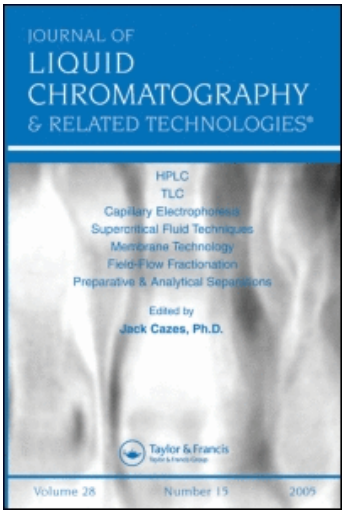

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Quantitative Estimation of Dehydroascorbic Acid and Ascorbic Acid by High-Performance Liquid Chromatography—Application to Human Milk, Plasma, and Leukocytes

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QUANTITATIVE ESTIMATION OF DEHYDRO-ASCORBIC ACID AND ASCORBIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—APPLICATION TO HUMAN MILK, PLASMA, AND LEUKOCYTES

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ABSTRACT

A "high-performance" liquid chromatographic (HPLC) method for quantitation of dehydroascorbic acid and ascorbic acid and its application to protein-free human milk, blood plasma and leukocytes (buffy layer) is described. In the method, DL-homocysteine was used to convert dehydroascorbic acid quantitatively to ascorbic acid that was measured by reversed phase liquid chromatography. Fresh human milk was found to contain ascorbic acid 54.3±6.5 mg/l (mean±SEM; n=4) and dehydroascorbic acid 21.0±9.1 mg/l (mean±SEM, n=4) when stored at +4°C. The concentration of both forms of ascorbic acid was found to detoriate in similar ratios during storage at +4°C, and pasteurization considerably increased the loss of vitamin C. After pasteurization the milk contained ascorbic acid 8.6±3.4 mg/l (mean±SEM, n=4) and dehydroascorbic acid 6.6±2.4 mg/l (mean±SEM, n=4). In plasma the dehydroascorbic acid content (0.16±0.03 mg/l, mean±SEM, n=23) was lower than that of ascorbic acid (9.96±0.75 mg/l, mean±SEM, n=23).

The ascorbic acid concentration in the leukocyte mixtures was 0.21±0.04 mg/10^9 cells (mean± SEM, n=10) and dehydroascorbic acid concentration 0.09±0.03 mg/10^9 cells (mean±SEM, n=8). A statistically significant ($r=0.599$, $p<0.05$) correlation was established between the concentrations of ascorbic acid in plasma and leukocytes.

INTRODUCTION

Vitamin C is a water-soluble vitamin that occurs as L-ascorbic acid (AA) and its oxidized form, dehydro-L-ascorbic acid (DHA). Biologically both forms are regarded equally active (1). The proportion of DHA in biological fluids, especially in plasma, has been reported to be low (2,3). Since DHA may be formed from AA during storage of the sample or food, it has been suggested that both of these two compounds should be measured together as total vitamin C (TAA) after conversion of the forms totally either into AA or DHA.

Recent data from our laboratory (5) show that plasma levels of vitamin C in preterm infants fed on pooled pasteurized human milk are low. This is in contrast to maintenance of normal plasma vitamin C levels during prolonged breast-feeding in full-term infants (6). In order to further study our finding, we have developed a simple and rapid HPLC method for quantitative estimation of AA, DHA and TAA in fluids and cell mixtures. We have tested the applicability of the method especially on human milk in which a significant decrease in the total vitamin C during 24 h refrigeration at +4°C has been described (7) - the phenomenon that was not detected after a long-term storage in a freezer is important to consider because preterm infants are fed on stored milk. A modification of the method can be used for determinations of TAA in plasma and leukocytes, which are commonly accepted as indicators of the tissue status of vitamin C (8).

