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Determination of plasma ascorbic acid by high-performance liquid chromatography with ultraviolet and electrochemical detection

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ABSTRACT

A convenient and reliable reversed-phase liquid chromatographic method for the routine determination of ascorbic acid with ultraviolet detection is described. This system avoids the use of modifier and ion-pairing reagent. The mobile phase consists of 20 m*M* ammonium dihydrogenphosphate with 0.015% metaphosphoric acid. This method enables the detection of plasma ascorbic acid at a concentration of 120 ng/ml within 5 min. The recovery and reproducibility were above 95%. A comparative study was also performed using ultraviolet and electrochemical detectors. Excellent agreement was observed between the two detection modes, with a correlation coefficient of 0.99. In addition, the storage conditions and stability of ascorbic acid in plasma and whole blood were investigated. The results showed that ascorbic acid was more stable in whole blood when stored below 4°C.

INTRODUCTION

Ascorbic acid (AA) plays a very important role in human metabolism. Its functions include collagen synthesis, amino acid metabolism, synthesis of adrenalin, synthesis of anti-inflammatory steroids and iron and copper metabolism. Its role in the prevention of nitrosamine formation and interaction with free radicals suggest that the vitamin may also have a role in the prevention and treatment of cancer [1–3]. There is also growing interest in recent years on the prevention of cardiovascular disease by AA. The AA concentration in plasma has been used as an index of antioxidant activity [4]. It is therefore important to have a reliable, rapid and sensitive method for the measurement of AA that can be used for community and epidemiological studies.

Numerous methods have been developed for the detection and quantification of AA in whole blood or plasma. Some involved oxidation of AA with 2,6-dichlorophenol–indophenol [5], enzymic methods using ascorbate oxidase (EC 1.10.3.3) [6,7], and condensation of AA [8,9] and spectrophotometric analysis. Gas chromatography [8–10] and high-performance liquid chromatography (HPLC) [11–22] have also been studied extensively in recent years. HPLC methods are preferred as they have higher resolution than spectrophotometric methods. They are more specific and less prone to interference from other substances. For HPLC quantification of AA in biological fluids, the modes of detection that are commonly used are UV [11–14], fluorimetric [15–18] and electrochemical [13,14,18–22]. These

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methods all have good precision and reproducibility, but the mobile phases used are usually complex.

The mobile phase used by Lyold *et al.* [11] consisted of sodium dihydrogenphosphate with acetonitrile, metaphosphoric acid (MPA) and *dl*-homocysteine for UV detection. For fluorimetric detection, Honegger *et al.* [15] used a mobile phase consisting of sodium dihydrogenphosphate and EDTA in addition to sodium *n*-octylsulphate and acetonitrile. In another study, Dhariwal *et al.* [20] used a mobile phase of sodium phosphate, sodium acetate, dodecyl trimethylammonium chloride and tetraoctylammonium bromide in methanol and water, for coulometric electrochemical analysis.

This paper reports a rapid, reliable and sensitive method with simple mobile phase for the determination of AA. The mobile phase consists of 20 m*M* ammonium dihydrogenphosphate with 0.015% MPA. The proposed method was compared with that of Behrens and Madere [19] and was found to be more suitable for AA determination. A comparative study of UV and electrochemical detection was also performed. In addition, the stability of AA in plasma and whole blood was investigated.

EXPERIMENTAL

Reagents

L-(+)-AA, MPA, methanol, phosphoric acid and perchloric acid (PCA) were purchased from E. Merck (Darmstadt, Germany) and ammonium dihydrogenorthophosphate was purchased from BDH (Poole, UK). Distilled and deionized water was used for the preparation of all solutions.

HPLC apparatus and conditions

A Hewlett-Packard 1050 series quaternary pumping system (Waldbronn, Germany) and a Gilson Model 231-401 autoinjector (Villers-le-Bel, France) were used. The analytical column was a replaceable cartridge (Partisphere 5 C_{18}, 110 mm × 4.7 mm I.D.) protected by a guard cartridge system (Whatman, Clifton, NJ, USA). For UV detection and quantitation of AA, a Hewlett-Packard Model 1050 detector was used. An electrochemical detector (LC-4B) with a glassy electrode and a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA) was used for the comparative study. A CR-5A integrator (Shimadzu, Kyoto, Japan) was used for peak-area integration.

