

Quantification of Plasma and Leukocyte Vitamin C by High Performance Liquid Chromatography with Mass Spectrometric Detection

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Abstract—In the present study a high performance liquid chromatographic–mass spectrometric (LC–MS) method was developed for the measurement of ascorbic acid in human plasma and white blood cells. Special attention was paid to the known difficulties of ascorbic acid stability, and a plasma sample preparation method was developed, accordingly. The novel objective of this work was to focus on the determination of plasma ascorbic acid concentrations considering the necessity of accurate determination for further clinical studies related to scurvy induced by various pathological conditions. The presented study describes in detail a validated LC–MS method with adequate accuracy and precision for quantitative determination of ascorbic acid in human plasma. The method was tested on human plasma and leukocyte samples.

Keywords: ascorbic acid, dehydroascorbic acid, human plasma, LC–MS, stability, scurvy

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Vitamin C is a mild organic acid, structurally similar to glucose from which most animals produce their own ascorbic acid. The most important chemical property of this vitamin is the reversible oxidation to dehydroascorbic (DHA) acid, which means its underlying physiological activity. Ascorbic acid (AA) can exist as a free radical, which explains the antioxidant activity. Under physiological conditions, primarily the reduced (ascorbate) form is present [1, 2].

Vitamin C has already been studied more than any other supplement or pharmaceutical drug in the history, a fact that is not surprising given that scurvy is a fatal disease and chronic hypoascorbemia is considered today's most widespread deficiency disorder [3, 4]. In diabetes, allergy, infectious diseases and toxic abuse, the manifestation of this avitaminosis is in most cases inevitable and intravenous administration of ascorbic acid is often a part of emergency care also in our days [5, 6]. Furthermore, the incidence of acute and subacute infantile scurvy needs a special attention. On the basis of epidemiological surveys, the number of scorbutic children is increasing [7, 8]. Based on all these facts, it is important to notice the opinion of all those physicians who declare that, especially in the mentioned pathological states, the accurate quantification of ascorbic acid is needed. Considering that the most important reducing system in humans is represented by the glutathione–ascorbic acid interaction

[9, 10], wherein the presence of both molecules is essential, and knowing also that in plasma the primarily used antioxidant is vitamin C, it can be affirmed that measuring the AA/DHA levels can give a real image of the human body reducing state [11].

At present, there are no functional markers that allow detection of the human body vitamin C level, so ascorbic acid quantification in plasma and leukocytes remains the possibility for such investigations. Generally, plasma AA concentration is measured, but this cannot reflect precisely vitamin C level of various tissues. Determination of leukocytes AA concentration is closer to cells/tissues AA store quantity [12]. However, the analytical methods for such purposes raise many problems. The spectrophotometric, titration or enzymatic methods do not provide sufficient selectivity, these measurements are highly influenced by other reducing substances such as iron and copper ions, sugars, glucuronic acid, hydrogen peroxide, etc. Consequently, the most favorable techniques are HPLC methods [13–29]. The majority of HPLC methods for the determination of ascorbic acid in human plasma use UV or electrochemical detection [13–18]. Concurrently with the latter, it is known that the highest selectivity could be provided by mass spectrometry (MS), but this kind of detection rises difficulty. Only a few methods with mass spectrometry detection is presented in the medical literature for AA and DHA

determination in human plasma; furthermore, in some articles MS detection techniques are only mentioned, without precise presentation [19–23]. Other similar methods are focused only on foodstuff, multi-vitamin tablet, fruits or vegetables [24–29].

The key issue in the AA/DHA determination is the stability, since these molecules are extremely unstable in solutions. Under natural or UV light excessive degradation of AA occurs. After 1 h of UV light influence, AA concentration decreases by 79.7%, and from exposure to natural light the AA concentration decreases by 95.6% in a transparent container. Temperature is considered a key factor of AA and DHA stability, but a lot of analytical methods did not take this factor into account. At room temperature the solution remains stable only for 1 h. Freezing the samples is an important step for proper detection. Consideration should also be given to pH of the solution, since the stability of AA is much better in acidic medium. pH value of about 2.1 is generally required for the preparation of the samples. The concentration of the solution is also a decisive factor – higher AA concentration results in greater stability. Analytical methods require stabilizing agents for AA/DHA determination. The most frequently used stabilizing agent is meta-phosphoric acid, or its combination with ethylenediaminetetraacetic acid (EDTA). Other used substances are trichloroacetic acid (TCA), ortho-phosphoric acid, homocysteine, oxalic acid, EDTA, trifluoroacetic acid, dithiothreitol, etc., or associations such as EDTA and TCA. The presence of metal ions is another important factor, thus EDTA enhances the stability of the AA solution. The effects of Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , and Ca^{2+} were studied, and Cu^{2+} was found to significantly affect the amount of AA [11, 12].