MATERIALS

Reagents

Standard L-ascorbic acid and DL-homocysteine were purchased from Sigma Chemical Co (St. Louis, MO, USA). Standard dehydro-L-ascorbic acid and tetrabutylammonium hydroxide (TBAH) were from Fluka AG (Buchs, Switzerland). Trichloroacetic acid (TCA) and metaphosphoric acid (MPA) were from E. Merck AG (Darmstadt, FRG). DL-homocysteine was also freshly synthesized in our laboratory from DL-homocysteinethiolactone hydrochlorid, which was purchased from Fluka Ag. Macrodex^{R} (6%) was from Leiras Oy (Turku, Finland).

High-Performance Liquid Chromatography

The chromatograph was equipped either with a LKB Bromma 2150 HPLC pump and a LKB Bromma 2151 variable wave-length monitor (LKB, Bromma, Sweden) or with a Perkin-Elmer Series 4 pump and an LC-90 UV/visible Spectrophotometer Detector (Norwalk, CT, USA). The detector was set at a wavelenght of 254 nm.

A Nucleosil^{R} 7 C 18 column (Macherey-Nagel, DÜren, FRG) was used for the analyses. The mobile phase was 2 mmol/l TBAH in water, pH 2.92. The flow-rate was 1.5 ml/min.

Some of the plasma samples were analysed using an amino column according to the method described previously (9).

Milk Samples

About 10 ml samples of either unpasteurized or pasteurized (Meiko HSS-100, 62.5° C, 30 min) fresh human milk were used for the analyses. The vitamin C content in the samples was analyzed immediately or after storage at +4° C. Samples were controlled not to contain excessive bacterial growth.

Blood Samples

Venous blood samples obtained from 23 healthy adult volunteers from 22 to 40 years or age were used for determinations of plasma TAA, AA and DHA. Samples were drawn using Venoject VT-050 HL heparin tubes (Terumo Corp., Yonezawa, Japan).

Leukocytes for determinations of TAA, AA and DHA were isolated from 10 ml of EDTA blood (Venoject VT-053 TKZ) that were obtained from 10 healthy subjects from 24 to 38 years of age.

METHODS

Preparation of Milk Samples

Aliquots of the milk samples (3 ml) were treated with 1 ml of a 12.5% TCA solution in order to precipitate the proteins. After centrifugation for 10 min at 2000 x g the supernatants were filtered and the filtrates assayed for AA. TAA was analysed on the filtered extract after addition of 2.0 ml of 0.8% freshly prepared DL-homocysteine solution into 0.5 ml of the extract in order to reduce all DHA to AA (10). The pH of the mixture was adjusted to 7.0 - 7.2 with a 45% K_2HPO_4 buffer. After incubation for 15 min in room temperature the samples were refiltered and assayed immediately. The samples were carefully protected from light during the handling procedure.

Preparation of Plasma Samples

For the assay of AA, 200 µl of fresh plasma was vigorously mixed with 300 µl of 12.5% TCA or 5% MPA and handled as described previously (9). For the analysis of TAA, 20 µl of freshly prepared homocysteine solution was added into 200 µl of plasma. After an incubation of

15 min at room temperature, 300 µl of 12.5% TCA or 5% MPA was added into the test tube. The mixture was centrifuged at 2000 x g for 10 min and an aliquot of 20 µl of the supernatant solution was injected into the HPLC column.

Preparation of Leukocytes

The method described by Evans et al. (11) was used for the preparation of leukocyte mixtures (buffy layer) and determination of AA except using 6% Macrodex instead of Ficoll-Paque. The leukocyte mixture was homogenized in TCA. DHA was determined from the TCA supernatant following homocysteine treatment: 250 µl of the supernatant was mixed with 1 ml of 0.8% homocysteine solution, pH was adjusted to 7.0 - 7.2 with K_2HPO, the mixture was incubated for 15 min at room temperature and 20 µl of it was injected into the HPLC column.

Cell Counting

A part of the leukocyte mixture was used for leukocyte and platelet counting (Coulter Counter® S-Plus IV, Coulter Electronics Inc., Hialcah, USA).