Buffers of different pH were used for UV and electrochemical detection. The pH was adjusted with phosphoric acid. For electrochemical detection, the buffer contained 20 m*M* ammonium dihydrogenphosphate with 0.015% (w/v) MPA (pH 2.55). For UV detection, the same buffer was used, but the pH was adjusted to 2.95. For electrochemical detection, the potential was set at +0.70 V, and for UV detection the wavelength was set at 245 nm. The flow-rate was 1.0 ml/min, and separation was carried out at room temperature.

Sample preparation

Blood was collected from healthy individuals in brown tubes with EDTA [8] as anticoagulant, and plasma was obtained by centrifugation at 1000 *g* for 10 min. A 100-μl aliquot of plasma was added to 100 μl of cold 10% (v/v) PCA containing 1% (w/v) MPA in a brown microcentrifuge tube. It was then allowed to stand at 4°C for more than 20 min to ensure complete deproteinization. Deproteinized samples that were to be analysed on another day were stored at −20°C. Prior to analysis, 200 μl of mobile phase were added to the deproteinized sample. The sample was then centrifuged at 12 000 *g* for 1 min. A 10-μl aliquot of the supernatant was then introduced into the HPLC system.

Standard preparation

The stock solution of AA (1 mg/ml) was prepared in the mobile phase and stored at −20°C. Owing to protein precipitation, the dilution factor of the deproteinized samples was not the same as that of the aqueous standards. In order to ensure accuracy of quantification, a standard additions method was used. The stock standard was further diluted with 10% (v/v) PCA containing

1% MPA, to give a range of concentrations from 0.5 to 20 μg/ml. A series of spiked samples was then prepared by deproteinizing the pooled plasma with equal volumes of these AA standards containing protein denaturant.

The calibration curve for each detector was obtained on four different days.

Stability study

The stability of AA at different concentrations in whole blood and plasma kept at 4°C was examined for a period of up to 24 h. This was done by dividing the blood of four healthy individuals into two portions, one kept in the form of whole blood and the other in plasma. The plasma sample (100 μl) was deproteinized immediately. The AA concentrations were determined and treated as original levels. Whole blood samples were stored individually in small aliquots (blood 300 μl) in brown microcentrifuge tubes at 4°C. The plasma of whole blood was separated only immediately before the deproteinization step. The AA concentrations of stored whole blood and plasma were analysed at hourly intervals for 5 h, and also a day later.

The stability of AA in deproteinized plasma stored at −20°C was also studied. Pooled fresh plasma was distributed into seven brown microcentrifuge tubes (100 μl each). The samples were then deproteinized, as described in above. One of the samples was analysed immediately, and the result was used as the initial (100%) concentration of AA. The rest of the tubes were kept at −20°C and analysed on six consecutive days. The percentage of AA remaining was determined.

The stability of AA in the standards was also studied. Seven small brown bottles containing 2 μg/ml AA in the mobile phase were prepared. One of the bottles was analysed immediately, and the AA concentration found was used as the initial (100%) concentration. The rest was kept at −20°C and analysed on six consecutive days. The percentage of AA remaining was determined.

RESULTS AND DISCUSSION

Mobile phase and chromatographic analysis

Several earlier studies have been conducted using HPLC for AA analysis [11–22]. The mobile phases used are usually complex, with more than two compounds plus an ion-pairing reagent and modifier. The use of ion-pairing reagents and modifiers tends to cause instability in the chromatographic separation [23]. In our earlier analysis, we followed the method described by Behrens and Madere [19]. The mobile phase used was 0.02 *M* acetic acid with 10% (v/v) methanol, 1 m*M* *n*-octylamine and 0.015% (w/v) MPA, at pH 4.8. This method gave satisfactory separation of AA, but the pressure in the column gradually increased with each subsequent injection of sample. It was noted that the guard column became clogged after *ca.* twenty injections. This might be due to the low solubility of AA in methanol and the precipitation of MPA with the ion-pairing reagent, *n*-octylamine.

