid; incompatibility; stabilization

Cyanocobalamin (VB12) and ascorbic acid (VC), both water-soluble vitamins, are essential nutrients involved in many physiological functions. These vitamins are extensively used in many pharmaceutical preparations and dietary supplements. An incompatibility of VB12 and VC in aqueous solutions has been reported.[1—7] However, it has not been fully elucidated how to stabilize these vitamins in a mixed solution yet. Many researchers have investigated the effect of VC on the stability of VB12 or cobalamin analogues in solution.[8—12] Most of them have focused only on the VB12 degradation, but not on VC. While Bartilucci *et al.*[6,7] have reported the interaction of VB12 and VC mixed condition in aqueous solutions evaluated by less sensitive methods, microbiological method for VB12 and titration for VC.

In the present study, we determined the stabilities of VB12 and VC in acetate buffer using high performance liquid chromatography (HPLC), which shows better accuracy and precision than microbiological method and titration. The effects of halide salts on the stabilities of these vitamins in the buffer were also studied for the improvement of their stability.

Experimental

Materials and Reagents VC and VB12 regulated by Japanese Pharmacopoeia were obtained from BASF Takeda Vitamins (Tokyo, Japan) and Aventis Pharma S.A. (Alsace, France), respectively. All other chemicals were purchased commercially. Water purified through a Milli-Q Labo (Millipore, Bedford, MA, U.S.A.) water system was used for all dilutions and sample preparations.

Determination of VB12 Using HPLC An LC-10AVP system (Shimadzu, Kyoto, Japan) equipped with SIL-10ADVP auto-injector, SPD-10AVVP detector, LC-10ADVP pump, DGU-14A degasser, CTO-10ACVP column oven and C-R6A data processor was used to determine VB12 in the solutions. The HPLC conditions were as follows: column, 150×4.6 mm TSK gel ODS-80TsQA (Tosoh, Tokyo, Japan); column temperature, 25 °C; detection wavelength, 361 nm; mobile phase, acetonitrile–50 mM acetate buffer, pH 4.5 (13 : 87, v/v); flow rate, 1.0 ml/min; injection volume, 50 μl. Sample mixtures were directly injected into HPLC.

Determination of VC Using HPLC An LC-10AVP system, the same composition system as described above, was used. The HPLC conditions were as follows: column, 150×6 mm YMC pack ODS-AQ312 (YMC, Kyoto, Japan); column temperature, 25 °C; detection wavelength, 290 nm; mobile phase, water–methanol–200 mM phosphate solution, pH 3.5 (8 : 1 : 1, v/v/v); flow rate, 1.0 ml/min; injection volume, 5 μl. Samples for HPLC analysis were prepared as follows: 0.3 ml of sample mixtures were transferred to vials for HPLC followed by adding 0.3 ml of 3% metaphosphoric acid containing 2% hydrochloric acid. The mixtures were analyzed immediately by HPLC.

Influence of VC on VB12 Stability Various concentrations of VC (0, 1, or 5 mM) with 0.25 μM VB12 in 100 mM ammonium acetate–acetic acid buffer (pH 4.8) were prepared in a 12-ml brown glass vial with cap, and the mixtures were kept at room temperature for 8 h. The concentrations of VB12 and VC were determined by HPLC after 2, 5, and 8 h of incubations.

Influence of NaCl on VB12 and VC Stabilities Various concentrations of NaCl (0, 50, 100, or 200 mM) with 0.25 μM VB12 and 5 mM VC in 100 mM acetate buffer (pH 4.8) were prepared in a 12-ml brown glass vial with cap, and the mixtures were kept at room temperature for 35 d. The concentrations of VB12 and VC were determined by HPLC after 1, 7, 21, and 35 d of incubations.

Effects of Sodium Halides on VB12 and VC Stabilities at Different pH The sample mixtures containing 0.25 μM VB12, 5 mM VC and 50 mM sodium halide (NaCl, NaBr, or NaI) were prepared in 100 mM ammonium acetate–acetic acid buffer (pH 3.5, 4.0, 4.5, 4.8, 5.0, or 5.3). These solutions were kept in a 12-ml brown glass vial with cap at room temperature for 48 h, and HPLC analyses were performed according to the methods as describe above.

Effects of Other Halide Salts on VB12 and VC Stabilities The mixture of 0.25 μM VB12 and 5 mM VC with 50 mM halides salts (sodium, potassium, magnesium, or calcium halides) in 100 mM acetate buffer (pH 4.8) were prepared in 12-ml brown glass vial with cap. The mixtures were kept at room temperature for 24 h, and HPLC analyses were performed according to the methods as describe above.

