Determination of ascorbic acid and dehydroascorbic acid in juices by high-performance liquid chromatography with electrochemical detection using L-cysteine as precolumn reductant

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ABSTRACT

Determination of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in juices was performed by high-performance liquid chromatography with electrochemical detection using L-cysteine as precolumn reductant. This method was suitable for the determination of AA and total AA (AA + DHAA) in juices. The mild reduction of DHAA to AA with L-cysteine took *ca.* 15 min, and the retention time of AA was *ca.* 15 min. The detection limit (signal-to-noise ratio = 2) was *ca.* 0.15 ng. The method was selective and reproducible (relative standard deviation 2.6–3.2% for AA and 2.1–3.2% for total AA). The calibration graph for AA was linear in the range 0.1–10 ng. The recovery of AA was over 90% by the standard addition method.

INTRODUCTION

A number of reports on the determination of ascorbic acid (AA) [1–11] have appeared in the last 10 years. Numerous efforts have been directed to the development of specific and sensitive detection systems in high-performance liquid chromatography (HPLC). HPLC with UV detection, electrochemical detection (ED) and fluorescence detection has proved useful for the determination of AA in foods such as juices, vegetables and potatoes [1–4] and in biological fluids [4–10]. HPLC with ED is valuable in the determination of trace compounds in complex matrices because of the excellent sensitivity and selectivity provided [12].

Usually, dehydroascorbic acid (DHAA) is determined as the difference between the total AA (AA + DHAA) after DHAA reduction and the AA content of the original sample. AA is easily oxidized to DHAA in the presence of reagents such as halogens, hydrogen peroxide and heavy metal ions, especially CU^{2+}, Ag^+ and Fe^{3+} and at alkaline pH [13]. DHAA is electrochemically inactive and most DHAA assays involve preliminary reduction of DHAA to AA with homocysteine [1–6] and subsequent measurement of total AA by HPLC with UV detection or ED. The reduction reagent requires the rapid and mild reduction of DHAA to AA, no response to ED monitoring of the applied potential and low cost. Commercially available and cheap L-cysteine has not been used as a precolumn reductant for the reduction of DHAA to AA.

A simple, rapid, sensitive, highly selective and reproducible method for the determination of AA and DHAA in foods, drugs and biomedical samples is required for quality control purposes and in clinical chemistry. We reported previously [11] the routine determination of AA in complex

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sample matrices (elemental diets) by HPLC with ED without the use of an internal standard. This paper describes the selective and sensitive determination of AA and DHAA in juices by HPLC with ED using an internal standard and L-cysteine as pre-column reductant.

EXPERIMENTAL

Reagents and materials

AA was purchased from Tokyo Kasei (Tokyo, Japan), L-cysteine from Ajinomoto (Kawasaki, Japan) and α-methyl-L-dopa was from Nacalai Tesque (Kyoto, Japan). Other reagents were of analytical-reagent grade. Membrane filters (0.45 μm) were obtained from Millipore (Bedford MA, USA). Lemon and grape juices were freshly prepared prior to use. Other juices were commercially available.

Apparatus and conditions

A Model 655 A-11 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model Σ 875 electrochemical detector (Irica, Kyoto, Japan) was used. The applied potential was set at 300 mV *versus* an Ag/AgCl reference electrode. The samples were applied by a Rheodyn Model 7125 sample loop injector with an effective volume of 20 μl. HPLC was carried out on a 15 × 0.46 cm I.D. Inertsil ODS-2 (5 μm) reversed-phase column (GL Sciences, Tokyo, Japan) using as the mobile phase 100 mM KH$_2$PO$_4$ (pH 3, adjusted with phosphoric acid)–1 mM ethylenediaminetetraacetic acid disodium salt (EDTA·2Na). The flow-rate was 0.6 ml/min at room temperature.

Standard AA preparation

Standard AA solution (10 μg/ml) was freshly prepared prior to use. AA in this solution was stable for 30 min at 5°C. The AA peak-height ratio was constant for 30 min and subsequently decreased.

Standard DHAA preparation

Standard DHAA solution was freshly prepared by the addition of 0.1 M iodine solution (250 μl) to the standard AA solution (10 μg) prior to use [14,15]. DHAA in this solution was

stable for 30 min at 5°C. The AA peak-height ratio after reduction of DHA to AA with L-cysteine was constant for 30 min and subsequently decreased periodically.

Sample preparation

Samples were preliminarily diluted to an estimated AA concentration of 10 μg/ml with deionized water, then the solution was filtered with a membrane filter (0.45 μm) and the filtrate was used for the two purposes of the determination of AA and of total AA. AA in this sample solution was stable for 30 min at 5°C. The AA peak-height ratio was constant for 30 min and subsequently decreased.