In this study we used a relatively simple mobile phase, 20 m*M* ammonium dihydrogenphosphate with 0.015% MPA, which was effective for the separation of AA from the complex matrix interferences. It was much easier to prepare than the others [11–22]. Over a hundred samples had been injected and the HPLC system did not appear to suffer from the problem mentioned above. It was observed that the lower pH of our mobile phase provided better retention of AA in C_{18} column. It was decided therefore to use a mobile phase of pH less than 3 for both modes of detection. At pH 2.95 there was a front peak very close to the AA peak when using electrochemical detection. However, AA was free from interference when using a mobile phase of pH 2.55. Separation at pH 2.55 was also possible for UV detection, although peak tailing of the front peak resulted in interference with AA determination, especially with samples of low concentration.

The chromatograms of plasma samples using electrochemical and UV detection are shown in Figs. 1a and 2a, respectively. Figs. 1b and 2b show the same samples spiked with AA. The AA was detected at 2.0 min using either an UV detec-

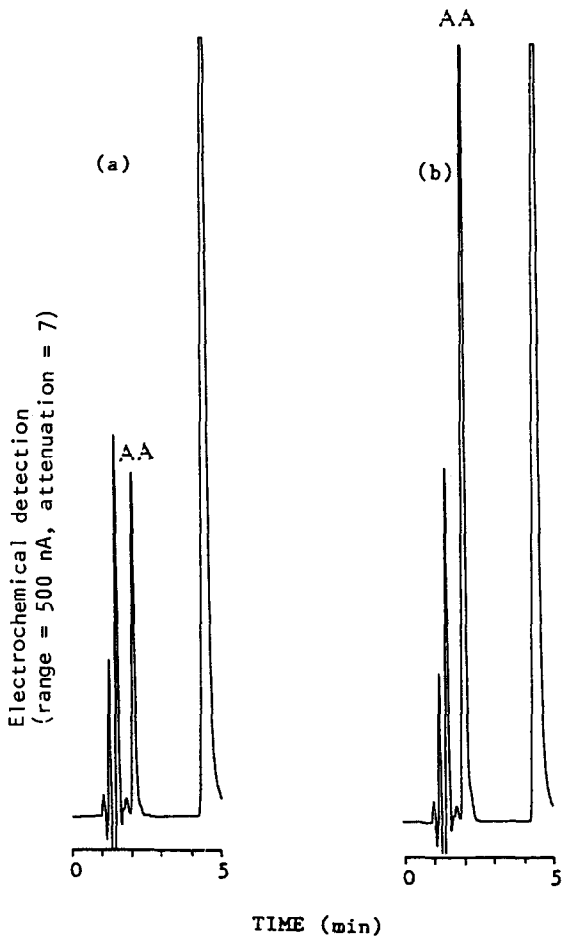

Fig. 1. HPLC elution profiles of the AA using electrochemical detection of (a) plasma and (b) plasma sample spiked with 5 μg/ml AA.

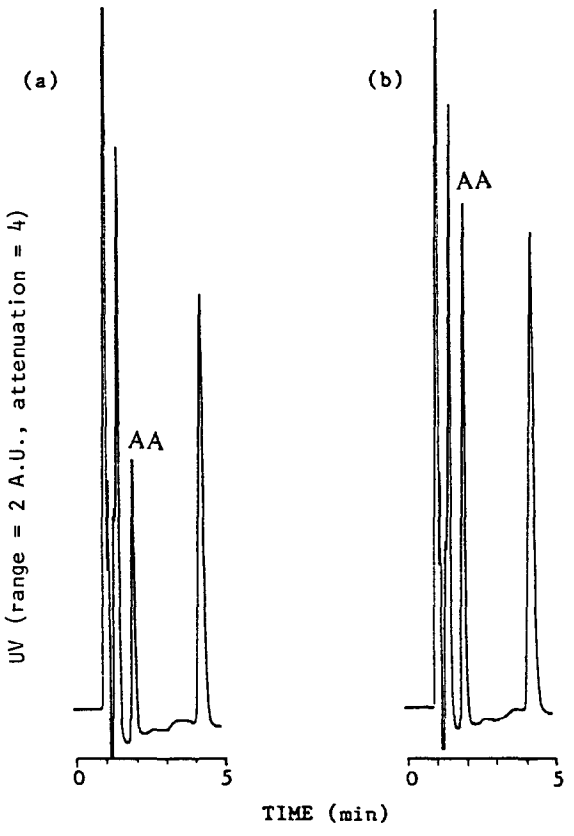

Fig. 2. HPLC elution profiles of the AA using UV detection of (a) plasma and (b) plasma sample spiked with 5 μg/ml AA.

tor or an electrochemical detector. The total analysis time was 5.0 min per injection of sample. With an injection volume of 10 μl, the detection limit for electrochemical detection was 0.3 ng; for UV detection, the limit was 1.2 ng.