Results and Discussion

Influence of VC on VB12 Stability Influence of various concentration of VC (0, 1, or 5 mM) on the stability of VB12 at 0.25 μM in acetate buffer (pH 4.8) were measured by HPLC. The time course of the concentration of VB12 was shown in Fig. 1a, and the one of VC was shown in Fig. 1b. No degradation of VB12 was observed in the absence of VC in the buffer solution. However, when VC was added to the

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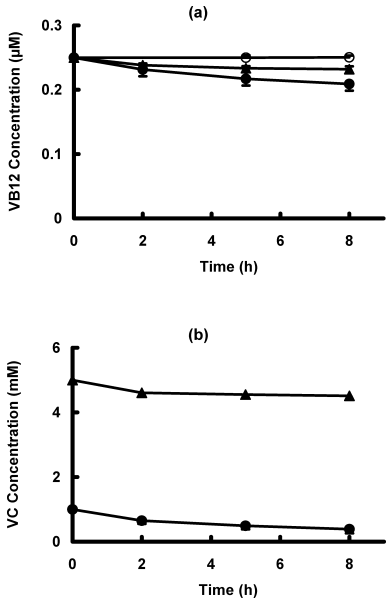

Fig. 1. Time Course Studies of the VB12 (a) and VC (b) in Acetate Buffer

Symbols: ○, without VC; ●, 1 mM; ▲, 5 mM. Various concentrations of VC (0, 1, or 5 mM) with 0.25 μM VB12 in 100 mM acetate buffer (pH 4.8) were prepared, and the mixtures were kept at room temperature for 8 h. The concentrations of VB12 and VC were determined by HPLC after 2, 5, and 8 h of incubation. Each value represents the mean±S.D. (n=3).

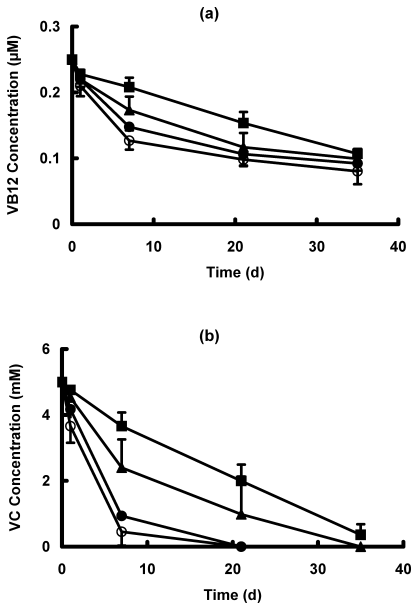

Fig. 2. Effects of NaCl Concentrations on VB12 (a) and VC (b) Stabilities

Symbols: ○, without NaCl; ●, 50 mM; ▲, 100 mM; ■, 200 mM. Various concentrations of NaCl (0, 50, 100, or 200 mM) with 0.25 μM VB12 and 5 mM VC in 100 mM acetate buffer (pH 4.8) were prepared, and the mixtures were kept at room temperature for 35 d. The concentrations of VB12 and VC were determined by HPLC after 1, 7, 21, and 35 d of incubation. Each value represents the mean±S.D. (n=3).

solution, VB12 concentrations decreased in accordance with VC degradation. There was a good correlation between the degradation of VB12 and VC (correlation coefficient more than 0.98). It has been previously reported that VC was decomposed by oxidation at higher pH than pK_{a1} (4.12).[13,14] Therefore, these data suggest that the oxidation of VC may interact with the VB12 degradation in mixed solution.

Influence of NaCl on VB12 and VC Stabilities Influence of various concentration of NaCl (0, 50, 100, or 200 mM) on VB12 and VC stabilities in acetate buffer (pH 4.8) were evaluated by HPLC. The time course of the concentration of VB12 was shown in Fig. 2a, and the one of VC was shown in Fig. 2b. NaCl inhibited both VB12 and VC degradations in a dose dependent manner. An inhibition of degradations of VB12 and VC was most significantly at 7 d with 200 mM NaCl. VB12 was only 17% decrease from the initial concentration compared to 49% reduction of control (without NaCl), and VC was only 27% reduction compared to 91% reduction of control.

