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Determination of Ascorbic Acid and Dehydroascorbic Acid in Plasma by High-Performance Liquid Chromatography with Coulometric Detection — Are They Reliable Biomarkers of Oxidative Stress?[1]

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The concentrations of the hydrophilic antioxidants ascorbic acid and dehydroascorbic acid in plasma for some time have been considered possible biomarkers of oxidative stress. However, several problems are associated with the accurate measurement of these two compounds. We have developed and validated a selective and reproducible high-performance liquid chromatographic method for the quantification of ascorbic acid and dehydroascorbic acid in plasma. The method meets the requirements of a reliable routine analysis. The plasma samples are stabilized with 5 mM *meta*-phosphoric acid, centrifugated at 4°C before HPLC analysis. For ascorbic acid analysis, the sample pH is adjusted to 2.6, whereas for total ascorbic acid measurement dehydroascorbic acid is reduced to ascorbic acid using dithiothreitol for 5 min at pH 6.2 after which the sample pH is adjusted to 2.6. The samples are analyzed on a reversed-phase system using coulometric detection. Dehydroascorbic acid concentrations are calculated by subtraction. Within- and between-day coefficients of variation for the complete assay were in the range of 4–8 and 3–6% for ascorbic acid and total ascorbic acid, respectively. The stability of ascorbic acid was monitored under various conditions including storage and the implications as well as the reliability of ascorbic acid as a biomarker are discussed.

Reactive oxygen species (ROS)[3] seem to be involved in the development of several diseases and possibly in aging and cancer in general (1) and the interest in their possible use as biomarkers of oxidative stress is therefore well-founded. The significance of AA and DHAA as an antioxidant couple in biological systems is well established (2–4). The relation between antioxidant levels and degenerative diseases such as cancer and atherogenesis has shown significant protective effect of ascorbic acid and other antioxidants (see Refs. (5,6) and references therein). Consequently, the relative content of DHAA and AA has been suggested to be a useful biomarker reflecting the ROS and overall radical level (7–10). However, a prerequisite for such considerations includes a reliable assay for the measurement of these two unstable compounds.

Reliable determination of AA and DHAA is challenging because of the unstable nature of the compounds, and the fact that they furthermore are in equilibrium with each other (Fig. 1). Numerous methods concerning the quantification of AA have been published most often involving spectrophotometric analysis, gas chromatography, or high-performance liquid chromatography (see Refs. (11,12) and references therein). However, several problems still remain associated with the determination of AA and DHAA: (a) sample stability with respect to the relative DHAA and AA content, (b) individual recovery, (c) chromatographic design, (d) reproducibility, (e) column durability, (f) choice of detection principle, (g) general autooxidation of AA, and (h) sensitivity and selectivity toward AA.

In the present paper we report our monitoring of AA stability under various conditions in aqueous standards as well as in plasma. We also report an optimized

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[3] Abbreviations used: AA, ascorbic acid; AA_{total}, total ascorbic acid; DHAA, dehydroascorbic acid; $DHAA_{total}$, total dehydroascorbic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MPA, *meta*-phosphoric acid; ROS, reactive oxygen species; S/N, signal-to-noise ratio; Trizma base, Tris(hydroxymethyl)aminomethane; CV, coefficient of variation.

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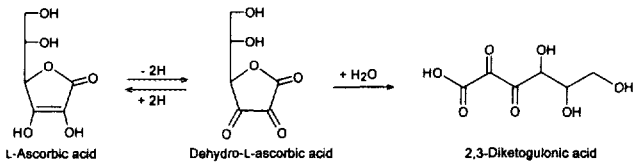

FIG. 1. L-Ascorbic acid is in equilibrium with its oxidized form dehydro-L-ascorbic acid. Dehydro-L-ascorbic acid is very unstable and is easily hydrolyzed irreversibly to 2,3-diketogulonic acid, which has no antiscorbutic effect.

assay procedure that meets the requirements of a reliable routine analysis for the assessment of the DHAA and AA content in plasma considering all of the problems mentioned. Finally, the reliability of ascorbic acid as a possible biomarker of oxidative stress is discussed.

