Determination of L-Ascorbic Acid in Tomato by Isotachoelectrophoresis. Application of Computer Simulation System for Setting of Determination Conditions to Avoid the Mixed-Zone Problem

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Summary When the isotachoelectrophoretical method is used with HCl-β-alanine as the leading electrolyte solution (pH 3.6), ascorbic acid forms a mixed zone with glutamic acid, and it is difficult to separate the two. Therefore, the application of a computer simulation system to separate the two acids was investigated.

This system can be used with compounds for which the absolute electrophoretical mobility and dissociation constant have already been determined. Values for glutamic acid alone were entered in the data bank “SIPS-1,” and then those for ascorbic acid were estimated from the values of similar compounds. Values for aspartic acid were used, and the electrophoretical behavior of ascorbic and glutamic acids was simulated at pH range from 3 to 9.9. Based on this simulation, separation appeared possible at pH 9.0 to 10.0, and indeed, experimental determination of ascorbic acid was successfully performed with a leading electrolyte solution composed of HCl-ethanolamine (pH 9.4) without any glutamic acid interference.

Even though ascorbic acid is highly susceptible to oxidation in an alkaline medium, because the ascorbic acid solution is injected at the interface of the leading and terminal electrolyte solutions in the electrophoretical procedure, it does not come in contact with air during electrophoresis. This explains why ascorbic acid can be determined in an alkaline medium.

Key Words: ascorbic acid, glutamic acid, isotachoelectrophoresis, mobility (Mo), computer simulation system, mixed-zone, tomato, leading electrolyte, terminal electrolyte

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The determination of L-ascorbic acid (AsA) is usually performed by either the 2,6-dichlorophenolindophenol or the 2,4-dinitrophenylhydrazine (DNP) method, although it is recognized that interference can occur due to contamination if either compound has the same redox potential as that of the AsA-dehydroascorbic acid (DHA) system or carbonyl compounds, such as sugars. Analysis of diketogulonic acid (DKG), one of the oxidized products of AsA, is also difficult. Mills et al. (1) applied the DNP method to DKG analysis. After reducing DHA in the sample solution with H₂S gas, the residual compound reactive with DNP was designated as DKG. However, the transformation of a part of the DHA to DKG during the reduction procedure with H₂S was thought to have caused a degree of error.

The isotachoelectrophoretical method has been successfully employed for the determination of AsA and DKG. In our previous report (2), 0.01 N HCl-β-alanine (pH 3.6) was used as the leading electrolyte and 0.01 N n-caproic acid as the terminal electrolyte. For the identification of compounds (RE), the ratio of potential gradient of the sample zone in the chart (RS) to that of the leading zone (RL) was employed (3), \( \frac{RE}{RL} = \frac{R_S}{R_L} \).

With this method, AsA and DKG in sample solutions were determined successfully, but in tomato juice, the AsA content obtained by the isotachoelectrophoretical method was quite a lot higher than that obtained by the DNP method, even though the recovery of added AsA was sufficient. It was assumed that a mixed zone of AsA and contaminants was formed (4).

In the isotachoelectrophoretical procedure, the degree of resolution of compounds with similar electrophoretic behavior is dependent on the selection of the electrolytic system, especially the nature of the buffer solutions and their pH values (5). Recently a computer simulation method was developed for the prediction of optimum separation conditions. This method (6,7) can simulate the electrophoretical behavior of any compound if its absolute electrophoretic mobility (Mo) and dissociation constant (pKₐ) are known.

In this report, the isotachoelectrophoretic behavior of a mixed solution of AsA and Glu was investigated using this method.

MATERIALS AND METHODS

Reagents. Ascorbic, glutamic and n-caproic acids, ethanolamine, hydroxypropyl methyl cellulose (HPC) and other reagents were purchased from Wako Chemical Co., Ltd. and all were of reagent grade.

Instruments: Isotachoelectrophoretic analyzer: Shimadzu Co., Ltd., Type IP-1B.

High performance liquid chromatograph: Nihon Bunkou Co., Trirrotar III, with the following operating conditions: column: Showdex OH pak M-614 (4.6φ × 250 mm) column; solvent: 0.055 M Na₂HPO₄ + 0.045 M KH₂PO₄ + 7% CH₃OH; flow rate: 0.8 ml/min; pressure: 3 kg/cm²; detector: UV-254 nm; chart speed: 5 mm/min; range: 1.28.
DETERMINATION OF VITAMIN C

Computer simulation data bank: “SIPS-1,” developed by Hirokawa and Kiso (3).

Personal computer: NEC-PC 9801-E and PC-PR 201CL printer.