In the present study, an LC–MS method was developed for the quantitation of ascorbic acid in human plasma and white blood cells. The vast majority of methods uses other detection techniques than MS for the determination of AA and DHA in human plasma [13–18]; only a few methods with mass spectrometric detection are presented in the literature [19–23] but in most cases they do not provide a precise description of the method; the study in hand presents in detail an LC–MS method by which it could be widely available for further clinical studies or even routine analysis related to ascorbic acid deficiency. The novelty of this work also consists in the short analysis time. At the same time, special attention was paid to the known difficulties of ascorbic acid stability, and a plasma sample preparation method was developed, accordingly.

EXPERIMENTAL

Chemicals. Ascorbic acid (95% purity) was provided by Vim Spectrum, Targu Mures. Acetonitrile,

methanol, and acetic acid were from Merck (Merck KgaA, Darmstadt, Germany). Ultrapure deionized water was produced by a Millipore Simplicity (Millipore SA, Molsheim, France) water system. Human plasma samples were collected from volunteers with no diagnosis of scurvy.

Standard solutions. Stock solutions of AA with concentration of 4 mg/mL were prepared by dissolving appropriate quantities (weighed on an analytical balance from Mettler Toledo) in 1 mL of ultrapure water and 4 mL of methanol with 50 μL of 0.1% acetic acid. Eight working AA solutions with concentrations of 0.1–4 mg/mL were obtained by diluting the proper volumes of stock solutions in methanol. These working solutions were used to prepare standard plasma samples resulting in 8 plasma standards with concentrations ranging between 20 and 808 $\mu\text{g}/\text{mL}$. Accuracy and precision of the method were verified using plasma standards with concentrations of 61, 404 and 646 $\mu\text{g}/\text{mL}$. Quality control samples (QC) with the same AA concentrations of 61 (QCA), 404 (QCB) and 646 (QCC) $\mu\text{g}/\text{mL}$ were used during sample analysis. The stock solutions were freshly prepared every day in amber flasks, and all the standards were prepared daily no more than 1 h prior to be injected in the LC–MS system.

Chromatographic and mass spectrometric system and conditions. The HPLC system was a 1100 series model (Agilent Technologies) consisting of a quaternary pump, an in-line degasser, a thermostated autosampler, a column thermostat, and a 6410 series model triple quadrupole mass spectrometric detector (Agilent Technologies). Chromatograms were processed using a MassHunter software.

The detection of the analytes was in the selected ion monitoring mode using an electrospray negative ionization. The monitored ions were 175 m/z for AA and 173 m/z for DHA (Fig. 1). We also tried working in multiple reaction monitoring mode with fragmentation of 175 to 115 m/z for AA (collision energy 10) and 173 to 113 m/z for DHA (collision energy 4), but the signals were very low for these fragments and not reproducible. Other detector parameters: dry temperature 350°C, nebulizer 40 psi, dry gas – nitrogen at 8 L/min and 3 min analysis run time.

Chromatographic separation was performed at 40°C on a Zorbax SB-C18 (100 \times 3 mm, 3.5 μm , Agilent Technologies) column, protected by an in-line filter.

The mobile phase consisted of a mixture of water containing 0.1% acetic acid and acetonitrile (75 : 25, v/v). The pump delivered the mobile phase at 0.4 mL/min. The injected volume was 2 μL .

Sample preparation. Standard plasma samples for validation were prepared as follows in order to be chromatographically analyzed. In Eppendorf tubes 80 μL of plasma with 20 μL of appropriate working solution were precipitated with 300 μL of methanol and vortex-

