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Note

Determination of ascorbic acid in its mixtures with thiols by reversed-phase high-performance liquid chromatography with electrochemical detection using a DC-TAST polarograph

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The important role of *l*-ascorbic acid (AA) in biological systems has stimulated wide interest in it and improved procedures have recently been reported for its determination; they are mainly high-performance liquid chromatographic (HPLC) methods. HPLC has been applied to the determination of AA with anion-exchange[1–5], bonded-phase NH_2[6–8], reversed-phase[9–12], ion-pair reversed-phase[13–19] techniques and UV[1,2,6–11,13,14,16–18], luminescence[4] or electrochemical detection (using glassy carbon or carbon-paste electrodes)[3,5,12,15,19].

The dropping mercury electrode (DME) has not previously been used in HPLC for AA measurements, although a large group of direct procedures of AA determination are based on polarography[20–22]. AA forms on an DME a two-electron anodic wave, which appears over the whole pH range; its height is directly proportional to concentration and the half-wave potential ($E_{1/2}$) depends on pH, *e.g.*, $E_{1/2}$ = 0.18 V (*vs.* normal calomel electrode; NCE) at pH 1.8 while $E_{1/2}$ = −0.06 V (*vs.* NCE) at pH 7.0[21]. Most of the compounds usually present in biological materials, including coloured compounds, do not influence the results of the determination. Only compounds such as thiols (present in some biological materials), which form an anodic wave at the range of potentials as AA, make the polarographic analysis incorrect.

To avoid this limitation we have developed a simple chromatographic method for AA measurements with polarographic detection using the sensitive DC-TAST technique. Reversed-phase chromatography was used for separation. Cysteine and glutathione (reduced) served as model interfering thiols.

EXPERIMENTAL

L-Ascorbic acid (puriss) was obtained from Polfa (Poland). All other materials were of analytical-reagent or laboratory grade and were used without further purification.

Chromatographic experiments were performed using a Type 302 apparatus equipped with a Type PP2 flow-through detector[23] and a Type PPW1 DC-TAST polarograph[24] (Institute of Physical Chemistry, Polish Academy of Science, Warsaw, Poland); pH measurements were performed using a Type N517 pH meter (Meratronic, Poland).

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For HPLC stainless-steel columns (250 × 4 mm I.D.) were used, packed with 10 μm LiChrosorb RP-18 (E. Merck, Darmstadt, F.R.G.). All measurements were carried out at 20 ± 1°C and at a constant flow-rate of 1.0 cm^3/min.

Aqueous mobile phase was prepared by dissolution of oxalic acid to a final concentration 0.25% (0.028 *M*) in 1/15 *M* phosphate buffer of pH 5.5. The solution, of pH 2.9, was deaerated with argon.

Synthetic samples, prepared from pure substances, were dissolved in 0.028 *M* oxalic acid. The natural materials were crumbled, treated with 0.056 *M* oxalic acid (acid to sample = 1:3, w/w) for about 1 h and then filtered. All samples introduced onto the column contained oxygen.

RESULTS AND DISCUSSION

Ion-pair reversed-phase HPLC with various ammonium salts as the source of counter ions has been the most frequently used technique for AA determination. In this study we found that a simple reversed-phase system (LiChrosorb RP-18 column) with an aqueous acidic mobile phase of pH 2.9 containing phosphate buffer (1/15 *M*, pH 5.5) and oxalic acid (0.028 *M*) also gives satisfactory results (Fig. 1). Moreover, under these conditions AA remained stable. Oxalic acid, a well known