The preparation of tomato juice and the determination of AsA by the 2,4-dinitrophenylhydrazine method were the same as in a previous report (4).

RESULTS AND DISCUSSION

Mo and pKₐ values of about 500 kinds of ionic compounds have been registered in the “SIPS” data bank which allows simulation of the isotachopherogram of these compounds in different electrophoretical buffer systems.

For the effective mutual separation of ionic compounds, the leading electrolyte is usually chosen so that its pH (pHₐ) produces the largest difference in Mo of the compounds. The pHₐ can be computed for compounds with known Mo and pKₐ values. But if the Mo and pKₐ values of an ionic compound are not known, the pHₐ value of a closely related ionic compound can usually be substituted.

Although the values for glutamic acid were entered in the data bank, no information about the AsA Mo was available. Since aspartic acid resembles AsA, these data were retrieved from the data bank and employed. The pKₐ values of AsA are as follows: pKₐ₁=4.17 (8) or 4.25 (9); pKₐ₂=11.57 (8).

Employing pKₐ₁ and pKₐ₂ values of 4.17 and 11.57, respectively, and the Mo values of aspartic acid (29.8 and 56.5), the isotachopherograms were simulated over the pH range from 3 to 6.5. For this simulation, Cl⁻ was employed as the leading ion, and the following counter ions were used: glycylglycine (pH 3.0), β-alanine (pH 3.6), creatinine (pH 4.5), and histidine (pH 6.0 and 6.5), and for the terminal ion, n-caproic acid was chosen due to its low mobility compared to other ions in the sample solutions.

In theory, it should be possible to separate AsA and Glu in the pH range from 3 to 4.5, but in practice, the isotachopherogram of AsA and Glu at pH 3.6 with β-alanine as the counter ion showed the formation of a mixed zone of these compounds. The same was seen at pHₐ of 3.0 and 4.3. The reason for this discrepancy was thought to be an error in the Mo value employed in the simulation procedure.

To clarify this point, it became necessary to determine the mobility of AsA where the RE of AsA and Glu would become the same or nearly the same as the assumed Mo value. At pH 3.6 (β-alanine), the mobility of AsA was investigated by the simulation method. Two sets of AsA pKₐ values were used: pKₐ₁=4.17, pKₐ₂=11.57 and pKₐ₁=4.25 and pKₐ₂=11.57. The simulated relationships between RE and Mo are shown in Fig. 1. As the RE of Glu under such conditions was 6.19, the Mo of AsA at the cross points of RE of AsA and Glu was 24.2 for the first system and 25.8 for the second system.

Using the former pKₐ values (pKₐ₁=4.17, pKₐ₂=11.57) and a tentative Mo (24.0), three-dimensional isotachopherograms were simulated between pH 3 and 9.9.
Fig. 1. Simulated relationship between $R_E$ and Mo of ascorbic acid. ○: $pK_1$, 4.17 and $pK_2$, 11.57; ●: $pK_1$, 4.25 and $pK_2$, 11.57; pH: 3.6.

Fig. 2. Simulated three-dimensional isotachopherograms of glutamic, ascorbic and caproic acids at pH range 3 to 6.5. For ascorbic acid, the simulated Mo values employed were: 24.0 ($z$, −1; $pK$, 4.17) and 48.0 ($z$, −2; $pK$, 11.57).

(Figs. 2 and 3). In this simulation, the following counter ions were employed: glycylglycine (pH 3.0), β-alanine (pH 3.6), creatinine (pH 4.5), histidine (pH 6.0 and 6.5), imidazol (pH 7.5), amediol (pH 8.5 and 9.0) and ethanolamine (pH 9.4 and 9.9). Yagi (10) reported that, for the separation of compounds having similar $R_E$ values, a minimum difference of 0.25 in the $R_E$ value was necessary, and in practice, more than 0.5 was recommended. From pherograms in an alkaline medium with pH greater than 9.0, it was assumed that these compounds would separate; at pH 9.4 the $R_E$ difference between two was over 0.5.

From these relations, the relative mobility of AsA and Glu in various pH ranges could also be simulated (Fig. 4). Figure 4 shows the difficulty of separating...
Fig. 3. Simulated three-dimensional isotachopherograms of glutamic, ascorbic and caproic acids at pH range 7.5 to 9.9. Mo values for ascorbic acid were the same as in Fig. 6.

AsA and Glu in an acidic medium, but at pH greater than 9.0, greater separation was possible. The same pattern was seen when the second set of pK_a values and a tentative AsA Mo value of 25.8 were substituted.

