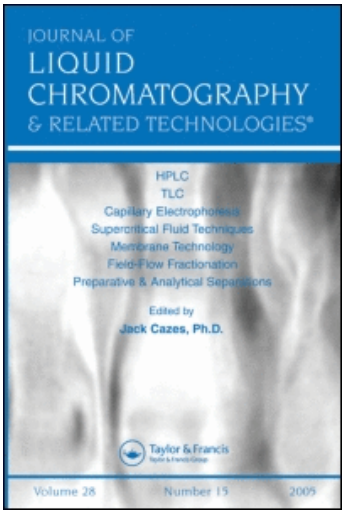

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SIMULTANEOUS ASSAY OF INORGANIC PHOSPHATES AND 2-POLYPHOSPHATE ESTERS OF L-ASCORBATE BY HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

High performance anion exchange chromatography with conductivity detection (HPAEC-CD) was used to separate and assay 2-mono-, 2-di-, and 2-triphosphate esters of L-ascorbic acid and four inorganic phosphates. The separation was performed on a Dionex AS-11 analytical column with a linear gradient of 20 mM to 80 mM aqueous sodium hydroxide in 10 min. The sensitivities (detection limits) were about 0.1-0.15 μg/mL (2-3 ng) for the inorganic phosphates and 0.2 μg/mL (4 ng) for each 2-phosphate ester of L-ascorbic acid, all calculated as free-acid forms. The method was applied to determine the major components in a commercial phosphorylated L-ascorbate.

INTRODUCTION

The instability of L-ascorbic acid (AsA) to oxygen and acidity in foods and feeds has led to the development of derivatives of AsA with enhanced stability. The 2-phosphorylated derivatives of AsA[1-4] are especially useful because they are equivalent in vitamin C potency to AsA.[5-7] One method to produce the 2-phosphorylated derivatives is to react AsA with 1.3 equivalents of sodium trimetaphosphate (STMP) in alkali at pH 10.7.[4,8] The predominant products are L-ascorbate 2-triphosphate (AsTP) and ortho- and pyrophosphates. However, upon storage at 25°C, the pH of the reaction mixture decreases gradually to 6.3, and AsTP undergoes hydrolysis to orthophosphate plus L-ascorbate 2-di- and 2-monophosphates (AsDP and AsMP). The latter ester resists both acid- and alkali-catalyzed hydrolysis. Thus AsA phosphorylated with STMP is a mixture of inorganic and organic phosphates.[8]

Reverse-phase high performance liquid chromatography with ultraviolet (UV) detection has been used previously to monitor the organic products when AsA was reacted with STMP.[4,8,9] In this paper, we describe a method of high performance anion exchange chromatography with conductivity detection (HPAEC-CD) to separate and assay simultaneously inorganic phosphates and 2-phosphate esters of L-ascorbate.

This method was applied to a commercial product of phosphorylated L-ascorbate that is used to fortify feeds with vitamin C.

MATERIALS

Deionized water purified with a Milli-Q system (Millipore, Bedford, MA) was used for the preparation of eluting standard, and sample solutions. All chemicals were analytical-reagent grade unless mentioned otherwise. Sodium hydroxide (50% solution) was obtained from Fisher Scientific Chemical Company (Pittsburgh, PA), and disodium phosphate (99.0% pure), tetrasodium pyrophosphate (99.0% pure), hexasodium tripolyphosphate (98.0% pure), and trisodium trimetaphosphate (95.0% pure) were purchased from Sigma Chemical Company (St. Louis, MO). L-Ascorbate 2-monophosphate (AsMP) magnesium salt (minimum 85% purity) was from Sigma (St. Louis, MO), and tricyclohexylammonium L-ascorbate 2-monophosphate (ASMP) (m.p. 174-176°C), tetracyclohexylammonium L-ascorbate 2-diphosphate (AsDP) (m.p. 148-150°C), and pentacyclohexyl ammonium L-ascorbate 2-triphosphate (AsTP) (m.p. 146-148°C) were prepared by the methods of Wang et al.[4]

Those cyclohexylammonium salts had been stored at 5°C in a desiccator for 18 months prior to use. A commercial phosphorylated L-ascorbate (ROVIMIX STAY C, Roche, Basel) was obtained from a commercial feed mill.