MATERIALS AND METHODS

Materials and Reagents

Water for all applications was obtained from an Elgastat Maxima water purification system (Elga Ltd., Bucks, UK). All compounds were of analytical grade. AA, DTT, ascorbic acid oxidase, DL-homocysteine, and Trizma base buffer were from Sigma (St. Louis, MO), while DHAA, *ortho-* and *meta*-phosphoric acid, disodium hydrogen phosphate, and disodium–EDTA were purchased from Merck (Damstadt, Germany). *n*-Dodecyltrimethylammonium chloride was obtained from Lancaster (Lancashire, UK). After overnight fast, blood samples were collected in the morning from four healthy male subjects, who did not take AA-containing supplements. Standard solutions of AA (100 μM were made in 5.0% MPA containing 0.5 mM homocysteine. The standard solutions were stored in aliquots of 200 μl at −20°C and for reference, daily injections of the standards showed no change for at least 30 days.

Sample Preparation

Blood samples (1 to 10 ml) were immediately centrifuged (4000*g*, 10 min, 4°C) and an aliquot of the top plasma of each sample was directly mixed with an equal volume of precooled 10% MPA. The formed precipitate was spun down by centrifugation (4000*g*, 10 min, 4°C) and the samples were kept at −20°C until analysis.

Ascorbic Acid Measurements

The MPA-treated plasma samples were gently thawed and kept on ice at all times. For the AA measurement, 100 μl of the sample supernatant was diluted with 100 μl 0.125 M Trizma buffer, pH 9.0, resulting in a pH of 2.6 to 2.7. Twenty microliters of this mixture was injected into the HPLC system.

AA_{total} was measured after reduction of the DHAA present in the sample. The reduction was carried out by adding 100 μl of the supernatant of the MPA-treated plasma sample to 50 μl 0.5 M Trizma buffer, pH 9.0, containing 10 mM DTT resulting in a pH ranging from 6.1 to 6.2. After a reduction period (25°C, 5 min), the reaction was quenched by addition of 50 μl of 0.2 M H_2SO_4. This resulted in a final pH of 2.6 to 2.7 and the sample was cooled to 0°C. Twenty microliters of the mixture was used for the HPLC analysis.

Stability Experiments

AA stability was investigated under various storing conditions and during sample preparation. The stability of blood samples and plasma before stabilization is of general interest when large clinical studies are prepared. Blood samples were divided into two portions. One part was left on ice and an aliquot of it was worked up approximately every 15 min as described in sample preparation. The other part was immediately centrifuged (4000*g*, 10 min) and the plasma was separated from the erythrocytes. The plasma was left on ice and, approximately every 15 min, an aliquot was added to an equal volume of precooled 10% MPA, worked up, and analyzed.

For the investigation of stored plasma sample stability, the samples were treated with MPA as described in sample preparation, divided into 200 μl aliquots, and stored at −20°C until analysis.

Chromatographic Analysis

The plasma samples were analyzed on an automated HPLC system consisting of the following Merck–Hitachi Instruments (San Jose, CA) units: AS-2000A refrigerated autosampler (4°C, samples protected from light), L-6000 Pump, T-6300 Column Thermostat operated at 30°C and a D-6000 Interface Unit. For peak detection, the chromatographic system was connected to an ESA Coulochem II coulometric electrochemical detector (ESA Inc., Bedford, MA) equipped with a Model 5020 guard cell operated at 200 mV and a Model 5010 dual analytical cell operated at 100 mV. All units were connected to a personal computer for control and for collection and analysis of data (HPLC-Manager, Version 2, Merck–Hitachi). The column was a Nova-Pak C_{18} (particle size 4 μm, pore size 60 Å, 75 × 3.9 mm i.d., Waters) operated at 0.50 ml/min and fitted with a Nova-Pak C_{18} precolumn (Guard-Pak system, Waters). The precolumn was changed when the back pressure had increased significantly after injection of ~400 to 600 samples. The mobile phase consisted of 0.1 M disodium hydrogen phosphate, 2.5 mM disodium–EDTA, and 2.0 mM *n*-dodecyltrimethylammonium chloride and the pH was adjusted to 3.0 with *ortho*-phosphoric acid. The mobile phase was filtered through a 0.45-μm filter under vacuum and degassed before use.