AA determination

To 20 μl of the above solution were added 10 μl of α-methyl-L-dopa (125 μg/ml) and 2% metaphosphoric acid solution (800 μl) for the determination of AA. AA in this solution was stable at room temperature for 30 min. The AA peak-height ratio was constant for 30 min and subsequently decreased. An aliquot (20 μl) was injected into the chromatograph.

DHAA determination

DHAA was determined as the difference between the total AA after DHAA reduction and the AA content of the original sample. To 20 μl of the above solution were added 10 μl of α-methyl-L-dopa (125 μg/ml) and L-cysteine (2.5 mg/ml) diluted in 10 mM phosphate buffer (pH 6.8) (800 μl) for a the determination of total AA. After this solution had been allowed to stand at room temperature for 15 min, an aliquot (20 μl) was injected into the chromatograph. AA in this solution was stable at room temperature for 30 min. The AA peak-height ratio was constant for 30 min and subsequently decreased periodically.

RESULTS AND DISCUSSION

Internal standard

At the beginning of the work, several internal standards were examined for the determination of AA in juices. Electrochemically active 4-hydroxy-3-methoxybenzoic acid, 2,5-dihydroxy

benzoic acid, N-acetyl-L-cysteine, N-acetyl-L-tyrosine, N-acetyl-L-tryptophan and α-methyl-L-dopa were examined. It was found that α-methyl-L-dopa was the most useful internal standard, because it has a suitable retention time (about 14 min) and shows excellent separation and applied potential (300 mV versus an Ag/AgCl reference electrode).

The relationship between applied potential and sensitivity of AA, α-methyl-L-dopa, cysteine, tyrosine and tryptophan was examined. A typical hydrodynamic voltammogram is illustrated in Fig. 1. The current (peak height) at each applied potential was divided by the current at the maximum possible potential to obtain the relative current ratio. This value was plotted against the applied potential. The detector gave a linear response up to 500 mV versus Ag/AgCl for α-methyl-L-dopa and 900 or 1000 mV versus Ag/AgCl for cysteine, tyrosine and tryptophan. When the applied potential was set at 400 mV or more versus Ag/AgCl AA could not be determined, because AA was present on the solvent front with a large shoulder and was not separated completely.

Based on the above, the applied potential was set at 300 mV versus Ag/AgCl for the selective determination of AA.
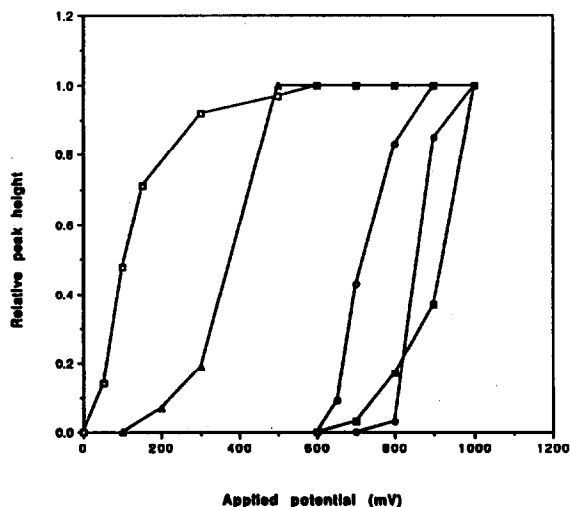
Reduction conditions

The effects of the pH of the solution, amount of L-cysteine and reaction time at room temperature on the reduction of DHAA to AA were examined by comparing the peak height of AA with that of α-methyl-L-dopa. It can be seen in Figs. 2–4 that all three parameters affect the reduction of DHAA to AA. The results that the optimum reduction pH was 6–7.5, amount of L-cysteine 2.5% and reaction time at room temperature about 15 min. Rapid and mild reduction was carried out as follows: DHAA (200 ng) diluted in 10 mM phosphate buffer (pH 6.8) (800 μl) containing L-cysteine (2.5 mg/ml) was allowed to stand at room temperature for 15 min. The reduction scheme is shown in Fig. 5.

Chromatography

The chromatograms obtained by HPLC with ED of AA and AA after reduction of DHAA using the proposed reduction conditions were examined.