Standardization and Calculation of the Results

The AA and DHA standards, 30 mg/l for milk analyses and 10 mg/l for plasma and leukocyte analyses, were made up daily in distilled water. DHA content of the samples was calculated by subtracting AA from TAA.

RESULTS

Milk Samples

The elution time for AA was 2.9 min in AA analysis and 4.0 min in TAA analysis, owing to the altered pH of

the injected sample aliquot (Fig. 1). Homocysteine-treated DHA standard behaved similarly to the homocysteine-treated AA standard. The chromatograms were practically free from interfering peaks.

Our method was applied to analyse the stability of vitamin C in human milk during storage. Both AA and DHA declined with time in an essentially same ratio when stored at +4° C (Table 1), whilst after pasteurization both of these were found in almost equal amounts the AA showing the most pronounced decrease. The table also shows that pasteurization and 24 h-storage destroy about 80% of the total ascorbic acid activity, whereas 24 h-storage at +4° C destroys only about 40% of the vitamin C activity.

Plasma and Leukocytes

Mean TAA, AA and DHA concentrations in plasma were 10.12 ± 0.74 mg/l (mean ± SEM, n=23), 9.96 ± 0.75 mg/l and 0.16 ± 0.03 mg/l, respectively.

AA concentration in the leukocyte mixture was 0.21 ± 0.04 mg/10^9 cells (mean ± SEM, n=10) and DHA concen-

TABLE 1

TAA, AA and DHA Content of Unpasteurized and Pasteurized Human Milk (Expressed as Mean ± SEM).

Unpasteurized sample (n=4) storage period (hours) at +4° C	mg/l TAA	AA	DHA
5	76.0±11.3	54.3±6.5	21.0±9.1
25	46.1±6.9	34.1±9.1	12.4±5.6
Pasteurized and storage for 20 h at +4oC (n=4)	17.7±1.9	8.6±3.4	6.6±2.4

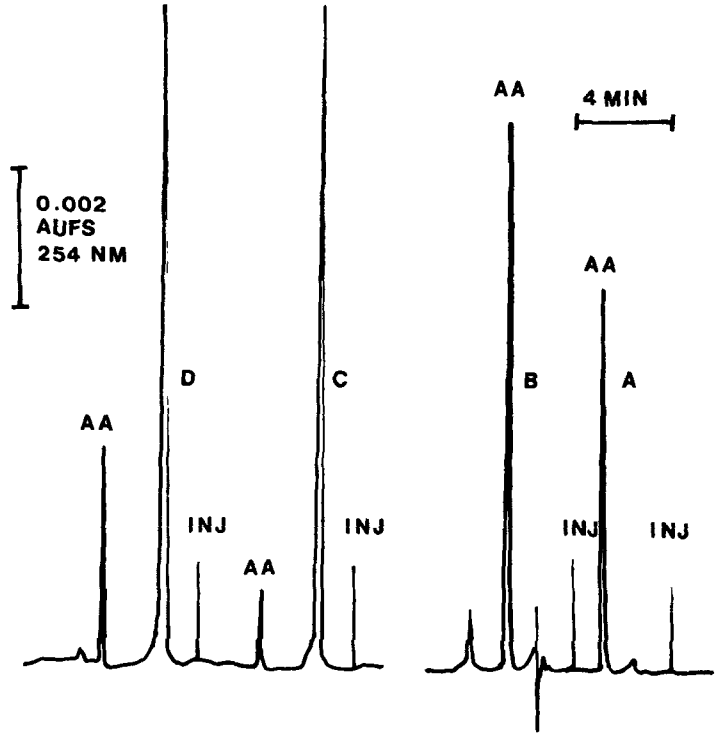

FIGURE 1. Representative chromatograms showing injections of 20 µl of (a) AA standard, (b) AA in filtered protein-free milk, (c) homocysteine-treated DHA standard and (d) a homocysteine-treated milk sample for TAA analysis. Column: Nucleosil® C18; mobile phase: 2 mmol/l TBAH, pH 2.92, flow rate 1.5 ml/min.

tration was 0.09 ± 0.03 mg/10^9 cells (n=8). In two samples the DHA content was below the limit of detection. A statistically significant positive correlation was observed between the AA concentration in leukocytes and plasma (r=0.599; Student's t-test for correlation coefficient, $p<0.05$) (Fig.2).