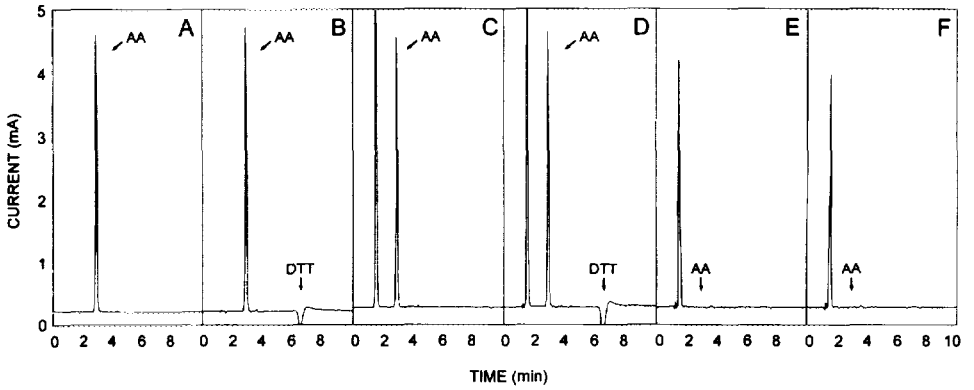

FIG. 2. Chromatographic analysis with coulometric detection of AA samples handled as described under Materials and Methods. The detector signal is expressed in mA. (A) Aqueous sample containing 35 μM AA (equals an actual injection of 17.5 pmol (3.1 ng) AA). (B) Aqueous sample reduced with DTT. (C) Typical plasma sample. (D) Reduced plasma sample. (E) Plasma sample after incubation with 5 μM $CuBr_2$ (5 min, 25°C). (F) Plasma sample after incubation with ascorbic acid oxidase (5 min, 25°C).

RESULTS

Chromatography

Figures 2A and 2C represent typical chromatograms of an AA standard and a plasma sample, respectively. The coefficient of variation (CV) of the retention time of AA was 0.99% (n = 20) within series of runs and 1.64% (n = 82) between series of runs. The retention times observed for the present system ranged from 2.88 to 3.03 min (n = 30) on a day-to-day basis. The CV of the peak area of AA in 10 μl injections of a plasma sample was 1.68% (n = 10).

For optimum sensitivity and selectivity, the detection of AA at 100 mV was based on the voltammogram in Fig. 3, which shows the response or current versus the potential of the cell. The low potential of 100 mV minimizes the possibility of interference from other compounds. The guard cell operated at 200 mV ensures that all possible interfering substances from the mobile phase are oxidized prior to entry of the analytical cell.

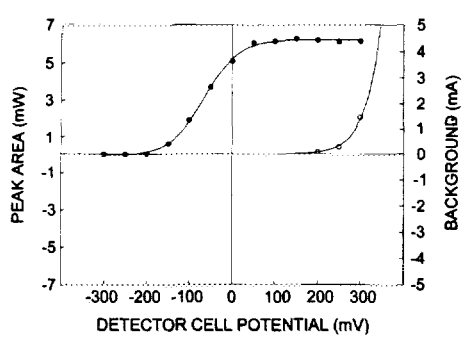

FIG. 3. Voltammogram of AA. Twenty-microliter standard solutions containing 80 μM AA and 0.5 mM homocysteine in 5% MPA were injected on HPLC with the electrochemical detector preset at −400 to 400 mV. AA (●) is expressed in mW. The background (○) is expressed as mA current. Sigmoidal curve fit ($r^2 > 0.999$).

In accordance with earlier observations stating no electrochemically activity of DHAA (11,13–19), the compound showed neither oxidative nor reductive capabilities in the range of −500 to +500 mV. Although the possibility of electrochemical activity at higher numeric potential exists, the general background of the mobile phase in the present chromatographic system was out of range and therefore practical peak measurement was not possible.

Peak Verification

Identical retention times were obtained for the measured plasma peak and an authentic aqueous AA sample, respectively. For confirmation of the peak identity, quantitative oxidation of AA was performed by addition of 5 μM copper(II)bromide to a 100-μl sample. In accordance with previous observations (Ref. (20) and references therein), the oxidation occurs in less than 1 min. In addition, the quantitative oxidation of AA was achieved by incubating the sample with ascorbic acid oxidase (EC 1.10.3.3) at 25°C for 5 min at pH 6.2. Figure 2 shows chromatograms of a plasma sample before and after oxidation. The quantitative oxidation reactions of AA leaving no peaks demonstrate the homogeneity of the measured AA peak. In addition to these specific experiments, a second coulometric detector cell was placed in series with the one used for detection of AA at 100 mV. This second cell showed no peak at or near the retention time of AA indicating total oxidation of AA at the first cell and no presence of interfering compounds.