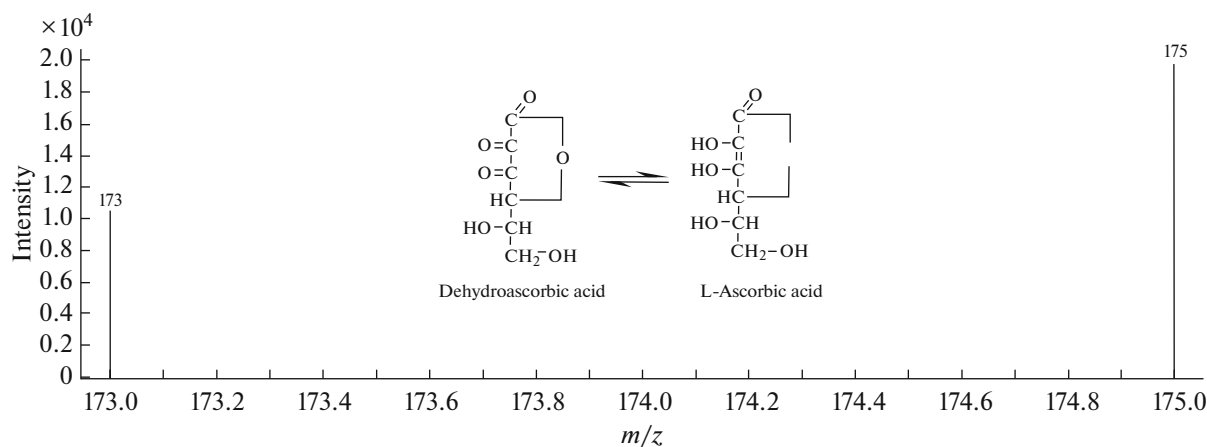


Fig. 1. Mass spectrum of ascorbic acid (m/z 175) and dehydroascorbic acid (m/z 173).

mixed for 10 s. After centrifugation at 15000 rpm for 5 min, 100 μ L of supernatant was stabilized with 250 μ L of acetic acid (0.1%), then injected to the LC–MS system. It took 40 min for the samples to be prepared, followed directly by LC–MS analyses for the method validation. Sample sets were prepared freshly for every analytical run.

Validation. The validation was performed according to the European Medicines Agency (EMA) bioanalytical method validation guideline [30].

Linearity. To determine linearity of the method, 8 standard samples were used at concentration levels of 20, 40, 81, 162, 242, 323, 606, 808 μ g/mL of AA. The standard solution sets were made of freshly AA spiked plasma samples for every separate analytical run. For the construction of the calibration curve, the applied linear calibration model was: $y = ax + b$, where y was peak area and x – concentration, with a weighting factor of $1/x$. Separate calibration curves were made for each analytical run. The calibration model was accepted if the concentrations of the calibration standards were within $\pm 15\%$ of the nominal value and within $\pm 20\%$ for lower limit of quantification (LLOQ). At least 75% of the calibration standards should meet this criterion including highest and lowest calibration levels.

Selectivity and lower limit of quantification. The lower limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%. The analyte signal of the LLOQ sample should be at least 5 times the signal of the blank sample.

In order to describe the selectivity of the method, three different blank plasma samples from not scorbutic volunteers were analyzed. The plasma samples were kept in the freezer (-20°C) for 3 months without stabilizing agent, so these were considered to be blank for AA.

Precision and accuracy. The within-run precision (expressed as coefficient of variation, CV, %) and accuracy (expressed in percentage as relative difference between obtained and nominal values of concentrations) of the procedure were determined by analyzing five different samples of the low (61 μ g/mL), medium (404 μ g/mL) and high (646 μ g/mL) levels of the considered concentration range on the same day. The inter-day accuracy and precision were obtained by repeating the measurements in three separate runs.

Matrix effect. The matrix effect was investigated by analyzing the ratio of the peak area in the presence of matrix (blank plasma spiked with AA) to the peak area in absence of matrix (pure aqueous AA solution).

Carry over was investigated by injecting blank sample after a high concentration (QCC, 646 μ g/mL) sample. The peak area detected at specified retention time should not be higher than 20% of LLOQ.

Stability. In the scientific literature, it is an old-established fact that AA is a very unstable molecule. As we know, AA first oxidizes reversibly to dehydroascorbic acid (DHA, m/z 173) which degrades irreversibly to 2,3-diketogulonic acid and this one degrades to threonic and oxalic acids. By these statements, simultaneous determination of AA and DHA must be carried out by an analytical method for the investigation of the stability of AA samples. We also know that the pH of the solution, temperature and light are the most decisive factors influencing the AA solutions stability.

With special attention to the well-known influencing factors, for the validation of the method the following tests were evaluated: long term stability of the analyte in matrix stored in the freezer, stability of the processed sample at autosampler temperature. Stability in matrix at room temperature was investigated for a very short period of time because we focused on a very quick sample preparation.

Table 1. Calibration curve parameters ($y = ax + b$)

| Run | N | a | b | R^2 |
|-----|---|-----|-------|-------|
| 1 | 8 | 445 | 1901 | 0.990 |
| 2 | 6 | 725 | 11424 | 0.991 |
| 3 | 6 | 725 | 11413 | 0.991 |

For the method validation, the stock solutions were processed in amber flasks and stabilized with 0.1% acetic acid. The preparation of the standard solutions ready to be injected required 40 min (from weighing the substance on analytical balance) followed by immediate injection in the LC–MS system for the final validation of the method used. The stability of AA samples was tested for 24 h at 20°C, and the frozen samples (–20°C) were tested for 3 months. Simultaneous determinations of AA and DHA were made by the proposed LC–MS method.

RESULTS AND DISCUSSION

Results of validation. *Linearity.* The analytical response was linear in AA concentration range of 20–808 µg/mL. The calibration curve constants for the three analytical runs are presented in Table 1.

