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Note

Analysis of ascorbic acid derivatives by high-performance liquid chromatography with electrochemical detection

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Many metabolites of ascorbic acid (AA, vitamin C) have been detected in the urine and tissues of animals, but only a few have been identified[1]. In addition to the well known ascorbic acid metabolites (dehydroascorbic acid, 2,3-diketogulonic acid, oxalic acid and carbon dioxide), ascorbic acid-2-sulfate (AAS) has been found in brine shrimp cysts, in the urine of man, monkeys, rats and guinea pigs and in rat tissues[1–7]. 2-O-Methylascorbic acid (OME) has been found in the urine of rats and in rat liver extract in the presence of catechol-O-methyltransferase[8,9].

The chemistry of ascorbic acid and its derivatives has been reviewed by Tolbert *et al.*[1] in 1975 and by Andrews and Crawford[10] in 1982. Some ascorbic acid derivatives have been shown to have vitamin C activities. AAS is antiscorbutic for fish[3,11]. Ascorbic acid-2-phosphate (AAP) is antiscorbutic for the rhesus monkey and the guinea pig[12]. A study on the effect of ascorbic acid on the growth of the tobacco hornworm *Manduca sexta* showed that AAP is as effective as ascorbic acid in promoting hornworm growth and that both AAS and 6-deoxy-6-bromoascorbic acid (6BR) have some ascorbic acid activity[13]. The chloro-compound 6-deoxy-6-chloroascorbic acid is also reported to have high antiscurvy activity[14].

In addition to its importance in nutrition, AA is commonly used as an antioxidant. Some of these AA derivatives have been shown to be relatively stable against oxidation and prolonged storage. These AA derivatives may find applications in food preservation and in non-food areas. It has been demonstrated that AAS is the preferred form of vitamin C for fish[11] and that AAP is used to maintain 2,3-diphosphoglycerate in human red blood cells during blood storage[15,16].

These AA derivatives which are potentially important metabolites of AA may exist in animal tissues and body fluids, perhaps in very small quantities. Paper chromatography and thin-layer chromatography are commonly used for the analysis of small amounts of these substances. AAS and AAP have been assayed by liquid chromatography and high-performance liquid chromatography (HPLC)[17–22]. AAP has been measured by an enzymetic method[22]. Recently, nanogram quantities of AA and AAS have been quantitatively differentiated by reversed-phase HPLC with electrochemical detection[23]. In the present report, we have utilized this method to analyze some electrochemically active AA derivatives available to us. The HPLC system described in Experimental is able to separate AA, AAS, AAP, OME, 6BR, 5-methyl-3,4-dihydroxytetrone (MDT), and two internal standards (3,4-dihydroxybenzoic acid

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and 3,4-dihydroxyphenyl acetic acid). The reductone MDT is a product of the non-enzymatic browning reaction of AA in foodstuffs during processing[24,25].

EXPERIMENTAL

HPLC

The methods and instrumentation were thoroughly described by Pachla and Kissinger[26,27]. All chromatograms were obtained using commercially available components. The electrochemical detector was an amperometric detector (BioAnalytical Systems, West Lafayette, IN, U.S.A.). The analytical column was a 25 cm × 4.6 mm I.D., Ultrasphere ODS (5 μm) Altex prepacked HPLC column. The eluent which consisted of 44 m*M* acetic acid, 16 m*M* sodium acetate and 1.8 m*M* 1,5-dimethylhexylamine in ethanol–water (6.7:93.3) was partially degassed by a simple water aspirator before use. A Milton Roy pump (Model 396) equipped with a pulse dampener was used to pump the eluent through the system at a constant flow-rate of 0.93 ml/min. The injector was a Rheodyne injection valve (Model 7125). The detector electrode was packed with a wax-graphite paste (CP-W, BioAnalytical Systems). The potential of the chromatographic detector was set at 0.95 V *vs.* Ag/AgCl reference electrode.

Chemicals and solutions

Ascorbic acid and 1,5-dimethylhexylamine were purchased from Sigma. Ascorbic acid-2-sulfate (dipotassium salt) was obtained from Hoffman-La Roche. 2-O-methylascorbic acid, 6-deoxy-6-bromoascorbic acid and tricyclohexylammonium ascoirbic acid-2-phosphate were kindly supplied by Professor Paul A. Seib. 5-Methyl-3,4-dihydroxytetrone was prepared by a slight modification of the method reported by Euler and Hasselquist[28]. The internal standards 2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid were purchased from Sigma; 3,4-dihydroxybenzylamine hydrobromide was purchased from Adrich.

