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Note

Determination of ascorbic acid and dehydroascorbic acid in plasma and cerebrospinal fluid by liquid chromatography with electrochemical detection

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The role of ascorbic acid (AA) in metabolism is complex. Its protecting action against the oxidizing effect of the free radicals is of crucial importance. Its presence is necessary for the activity of dopamine β-hydroxylase (DβH) as well as for the synthesis of collagen [1–3]. It is known that the human organism does not synthesize AA and it is ingested with foods. Owing to the presence of two hydroxyl groups, AA can easily be oxidized to 1-dehydroxyascorbic acid (DHAA), and its activity is based on this property. The concentrations of AA and DHAA and their ratio can be an indicator of the redox processes taking place in the organism.

The AA content in the body fluids can be determined by high-performance liquid chromatography (HPLC) with UV [2–10], fluorimetric [7, 11–15] or electrochemical [7,9,16–20] detection. DHAA has been determined directly or after reduction to AA [5,7,9,15,21].

This paper reports a method for the measurement of AA and DHAA concentrations in plasma and cerebrospinal fluid (CSF) by using HPLC with an electrochemical detector.

EXPERIMENTAL

Chemicals

l-Ascorbic acid was purchased from Reanal (Budapest, Hungary) and *l*-de-

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hydroascorbic acid and *dl*-dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.). Water used was demineralized and filtered through a 0.45-μm filter.

Chromatography

Isocratic analyses were carried out with a Perkin-Elmer Series 10 high-performance liquid chromatograph (Norwalk, CT, U.S.A.), equipped with a Rheodyne 7125 sample injector (Cotati, CA, U.S.A.) and a Model LC-4B thin-layer electrochemical detector with a TL-5A cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The separation was carried out on a reversed-phase, Spherisorb ODS-2 column (average particle size 5 μm; 250 mm $\times$ 4.6 mm I.D.) from Latek (Heidelberg, F.R.G.). The potential of the glassy carbon working electrode was set at +0.80 V versus an Ag/AgCl reference electrode. Chromatograms were recorded with a Shimadzu CR 1B (Kyoto, Japan).

The mobile phase was a mixture of a buffer and 5% (v/v) methanol. The buffer consisted of 0.1 m*M* disodium hydrogenphosphate (Na_2HPO_4), 0.07 m*M* EDTA and 0.15 m*M* sodium octyl hydrogensulphate, adjusted to pH 3.1 with orthophosphoric acid. The mobile phase was continuously degassed by sparging with nitrogen. The flow-rate was 0.5 ml/min, resulting in a pressure drop of ca. 21 MPa. The column temperature was maintained at 28°C.

Standard preparation

A standard stock solution of AA (4 μg/ml) was prepared in 0.2 m*M* metaphosphoric acid and 5 m*M* EDTA solution. This solution was further diluted in the same solvent to give a series of working standards of AA. Their concentrations were 400, 200, 100 and 50 ng/ml. The stock solution was stored at −34°C. This solution was stable for at least three weeks.

Sample preparation

Samples were obtained from healthy controls and from patients with various mental diseases in our psychiatric department. Lumbar punctures were performed with the consent of the patients between the fourth and fifth lumbar vertebrae. The patients did not take any medicine that could influence the AA levels. After bed rest in sitting posture, 2 ml of blood and 3 ml of CSF were taken. Blood was poured into a centrifuge tube containing 2 mg of sodium metabisulphite and one drop of heparin. After centrifuging, at 1000 *g*, 0.1 ml of plasma was added to a solution of 0.2 m*M* metaphosphoric acid and 5 m*M* EDTA. The mixture was centrifuged at 1000 *g* for 10 min at 0°C, then the pure supernatant was stored at −34°C until the analysis. The CSF samples (0.1 ml) were prepared and stored as described for the plasma.

For the determination of the DHAA, 2.5 m*M* DDT was added to 50 μl of plasma and CSF samples were mixed with 0.2 m*M* metaphosphoric acid and 5

m*M* EDTA. The quantitative reduction of DHAA was performed for 10 min after mixing the components.

