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Short communication

Simultaneous determination of vitamin C and its carbamylated derivatives by high-performance liquid chromatography with post-column derivatization

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Abstract

A highly sensitive method for the simultaneous determination of ascorbate (AsA), dehydroascorbate (DHA), 2,3-diketogulonate (2,3-DKG), carbamyl ascorbate (CAA) and carbamylated dehydroascorbate derivative (CDA) was developed by high-performance liquid chromatography with post-column derivatization. The successful separation of these substances was achieved by an adsorption chromatography using poly(ethylene glycol) copolymer as a packing material in the separation column. For the detection, each substance was boiled with benzamidine in alkaline solution, producing fluorescence products. Both CAA and CDA were alkaline-labile, degrading to AsA and 2,3-DKG, so that these carbamylated derivatives could be detected in a similar manner as AsA and 2,3-DKG, respectively. The detection limits for quantitative determination of these substances were less than 0.5 μM, and the coefficients of variation of the peak areas were in the range of 2.2 to 2.8%. The usefulness and practicability of the present method were verified by application to the determination of these substances in plant leaves soaked in 0.5 *M* sodium cyanate solution. © 1998 Elsevier Science B.V.

Keywords: Vitamins; Ascorbate; Dehydroascorbate; 2,3-Diketogulonate; Carbamyl ascorbate

1. Introduction

Cyanate is widely known to prevent the physiological reactions in organisms including plants and mammals as a herbicide [1,2] and an uremic toxin [3–5], respectively. Cyanate is electrophilic, and thus it has been studied as an agent for carbamylation of amino groups and sulfhydryl groups [6–11]. Recently, we found a fact that cyanate irreversibly reacts with both ascorbate (AsA) and dehydroascorbate (DHA) in neutral solution at ordinary temperature, resulting in the production of carbamyl ascorbate (CAA) [12] and carbamylated dehydroascorbate derivative (CDA) [13], respectively (Scheme 1). The AsA–DHA redox cycle is one of antioxidant systems, which protect organisms from oxidative stress. This fact coupled with our finding suggests that, if the extent of these reactions are greatly increased in vivo, organisms would be significantly damaged through a declining of the antioxidant system [12].

In the present study, we established a method for simultaneous determination of AsA, DHA, 2,3-DKG, CAA and CDA by means of high-performance liquid chromatography (HPLC) with fluorometric post-column derivatization using benzamidine as a fluorescence reagent, in which CAA and CDA were de-

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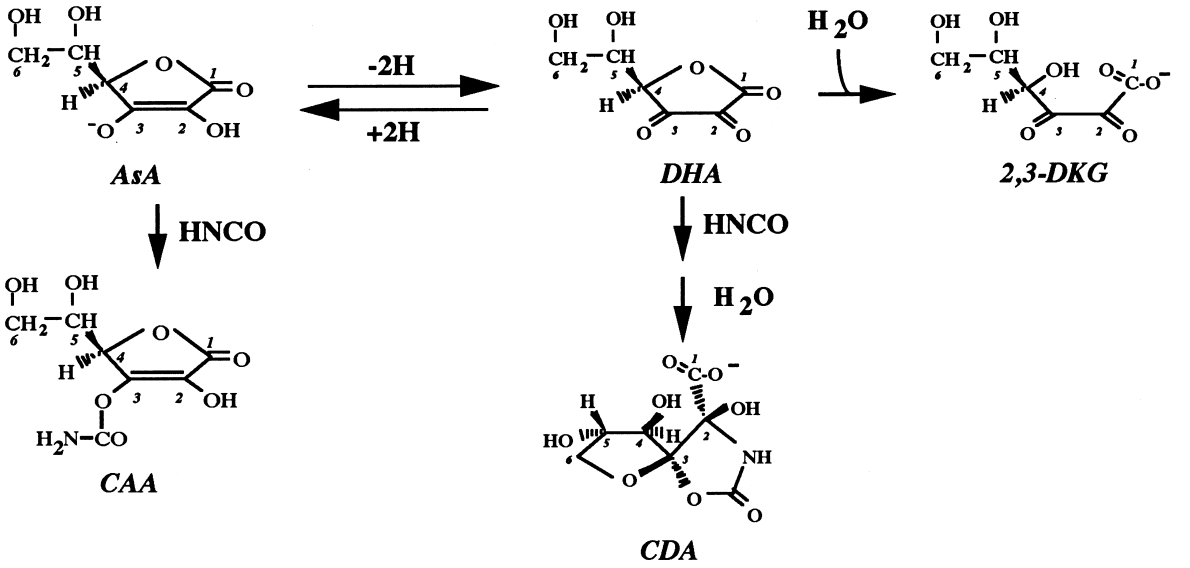


Scheme 1. Reactions of cyanate with AsA and DHA.

graded to AsA and 2,3-DKG, respectively, and subsequently converted to fluorescence products.

