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Short Communication

Simultaneous high-performance liquid chromatographic determination of ascorbic acid and dehydroascorbic acid in biological samples

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ABSTRACT

The ascorbic acid (AA)–dehydroascorbic acid redox couple is an important component of many biological systems, and various physiological roles have been described for this vitamin. Simultaneous measurement of both AA and dehydroascorbate using high-performance liquid chromatography (HPLC) has proven difficult owing to detection problems. A simple, single-step HPLC assay for the simultaneous detection of both AA and dehydroascorbate was developed without the burden of derivatization of either compounds. This has proven to be a reliable technique and should be applicable to a wide variety of biological samples.

INTRODUCTION

There are no ideal single-step high-performance liquid chromatographic (HPLC) assays for both ascortic acid (AA) and its direct oxidation product dehydroascorbic acid (DHA). The major problem is the simultaneous detection of both constituents: AA and DHA do not absorb at the same wavelength in the UV region, DHA cannot be oxidized or reduced electrochemically, and derivatization reactions for fluorescence or phosphorescence are time-consuming and difficult to carry out. Some workers have collected fractions during HPLC analysis of AA, reduced the DHA in the fractions using dithiothreitol, and then re-injected the sample for quantitation of total AA [1]. Such methods require considerable oper-

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ator time and lead to increased uncertainties for small DHA levels due to the difference estimation. Derivatization of DHA has been successful for separation from AA [2], but interferences seem to be a substantial problem. The need for a practical, rapid and direct HPLC assay for both compounds is evident.

Such an assay was developed, for both small animal and human brain tissue samples, and is presently in use in this laboratory. This methodology enables detailed examination of the ascorbate system in the brain and is applicable to any of a wide variety of biological as well as other types of samples.

As conditions have previously been worked out for the separation and quantitation of AA and DHA [3], modifying this system for simultaneous analysis of AA and DHA was relatively straightforward. The maximum UV absorbance of DHA is at approximately 215 nm with no intereference from AA whose maximum absorbance is near 254 nm. This allows for dual measurement of DHA at 215 nm by UV absorbance, and downline detection of AA at an electrochemical detector maintained at 0.60 V*versus* an Ag/AgCl reference electrode.

EXPERIMENTAL

The chromatographic eluent was prepared as follows: 1.14 g of dibasic sodium phosphate per liter; 700 μl tributylamine per liter, pH adjusted to 5.5. This was then vacuum-filtered and sparged with argon to remove any excess oxygen. Samples were directly injected onto a reversed-phase C_{18} Ultraspere column (average particle size 5 μm; 150 mm × 4.6 mm I.D.) from Beckman (Alltech Assoc. Deerfield, IL, U.S.A.). This column was preceded by a Brownlee C_{18}, Spheri-5 guard cartridge column (average particle size 5 μm; 30 mm × 4.6 mm I.D.) from Anspec (Ann Arbor, MI, U.S.A.) to prevent excessive build-up of protein and soluble material on the analytical column when performing direct injections as described. This guard column was replaced after approximately 100 brain sample injections. Stock AA and uric acid (UA) solutions were prepared from solid standards, and DHA was prepared as decribed by Ohmori and Takagi [4]. Samples were routinely prepared in thoroughly deaerated mobile phase (without the tributylamine) and involved sonication of previously frozen brain samples (10–50 mg), which had thawed to approximately room temperature, in 500 μl of this buffer. This was followed by centrifugation at 39 000 *g* and direct injection of the supernatant on this system.

RESULTS AND DISCUSSION

Fig. 1 shows, for standards, that under these chromatographic conditions the electrochemically detected AA is well resolved from an approximately equimolar concentration of UA. The latter is a frequent interference for AA detection in many biological samples. The DHA, detected by UV absorbance is well resolved from the void volume signal. In brain tissue samples, UA is small in comparison

