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THE DETERMINATION OF DEHYDROASCORBIC ACID AND ASCORBIC ACID IN THE SERUM AND SYNOVIAL FLUID OF PATIENTS WITH RHEUMATOID ARTHRITIS (RA)

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Using a novel high performance liquid chromatography (HPLC) determination of ascorbic acid and dehydroascorbic acid, we have measured the relative amounts of ascorbate and dehydroascorbate in 20 normal controls and in paired sera and synovial fluid from 13 patients with rheumatoid arthritis (RA).

In complete contrast to previous published findings we were able to detect dehydroascorbate in normal human sera (12.0 ± 3.7 μmol/l), while the mean and range of ascorbate measured in normals was 69.6 ± 20.6 μmol/l.

These findings were completely reversed in rheumatoid sera (21.8 ± 8.6 μmol/l and 5.1 ± 5.0 μmol/l for dehydroascorbate and ascorbic acid respectively). In several rheumatoid sera no ascorbate could be detected.

In paired synovial fluid and serum samples, there was always more dehydroascorbate detected in synovial fluids than in the corresponding sera ($p < 0.01$).

The data suggests that the reduced level of ascorbate and increased level of dehydroascorbate may be a reflection of the increased antioxidant and free-radical scavenging activity of the vitamin in RA, especially within the inflamed joint.

Key words: ascorbic acid, dehydroascorbic acid, high performance liquid chromatography, rheumatoid inflammation

INTRODUCTION

The ascorbic acid content of serum, leuokocytes and synovial fluid of patients with rheumatoid arthritis (RA) has been studied extensively[1-5]. Several reports have suggested that there is an increased catabolism of ascorbic acid in these patients because they have a consistently lowered level of the vitamin in their blood, which is difficult to elevate into the normal range even by the administration of large doses of ascorbate[6,7].

Ascorbic acid and dehydroascorbic acid are the biologically active forms of vitamin C in man. Dehydroascorbic acid is the more lipophilic of the two compounds and it is

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thought that ascorbic acid is converted into dehydroascorbate enzymatically in order that it may be freely available for intracellular metabolism[8]. Ascorbic acid is also a very good reducing agent, and as such may be easily oxidised, in vivo, in the presence of abnormal oxidative reactions. Present methods used for measuring total ascorbate cannot differentiate between these two compounds, and when differential measures are made they are usually derived by calculation from total measurements. The purpose of the present study was to develop a high performance liquid chromatography method for measuring, simultaneously, the relatively amounts of ascorbate and dehydroascorbate in serum and synovial fluid of rheumatoid patients, in order to investigate the possible antioxidant role of ascorbate in rheumatoid disease.

MATERIALS AND METHODS

Chemicals

Methanol and water were of HPLC grade and obtained from BDH chemicals, Poole, Dorset. 1,2 phenylene diamine, EDTA were purchased from Sigma Chemical Company and dehydroascorbic acid was obtained from Koch Light Laboratories. All other chemicals were of Analar grade and obtained from BDH laboratories.

Clinical Subjects

Sera were collected from normal human volunteers (laboratory staff) mean age: 49 years, range 35–63. Paired sera and synovial fluids were collected from 13 patients with classical or definite rheumatoid arthritis, during the course of therapeutic joint aspiration. Mean age: 41 years, with an age range 21–81 years.

HPLC measurement of ascorbate and dehydroascorbate

Sera and synovial fluids were extracted with an equal volume of 10% trichloroacetic or metaphosphoric acid (MPA) within 30 minutes of collection. The extracts were then frozen at -25°C until required for assay. After thawing at room temperature the extracts were vortex-mized for 1 minute with 100 μl of 10 mM ethylenediaminetetraaceticacid (EDTA) to chelate traces of iron which might catalyse the oxidation of ascorbate to dehydroascorbate. The samples were then centrifuged at 2,500 g and mixed with 500 μl of 1 mM 1,2 phenylene diamine, gassed with nitrogen for 30 seconds, capped, and allowed to stand for 60 minutes at 4°C until the reaction of dehydroascorbate with 1,2 phenylene diamine was complete. Determination of ascorbic acid and dehydroascorbate was performed simultaneously by HPLC, according to a modification of the procedure of Keating and Haddad[9]. The liquid chromatograph used was an LDC (Infrotronics) modular system consisting of a pump, a UV detector and fluorescence detector. Samples (200 μl) were injected onto a lichrosorb NH_2 column and eluted with 30:70 methanol-water at a flow rate of 1 ml/min. Elution of ascorbic acid was monitored at 265 nm while detection of the quinoxaline resulting from the reaction between dehydroascorbate acid and 1,2 phenylenediamine (figure 1) was detected by its fluorescence at 425 nm when excited at 350 nm.