Stability of AA in standards, whole blood and plasma

Many earlier reports had shown that PCA and MPA are good stabilizers for AA determination [24–26]. A 5% proportion of MPA was generally used for sample preparation. However, it is known that MPA precipitates with many ion-pairing reagents and can cause high column back-pressure or extraneous chromatographic peaks [27–29]. In the present investigation, it was observed that MPA was not as effective as PCA as a protein denaturant. Nevertheless, AA was found to be unstable in PCA. A compromise was to use 10% (v/v) PCA containing 1% (w/v) MPA as protein denaturant as well as stabilizer. AA in plasma samples treated with this acidic combination was found to be stable for up to six days when stored at −20°C (Fig. 3). Fig. 3 also shows the rapid decay of AA standard (2 μg/ml) at room temperature. On the contrary, a drop of less than 2% in the AA concentration was noted after three days storage at −20°C. The percentage remaining was less than 85% after six days. Because increased temperature accelerates the

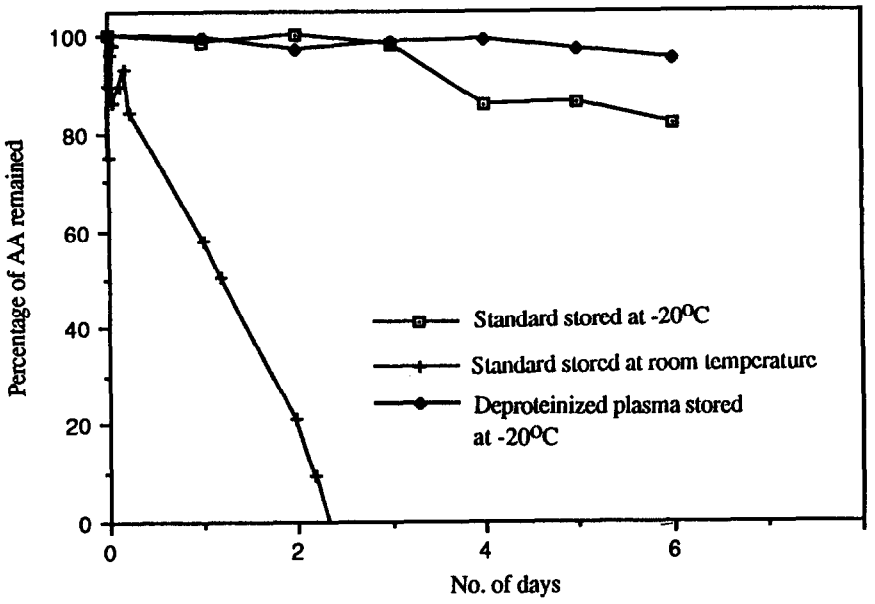

Fig. 3. Stability of AA in standards (2 μg/ml) kept at room temperature and −20°C, and in deproteinized plasma kept at −20°C. Analysis was performed with UV detection.

oxidation of AA, it is important to keep the samples at −20°C before analysis and below 4°C during analysis. Hence, we recommend the use of an autoinjector with a low-temperature incubator for multiple determinations of AA.