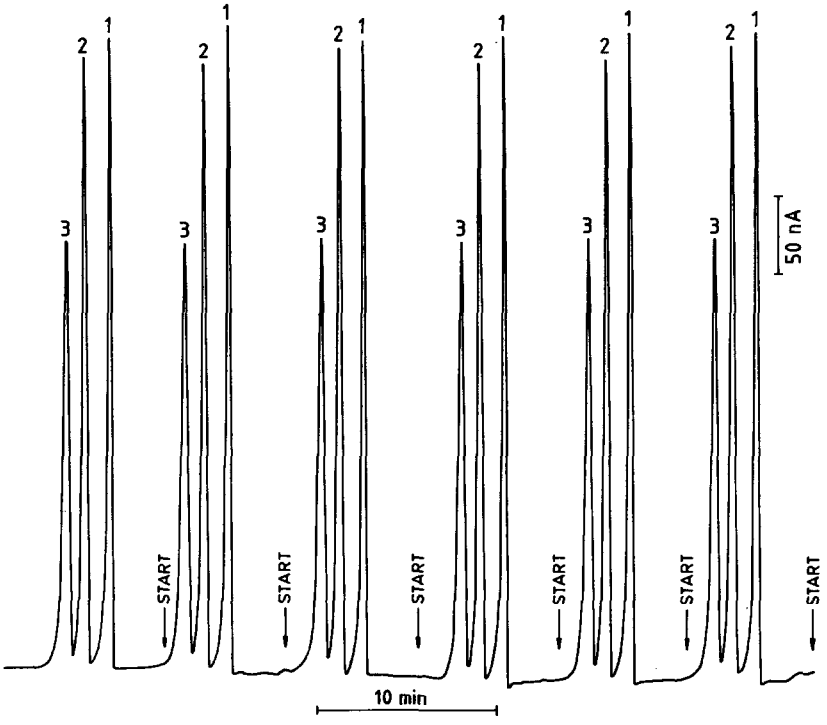

Fig. 1. Six consecutive chromatograms of a mixture of $1.2 \cdot 10^{-3}$ *M* cysteine (1), $7 \cdot 10^{-4}$ *M* AA (2) and $1.5 \cdot 10^{-3}$ *M* glutathione (red.) (3). Column (250 × 4 mm I.D.) packed with 10 μm LiChrosorb RP-18. Mobile phase: 1/15 *M* phosphate buffer (pH 5.5), 0.028 *M* oxalic acid in water, pH 2.9. Sample size, 5 μl; flow-rate, 1 cm^3/min. Potential of detection, 500 mV *vs.* Ag/AgCl.

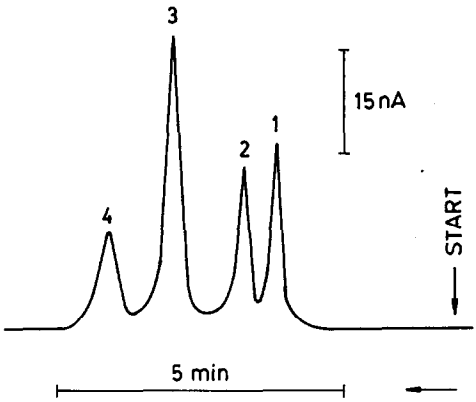

Fig. 2. Chromatogram of an onion extract (30 g of onion pulp and 10 g of 0.028 *M* oxalic acid mixed and filtered after 1 h) performed under the same conditions as in Fig. 1. Peaks: 1 = unknown; 2 = cysteine; 3 = AA (concentration in the injected sample evaluated as $1 \cdot 10^{-4}$ *M*); 4 = glutathione.

stabilizer of AA in solution, not only was added to the samples but also acted as one of the components of the mobile phase. In such mixtures the AA content does not change measurably for about 8 h. A similar solution but using metaphosphoric acid has been described for the determination of AA in urine[9].

Taking into acount the pH of the mobile phase (2.9) and the dissociation constant of AA ($pK_1 = 4.15$)[25], one can conclude that the mechanism of retention of AA on a reversed-phase column is based on adsorption of its undissociated molecules. As shown in Fig. 1, the complete separation of AA, cysteine and glutathione (red.) occurs in a comparatively short time (about 7 min).

We found a linear dependence of the height of the AA peak on concentration in the range $0–5 \cdot 10^{-3}$ *M*. The reproducibility of the determination was satisfactory; each value of peak height was determined as the mean of six measurements (injections) with a relative standard deviation of better than 1.6%. Under the conditions recommended, the detection limit (defined as the amount of an injected substance giving a signal twice that of the baseline noise) is $35 \cdot 10^{-12}$ mol (*i.e.*, 5 μl of $7 \cdot 10^{-6}$ *M* solution). This favourable detection limit with DC-TAST polarography cannot be achieved by simple d.c. polarography. The method is simple and reproducible owing to the continuously renewing surface of the DME. It can be used for the determination of AA in various natural materials, as shown in Fig. 2. The simultaneous quantitative determination of cysteine and glutathione can be also performed with the same procedure.

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