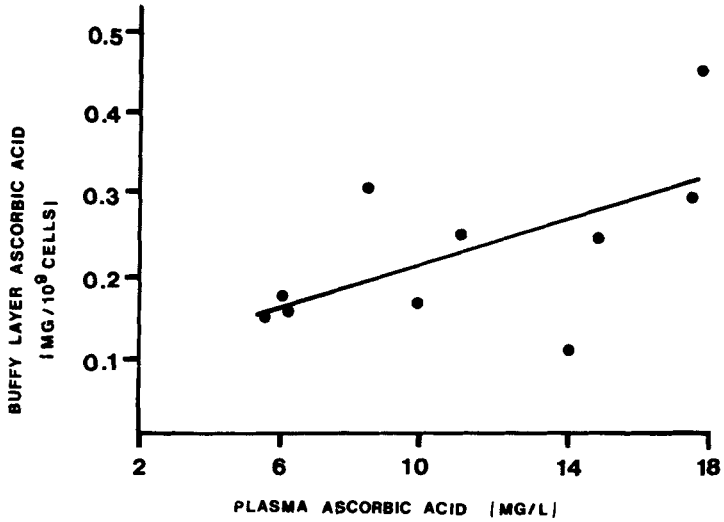

Fig. 2. Relationship between total ascorbic acid concentration in leukocytes (y-axis) and plasma (x-axis) ($r=0.599$, $n=10$, $p<0.05$).

Analytical Imprecision and Recoveries

The within-day and day-to-day coefficients of variation for AA determination were 2.5% (n=6) and 3.5% (n=6), respectively. The absolute recoveries of DHA and TAA analyses in milk were 76.5 ± 10.6% (mean ± SD, n=7), and in plasma analyses 101.0 ± 6.3% (n=6).

DISCUSSION

Reduction of DHA to AA is a commonly used method for analysing total vitamin C in biological fluids. One reason for this is that DHA poorly absorbs light at 254 nm and short wavelenghts of UV light are required for

detection by HPLC. Dithiotreitol (2,12) and homocysteine (13-15) are the reducing agents commonly used before determination of TAA by colorimetric or UV-detection. Usually TCA or MPA is added to the sample before homocysteine treatment in order to prevent AA and DHA from oxidizing to diketogulonic acid and other degradation products (16). In plasma samples, the physiological pH is close to the optimum required for homocysteine to reduce all DHA to AA (13). Because of this the acid was added into the test tubes after the homocysteine treatment. This facilitates greater sensitivity for the method since the sample is not diluted as a result of neutralization. When leukocyte mixtures are analyzed, the cell homogenization usually requires an acidic environment and neutralization is indicated.

Our results for the AA concentrations in leukocytes of healthy subjects agree well with those of previous studies (11,18). The DHA content in lymphocytes (12,19) is somewhat lower than our results in the total leukocyte mixtures (buffy layer). Our higher results are presumably due to granulocytes and platelets present in the leukocyte mixture. The intracellular concentration of AA in platelets has been verified to be between that of lymphocytes and granulocytes (11).

Human milk vitamin C activity was found to be labile especially after storage or pasteurization. These results might explain our own findings of declining plasma levels of AA in low birth-weight infants fed exclusively with pasteurized breast milk (5).

SalmenperÄ has found that exclusively breast-fed full-term infants are well protected against vitamin C deficiency in spite of very low maternal plasma or milk concentration (6). Moreover, Byerley et al. found that vitamin C levels in milk did not increase in response

to increasing maternal intake of vitamin C (20). In conclusion, the infant intake and plasma concentration of vitamin C are adequate, when the infant is breast-fed from the day of the birth and breast-feeding continues long enough. When human milk is pasteurized, intake may be insufficient and vitamin C deficiency may develop.

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