The stability of AA in whole blood and plasma after collection and before treatment were also studied. As shown in Fig. 4, AA was more stable in whole blood than in plasma when stored below 4°C. Although AA was less stable in plasma samples, they were found to be more suitable for chromatographic analysis. This was because the

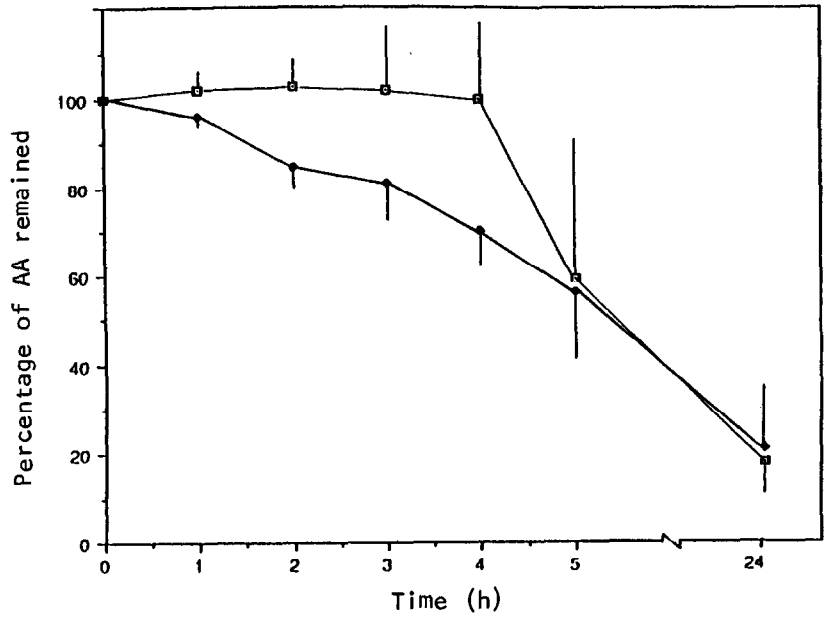

Fig. 4. Stability of AA in plasma (◆) and whole blood (□) at 4°C. Analysis was performed with UV detection.

analysis of deproteinized whole blood caused more interferences. In addition, the concentration of AA detected in whole blood might be lower, because AA could be easily oxidized during the deproteinization step as oxygen is liberated by oxyhaemoglobin [19]. Therefore, it is also recommended that treatment of plasma samples should be performed as soon as possible after isolation from red blood cells. They should be prepared in acidic media, otherwise they should remain as whole blood, stored at 4°C treated within 4 h after collection.

Recovery and standard calibration

Recovery of AA from the deproteinized plasma was determined by spiking a known amount of AA and determined from the calibration curve of aqueous standards. It was found that the recoveries at various concentrations were above 100% for the spiked samples (Table I). This was due to the precipitation of protein, which caused the dilution factor of the deproteinized samples to differ from the aqueous standards [30].

The day-to-day variation was studied by comparing the slopes of the standard curves plotted on four different days. The regression lines were linear in the concentration range 0.5–20 μg/ml AA, and the variation of slopes for day-to-day analysis was less than 3% for electrochemical detection and 5% for UV detection (Table II). It was noted that the mean value for the *y*-intercept had a large standard deviation. This was probably due to the oxidation of AA during storage.

Plasma samples from 38 healthy individuals were analysed using the proposed method. The mean concentration of AA in plasma was 7.03 μg/ml, and the range was 2–17 μg/ml.

TABLE I

RECOVERIES OF AA OF VARIOUS CONCENTRATIONS IN PLASMA (n = 5).

Concentration (μg/ml)	Recovery (mean ± S.D.) (%)	C.V. (%)
0.5	103.9 ± 0.021	2.02
2.0	109.9 ± 0.046	4.17
10.0	111.5 ± 0.043	3.83

TABLE II

CALIBRATION CURVE OF SPIKED SAMPLES

Detection	Mean[a]	R^a
Electrochemical	0.000 1378x − 984 Slope S.D. = 0.000 003 175 C.V. = 2.30% *y*-Intercept S.D. = 46.69 C.V. = 4.74%	0.9996
UV	0.002 92x − 540.5 Slope S.D. = 0.000 134 68 C.V. = 4.62% *y*-Intercept S.D. = 38.04 C.V. = 7.04%	0.9997

[a] n = 4.

