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Original Article

Determination of Vitamin C in Small Volumes of Blood by HPLC/EC

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Abstract
A sensitive procedure for determining total vitamin C (ascorbic acid + dehydrate ascorbic Acid) in a blood drop from a finger prick, before and after the administration of a vitamin C tablet is described. Analysis was carried out by high performance liquid chromatography with electrochemical detection (HPLC/EC). Measurements were taken one hour, two hours and six hours after the administration of a 500 mg vitamin C tablet. D-Isoascorbic acid was used as the internal standard and analysis was carried out using two C-18 columns connected in series and a phosphate buffer mobile phase. Dehydroascorbic acid in the samples was converted to ascorbic acid by incubation with DL-homocysteine for 30 minutes. The level of vitamin C in blood reached a maximum concentration after two hours.

Keywords: Vitamin C, Electrochemical detection, HPLC, Blood.
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1. Introduction

The physiologic importance of vitamin C to human health is well known, e.g., as a potent antioxidant, ascorbic acid (AA) has the capacity to eliminate reactive oxygen species [1, 2]. The total vitamin C content in blood plasma and leucocytes is widely accepted as an indicator of tissue status of vitamin C [3]. A number of techniques have been used to quantify vitamin C in foods and biological samples including: a colorometric assay the ascorbic acid reaction with 2,4-dichlorophenol-indophenol [4], or folin-phenol reagent [5], capillary zone electrophoresis [6], several HPLC methods using UV detection [2, 7] and HPLC with electrochemical detection (EC), which is currently the method

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of choice for quantification of ascorbic acid in foods, tissues and biological fluids [8-11]. The current study reports a method sufficiently sensitive to determine AA plus dehydroascorbic acid (DHA) in 50 l of blood using HPLC-EC.

2. Materials and methods

2.1. Chemicals

Ascorbic acid, D-isoascorbic acid (IAA), DL-homocysteine, potassium dihydrogen phosphate, and trichloroacetic acid were obtained from Sigma-Aldrich Chemical Company, Gillingham, Dorset. Vitamin C tablets (500 mg, Pharmadass) were purchased locally.

2.2. Finger tip blood samples

One or two drops of blood were collected from healthy volunteers using a lancet directly into eppendorf tubes. Samples were taken before and one h, two h, and six h after the administration of a 500 mg vitamin C tablet. Immediately after collecting blood samples, 50 l blood was measured from the drop using a Hamilton syringe and was transferred into another eppendorf tube. Then 100 l of phosphate buffer (100 mM, pH 4.7), containing 1 mM EDTA was added to the tubes followed by 30 l of 1% DL-homocysteine solution. The samples were left for 30 minutes at the room temperature, and then 40 l of a 30% trichloroacetic acid solution was added to the samples. The samples were then centrifuged to remove the precipitated cells and proteins, and 200 ng of IAA (20 µl of 10 µg/ml) was added to 100 l of the supernatant, and 50 l of the final solution was injected into the HPLC system.

2.3. Instrumentation

A P100 Spectra-Physics isocratic HPLC system was used. The Rheodyne injection valve was fitted with a 50 l loop. Separation was achieved by using two Prodigy 5 m ODS C18 reversed-phase columns (250 4.6 mm i.d., Phenomenex UK, Macclesfield) which were connected in series. Detection was carried out using a LC-4A electrochemical detector (Bioanalytical Systems). The potential of detector was set at 0.4 V versus an Ag-AgCl reference electrode . The mobile phase was composed of 100 mM phosphate buffer, pH 3, containing 1 mM EDTA, and the flow rate was 1 ml/min.

2.4. Calibration and precision

A calibration curve was prepared by dissolving different amounts of AA + 2 µg of IAA in 1 ml of the mobile phase. The standard curve between 1-6 g/ml of AA was linear (r = 0.998). If required, samples were diluted with the mobile phase to fall within the range of the calibration curve. The precision of the method was determined by injecting 5 aliquots of the same blood sample containing 13.1 µg/ml of AA which gave precision of 1.4 % for the measurement of the sample.

3. Results

By using two C18 reversed columns in series, baseline separation between the L-ascorbic acid present in the blood and IAA used as an internal standard was possible. The exact mechanism permitting this separation is unclear, but IAA is the ideal internal

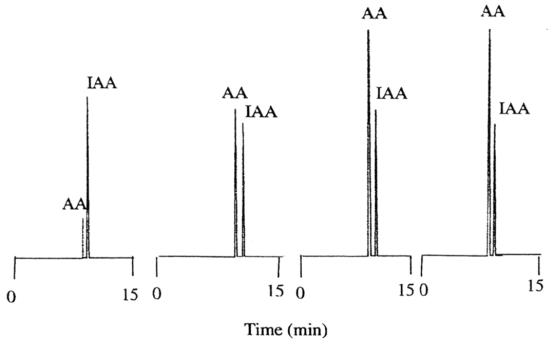

Figure 1. HPLC chromatogram of ascorbic acid (AA), measured in a blood drop. Samples were taken before and at 1, 2 and 6 h (left to the right, respectively) after taking a 500 mg vitamin C tablet by volanteers. Two hundred nanograms of isoascorbic acid (IAA) was added to 100 l of each sample prior to the injection to the HPLC.

standard for this assay because of its close similarity to AA. The vitamin C level was measured in six healthy volunteers (5 male and 1 female). Typical chromatograms are shown in Figure 1 for vitamin C in blood samples obtained from one of the volunteers. The mean concentrations for vitamin C in the blood samples before and at 1 h, 2 h, and 6 h after the administration of a vitamin C tablet are shown in Table 1.

Table 1. Mean (SD) concentrations of vitamin C (AA+DHA) in the blood samples before and at different time points after taking a 500 mg vitamin C tablet.

Time (h)	Concentration of AA in the blood (μg/ml)
0	7.74±3.5
1	11.0±3.3
2	19.1±6.6
6	13.4±7.1

In order to determine whether any peaks corresponding to DHA appeared in the chromatogram, a sample containing 5 g/ml DHA and 2 μg/ml IAA was injected into the HPLC. Consequently, only the peak corresponding to the IAA was observed, and the injection of DHA alone did not reveal any peak.

4. Discussion

The method described above has the potential to detect <1 g/ml of ascorbic acid. However, in practice, there is a cut off point where sensitivity falls off abruptly below this level. This is probably due to the rapid oxidation of ascorbic acid at <50 ng on a column by the chromatographic system prior to reaching the detector. In the current work, metaphosphoric acid was avoided as a preserving agent because it was found to inhibited the reduction of DHA to AA.

Previous work using HPLC with UV detection determined vitamin C (AA+DHA) concentration in human plasma to be 15.3 g/ml without supplementation [3]. In another study, it was shown that in fresh blood plasma samples, the DHA concentration was less than 5% total AA [8]. Various methods have been described for the reduction of DHA to AA e.g. incubation of DHA with dithiothreitol [3], 2-mercaptoethanol [12], and DL-homocysteine [9]. In the current study, 2-mercaptoethanol in sodium phosphate buffer, pH 7.4, with 1mM thiourea and 0.1 mM EDTA [12] was tested but the efficiency of reduction was not high, and better results were obtained with DL-homocysteine as a reducing agent.

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