The chromatograms in Fig. 6a and b of AA (200 ng) diluted in 800 μl of deionized water and 10 mM phosphate buffer (pH 6.8) containing 0.25% L-cysteine show that the standard AA used here did not contain DHAA, because both AA peak heights were almost identical. When
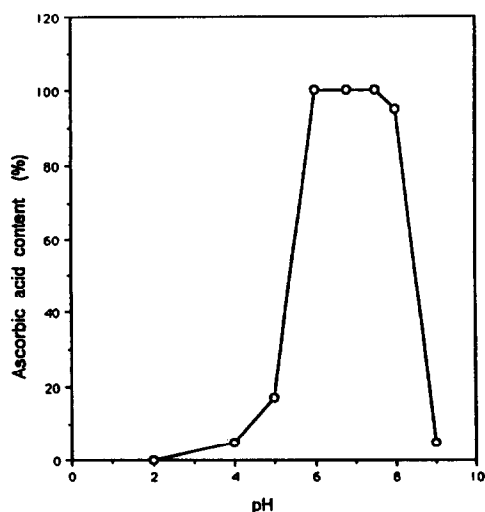
Fig. 1. Hydrodynamic voltammograms of AA, cysteine, tyrosine and tryptophan. ▲ = α-Methyl-L-dopa (internal standard); □ = AA; ■ = L-tryptophan; ○ = L-cysteine; ● = L-tyrosine.

Fig. 2. Effect of pH on the reduction of DHAA to AA. Conditions: DHAA (200 ng) diluted in 10 mM phosphate buffer (800 μl) containing L-cysteine (2.5 mg/ml) was allowed to stand at room temperature for 15 min.
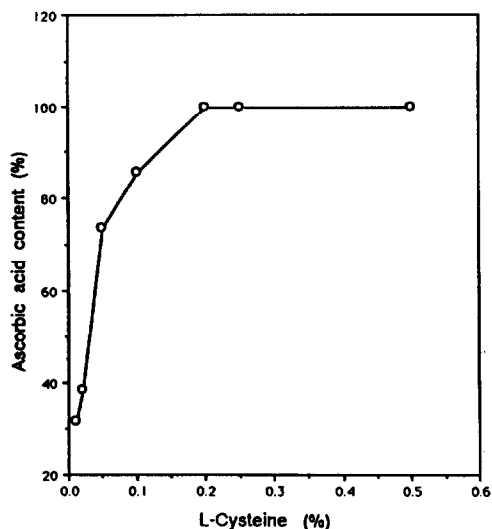
Fig. 3. Effect of amount of L-cysteine on the reduction of DHAA to AA. Conditions: DHAA (200 ng) diluted in 10 mM phosphate buffer (pH 6.8) (800 μl) containing L-cysteine was allowed to stand at room temperature for 15 min.

0.1 M iodine solution was added to AA (200 ng) in deionized solution, no AA peak was observed on the chromatogram (Fig. 6c) because of the oxidation of AA to DHAA by the iodine [14,15]. On the other hand, after further addi-
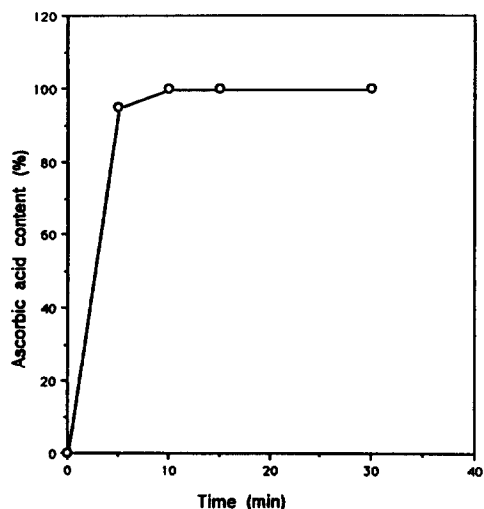

Fig. 4. Effect of reaction time on the reduction of DHAA to AA. Conditions: DHAA (200 ng) diluted in 10 mM phosphate buffer (pH 6.8) (800 μl) containing L-cysteine (2.5 mg/ml) was allowed to stand at room temperature.

tion of L-cysteine to the above oxidized AA (Fig. 6c) an AA peak was observed on the chromatogram (Fig. 6d) because of the rapid reduction of DHAA to AA by the L-cysteine. The recovery in the reduction of DHAA to AA was over 95%.