Selectivity and lower limit of quantification. The LLOQ was set at AA concentration in plasma of 20 µg/mL corresponding to 1.4 µg/mL effective concentration in sample vials and 2.9 ng of AA injected on column. The analyte signal was 5.3 times higher than the signal of a blank sample (Fig. 2). The signal to noise ratio was 40 for LLOQ.

Three different human blank plasma samples were analyzed, and no significant interference was observed at the retention time of analyte. Peak areas at RT of

Table 2. Intra-day accuracy and precision results

| Run | LLOQ, µg/mL | Concentration, µg/mL | | |
|-------------|-----------------|----------------------|------------------|------------------|
| | | QCA | QCB | QCC |
| 1 | 21 | 52 | 378 | 566 |
| 2 | 26 | 55 | 382 | 586 |
| 3 | 23 | 53 | 389 | 799 ^a |
| 4 | 28 ^a | 55 | 350 | 586 |
| 5 | 27 ^a | 59 | 318 ^a | 655 |
| Mean | 24 | 55 | 374 | 598 |
| SD | 2.5 | 2.7 | 17 | 39 |
| CV, % | 11 | 5.0 | 4.6 | 6.5 |
| Accuracy, % | 118 | 90 | 93 | 93 |

^a Outlier.

1.1 min were calculated and these were maximum 20% of peak area of LLOQ.

Precision and accuracy. The intra-day accuracy was in the range of 90–93% for QC samples and 118% for LLOQ. The intra-day precision for QC samples for all of the concentration levels was below 10.5% (Table 2).

The inter-day accuracy was in the range of 91–102% for QC samples and 100% for LLOQ. The inter-day precision for QC samples was below 5.2 and 18.4% for LLOQ (Table 3).

Matrix effect. The response of human plasma sample compared to a pure aqueous solution (each of them was prepared using the same procedure) was around 30%, which was low, but constant and reproducible

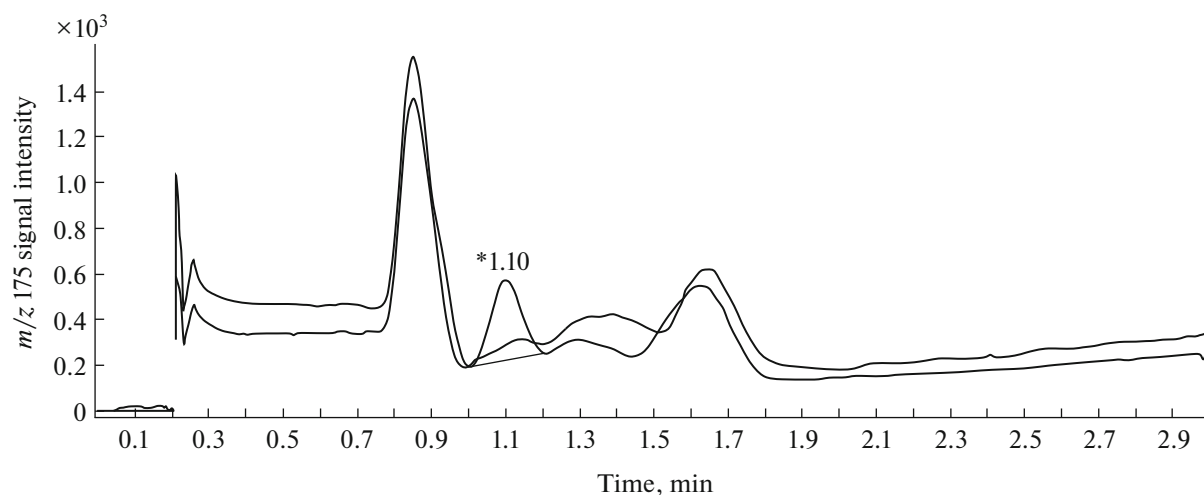


Fig. 2. Chromatogram of a blank human plasma sample at retention time of 1.1 min (area = 993) and an LLOQ sample (20 µg/mL) at retention time of 1.1 min (area = 6214).

Table 3. Inter-day accuracy and precision results

| Run | LLOQ, $\mu\text{g/mL}$ | Concentration, $\mu\text{g/mL}$ | | |
|-------------|------------------------|---------------------------------|-----|-----|
| | | QCA | QCB | QCC |
| 1 | 16 | 58 | 391 | 660 |
| 2 | 22 | 54 | 363 | 638 |
| 3 | 22 | 55 | 355 | 679 |
| Mean | 20 | 56 | 369 | 659 |
| SD | 3.7 | 1.9 | 19 | 21 |
| CV, % | 18 | 3.5 | 5.1 | 3.1 |
| Accuracy, % | 100 | 93 | 91 | 102 |

during method validation in all of the analytical runs (Fig. 3).