RESULTS AND DISCUSSION

The optimal oxidation potential for the analysis of AA was found to be +0.80 V (Fig. 1) The potential curve of AA shows maximum value above +0.75 V. Therefore, the measuring potential of +0.80 V was used, with a 2 nA background current. The calibration curve was linear in the concentration range 50–400 ng/ml, and the linear regression equation was $y=0.249x+0.036$, $r=0.998$, $p<0.001$. The detection limit was 5 ng per injected volume at a signal-to-noise ratio of 3:1. The corresponding coefficient of variation (C.V.) was 2% for ten replicate analyses.

AA decomposes very rapidly, so appropriate conditions are necessary for its storage. According to our observations, oxidation of AA was accelerated by increased eluent pH, increased temperature and the presence of red blood cells.

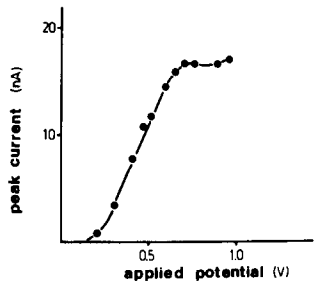

Fig. 1. Current–voltage curves for ascorbic acid. The optimum operating potential is +0.8 V using an Ag/AgCl reference electrode.

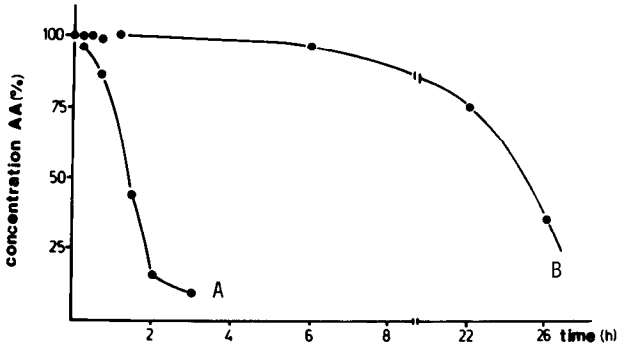

Fig. 2. Degree of oxidation of the ascorbic acid in CSF at room temperature without antioxidant (A) and after addition of 0.2 m*M* MPA and 5 m*M* EDTA (B).

The decrease of the AA in the plasma is even more pronounced since it can be oxidized during deproteinization if oxygen is liberated from oxyhaemoglobin.

It is known from the literature that, owing to its rapid oxidation, the measurement of AA is difficult. Stabilizing solutions are, therefore, used to inhibit its oxidation: perchloric acid [1,17,20], trichloroacetic acid–orthophosphoric

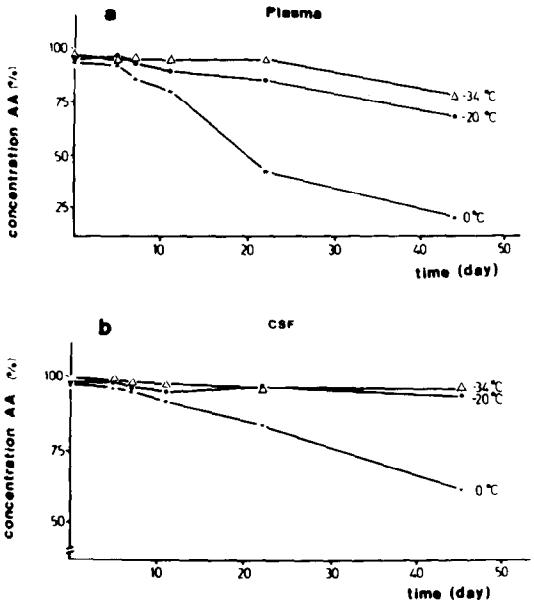

Fig. 3. Change of the ascorbic acid content in plasma (a) and in CSF (b) at different temperatures in a mixture of 0.2 m*M* MPA and 5 m*M* EDTA.