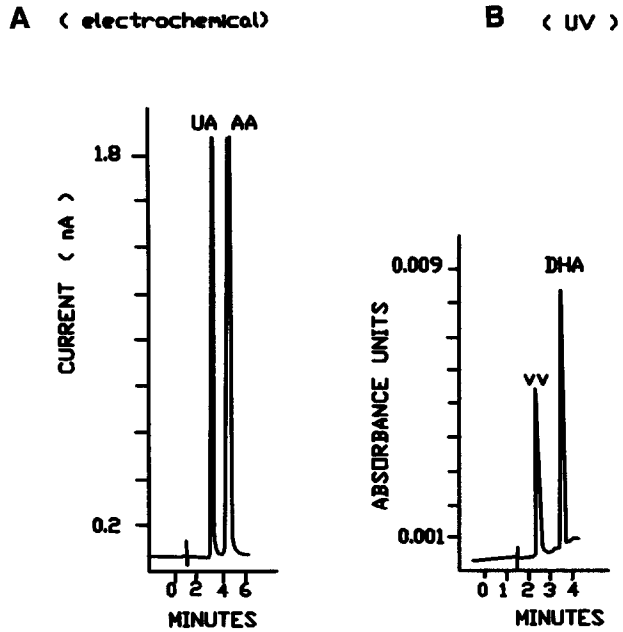

Fig. 1. (A) Standard ascorbate and uric acid chromatogram. A 10-μl volume (1 μM in both AA and UA) was directly injected on-column with conditions decribed in the text. (B) Dehydroascorbic acid standard (20 μM), signal from UV detection. In all cases, vv corresponds to the void volume or solvent front.

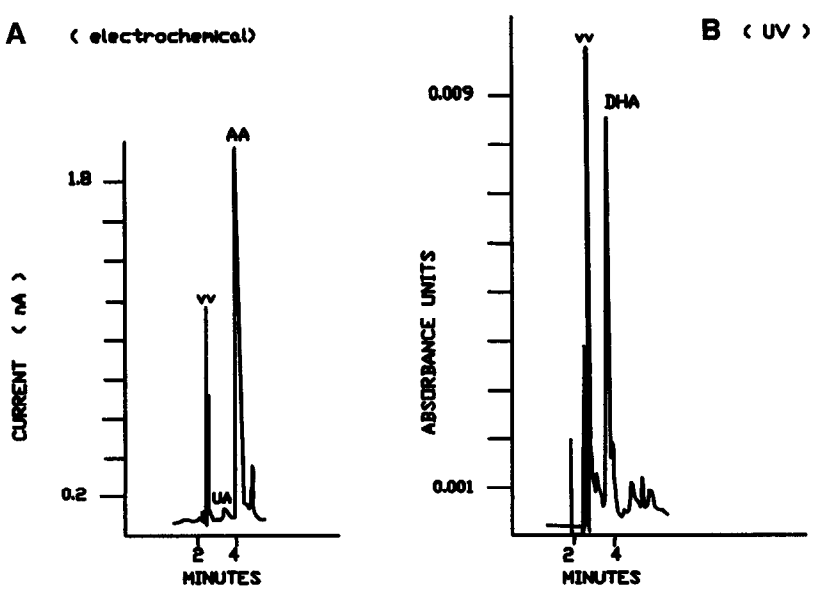

Fig. 2. Brain sample chromatogram for a 10-μl direct injection on-column where (A) displays ascorbic acid and urate and (B) shows the dehydroascorbate analysis. Conditions are as described in the text.

to AA (see Fig. 2) and represents no complications under these conditions. The UV signal for DHA is still well separated from any interferences in brain homogenates also. In order to simultaneously determine UA in brain tissue with AA and DHA, much higher detector sensitivity can be used.

For applications to brain samples, calibration curves from 0.02 to 20 μg AA injected on-column (10-μl injections) were run with linear regression coefficients of 0.922 (the sensitivity for AA is actually far greater, linear calibrations down to 100 pg on-column are routinely carried out). Linear calibrations for DHA were obtained between 10 ng and 2 μg (correlation coefficient 0.889) DHA injected on-column. Since DHA in brain tissue is typically 10% of AA, this calibration is satisfactory. The within-run precision for these measurements was determined to be 3% for AA (coefficient of variation, for six 2-μg on-column injections) and 5% for DHA (seven 500-ng on column injections). It should be noted that the present analysis offers no new improvement in DHA sensitivity. When brain analyses for AA are carried out, the decay of endogenous content does not appear to be significant. Indeed, brain tissue left exposed to air up to 18 h does not appear to lose an appreciable amount of its initial concentration [5]. We attribute this to an endogenous "protective factor" described by Mishra and Kovachich [6] which prevents excessive auto-oxidation of AA, and is present in central nervous system tissues. In addition, we have observed that over time, with repeated injections of identical brain samples, the ascorbate concentration does not significantly decrease. Also, the DHA content does not rise as would be expected during air auto-oxidation which occurs with the ascorbate/dehydroascorbate redox couple in beaker solutions. For quantitation in other sources (plasma, urine, juices, etc.) samples could be placed in an appropriate media such as perchloric acid which will prevent this deleterious auto-oxidation.

This methodology has proven to be quite reliable and is a very simple, straightforward analysis for accessing information on the ascorbate system. The procedure is easily automated, and laboratories investigating functional and physiological roles of ascorbic acid should be able to employ this technique with relative ease.

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