Carry over. When blank sample was injected after QCC (646 $\mu\text{g/mL}$) sample, the peak area detected at the retention time of 1.1 min was not higher than 20% of the LLOQ (Fig. 4).

Stability. After several investigations, we have concluded that at room temperature in neutral medium (pH 7) the amount of AA decreases drastically after 1 h. AA concentrations in the samples stored in the freezer (-20°C) without stabilizing agents after 3 months were equal to zero. The samples can be stabilized with 0.1% acetic acid. It can be affirmed that fast sample preparation and also a fast LC–MS determination are needed. The validated method has a 3 min analytical run, which is fast enough for proper determination of AA and DHA in the entire sample set stabilized with acetic acid for the method validation. Analyzing the acetic acid stabilized plasma and prop-

erly diluted working samples at the same concentrations which were kept at room temperature for 24 h, it is very important to notice that the amount of AA decreased by 84% and DHA amount increased by 4 times in the diluted working solution (Fig. 5), and in the plasma samples AA concentration decreased by 98% and DHA content increased by 1.8 times (Fig. 6).

Application of the method to the analysis of dietary supplements. The validated method was applied for the quantification of AA in a dietary supplement containing calcium-ascorbate and flavonoids (Fig. 7) for investigation of AA concentrations in plasma in healthy volunteers and for the quantification of AA in human leukocytes.

One capsule of the dietary supplement containing 90 mg of calcium-ascorbate and flavonoids was diluted in 25 mL of water, filtered and immediately analyzed by LC–MS.

Four volunteers were implicated for measuring AA concentrations in plasma and leukocytes after administration of two different vitamin C dietary supplements (one containing calcium ascorbate and flavonoids and the other a slow release formulation of ascorbic acid). For the quantification of plasma AA, blood collections were carried out at 0, 1, 2, 3, 4, 6, 8 h. The plasma samples were stabilized with acetic acid and frozen (-20°C). Then, 100 μL of plasma sample was precipitated with 300 μL of methanol and prepared according to the previously presented procedure prior to the LC–MS analyzes.

Mononuclear cells were separated using LymphoprepTM with density-gradient centrifugation. For a fast separation and sample preparation, it was a one-step method.

Mean plasma concentration curves ($n = 2$) for 4 volunteers are presented in Fig. 8. There is no significant difference between the 2 preparations, calcium

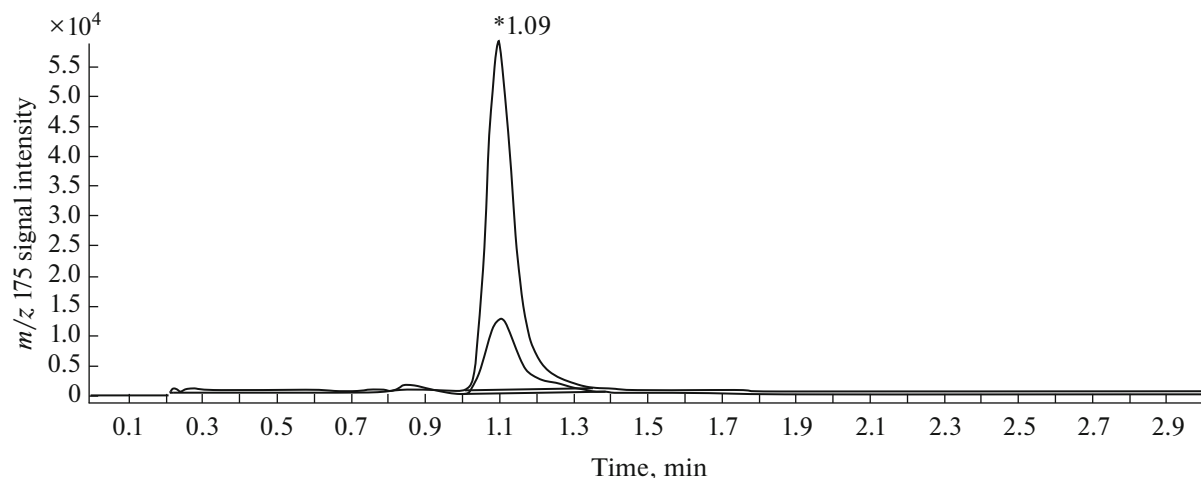


Fig. 3. Human plasma sample (808 $\mu\text{g/mL}$) compared to a pure aqueous solution with the same ascorbic acid concentration.