2. Experimental

2.1. Materials

Ascorbic acid was purchased from Wako Pure Chemicals (Osaka, Japan). Dehydroascorbic acid was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were of reagent grade. Asahipak GS-320 7E (250×7.6 mm I.D.) was purchased from Showa Denko (Tokyo, Japan). A standard solution of dehydroascorbate (100 m*M*) was prepared as follows: dehydroascorbic acid was dissolved in 0.1 *M* acetate buffer (pH 4.0), and the solution was standardized by HPLC using solution of known ascorbic acid concentration as standard. Peak response of dehydroascorbate in the solution was compared with that of dehydroascorbate obtained from a standard solution of ascorbic acid after oxidation in 0.1 *M* acetate buffer containing 10 m*M* cupric sulfate for 1 h at room temperature.

2.2. Apparatus

The HPLC assembly consisted of a HPLC pump (Hitachi; L-6000), a sample injector (Rheodyne; 7725), a double-plunger pump (Shimamura Instrument, Tokyo, Japan; PSU-2.5W), a dry reaction bath (Shimamura Instrument; DB-5), a fluorescence spectrophotometer (Hitachi; F-1050) and a chromatointegrator (Hitachi; D-2500).

2.3. Syntheses of carbamylated derivatives

2.3.1. Carbamylated dehydroascorbate derivative [13]

Dehydroascorbate was prepared from ascorbic acid by oxidation using cupric ion. Ascorbic acid powder (52 g) was added to 2 l of 0.15 *M* copper(II) acetate solution, and then the reaction solution was stirred vigorously at room temperature. After standing for 10 min, 1 l of 0.75 *M* sodium cyanate solution was added dropwise to the solution, and the reaction mixture was stirred overnight. The reaction mixture was filtered through filter paper (Advantec Toyo; 51A), and the filtrate was passed through Dowex 1-X8 (Cl^- form, 50–100 mesh, 10×5 cm I.D.). The eluate was concentrated to 200 ml by evaporation, and the solution was added dropwise to 800 ml of 99% ethanol. The CDA precipitate was recovered by filtration. The precipitate was dissolved in water, and the volume was adjusted to 200 ml. CDA in the solution was crystallized by mixing with

800 ml of 99% ethanol. CDA was recrystallized four times from ethanol–water. CDA has the molecular formula, $C_7H_8NO_8Na$, and contains two water molecules of crystallization. The result of the elemental analysis was as follows; calculated for $C_7H_8NO_8Na \cdot 2H_2O$: C, 28.70%; H, 4.09%; N, 4.78%; O, 54.61%; Na, 7.85%. Found: C, 28.70%; H, 3.89%; N, 4.55%; Na, 7.70%; other elements, 55.16%.