Reduction Conditions

Determination of the optimal reaction time for the DHAA to AA reduction must include considerations regarding the irreversible conversion of DHAA to 2,3-

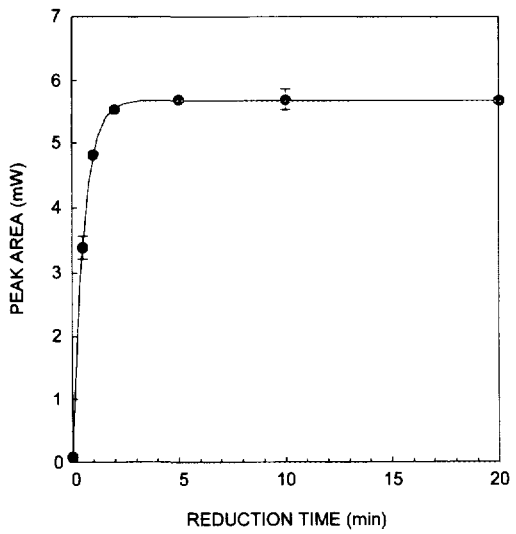

FIG. 4. Reduction of DHAA to AA. DHAA solutions (50 μM) were reduced to AA at pH 6.2 as described under Materials and Methods. Each data point represents mean with SD of four individual experiments. AA content (●) is expressed in mW. Sigmoidal curve fit (r^2 > 0.9999).

diketogulonic acid at elevated pH. Consequently, the reaction time should be minimized and the pH controlled. In accordance with earlier observations (21), we found that pH 6.2 offers optimum conversion. Figure 4 shows the formation of AA as a function of reaction time in a 50 μM solution of DHAA treated as described above. From the experiment it can be concluded that 3 min is a sufficient reduction period. However, 5 min has been chosen as the standard reduction period since it permits a simultaneous and thorough preparation of approximately 10 plasma samples. In Fig. 2, chromatograms of a reduced standard sample and a reduced plasma sample are displayed. The reducing agent DTT does not interfere with AA and, consequently, tedious extraction procedures are not necessary.

Standard Curves

Standard curves were obtained from solutions prepared and processed by three different methods: direct injection of known concentrations of AA onto the HPLC system, normal sample preparation of aqueous solutions of known concentrations of AA, and finally normal sample preparation using a plasma sample to which various amounts of AA had been added before workup. From these experiments the possible effects of the workup procedure and the matrix could be observed.

Standard solutions containing known concentrations of AA (5.0–80.0 μM equal to 25–400 pmol per injection) were injected directly. Excellent linear correlation (r > 0.9999) between the concentration of AA and the peak area as measured by the computer software was confirmed over the entire range. It should be noted that the detection limit of the assay is lower than 1.0 nM (S/N = 15, equal to 20 fmol per injection). However, the standard solution range presented here has been chosen as representative for possible plasma concentrations.

Experiments were also made comparing the workup of plasma samples and aqueous solutions. Again AA was added giving final concentrations of 5.0–80.0 μM, and AA and AA_{total} were measured in both cases. Figure 5 shows excellent linear correlation (r > 0.999 in all cases) and the recovery was 100.8% (AA) and 101.4% (AA_{total}), respectively, as calculated from the slopes. In the case of aqueous samples, no oxidation is observed as the data points corresponding to reduced and nonreduced samples are virtually identical. In the case of plasma samples, the numeric difference between the corresponding data points (AA and AA_{total}) is maintained offering two parallel lines. This demonstrates that the initial equilibrium has been successfully blocked allowing no further oxidation. The experiment also demonstrates that standards can be made as aqueous solutions.

