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Note

Quantitative analysis of ascorbic acid-2-sulfate by high-performance liquid chromatography with electrochemical detection

C.S. TSAO*, M. YOUNG and S.M. ROSE

Linus Pauling Institute of Science and Medicine, 440 Page Mill Road, Palo Alto, CA 94306 (U.S.A.)

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Ascorbic acid-2-sulfate (AAS) was found in brine shrimp cysts, in the urine of humans, monkeys, rats and guinea pigs [1—4] and in rat bile [5] and tissues [6, 7]. AAS has been reported to be one of the metabolites of ascorbic acid (AA, vitamin C). It has been suggested that AAS was involved in some physiological functions [4].

AA and AAS have been quantitatively differentiated by their different rates of osazone formation of the oxidized AA and AAS with 2,4-dinitrophenylhydrazine [8] and by their different rates of oxidation with 2,6-dinitrophenolindophenol and $KBrO_3$ [9]. Paper chromatography [1, 3, 5, 10—13] and thin-layer chromatography [1, 3, 5, 11—17] have been commonly used for the separation of these two substances in microgram quantities. High-voltage paper electrophoresis [5] has also been used. AAS has been quantitated by measuring the UV absorption of the eluent from a liquid chromatography column after isolation from animal tissues [1, 10, 18—20]. High-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector has also been used for the separation of AA and AAS [21].

HPLC has been used for the measurement of AA with anion-exchange columns [21—27], LiChrosorb-NH_2 [28—31] reversed-phase C_{18} [24] and ion-pair reversed-phase C_{18} [32—34] columns with UV detection [27—31, 34, 35] and electrochemical detection [22—26, 32, 33].

In the present study, AAS is analyzed using reversed-phase HPLC with electrochemical detection. AAS has been shown to oxidize at an applied potential of 0.88 V vs. Ag/AgCl [33]. An ion-pairing agent, octylamine, is added to the acetate mobile phase. The procedure is simple and rapid and involves only homogenization of the sample, centrifugation and direct quanti-

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tation by HPLC. The method is reliable, highly sensitive, and can be used to detect accurately as low as 2 ng of AAS in a sample.

EXPERIMENTAL

HPLC

The equipment and instrumentation were thoroughly described by Pachla and Kissinger [22, 33]. All chromatograms were obtained using commercially available components and an amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The analytical column was a 7.5 cm × 4.6 mm I.D. Ultrasphere ODS 3-μm, Altex prepacked column (Cat. No. 244 254). The eluent which consisted of 30 m*M* acetic acid—acetate (22 m*M* glacial acid and 8 m*M* sodium acetate) plus 1.5 m*M* octylamine in ethanol—water (9.5:90.5) was partly 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 0.7 ml/min. The injector valve 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 0.91 V vs. Ag/AgCl reference electrode. The concentration of AAS in the samples was determined by comparing the peak height or the area to that of a calibration standard.

Preparation of solutions

The stock AA or AAS solution was prepared freshly with 50 m*M* perchloric acid and diluted immediately before use. Urine samples were diluted 1:10 with cold 50 m*M* perchloric acid and analyzed immediately. A volume of 5 μl was injected into the HPLC column.

RESULTS

We have obtained chromatograms of AAS at detector potentials ranging from 0.85 to 1 V vs. Ag/AgCl. When the HPLC was run with an applied potential of 1 V vs. Ag/AgCl, the chromatograms of a urine sample showed the occurrence of many interfering substances in the sample, and a detector potential of 0.85 V vs. Ag/AgCl was not sufficient for the detection of very small quantities of AAS in the urine. Thus, an applied potential of 0.91 V vs. Ag/AgCl was used for the operation of the chromatographic detector, which would allow the electro-oxidation of AAS but would prevent the oxidation of many interfering substances in the urine unrelated to AAS analysis.