Effects of Sodium Halides on VB12 and VC Stabilities at Different pH Since NaCl has significant impact on VB12 and VC stabilities, other sodium halides tested. Effects of several sodium halides (NaCl, NaBr, or NaI) on VB12 and VC stabilities in various pH level of acetate buffer (pH 3.5 to 5.3) were studied. Figure 3 shows VB12 and VC concentrations after kept with various sodium halides at room temperature for 48 h. When sodium halides were not added to the solutions, both VB12 and VC showed better stability at lower pH, due to existence of stable non-dissociated VC derived from pK_{a1} (4.12) in the solution. On the other hand, sodium halides inhibited both VB12 and VC degradations, and their stability was improved by increasing atomic number of halogen (Cl<Br<I) at even higher pH levels. Both VB12 and VC concentrations at pH 5.3 without sodium halide showed reductions, 20% and 46%, respectively. The stabilities of

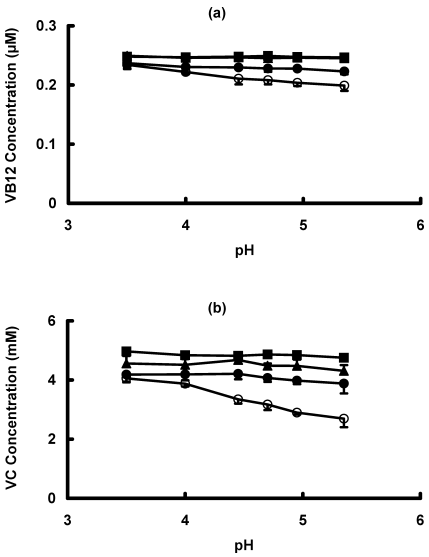

Fig. 3. Effects of Sodium Halides on VB12 (a) and VC (b) Stabilities at Different pH

Symbols: ○, without sodium halides; ●, NaCl; ▲, NaBr; ■, NaI. The sample mixtures containing VB12 (0.25 μM), VC (5 mM) and sodium halide (50 mM) were prepared in 100 mM ammonium acetate–acetic acid buffer (pH 3.5, 4.0, 4.5, 4.8, 5.0, or 5.3). These solutions were kept at room temperature for 48 h, and then evaluated by HPLC analysis. Each value represents the mean±S.D. (n=3).

both vitamins at pH 5.3 was significantly improved by adding NaI, and increased 19% of VB12 and 41% of VC concentrations compared to the control (without sodium halide).

Effects of Other Halide Salts on VB12 and VC Stabilities Furthermore, in order to confirm the above-described in detail, effects of other halide salts such as potassium, calcium, or magnesium halides on VB12 and VC stabilities in acetate buffer (pH 4.8) were also investigated. Table 1 shows VB12 and VC concentrations after kept with various halide salts at room temperature for 24 h. When halide salts were

Table 1. Comparison of the Stability of VB12 or VC under the Various Halide Salt Conditions in the Mixed Solution[a)]

Salt	VB12 concentration (μM)	VC concentration (mM)
Control	0.225±0.006	4.22±0.13
NaCl	0.238±0.002	4.40±0.07
NaBr	0.247±0.002	4.70±0.20
NaI	0.250±0.002	4.93±0.06
KCl	0.238±0.009	4.51±0.15
KBr	0.250±0.004	4.69±0.14
KI	0.250±0.003	4.92±0.08
$MgCl_2$	0.243±0.004	4.67±0.15
$MgBr_2$	0.244±0.005	4.82±0.11
MgI_2	0.246±0.002	4.88±0.06
$CaCl_2$	0.235±0.004	4.56±0.12
$CaBr_2$	0.243±0.005	4.71±0.16
CaI_2	0.245±0.002	4.89±0.09

a) The mixture of 0.25 μM VB12 and 5 mM VC with 50 mM halides salts in 100 mM acetate buffer (pH 4.8) were kept at room temperature for 24 h. The concentrations of VB12 and VC were determined by HPLC. Each value represents the mean±S.D. (n=3).

not added to the solution, both VB12 and VC concentrations showed reductions, 10% and 16%, respectively. However, all halide salts used in this experiment inhibited both VB12 and VC degradations, and the stabilities were proportionally associated with higher molecular weights of halide salts, shown in order of Cl<Br<I. There was a consistent relationship between VB12 and VC stabilities with existence of halide salts. These results indicated that halide anions and their atomic number were significantly related with the stabilities of both vitamins in solution.

Conclusion

Incompatibility of VB12 and VC in acetate buffer was studied using HPLC. In the presence of VC, the decrease of VB12 was observed with VC degradation in acetate buffer at pH 4.8. However, adding sodium halides to the buffer inhibited VB12 and VC degradations. These stabilization effects were also observed in the range from pH 3.5 to 5.3 and by adding potassium, magnesium, and calcium halides. These data demonstrate that increases in the halide anion concentrations and atomic number ($Cl^-<Br^-<I^-$) showed better stabilities of VB12 and VC in acetate buffer. Therefore, choosing an appropriate condition with a certain halide salt may be useful for stabilizing pharmaceutical products and dietary supplements when VB12 and VC are combined in solution.

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