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Short communication

Determination of ascorbic and dehydroascorbic acids in blood plasma and serum by liquid chromatography

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Abstract

A liquid-chromatography (LC) method with ultraviolet detection for measuring ascorbic (AA) and dehydroascorbic acid (DHA) in human blood and serum was studied. The method used an ODS reversed-phase column and cetyltrimethylammonium bromide as an ion-pairing agent. AA was measured before and after the reduction of DHA with dithiothreitol. The absence of interferences resulting from hemolysis products was verified and also the stability of the ascorbic acid in metaphosphoric acid extracts. The analytical parameters, linearity (1–80 μg/ml), accuracy (recovery, 96.7–100.7%) and precision (C.V.=3.1%), show that the method is reliable and adequate for measuring the total vitamin C content in serum and plasma.

Keywords: Ascorbic acid; Dehydroascorbic acid

1. Introduction

The total vitamin C content in blood plasma and leucocytes is widely accepted as an indicator of the tissue status of vitamin C [1]. The total vitamin C of a sample is the sum of L-ascorbic acid and its oxidized form, L-dehydroascorbic acid, because their biological activities are similar.

Ascorbic acid is a labile substance that readily oxidizes to dehydroascorbic acid and finally to diketogulonic acid. This last oxidation step is irreversible and is found to be much more temperature sensitive than the oxidation of ascorbic acid to dehydroascorbic acid [2].

Several reviews of the methods used to determine vitamin C have been published [3–7]. In all of them the advantages of enzymatic and chromatographic methods in comparison with the methods based on redox or derivatization reactions are stressed.

Different liquid chromatography (LC) methods have been used to measure AA and DHA. Some of them require electrochemical or fluorimetric detection, because of the low absorptivity of DHA in the ultraviolet range of the spectrum, but the equipment needed is not always available in hospital laboratories. To solve this problem some authors propose the previous reduction of DHA to AA using homocysteine [1,8,9] or dithiothreitol [10,11]. Others carry out a post-column derivatization of DHA with *o*-phenylendiamine [12,13].

The purpose of our study was to examine a high-performance liquid chromatographic method for

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measuring AA and DHA in blood plasma and serum with ultraviolet detection (254 nm). The reduction of DHA to AA was carried out with dithiothreitol.

With regard to the high-performance chromatographic method, our starting point was the method proposed by us [14] for determining ascorbic and isoascorbic acid in fruit juice. This procedure uses an octadecyl reversed-phase column, which is often available in laboratories and its mobile phase does not include organic solvents. The adaptation of this procedure to our objective implies the selection of the conditions of sample preparation, which should be minimal, including the reduction of DHA to AA, and the determination of the analytical parameters and possible interferences in the proposed high-performance liquid chromatographic method in order to ascertain whether it can be used to determine the AA and DHA content of blood plasma and serum.

2. Experimental

2.1. Instrumentation

The LC system consisted of an LC-6A pump with a 20-μl injection valve, an SPD-6A ultraviolet detector, an SCL-4A chromatopac integrator, and an SCL-6A control system (Shimadzu, Japan). A Spherisorb ODS C_{18} column (5 μm), 250×4 mm I.D. (Teknochroma, Barcelona, Spain) was used. The filters of 0.22 μm, 13 mm and 47 mm diameter were from Magna Nylon, MSI (Micron Separations, Westborough, USA).

2.2. Chemicals

Only analytical grade substances were used: ascorbic acid, 4-hydroxyacetanilide (Merck, Darmstadt, Germany); dehydroascorbic acid, dithiothreitol (Sigma, Taufkirchen, Germany); potassium dihydrogenphosphate, cetyltrimethyl ammonium bromide (Panreac, Barcelona, Spain); metaphosphoric acid (Aldrich, Poole, UK); deionised water, Milli-Q water system (Millipore, Jaffrey, MA, USA)

The ascorbic acid and dehydroascorbic acid standard solutions, 10 mg/l, were prepared daily in deionized water Millipore-Milli Q.

2.3. Chromatographic conditions

The method used is based on the work published by Benlloch et al. [14] as mentioned above. The method uses an octadecyl-silica reversed-phase column, a mobile phase containing 5 m*M* cetyltrimethylammonium bromide as the ion-pairing agent and 50 m*M* potassium dihydrogen phosphate as a buffer, at pH 4.5 (the solution was filtered through a 0.22-μm filter for use in the chromatograph), and 4-hydroxyacetanilide as the internal standard. All measurements were done at room temperature with a flow-rate of 1 ml/min.