METHODS

The HPAEC-CD system was equipped with a gradient pump (GMP-2), a PED-II detector (conductivity mode, Dionex, Sunnyvale, CA, USA), and a 20 μL volume injector (Rheodyne 7010 valve, Cotati, CA). Anions were separated on an AS11 analytical column (4 X 250 mm) protected by an AG11 guard column (4 X 50 mm; Dionex, Sunnyvale, CA, USA). The eluting solutions were water and 200 mM NaOH, which were combined to form a linear gradient of aq. NaOH from 20 mM to 80 mM over a 10 min period at a flow rate of 2 mL/min. An Anion Self-Regenerating Suppressor (ASRS-I, Dionex, Sunnyvale, CA) with autosuppression recycle mode was used to suppress conductivity of the eluting solution. The conductivity detector scale was set at 10 μS for full scale. Concentrations of all phosphates were calculated in their free-acid forms.

RESULTS AND DISCUSSION

Standard Inorganic Phosphates and 2-Phosphorylated L-Ascorbates

The chromatogram of a standard solution of four inorganic phosphates and AsMP and AsDP (cyclohexylammonium salts) shows that the components were separated within 10 min without overlap (Fig 1). The peaks eluting before retention time (Rt) 1.5 min are believed to be contaminants from glassware, cyclohexylammonium ions, or possibly L-ascorbate (AsA). The AsA solubilized in water showed a response at Rt 1.3 -1.4 min (data not shown). However, no attempts were made to determine the presence or absence of AsA in the 2-phosphate of AsA used in this study.

When 20 μL of the cyclohexylammonium salts of AsMP or AsDP at a level of 30 μg/mL were injected, which was three times more than the amount injected in the solution with the inorganic phosphates (Fig.1), each chromatogram showed only one peak, except for the peaks within Rt of 1.5 min (data not shown). Thus, both AsMP and AsDP were pure compounds, a fact

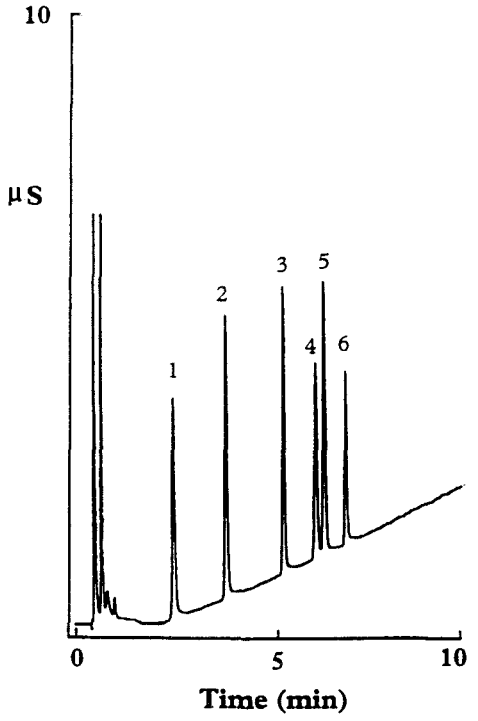

Figure 1. Separation of four inorganic phosphates and two 2-phosphorylated L-ascorbates by HPACE-CD. Injection volume was 20 μL of an aqueous solution containing 1. orthophosphate, 5 μg/mL; 2. 2-monophosphate L-ascorbate (AsMP), 10 μg/mL; 3. pyrophosphate, 5 μg/mL; 4. tripolyphosphate, 5 μg/mL; 5. 2-diphosphate L-ascorbate (AsDP), 10 μg/mL; 6. trimetaphosphate, 5 μg/mL. All concentrations were calculated as the free-acid forms of the phosphates.

supported also by their elemental analyses and relatively sharp melting points.[4] As expected, no detectable difference occurred in the conductive responses of equivalent levels of AsMP as its cyclohexylammonium salt or its magnesium salt, a commercial product with a purity of 85%.