The ion-pair complex derived from AAS is relatively strongly retained on the column. Although AAS elutes in a relatively broader band, we found that either peak heights or peak areas may be used for the construction of the calibration curve. A calibration in terms of peak height or peak area versus nanogram amounts of AAS is linear for the concentration range of 2—50 ng AAS. The precision of this method was checked by multiple analysis on a single urine sample containing AAS. Typically, the relative standard deviation for six measurements is calculated to be better than 3%. We found that AAS in solution is much more stable than AA.

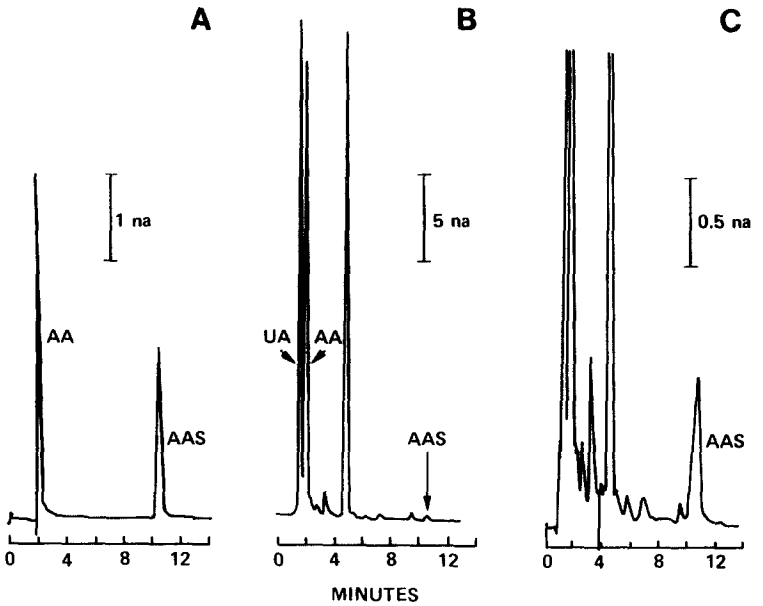

Fig. 1. Chromatograms for L-ascorbic acid (AA) and ascorbic acid-2-sulfate (AAS) in standard and in human urine samples, diluted 1:10, 5 μl injected. (A) 10 ng AA and 20 ng AAS standard injected; (B) urine sample containing uric acid (UA), AA and AAS; (C) urine sample, increased detector sensitivity and with 8 ng AAS standard addition. Conditions: Altex C_{18} reversed-phase 7.5 cm × 4.6 mm stainless-steel column; mobile phase: 30 m*M* acetate buffer plus 1.5 m*M* octylamine in ethanol—water (9.5:90.5) at a flow-rate of 0.7 ml/min; applied potential: 0.91 V vs. Ag/AgCl.

The advantages of the electrochemical detector are obvious in the analysis of biological samples. The UV detector for the detection of AAS [21] is far less sensitive and the chromatograms show interfering UV-absorbing components in biological samples [28—30].

The daily excretion of AAS in human urine has been reported to be 30—60 mg of the dipotassium salt [3, 35]. Fig. 1 illustrates the use of our method on the detection of AAS on urine samples. A parallel experiment of AA and AAS standards is shown in Fig. 1A for a chromatographic comparison. The chromatogram of the urine sample (Fig. 1B) shows the presence of uric acid (UA), AA and AAS as well as some unidentified electrochemically active compounds and the chromatography column is able to separate them. Two unidentified substances with elution times of 15 and 25 min, respectively are not shown in the figure. When the chromatograms were obtained under the same conditions but at a lower flow-rate of the mobile phase (0.5 ml/min), UA and AA were completely resolved. Fig. 1C shows the chromatogram of the urine sample with increased detector sensitivity and with standard AAS addition. The urine donor was a 28-year-old male subject. We found that 5 μl of the diluted urine sample contained 66 ng of AA and 1.5 ng of AAS. Thus, the concentrations of AA and AAS in the original urine of this subject were calculated to be 132 mg/l and 3 mg/l of the free acid, respectively.

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