2.3.2. Carbamyl ascorbate [12]

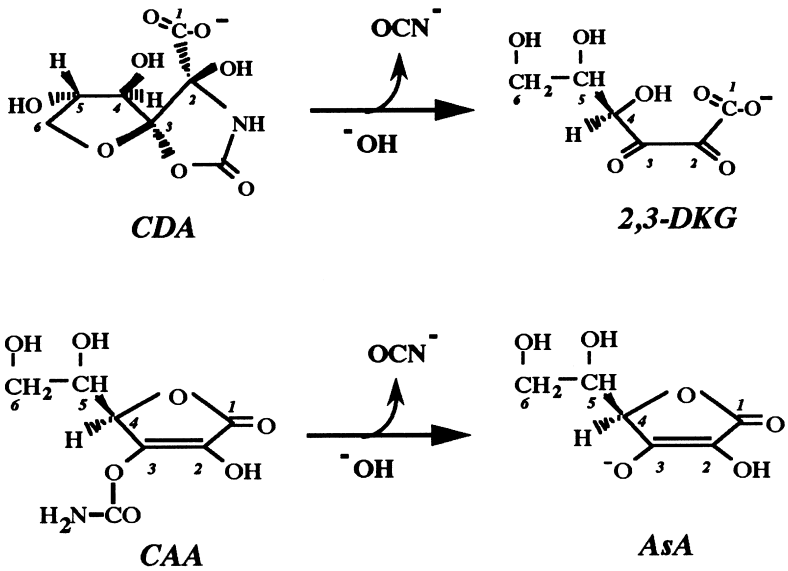
Ascorbic acid powder (52 g) and sodium cyanate (48.8 g) were dissolved in 1.0 l of water, and then the solution was adjusted to pH 6.0 by acetic acid. The solution was maintained at a pH in the range of 5.5 to 6.0 by acetic acid for 3 h at room temperature with stirring by magnetic stirrer. The reaction solution was concentrated to 150 ml by evaporation, and then the solution was left in an ice-water bath overnight. The solution was filtered through filter paper (Advantec Toyo; 51A), and the filtrate was added dropwise to 300 ml of 80% ethanol. Furthermore, the mixture solution was added dropwise to 1.2 l of acetone with stirring. CAA was separated from solvent as a colorless oily liquid. The oily phase was washed with 500 ml of acetone three times, and then dried in vacuo.

2.3.3. Simultaneous determination of AsA, DHA, 2,3-DKG, CAA and CDA

AsA, DHA, 2,3-DKG, CAA and CDA were determined by HPLC with post-column reaction fluorometric detection using the following chromatographic conditions: column, Asahipak GS-320 7E; eluent, 17 m*M* acetic acid containing 0.5 m*M* EDTA (1.0 ml/min). The post-column reaction conditions were as follows; reagent 1, 0.02 *M* benzamidine solution (0.25 ml/min); reagent 2, 0.75 *M* borate buffer containing 0.2 *M* potassium sulfite, pH 10.5 (0.25 ml/min); reaction temperature, 100°C; reaction time, 1.5 min; detection, fluorescence spectrophotometer (Ex. 325 nm, Em. 400 nm).

3. Results and discussion

For most fluorometric detections of sugars, they are commonly boiled with reagent in alkaline solution. Advantageously, CAA and CDA are alkaline-labile, degrading to AsA and 2,3-DKG, respectively (Scheme 2). Benzamidine is a popular reagent for fluorometric detection of sugars including AsA, DHA and 2,3-DKG [14]. Taken together, these facts suggest that it is possible to detect AsA, DHA, 2,3-DKG, CAA and CDA fluorometrically and



Scheme 2. Degradation of CAA and CDA in an alkaline solution.

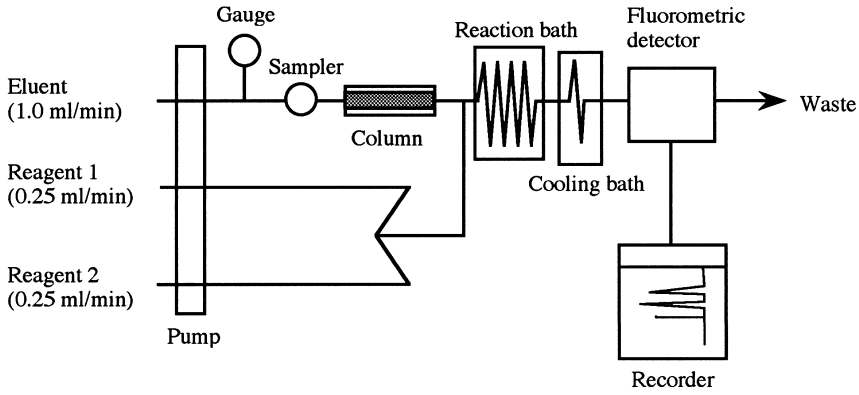


Fig. 1. Schematic diagram of HPLC for the determination of AsA, DHA, 2,3-DKG, CAA and CDA. Chromatographic conditions as described in Section 2.

simultaneously by boiling them with benzamidine in alkaline solution. A schematic diagram of the post-column derivatization system for HPLC is shown in Fig. 1. The relative fluorescence intensities of reaction products obtained from these substances via flow injection analysis are shown in Table 1. The response of CDA was almost similar to that of 2,3-DKG.