The detection limits estimated by the signal to noise ratio (S/N ≅ 3) of the conductivity detector under the chromatographic conditions used were about 0.1 μg/mL (2 ng) for ortho- and pyrophosphates, 0.15 μg/mL (3 ng) for tripoly- and trimetaphosphates, and 0.2 μg/mL (4 ng) for AsMP and AsDP.

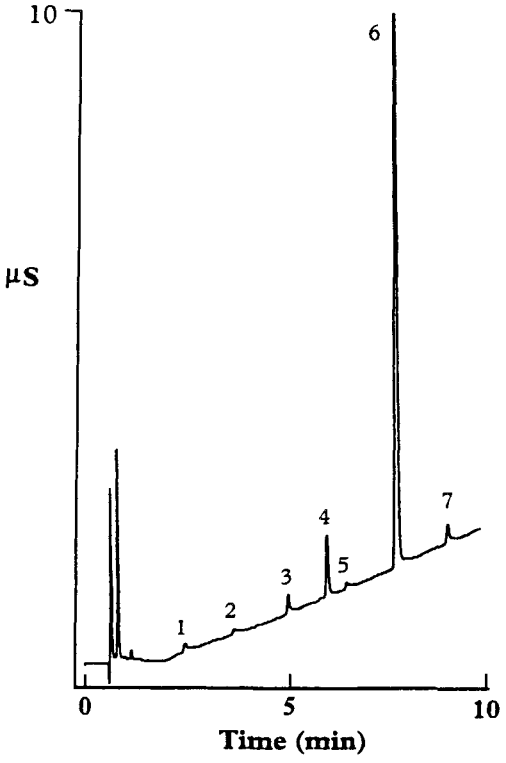

Figure 2. A chromatogram of L-ascorbate 2-triphosphate (AsTP) at 30 μg/mL (injection 20 μL). The peaks were identified as follows : 1. orthophosphate; 2. AsMP; 3. pyrophosphate; 4. AsDP; 5. trimetaphosphate; 6. AsTP; 7. Unknown.

Calibration curves of four inorganic phosphates, AsMP, and ASDP were established from a series of standard solutions. Relationships between the conductive responses (peak heights) and the concentrations in the range of 0.1-20 μg/mL were found to be linear, and all had correlation coefficients greater than 0.998.

The chromatogram of the sample of AsTP (Fig. 2) indicates that the amine salt of AsTP, which, like the amine salts of AsMP and AsDP, had been stored at 5°C in a desiccator for 18 months, was a mixture of 0.7% orthophosphate, 0.5% AsMP, 1.7 % pyrophosphate, 5.2% AsDP, 0.4 %

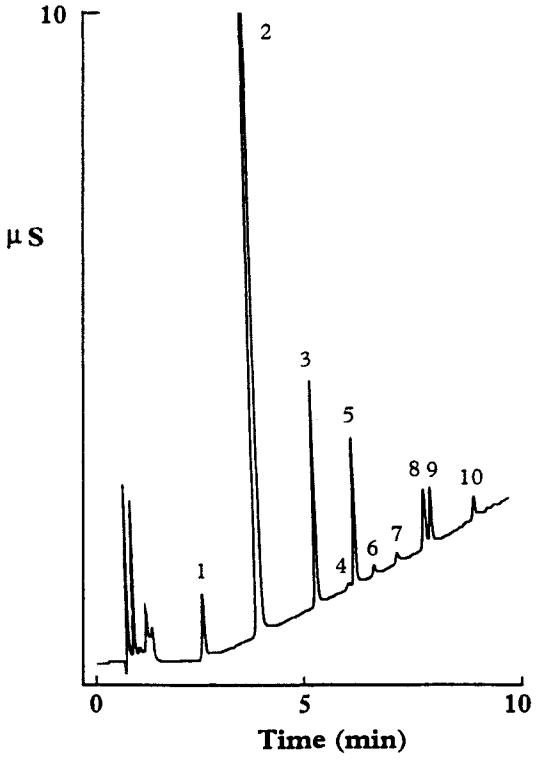

Figure 3. A chromatogram of a commercial product of 2-phosphorylated L-ascorbate injected (20 μL) at the concentration of 50 μg /mL. Peaks were identified as follows: 1. orthophosphate; 2. AsMP; 3. pyrophosphate; 4. tripolyphosphate; 5. AsDP; 6. trimetaphosphate; 7. unknown; 8. AsTP; 9. unknown; 10. unknown.