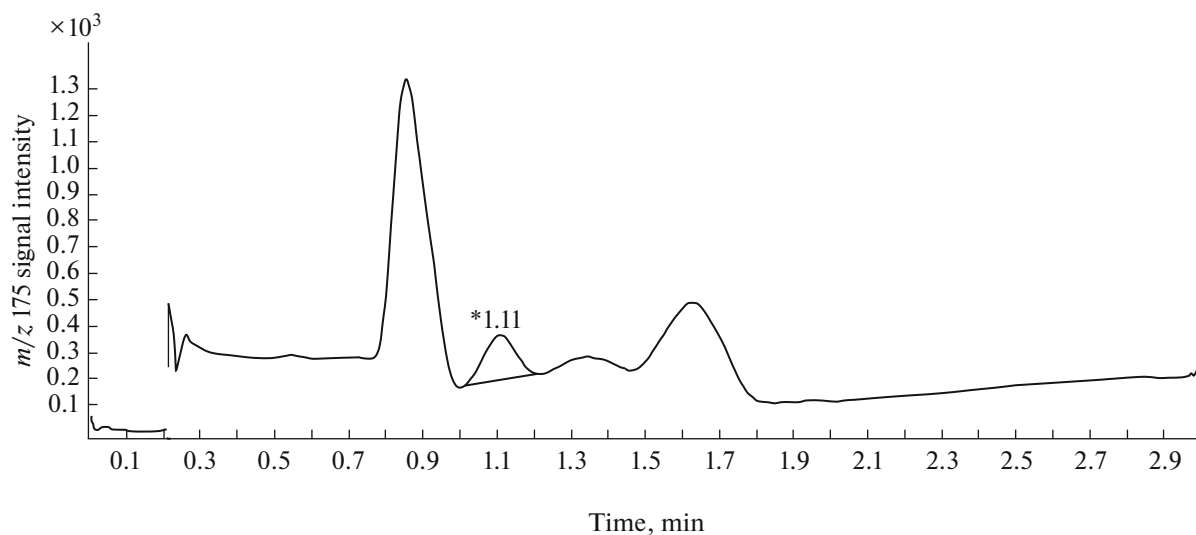


Fig. 4. Chromatogram of a blank plasma sample injected after QCC.