The chromatographic separation of AsA, DHA, 2,3-DKG, CAA and CDA was studied. As for ion-exchange chromatography and partition chromatography, a satisfactory result was not obtained. In contrast, a successful separation of these compounds was achieved by an adsorption chromatography using poly(ethylene glycol) copolymer as a packing material in the separation column. Asahipak GS-320 7E is a column packed with poly(ethylene glycol) copolymer. A chromatogram of standard substances is shown in Fig. 2A. The detection limits ($S/N=3$) were as follows: DHA, 0.1 μM; CAA, 0.4 μM; the others, 0.2 μM. In order to confirm the reproducibilities of the peak areas of these substances, the standard solutions (10 μM) were injected repeatedly five times, respectively. The coefficients of variation of the peak areas of DHA, AsA, 2,3-DKG, CDA and CAA were 2.8, 2.5, 2.2, 2.5 and 2.7%, respectively. The correlations between peak responses and the concentrations were found to be linear in the following ranges: DHA, 0.3–100 μM; CAA, 1.0–200 μM; the others, 0.5–100 μM.

Effect of cyanate on the AsA–DHA redox cycle in leaves of *Erigeron canadensis*, the most common weed in our campus, was studied. In the leaves of *Erigeron canadensis* exposed to sunlight, the concentration of DHA was higher than that of AsA. The leaves of *Erigeron canadensis* were soaked in 0.5 *M* sodium cyanate solution (0.5 *M* sodium cyanate solution is generally sprinkled on plants as herbicide)

Table 1
Relative fluorescence intensities of reaction products obtained from AsA, DHA, 2,3-DKG, CAA and CDA by boiling with benzamidine in alkaline solution

Substance	Concentration (μM)	Relative fluorescence intensity
Ascorbate	10	100
Dehydroascorbate	10	247
2,3-Diketogulonate	10	112
Carbamylated dehydroascorbate derivatives	10	109
Carbamyl ascorbate	10	34
Ascorbate-2-phosphate	10	0

Relative fluorescence intensities of reaction products were determined via flow injection analysis. The post-column reaction conditions are described in Section 2.

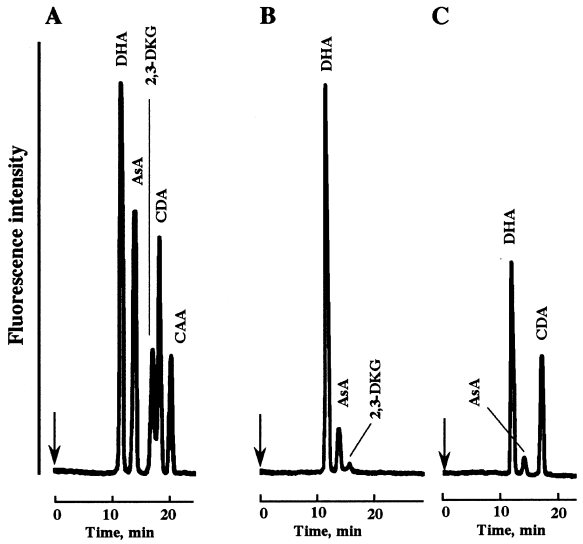


Fig. 2. Analysis of AsA, DHA, 2,3-DKG, CDA and CAA in cyanate-treated leaves of *Erigeron canadensis*. Chromatographic conditions as described in Section 2. (A) Mixture solution of AsA (10 μ*M*), DHA (5 μ*M*), 2,3-DKG (5 μ*M*), CDA (10 μ*M*) and CAA (20 μ*M*). The leaves of *Erigeron canadensis* were soaked in 0.5 *M* sodium cyanate solution at room temperature for 30 min. The non-treated leaves (B) and cyanate-treated leaves (C) were homogenized in 1% (w/v) metaphosphoric acid, and then the homogenate was centrifuged at 5000 *g* for 30 min. The supernatant was subjected to HPLC.

at room temperature for 30 min. The leaves were homogenized in 1% (w/v) metaphosphoric acid, and then the homogenate was centrifuged at 5000 *g* for 30 min. The supernatant was submitted to HPLC. Chromatograms are shown in Fig. 2B,C. By soaking the leaves in the sodium cyanate solution, the DHA level was significantly lowered with producing equimolar amounts of CDA. In this experiment, CAA was not detected in the plant leaves. When cyanate penetrated into plant leaves, the reaction of cyanate with DHA preceded that with AsA.

The present method is sensitive and reliable for the simultaneous determination of AsA, DHA, 2,3-DKG, CAA and CDA in plants, and makes it possible to study the interaction of cyanate with C vitamers.

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