trimetaphosphate, 90.3% AsTP, and 1.2% unknown. Those concentrations, except for AsTP and the unknown component, were obtained from the standard curves of the anions discussed previously. The value of the unknown component was based on the assumption that it was a positional isomer of AsTP and had the same response. Accordingly, the level of AsTP in the sample was calculated by difference to be about 90.3%.

When pentacyclohexylammonium AsTP was exposed to a humidity of about 60% at room temperature for 10 days, it was converted from a free-flowing powder into a sticky solid. Chromatographic analysis indicated that

Table 1

Levels (%, w/w) of Inorganic Phosphates and 2-Phosphorylated L-Ascorbate in a Commercial Product of 2-Phosphorylated L-Ascorbate

Component	Percentage (%, w/w)
Orthophosphate	1.4 ± 0.1[a]
Pyrophosphate	3.7 ± 0.1
Tripolyphosphate	Trace
Trimetaphosphate	0.2 ± 0.1
AsMP	54.9 ± 2.7
AsDP	3.4 ± 0.2
AsTP	1.7 ± 0.1
Unknown (peak 7)	0.2 ± 0.1
Unknown (peak 9)	1.2 ± 0.1
Unknown (peak 10)	0.6 ± 0.1

[a] Each value is the mean and standard deviations of four measurements and is calculated using hydrogen ion as cation.

about 50% of the AsTP had hydrolyzed to give inorganic phosphates, AsDP, and AsMP (data not shown). These results show that the HPAEC-CD method is useful in studying the hydrolytic stability of 2-polyphosphate esters of L-ascorbate, as well as the oxidative stability of AsMP.[10]

A standard curve for AsTP was derived from its amine salt based on a purity of 90.3%. The curve showed a linear relationship between the conductive response (peak height) and the concentration range of 0.1-20 μg/mL. The detection limit of AsTP was about 0.2 μg/mL (4 ng), similar to that of AsMP and AsDP.

The Rt of AsTP was about 8.3-8.4 min (Fig. 2). When AsTP was added to the standard solution of phosphates (data not shown), it was eluted about 1.4 min after trimetaphosphate (Rt ~ 7.1 min), indicating that AsTP could be separated clearly from the other phosphates (see also Fig. 3).

This method also gave a good separation of a mixture of standard AsMP and L-ascorbate 2-sulfate (AsS) using isocratic elution with 20 mM NaOH.[10] The Rts of AsMP and AsS were 5.4 and 4.7 min, respectively.

Commercial Polyphosphorylated L-Ascorbate

Figure 3 shows a chromatogram of a commercial product at a concentration of 50 μg /mL; 10 peaks (components) were observed with Rts greater than 1.5 min. Seven peaks were assigned based on the Rt values of phosphate standards, but three minor peaks labeled 7, 9, and 10 were unknown. The composition of the commercial product (Table 1) was calculated from the various standard curves of known components and by assuming the same conductive response as AsTP for the unknown peaks 7, 9 and 10, which again were thought to be positional isomers of triphosphorylated AsA.

CONCLUSIONS

The chromatographic method described here is able to separate simultaneously a mixture of inorganic phosphates and L-ascorbate 2-phosphates with high resolution in 10 min of operating time. The method could be used to monitor the progress of phosphorylation of L-ascorbic acid; to determine purity of AsMP, AsDP, AsTP, and reference standards; and to monitor levels of L-ascorbate 2-phosphates in pharmaceuticals, feeds, and foods.

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