Peak areas were used for quantitative analysis. Internal standardization was applied and calibration curves were prepared between 2 and 25 μg of ascorbic acid/ml in 5% metaphosphoric acid. All the standards contained the same known amount of 4-hydroxyacetanilide.

2.4. Samples (plasma and serum)

Venous blood samples were drawn from a forearm vein of volunteers using a syringe, and were transferred to tubes with or without heparin to obtain plasma or serum, respectively. Determinations were carried out immediately after the separation of plasma or serum by centrifugation at 1850 *g* for 15 min.

2.5. Sample treatment

2.5.1. Ascorbic acid

An aliquot of plasma or serum was diluted (1:1) with 10% metaphosphoric acid. Internal standard (4-hydroxyacetanilide) in the amount needed to obtain a final content of 4.5 μg/ml was added, and the mixture was then centrifuged at 3300 *g* for 10 min at room temperature. The supernatant was filtered through a 0.22-μm filter, and a 20-μl aliquot was injected directly into the HPLC system.

2.5.2. Dehydroascorbic acid reduction and determination as ascorbic acid

Internal standard was added to a 300-μl sample to obtain a 4.5 μg/ml concentration. Then 200 μl of 10 m*M* dithiothreitol was added and the mixture was kept at room temperature for 10 min. The same

volume of 10% metaphosphoric acid was added to this solution and the procedure was followed up as mentioned for the ascorbic acid determination.

The reduction of dehydroascorbic acid by dithiothreitol occurs at about pH 7, and, therefore, a buffer solution was not required either in plasma or in serum [15]. After the reduction the procedure was identical to the one applied for ascorbic acid. The total vitamin C content (AA+DHA) was determined, and the DHA content was obtained by subtracting the AA content.

3. Results

Chromatograms of the standard and the sample are shown in Fig. 1. The resolution of the peaks is good, the relative retention time is 8.7 min, and the baseline is stable.

In order to find out whether any peaks corresponding to DHA appear in the chromatogram when AA is measured, the procedure described for measuring AA in serum was applied, but the sample was replaced by 300 μl of DHA standard solution (100 μg/ml). The chromatogram showed only the peak corresponding to the internal standard.

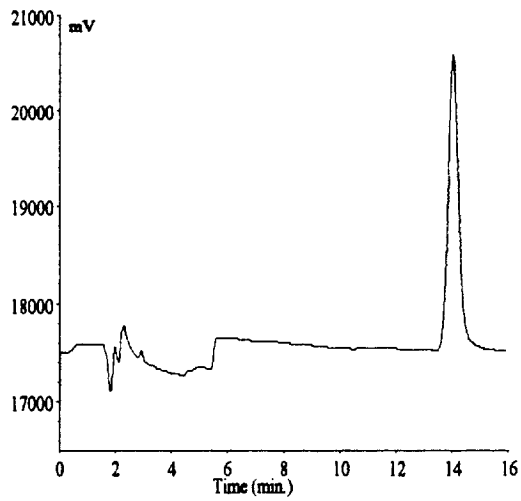

Fig. 2. Chromatogram of internal standard after dithiotreitol reduction. Internal standard, t_r=13.9 min.

The response of the internal standard did not change during the reduction of DHA, as can be seen in the chromatogram corresponding to the internal standard treated with dithiothreitol as described (see Fig. 2). Moreover, neither the reduced nor the oxidized form of dithiothreitol absorbs at 254 nm.