Usually, AA in foods and biomedical samples is determined by HPLC with ED at +600 mV or more *versus* an Ag/AgCl reference electrode or HPLC with UV detection at 245 nm [1–6]. When the determination of AA was performed by HPLC with ED at $\geqslant$600 mV *versus* Ag/AgCl or by HPLC with UV detection at 245 nm, not only AA but also cysteine, tyrosine and tryptophan were detected, as described previously [11]. It took about 60 min after the retention time of AA for complete elution. On the other hand, the chromatography for AA can be highly selective with rapid detection (retention time *ca.* 15 min) in the presence of other compounds by ED at 300 mV *versus* Ag/AgCl. This allows analysis without the need for clean-up. This procedure was simple and suitable for routine work. A typical chromatogram of AA (detection limit *ca.* 0.15 ng at a signal-to-noise ratio of 2) in orange juice is shown in Fig. 7.

From the above results, it was concluded that the method established here was advantageous for the routine determination of AA and total AA in juices, because of the simple, rapid (reduction time of DHAA to AA *ca.* 15 min; retention time of AA *ca.* 15 min) and highly selective analysis without the need for clean-up.

Determination of AA

A calibration graph for AA was constructed by plotting the peak-height ratio against the amount of AA in the internal standard. Satisfactory linearity was obtained over the range 0.1–10 ng ($y = 0.3421x - 0.023$; y = peak-height ratio, x = amount of AA in ng).

A known amount of AA and DHAA was added to orange juices and the overall recoveries were calculated by the standard addition method. As shown in Table I, the AA recovery was over 90% and the DHAA recovery was *ca.* 80%.

Table II gives the analytical data for AA and DHAA in juices. The relative standard deviation (R.S.D.) was 2.6–3.2% for AA and 2.1–3.2% for total AA with no addition of AA and
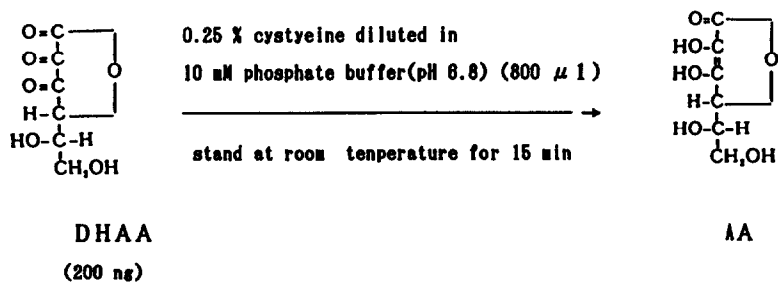
O=C—
O=C
O=C   O
H-C—
HO-C-H
CH₂OH

0.25 % cystyeine diluted in

10 mM phosphate buffer(pH 6.8) (800 μ l)

⟶

stand at room temperature for 15 min

O=C—
HO-C
HO-C   O
H-C—
HO-C-H
CH₂OH

DHAA

(200 ng)

AA

Fig. 5. Reduction scheme.

DHAA. Between-day R.S.D.s were not ex-
amined because AA and DHAA were unstable.

CONCLUSION

The use of commercially available and cheap
L-cysteine for the rapid and mild reduction of
DHAA to AA seems very useful for the de-
termination of AA and total AA by HPLC with
ED. The proposed method is satisfactory with
respect to selectivity, rapidity and cost in com-
parison with published methods [1–6]. This
method established here seems to be applicable
to the routine analysis of AA and total AA in

juices because of the simple, rapid (mild reduc-
tion time of DHAA to AA with L-cysteine ca. 15
min; retention time of AA ca. 15 min), sensitive
[detection limit ca. 0.15 ng (signal-to-noise
ratio = 2)], reproducible (R.S.D. = 2.6–3.2% for
AA and 2.1–3.2% for total AA) and highly
selective analysis without the need for clean-up
and with recoveries of over 90% for AA and
about 80% for DHAA. The application of the
proposed method to the determination of AA
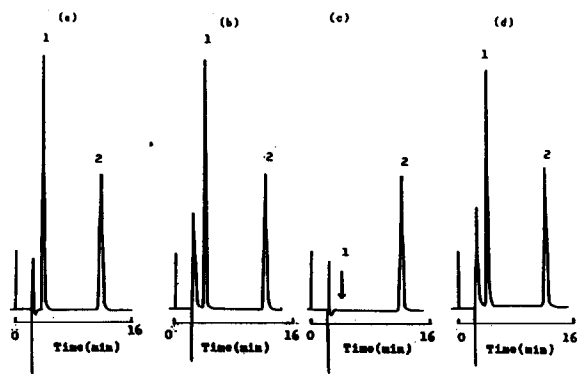and total AA in biological fluids is being studied.