The isotachopherogram of this mixed solution of AsA and Glu is shown in Fig. 5, simulated with pK_{a1} = 4.17. Using pK_{a1} = 4.25, a similar figure was obtained. From these results, the R_E of AsA was simulated at 3.41 and 3.16 to correspond to pK_{a1} values of 4.17 and 4.25, respectively, and the R_E of Glu used was 2.25.
Fig. 5. Simulated isotachopherograms of mixed solution of glutamic and ascorbic acids. Simulated conditions: pK and Mo of ascorbic acid: pK"1=4.17; pK"2=11.57; Mo: 24.0, 48.0; pH**: 9.4; electric current: 100 µA.

Table 1. Isotachoelectrophoresis conditions for analysis of ascorbic acid.

| Leading electrolyte | Concentration | Counter ion | pH | Terminal electrolyte | Concentration | pH | Electric current (µA) | Additive | Capillary tube | Controlled temperature (°C) |
|---------------------|---------------|-------------|----|----------------------|---------------|----|----------------------|----------|---------------|-----------------------------|
| Cl"-                | 0.01 N        | Ethanolamine| 9.4| β-Alanine            | 0.01 N        | 10.0| 100                  | HPC 0.1% | "Φ 0.5 mm, 20 cm" | 20                           |

AsA is easily oxidized in an alkaline medium, and the presence of heavy metal ions such as Cu²⁺ or Fe³⁺ accelerates this oxidation. Thus, even though the separation of AsA and Glu at pH 9.4 is possible under simulated conditions, this is not useful from a quantitative viewpoint. In confirmation, the determination of AsA in a solution adjusted to pH 9.3 with Na₂HPO₄, KH₂PO₄ and ethanolamine was carried out by high performance liquid chromatography (HPLC), and the results showed that AsA is labile in alkaline medium.

Even though AsA solution is labile in an alkaline medium, the isotachoelectrophoresis was undertaken in the conditions listed in Table 1. The isotachopherogram (Fig. 6) showed perfect separation of Glu from AsA, and also, as shown in Fig. 7,
Fig. 6. Isotachoelectrophoresis of glutamic and ascorbic acids at pH 9.4.

Fig. 7. Linear relationship between ascorbic acid concentration and zone length.

there was a linear relationship between the concentration of AsA and the zone width. This relationship was calculated by the least squares method and the following equation was obtained:

\[ y = 0.50x - 0.26. \]

When the determination of AsA was made at pH 3.6, the equation obtained by the same method was as follows (2):

\[ y = 0.50x + 0.05. \]

Both equations had the same slope.

In isotachoelectrophoresis, the sample solution is injected at the interface of the leading and the terminal electrolyte solutions and a current is passed through both electrodes, while in electrophoresis, the AsA solution migrates inside a small tube so that the AsA solution never comes in contact with air. This explains why AsA is not
The AsA content of tomato juice was determined by this electrophoretical method, and the separation of AsA from Glu was further confirmed (Fig. 8). The AsA contents of some vegetables (Table 2) was compared using DNP and isotachoelectrophoresis run with both acidic and alkaline media. The results clearly indicate that the determination of AsA can be performed by isotachoelectrophoresis even in an alkaline medium.

With the electrophoresis of tomato juice, peaks of organic acids with greater mobility than AsA appeared before the AsA peak, meaning that a longer time was required. Kaniansky and Everaerts (11) proposed the application of complex salts to electrophoresis to assist in the separation of compounds. Yagi (12) applied this method to the determination 5'-nucleotides, such as UMP, IMP and GMP, and found that separation was achieved with complexes with Cu²⁺ or Ni²⁺. Thus, by adding 2 mM of Ca²⁺ to the leading electrolyte solution, these organic acids were transformed into their complex salts and their mobility became less than that of

Table 2. Comparison of determination methods for ascorbic acid contents in foods.

| Sample                  | Hydrazine method | I.P. method pH 3.6 | I.P. method pH 9.4 |
|-------------------------|------------------|--------------------|--------------------|
| Green pepper            | 103.1            | 103.6              | 103.0              |
| Tomato                  | 21.3             | 212.1              | 21.4               |
| 100% Orange juice       | 28.2             | 30.0               | 30.3               |
| 50% Orange juice        | 10.9             | 10.0               | 10.3               |

oxidized even in a highly alkaline medium. The AsA content of tomato juice was determined by this electrophoretical method, and the separation of AsA from Glu was further confirmed (Fig. 8). The AsA contents of some vegetables (Table 2) was compared using DNP and isotachoelectrophoresis run with both acidic and alkaline media. The results clearly indicate that the determination of AsA can be performed by isotachoelectrophoresis even in an alkaline medium.
AsA. This method shortened the determination time, and the addition of Ca$^{2+}$ did not affect the determination of AsA.

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