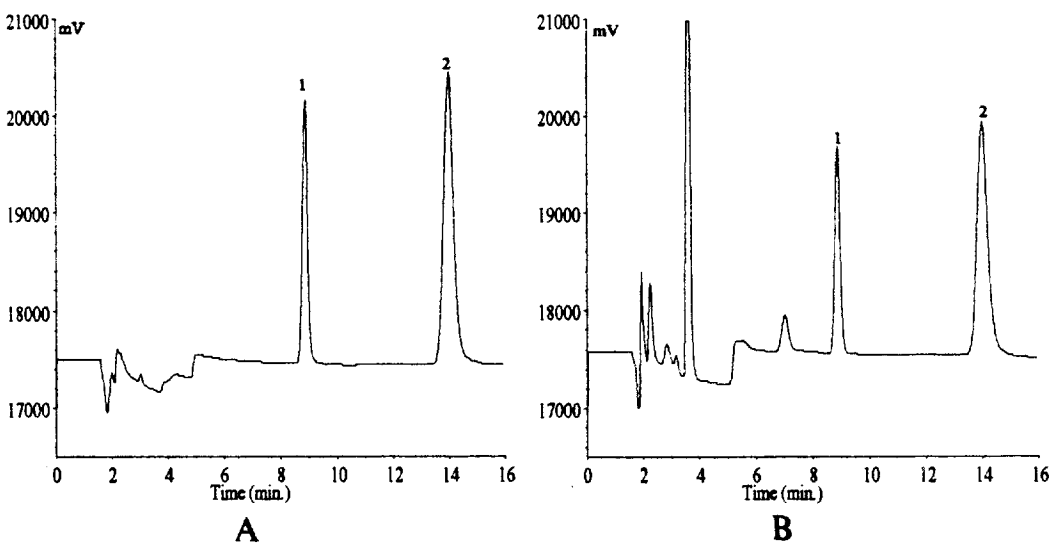

Fig. 1. Chromatograms of standard (A) and sample (B). (A) Ascorbic acid: t_r=8.9 min; internal standard: t_r=14.0 min. (B) Ascorbic acid: t_r=8.9 min; internal standard: t_r=14.1 min.

3.1. Analytical parameters

To check the reliability and usefulness of the proposed method the analytical parameters were determined.

3.1.1. Linearity and sensitivity

The relationship between the peak area and the AA concentration was evaluated over the range 1–80 μg/ml and was found to be linear ($y=0.30x-0.57$; $r=0.999$; $n=6$).

The sensitivity, calculated by applying the method proposed by Knoll [16], was 0.031 μg/ml.

3.1.2. Accuracy

The accuracy of the method was verified by means of recovery assays. Adequate amounts of AA and DHA were spiked to plasma in order to obtain added concentrations of 10 and 20 μg/ml. The recovery values of the added AA and DHA ranged from 97.8 to 100.7% (98.9±1.6, $n=3$) and 96.7–100.1% (98.4±1.7, $n=3$), respectively.

3.1.3. Precision

The repeatability of the method was 3.0% (9.14±0.27 μg/ml, $n=6$) and 3.1% (5.19±0.16 μg/ml, $n=6$) for AA and DHA, respectively.

3.1.4. Hemolysis interferences

In order to detect possible interferences caused by hemolysis in the AA and DHA determination, these compounds were measured in plasma, a hemolysate and a mixture of hemolysate and plasma (1:1).

The erythrocytes were washed several times and finally diluted with physiological solution (1:10). To obtain the hemolysate, 1 ml of erythrocytes was taken and diluted with 10 ml of water; 1 ml of hemolysate was added to 1 ml of blood plasma. Hemolysis was visible in the plasma.

In the chromatogram corresponding to the hemolysate, no chromatographic peak with the retention time corresponding to ascorbic acid can be seen. As can be inferred from the results summarized in Table 1, the presence of a visible hemolysis did not interfere with the determination of AA and DHA in blood plasma or serum.

Table 1
Hemolysis interferences

Sample	Concentration (μg/ml)	
	AA	AA+DHA
Hemolysate	N.D.[a]	N.D.[a]
Plasma	3.49	15.30
Hemolysate–plasma (1:1)	1.75	7.51

[a] N.D.=not detected.

3.1.5. Sample stability

Due to the instability of vitamin C it should be determined immediately after obtaining the samples. Since this is not always possible, it is useful to check the stability of the stored samples. The stability of AA and DHA in blood plasma stored at −18°C was studied and compared to that of its metaphosphoric extract.

An aliquot of blood plasma was split into 1-ml aliquots, which were immediately frozen at −18°C.

The same volume of an aqueous solution of metaphosphoric acid 10% containing the appropriate amount of internal standard was added to another aliquot of plasma. The mixture was shaken and centrifuged. The supernatant was taken and split into five aliquots. Ascorbic acid was measured immediately in one of them and the other four were frozen at −18°C.

Ascorbic acid was measured in blood plasma aliquots and in metaphosphoric acid extracts 2, 4, 6 and 8 days after freezing.

The calibration curves were obtained in the range 2–25 μg of ascorbic acid/ml in 5% metaphosphoric acid.

Blood plasma and metaphosphoric acid extracts were left to thaw at room temperature, and the above-described methods were applied to measure ascorbic acid.

From the results obtained summarized in Table 2, it can be concluded that plasma ascorbic acid content was stable for the first 2 days, and decreased on the fourth day after the extraction, whereas the ascorbic acid was stable in metaphosphoric acid extract at least until the sixth day. Therefore, when it is not possible to analyze the samples immediately, they should be extracted with metaphosphoric acid and the extracts should be frozen and kept at −18°C until analysis.

Table 2
Ascorbic acid stability

Time (days)	Concentration (μg/ml)	
	Plasma	Extract
0	16.73	16.73
2	16.87	16.76
4	13.14	16.60
6	11.10	17.07
8	10.24	15.45

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