Precision

The within-day and between-day precision of the assay with respect to the DHAA and AA content were calculated from series of experiments made with 200-μl aliquots of an average plasma sample with a content of 38.2 μM (AA) and 38.7 μM (AA_{total}). The within-day CVs were 4.39% (n = 10) for the AA measurement and 3.35% (n = 25) for the AA_{total} measurement, while the between-day CVs were calculated to be 8.55% (n = 5)

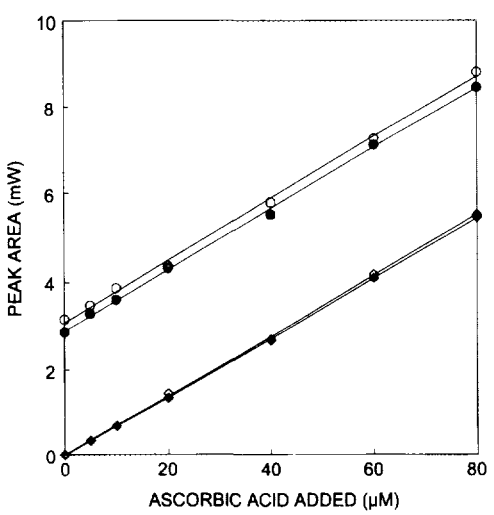

FIG. 5. Standard curves for aqueous AA solutions and plasma solutions to which known amounts of AA have been added. (●) Plasma samples; (○) plasma samples, reduced; (♦) aqueous samples; (◇) aqueous samples, reduced. Data are expressed in mW.

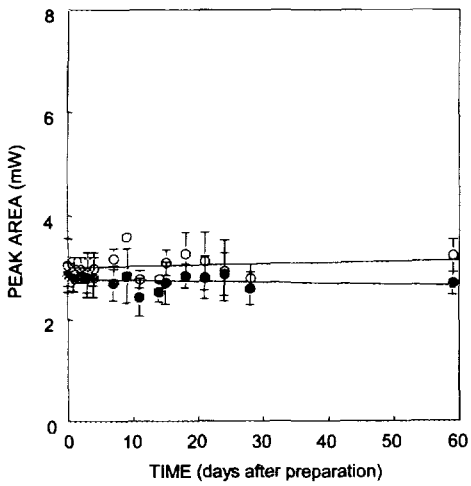

FIG. 6. Stability of AA samples stored at −20°C monitored by repeated measurement of AA (●) and AA + DHAA (○). Each data point represents four individual samples.

for the AA measurement and 6.26% (n = 5) for the AA_{total} measurement. These determinations reflect the complete assay from sampling to the final result. The CVs of the AA measurement were consistently larger than for the AA_{total} measurement, and we experienced a minimum of the CV of the AA measurement when about 10 samples were prepared and handled simultaneously until they were placed in the refrigerated autosampler. When stored in the autosampler at 4°C in the dark, only a 1.8% average deviation between a set of plasma samples (n = 20) analyzed at t = 0 and reran at t = 24 h was observed. This means that as much as 6 × 10 samples can be prepared in 1 day. If only interested in AA_{total}, as many as 60 samples can be prepared simultaneously.

Reference Range

We analyzed the plasma samples of four fasting and apparently healthy male subjects who did not take AA-containing supplements. The AA content was measured to 27.8 ± 1.32 μM (SD), while AA_{total} was measured to 28.1 ± 0.96 μM (SD) corresponding to a DHAA content of 1.1% of AA_{total}.

Stability of Prepared Ascorbic Acid Samples

In a set of plasma-sample aliquots analyzed at intervals during storage at −20°C for 2 months, no significant decrease in AA_{total} or AA was observed (Fig. 6). According to the slope estimated by linear regression of the detector signal on days after preparation, the change in AA_{total} was 0.05% per day (−0.07 to 0.17%; 95% confidence interval; P = 0.7). The ratio between AA and AA_{total} was estimated to initially 93% (89 to 97%) and according to the slope of the linear regression, the change during storage was −0.1% per day (−0.3 to 0.1%); P = 0.17). A similar experiment was carried out storing plasma samples at −80°C, however, no difference compared to the −20°C experiment was observed.

Stability of Nonacidified Blood and Plasma Samples

The sample preparation procedure was tested by monitoring the stability of nonacidified blood and plasma samples. Figure 7 shows the consequence of prolonged storage of an average blood or plasma sample on ice without stabilization. The level of oxidation ranged from approximately zero to 20% per hour. No significant difference between the oxidation in plasma vs whole blood was observed (Fig. 8).