All chemicals used in this assay were reagent grade. All standard solutions were prepared freshly with 50 m*M* perchloric acid and diluted before use. A volume of 5 μl was injected into the HPLC column.

RESULTS

Among the six ascorbic acid derivatives, AA, OME, MDT and 6BR are oxidized at a relatively lower potential (750 mV *vs.* Ag/AgCl). Fig. 1A–C shows the comparison of peak heights of various ascorbic acid derivatives at three different detector applied potentials. When HPLC was run at an applied potential of 800 mV *vs.* Ag/AgCl, the detector was able to detect all the components. At a potential of 950 mV all compounds injected show decent peak height and the separation was excellent. Several organic amines we tested are suitable ion-pairing reagents for the analysis of these substances but the best separation is obtained using 1,5-dimethylhexylamine. We have also examined various chromatographic conditions and eluent compositions. The best results were obtained using the system described in Experimental.

We have tested four frequently used internal standards for the HPLC analysis

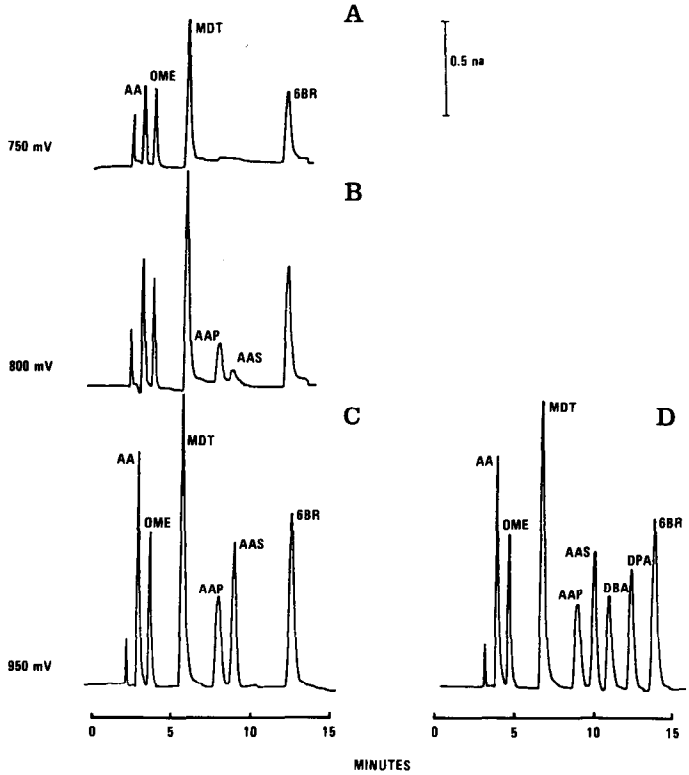

Fig. 1. Chromatograms of L-ascorbic acid (AA, 4 ng), 2-O-methylascorbic acid (OME, 14 ng), 5-methyl-3,4-dihydroxytetrone (MDT, 4 ng), ascorbic acid-2-phosphate (AAP, 60 ng), ascorbic acid-2-sulfate (AAS, 40 ng) and 6-deoxy-6-bromoascorbic acid (6BR, 4 ng). Internal standards: 3,4-dihydroxybenzoic acid (DBA, 4 ng) and 3,4-dihydroxyphenyl acetic acid (DPA, 4 ng). Chromatographic column: Altex C_{18} reversed-phase 25 cm × 4.6 mm I.D. stainless-steel column. Mobile phase: 60 m*M* acetate buffer, 1.8 m*M* 1,5-dimethylhexylamine in ethanol–water (6.7:93.3) at a flow-rate of 0.93 ml/min. Applied potential: (A) 750 mV; (B) 800 mV; (C,D) 950 mV *vs.* Ag/AgCl.

of ascorbic acid. 3,4-Dihydroxybenzylamine eluted between AA and OME and the three peaks were not completely resolved under the conditions we used. 2,5-Dihydroxybenzoic acid, with elution time of 23 min, was well separated from all other peaks and is not shown in the figure. Fig. 1D shows the mixture of AA and five derivatives with two internal standards 3,4-dihydroxybenzoic acid (DBA) and 3,4-dihydroxyphenylacetic acid (DPA). All components in the mixture are well resolved.

The precision of this method was checked by multiple analysis on a single sample containing all components. Typically, the relative standard deviation of each peak for six measurements is calculated to be better than 3%. We found that the assay solution for HPLC can also be prepared with cold 3% metaphosphoric acid with no apparent difference in the chromatograms. The derivatives of AA are more stable than AA itself both in perchloric acid and in metaphosphoric acid.

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