Fig. 6. Chromatograms of standard AA under various con-
ditions obtained by HPLC with ED at 300 mV versus an
Ag/AgCl reference electrode. AA concentration = 200 ng in
800 μl; internal standard (I.S.) concentration = 1.25 μg in
800 μl. (a) AA (200 ng) and I.S. (1.25 μg) diluted in
deionized water (800 μl); (b) AA (200 ng) and I.S. (1.25
μg) diluted in 10 mM phosphate buffer (pH 6.8) containing
0.25% L-cysteine (800 μl); (c) AA (200 ng) and I.S. (1.25
μg) and 0.1 M I₂ solution (50 μjl); diluted in deionized
water (800 μl); (d) AA (200 mg), I.S. (1.25 μg) and 0.1 M
I₂ solution (50 μl) diluted in 10 mM phosphate buffer (pH
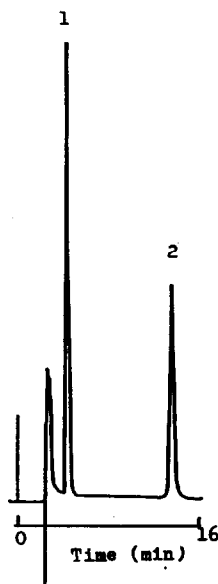6.8) containing 0.25% L-cysteine (800 μl). Peaks: 1 = AA;
2 = I.S.

Fig. 7. Chromatogram of AA in orange juice obtained by
HPLC with ED at 300 mV versus an Ag/AgCl reference
electrode. Amount of AA injected, 6.33 ng in 20 μl. HPLC
was carried out on a 15 × 0.46 cm I.D. column of Inertsil
ODS-2 (5 μm) using 100 mM KH₂PO₄ (pH 3, adjusted with
phosphoric acid)–1 mM EDTA·2Na as the mobile phase at
a flow-rate of 0.6 ml/min at ambient conditions. Peaks:
1 = AA; 2 = I.S.

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TABLE I

RECOVERIES OF AA AND DHAA ADDED TO ORANGE JUICE

Compound	Amount (mg per 100 ml)		R.S.D. (%)[a]	Recovery (%)
	Added	Found		
AA	0	25.3	3.2	–
	5	29.9	3.1	92
	10	35.0	2.9	97
	20	44.9	2.8	98
	40	65.5	2.9	100.5
Total AA (AA + DHAA)	0	28.1	3.3	–
	10	36.3	3.1	82

[a] n = 3.

TABLE II

CONTENTS OF AA AND DHAA IN JUICES

Sample	AA		DHAA concentration (mg per 100 ml)
	Concentration (mg per 100 ml)	R.S.D. (%)[a]	
Orange juice A	25.3	3.2	1.8
Orange juice B	15.3	3.1	16.4
Apple juice	27.9	2.9	5.8
Lemon juice	39.2	2.6	0
Japanese tea	5.2	3.2	0.3
Grape juice	0	–	2.2

[a] n = 3.

REFERENCES

1 D.B. Dennison, T.G. Brawley and G.L.K. Hunter, J. Agric. Food Chem., 29 (1981) 927.
2 W.A. Behrens and R. Madere, J. Liq. Chromatogr., 15 (1992) 753.
3 W.D. Grahham and D. Annette, J. Chromatogr., 594 (1992) 187.
4 W.A. Behrens and R. Madere, Anal. Biochem., 165 (1987) 102.
5 R.R. Howard, T. Peterson and P.R. Kastl, J. Chromatogr., 414 (1987) 434.
6 L.S. Liau, B.L. Lee, A.L. New and C.N. Ong, J. Chromatogr., 612 (1993) 63.
7 K. Iriyama, M. Yoshiura, T. Iwamoto and Y. Ozaki, Anal. Biochem., 141 (1984) 238.
8 M. Yoshiura, T. Iwamoto and K. Iriyama, Jikeiika Med. J., 32 (1985) 21.
9 M.A. Kotnink, J.H. Skala, H.E. Sauberlich and S.T. Omaye, J. Liq. Chromatogr., 8 (1985) 31.
10 M. Yoshiura and K. Iriyama, J. Liq. Chromatogr., 9 (1986) 177.
11 H. Iwase, J. Chromatogr., 505 (1992) 277.
12 P.T. Kissinger, Anal. Chem., 49 (1977) 477 A.
13 A.P. De Leenheer, W.E. Lambert and H.J. Nelis, Modern Chromatographic Analysis of Vitamins, Marcel Dekker, New York, 2nd ed., 1992, p. 235.
14 The United States Pharmacopeia, XXII Revision, US Pharmacopeial Convention, Rockville, MD, 1990, p. 109.
15 The Pharmacopeia of Japan, Hirokawa, Tokyo, 12th ed., 1992, p. C-37.