DISCUSSION

The lack of control of sample stability has been the key problem in AA analysis. AA is easily oxidized to DHAA in biological solutions. DHAA is even more unstable and reactive and is rapidly hydrolyzed irreversibly to 2,3-diketogulonic acid with a half-life of 6 min (pH 7.0, 37°C) (22). However at low pH, both compounds are relatively stable. The main issues therefore become the treatment of the sample before acidification and the storage temperature and period. As shown in Figs. 7 and 8, the oxidation of AA to DHAA occurs at a constant rate, except for an apparent 15- to 30-min lag period. This stresses that a considerable standard-

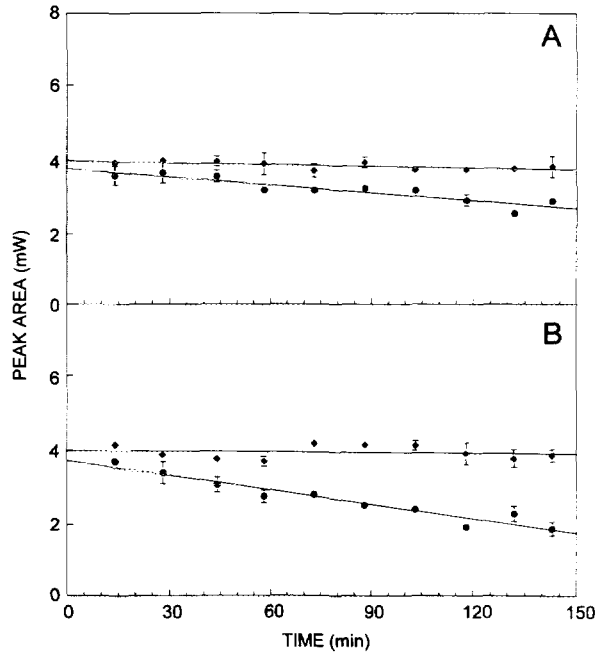

FIG. 7. Stability of nonacidified plasma (A) and blood (B) samples. Plasma and blood samples were left on ice until addition of MPA, after which they were worked up and analyzed. (●) AA; (◆) AA + DHAA. Data are expressed in mW.

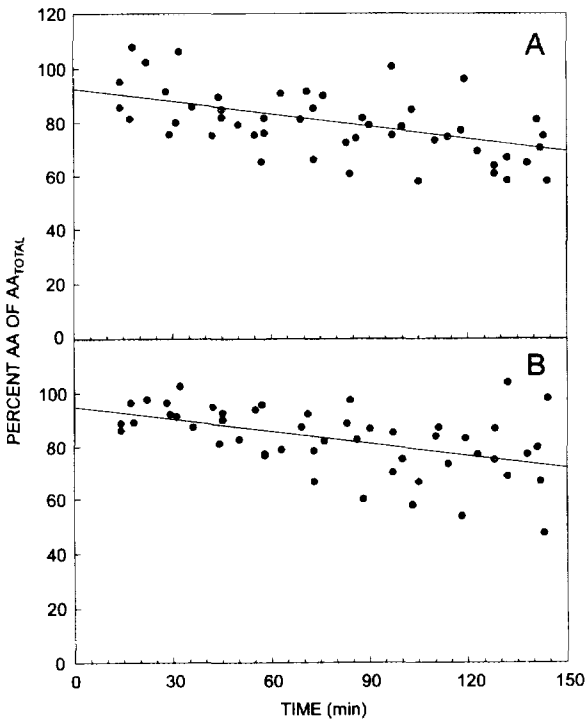

FIG. 8. Stability of nonacidified plasma (A) and blood (B) samples as percent AA of AA_{total}. The stability of individual samples may vary considerably, but no significant difference between the stability of plasma vs whole blood is observed.

ization of the sample preparation procedure must be implemented, both within the laboratory and even more for interlaboratory comparisons. The blocking of the equilibrium between DHAA and AA is crucial, and a reliable method depends on steps that ensure not only the general stability but also the equal recovery of both compounds. As shown in Figs. 5 and 6, it is possible to maintain the true DHAA content during both sample preparation and sample storage at −20°C.