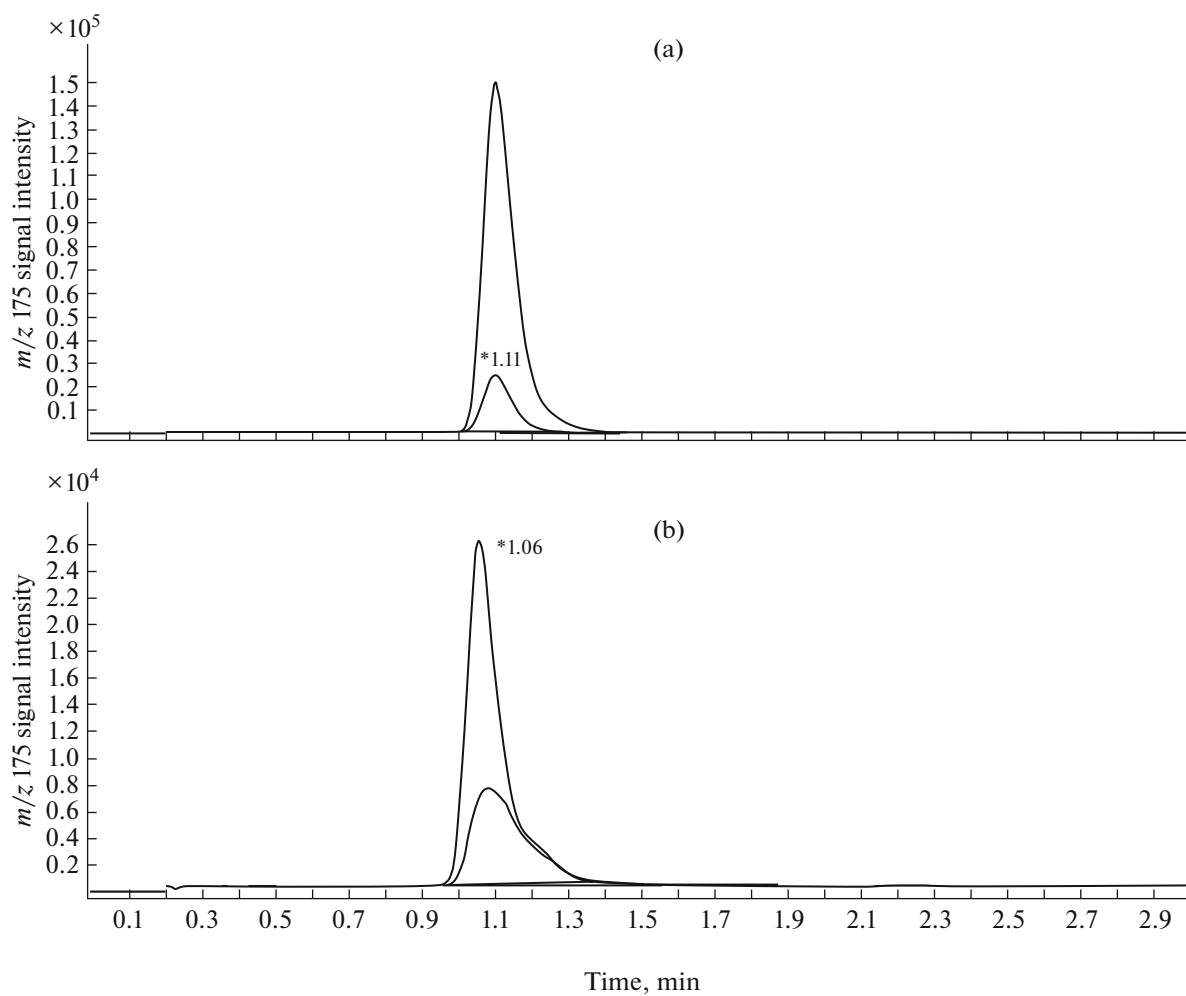


Fig. 5. Autosampler stability at 20°C of diluted working solutions: decrease in ascorbic acid concentration (a); increase in dehydroascorbic acid concentration (b).

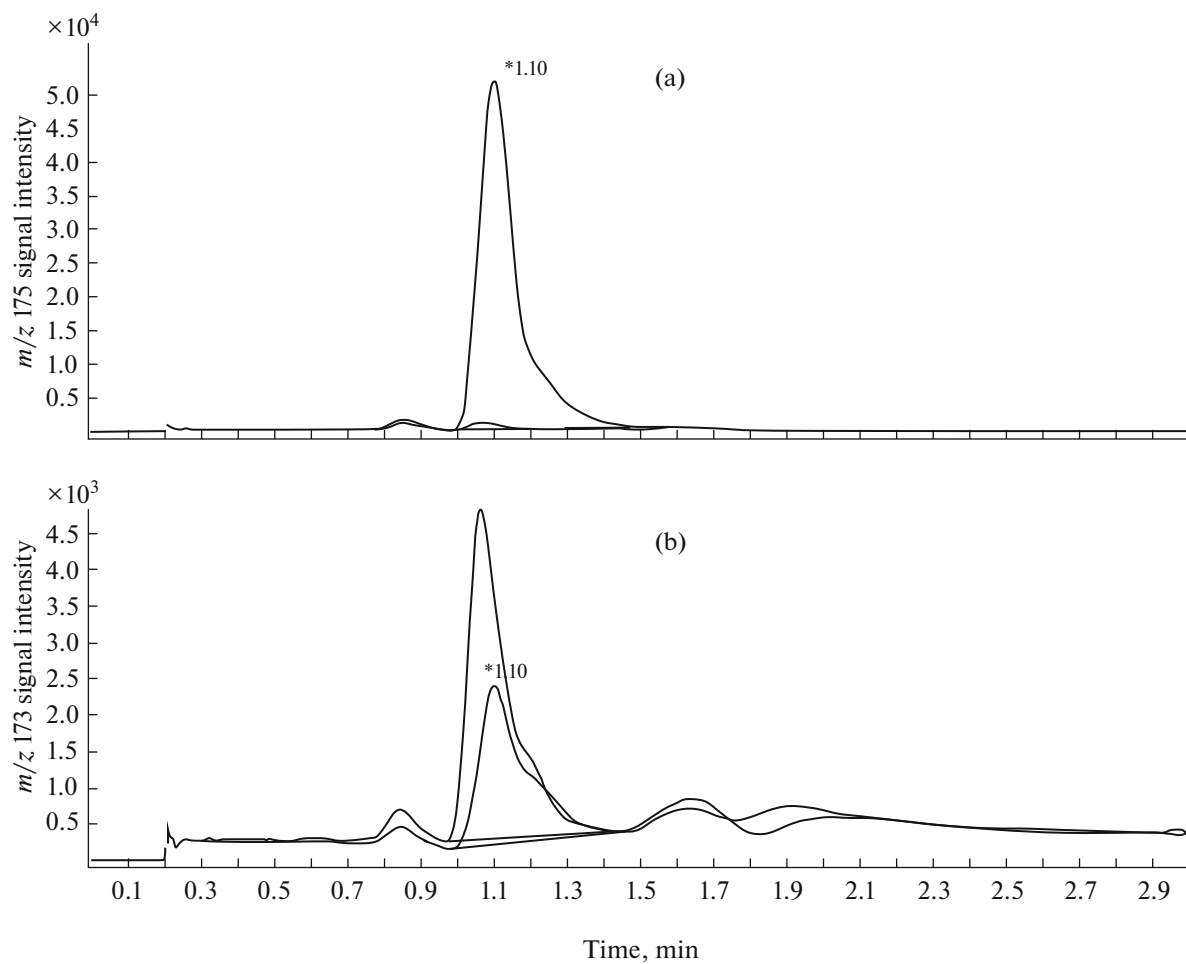


Fig. 6. Stability of plasma samples (808 µg/mL) at room temperature: decrease in ascorbic acid concentration (a); increase in dehydroascorbic acid concentration (b).