Determination of total vitamin C

Vitamin C is a redox system that consists of AA and dehydroascorbic acid (DHAA). We employed the method suggested by Behrens and Madere [19] to convert DHAA into AA with homocysteine, and determined the total vitamin C level. In this method, one volume of homocysteine in dipotassium hydrogenphosphate buffer was introduced into the deproteinized sample. The mixture was then incubated at room temperature for 30 min. As for the determination of AA, the method used was as described in *Sample preparation*. After centrifugation at 12 000 *g* for 1 min, the supernatants were analysed by the proposed method: 20 m*M* ammonium dihydrogenphosphate with 0.015% (w/v) MPA and UV detection. It was observed that the use of homocysteine to reduce DHAA to AA did not lead to an increase in the total vitamin C. The total vitamin C concentration obtained by using Behrens and Madere's method [19] was lower than that obtained with the present method (Table III). The lower AA concentration detected was probably due to the non-acidic incubation conditions (pH 7.1), which did not inhibit the oxidation of AA.

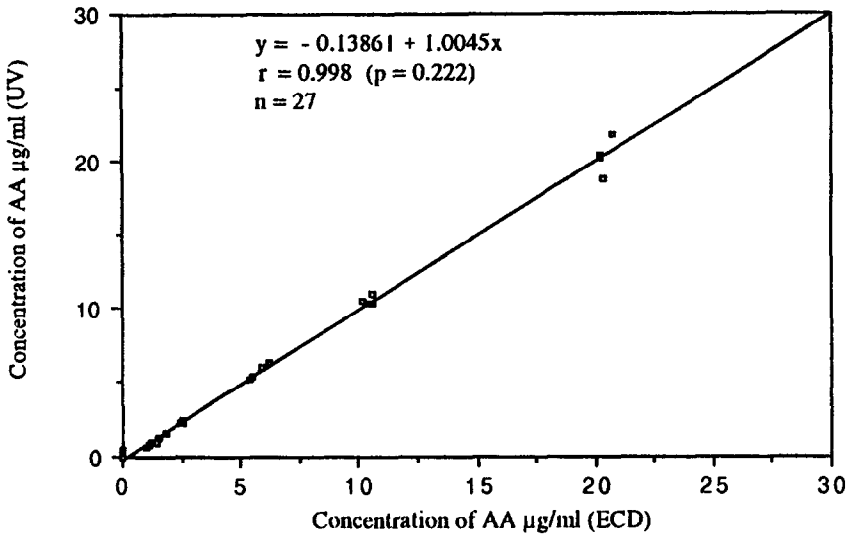

Fig. 5. Correlation between electrochemical (ECD) and UV detection.

Comparison of UV and electrochemical detection

Substantial work has been conducted on AA determination using electrochemical and UV detection [11–14,18–22]. In this study, we found that the two detection modes were comparable. As shown in Fig. 5, the coefficient of correlation (r) of 27 samples was equal to 0.998. The coefficient of variation (C.V.) for the within-day assay (n = 4) was less than 6%, and 12% for between-day analysis (n = 4) using electrochemical detection. For determination by UV detection, the within-day and between-day C.V. were less than 4 and 5%, respectively. The present HPLC method with UV detection has a good sensitivity (120 ng/ml) and is thus sufficient for AA determination of plasma vitamin C, which is usually in the 8–10 µg/ml [31]. In addition, the UV detector took less time to stabilize. Furthermore, a higher pH was used for separation when using the UV detector, 2.95 rather than 2.55 for electrochemical detection. At lower pH, there is a higher chance of column deterioration. UV detection is apparently a better choice for fast routine screening of AA concentrations. Electrochemical detection, on the other hand, is more sensitive with a lower detection limit of *ca.* 30 ng/ml. It is thus preferred when the samples contain extremely low levels of AA.

TABLE III

AA CONCENTRATIONS FOUND IN PLASMA TREATED WITH PCA AND MPA OR HOMOCYSTEINE

Sample	AA (µg/ml)	
	Treated with PCA and MPA	Treated with homocysteine
1	5.13	3.54
2	3.04	2.16
3	7.52	7.38
4	3.34	3.07

CONCLUSION

The concentration of AA in plasma can be quantified using a relatively simple mobile phase for HPLC separation with a UV detector. It involves a single-step deproteinization and direct injection of sample. A 100-µl aliquot of plasma was sufficient for the analysis, and after more than 100 injections no change of column efficiency was noted. With a total separation time of 5 min between each injection, 60–80 samples can be

analysed within a day. The method can be used for routine screening of vitamin C deficiency and for community study of this micronutrient.

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