During the development of the present assay, some technical problems were encountered. Ion-paring reagents are popular in ascorbic acid analysis due to the hydrophilic nature of the compound. However, we found that the commonly used myristyltrimethylammonium bromide and several other detergents were unsuitable as ion-pair reagents when samples were stabilized with MPA. The detergent precipitated within the chromatographic system when the MPA sample was injected on the HPLC. This resulted in the frequently observed fluctuating baseline with ultraviolet detection and total baseline instability with coulometric detection. The hydrophilic nature of AA has often been neglected in the development of the chromatographic methods for quantification. Even when ion-pairing reagents are used, the capacity factor (k') of AA is often between zero and 0.5 (12,14,17,18), favoring neither chromatographic separation of AA from possible hydrophilic impurities nor accurate integration. In the present work, the unorthodox use of an all-water-based mobil phase on a reversed-phase chromatographic system most certainly makes the aliphatic C_{18} chains clot, nevertheless maintaining a reproducible retainment of the ion-paired hydrophilic compound giving a $k' > 1$. Reproducibility of AA assays is often poor due to inadequate equilibration of the column. However, another important reason for reproducibility problems is degeneration of the column caused by the high acidity of sample or mobile phase. The most often used 5% MPA-containing plasma solutions have a pH of approximately 1.5. In the present method, the column stability was maintained by increasing the pH of the sample and the mobile phase to 2.6 and 3.0, respectively. While maintaining sample stability, this prevents hydrolysis of the column material and ensures reproducible retention times.

Other important points are the difficulties encountered in the detection of DHAA in general. Refractive index detection is generally not sensitive enough to satisfy all applications (13). DHAA is electrochemically inactive and has an ultraviolet absorbance maximum at approximately 215 nm (19). Although theoretically possible, measuring DHAA with a uv detector at the necessary low wavelength is not practical because of the high background present in that area and elevated risk of interference from other compounds. Thus most of the analytical procedures previously presented quantify AA first, reduce the present DHAA to AA with an appropriate reducing agent, and then repeat the analysis, this time measuring the total content of AA (AA_{total}) (14–16,18,23–28). Subsequently, DHAA can be calculated as AA_{total} − AA. Alternatively, methods based on the opposite strategy of quantifying DHAA and subsequently $DHAA_{total}$ after oxidation of the AA present include derivatization of DHAA with dinitrophenylhydrazine (29) or *o*-phenylenediamine (30) often followed by spectrophotometric detection. However, both of the latter reagents react with other compounds, including 2,3-diketogulonic acid, and this can lead to measurement errors in simple chromogenic assays (31). The obvious drawback of the subtraction methods in general is the decreased reliability of results obtained, when the substance to be derived constitutes only a small fraction of the parent compound. Nevertheless, quantification of AA and AA_{total} by HPLC remains the best available strategy.

Considerable discrepancy between the relative DHAA and AA reference concentrations for healthy adults in previous studies (24,25,28,32–39) is found with DHAA contents ranging from zero to 20% of the total AA. Considering the points raised above, the wide range of ratios could well indicate methodological problems in maintaining the original DHAA/AA ratio rather than a considerable physiological variation, and in agreement with our results, the most recent reports

seem to support a normal plasma content of DHAA in the range of zero to a few percent of AA_{total}. Consequently, the reliability of AA and DHAA as biomarkers of oxidative stress depends on the experimental design. When the DHAA content is less than 5% of the total AA, the results of large epidemiological studies become ambiguous if the likely differences are smaller or of the same order of magnitude as the coefficient of variation of the assay. The number of samples often required from each person in this type of studies may also practically exclude the proper handling of the sensitive AA samples. In these cases, differences in AA_{total} would be a far more reliable biomarker. However, in carefully designed studies monitoring the plasma response of individuals to, e.g., a high antioxidant diet, the relative concentrations of AA and DHAA may provide useful information.

The present analysis was developed to overcome several problems. The high recovery and stability of the samples are combined with coulometric detection offering high selectivity and sensitivity toward AA. Moreover, extraction of the reducing agent is not required. The detection limit (<20 fmol per injection) is low enough to meet the demands of biopsy analysis, etc. However, the possible stability problems prior to acidification must be solved in order to ensure a reliable measurement of DHAA in the specific matrix. In short, the present method offers reliable as well as highly selective and sensitive DHAA and AA analysis.

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