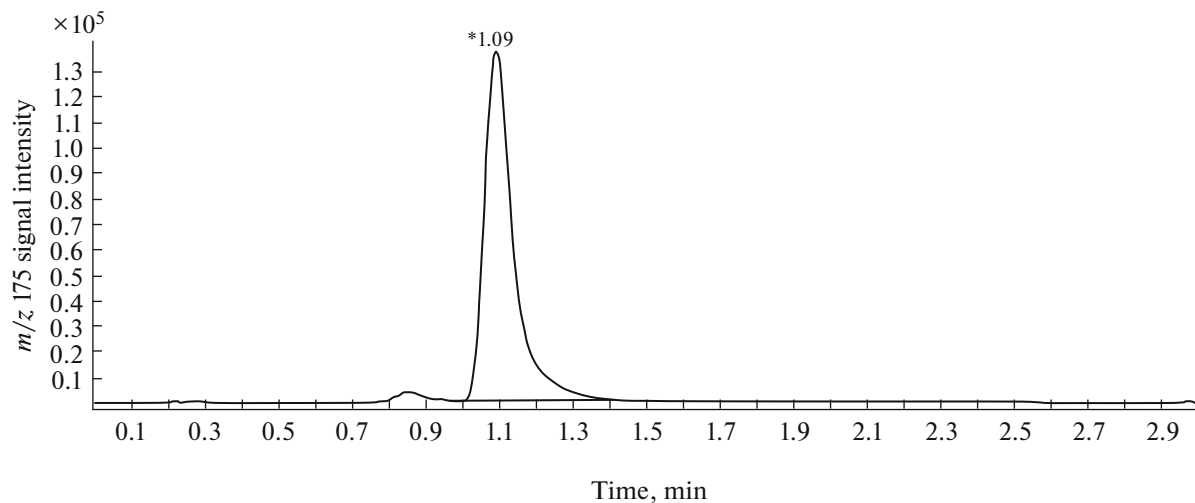


Fig. 7. Dietary supplement ascorbic acid concentration.

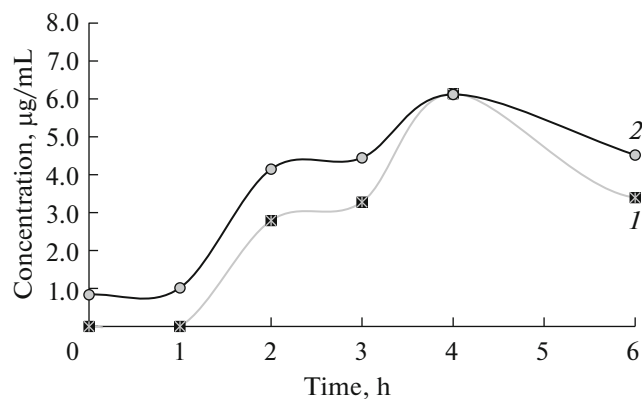


Fig. 8. Mean plasma ascorbic acid concentration curves ($n = 2$): (1) calcium-ascorbate + flavonoids, (2) slow release ascorbic acid.

ascorbate with flavonoids and a slow release formulation of ascorbic acid in the means of c_{\max} (around 6 $\mu\text{g/mL}$) and t_{\max} (4 h) in both cases. In human leukocytes, AA concentration was very low (not measurable) in the range of 0.5–2.5 $\mu\text{g/mL}$. In the future, special attention has to be kept on leukocyte sample preparation in order to maintain AA stability.

CONCLUSIONS

As we expected, AA and DHA determination in human plasma samples is a great analytical challenge. The motivation of this study was to describe in detail an LC–MS method for AA and DHA determination suitable for clinical studies. As we know, such measurements could be of a major importance in our days according to some pathological conditions that can lead to evident clinical manifestation of scurvy. The presented study describes a validated LC–MS method with adequate accuracy and precision for AA quantification in human plasma. The method was tested on human plasma and leukocyte samples collected from healthy volunteers (with no diagnosis of clinical scurvy). Further, plasma and leukocyte samples should be evaluated with special attention to the leukocyte samples preparation regarding a better AA stability. The paper focuses on the detailed presentation of LC–MS method for AA and DHA quantification in human plasma, therefore it completes data in the scientific literature regarding this topic.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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