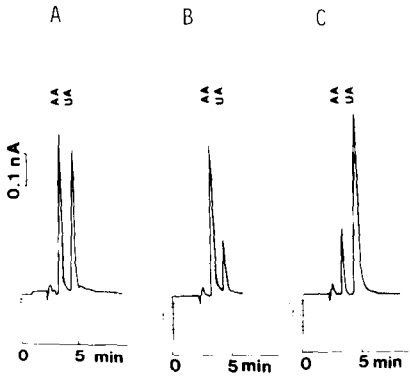

Fig. 4. Chromatograms of ascorbic acid in (A) a standard solution (4 μg/ml), (B) CSF and (C) plasma. Conditions as described in text. Peaks:AA = ascorbic acid; UA = uric acid.

acid [18], metaphosphoric acid [8,10,16,19,21] solutions, sometimes containing *dl*-homocysteine [3,10] and sodium metabisulphite [18] are preferred. Despite these conditions, AA is stable only for a short period of time.

For the stabilization of AA in plasma and CSF the most convenient mixture was 0.2 m*M* metaphosphoric acid (MPA) and 5 m*M* EDTA solution (Fig. 2). A low pH was produced by the MPA and EDTA bound metal ions present. In CSF the AA concentration decreased by 10% after 30 min and by 25% after 60 min, but it remained unchanged at room temperature for 6 h after addition of this mixture. CSF samples stabilized by this solution are stable for seven to eight days at 0°C and for forty days both at −20°C and −34°C (99.1%). In plasma, AA can be stored without decrease for four to five days at 0°C, for eight days at −20°C and for twenty-two days at −34°C (98.7%, Fig. 3).

Since DHAA is electrochemically inactive it cannot be directly determined

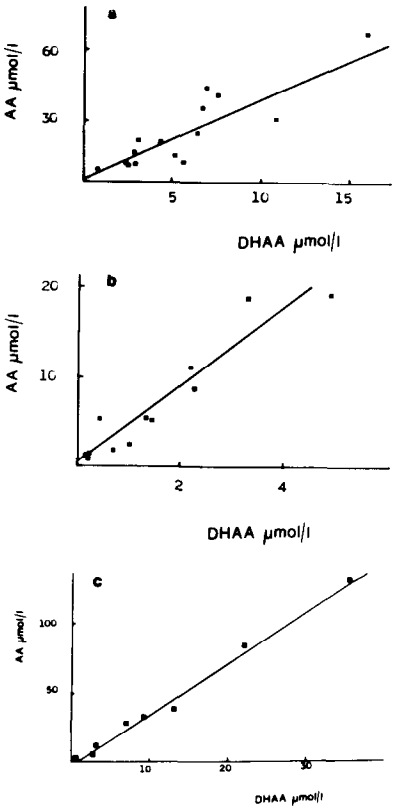

Fig. 5. Relationships between the concentrations of ascorbic acid and dehydroascorbic acid (a) in the plasma of the control group ($y=0.189x+1.749$; $r=0.801$; $p<0.001$), (b) in the plasma of the patients group ($y=0.196x+0.218$; $r=0.904$; $p<0.001$) and (c) in the CSF of the patients group ($y=0.274x+0.424$; $r=0.997$; $p<0.001$).

by electrochemical detection. DHAA was reduced to AA by 2.5 m*M* DTT and the combined amount of AA and DHAA was determined (Fig. 4).

Plasma AA values of 10–110 μM [2,11,16,19] and a DHAA level of 4.5 μM [2] were reported. In the course of our studies AA and DHAA have been determined in the blood of fifteen healthy controls (average age 35 ± 5 years) and in the blood and CSF of twelve patients (average age 71 ± 11 years).

A significant correlation was found between the AA and DHAA levels in the plasma and the CSF (Fig. 5). In the plasma of the control group, AA was found to be 23.2 ± 17.3 μM and DHAA 5.8 ± 2.7 μM, whereas among the patients AA was 8.1 ± 5.7 μM and DHAA 1.3 ± 0.9 μM. Plasma values of the healthy control group are in good agreement with the literature data. In the CSF of the patients, the average value for AA was 21.5 ± 4.4 μM and that for DHAA was 5.8 ± 5.1 μM.

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