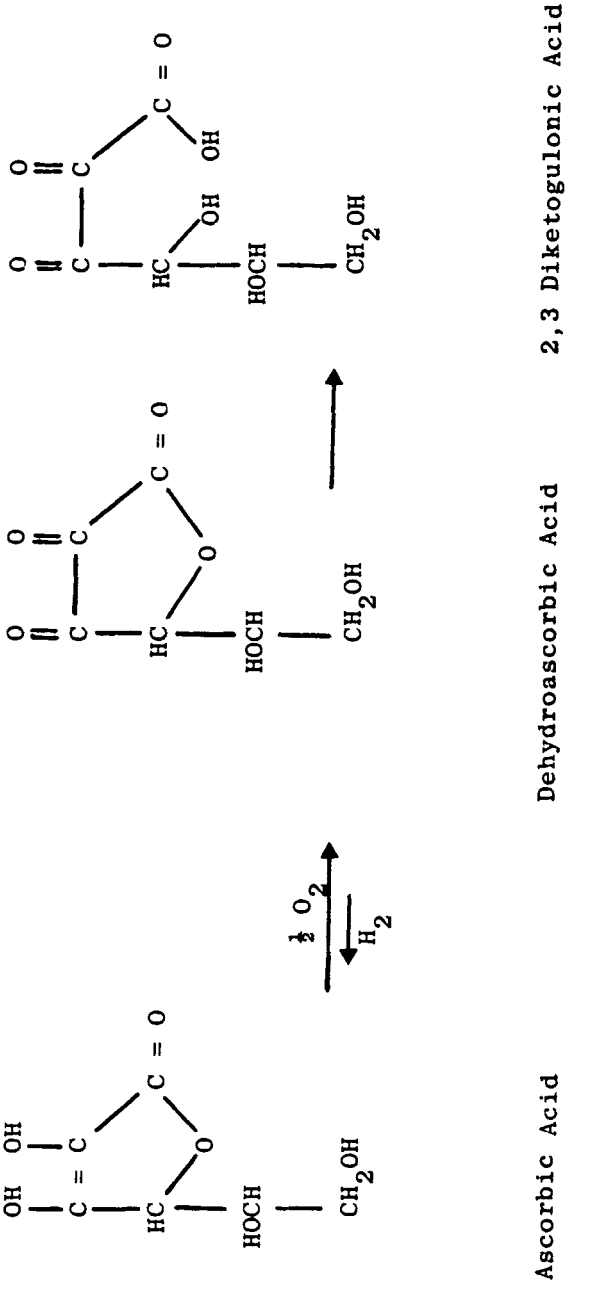


FIGURE 1 Reaction scheme for the pre-column derivatisation of dehydroascorbate acid using 1,2 phenylene diamine.

RESULTS

Calibration Curves

A linear standard curve was obtained by plotting the mean ascorbate and dehydroascorbate peak areas (duplicate injections) against concentration. Linear regression analyses of typical standard curves gave a result of $y = 201.74x + 733$, with a correlation coefficient (r) or 0.9994 for ascorbate and $y = 302.5x + 520$ ($r = 0.9987$) for dehydroascorbate.

Precision

The inter and intra batch precision was obtained by separate analysis and extraction of 10 aliquots of sera from a normal and RA pool. The results of precision analysis are shown in Table 1.

TABLE I
Precision analysis

	Within Batch				Between Batch			
	Normal (mean)	CV	Rheumatoid (mean)	CV	Normal (mean)	CV	Rheumatoid (mean)	CV
Ascorbate (μmol/l) n = 10	74.5	3.1%	8.7	3.9%	75.5	3.8%	9.3	4.1%
Dehydroascorbate (μmol/l) (n = 10)	15.8	4.0%	23.3	3.7%	16.2	4.5%	24.3	5.3%

Specificity

The influence of the plasma constituents, urea, uric acid, creatinine, cysteine, glutathione, glucose and bilirubin, on the recovery of ascorbic acid and dehydroascorbic acid were investigated. None of these compounds was found to interfere with the recovery of either ascorbic acid or dehydroascorbic acid measured by HPLC. The oxidised form of ascorbic acid, diketo gulonic acid, was also checked for interference, particularly in the derivatisation reaction of dehydroascorbic acid, but was found not to interfere with its recovery from a serum pool.

Recovery

The recovery of both ascorbate and dehydroascorbate in the extraction procedure and subsequent reaction with phenylene diamine was determined, by extracting sera containing known added amounts of ascorbate and dehydroascorbate (10 μM, 25 μM and 50 μM) with 10% MPA. The recoveries were found to be in the range 95–110% with a mean of 98% for ascorbate and 96% for dehydroascorbate. These results are expressed as a mean of 5 separate experiments.

TABLE II
Stability of serum ascorbic acid and dehydroascorbic acid on storage in MPA extracts -25°C

	Ascorbate μmol/l	Dehydroascorbate μmol/l
Day 0	15.6	20.5
Day 5	14.5	20.3
Day 10	15.7	21.5
Day 15	15.8	21.2
Day 20	14.6	20.8
Day 25	14.9	20.8
Day 30	15.0	21.8

Storage

Separate aliquots of MPA extracts of serum were assayed at regular intervals to determine the stability of ascorbate and dehydroascorbate in extracts (Table 2). The reproducibility of the results, taking into account the inter-batch variations (see Table 2), indicated that ascorbic acid and dehydroascorbic acid were stable in MPA extracts for up to a period of 3–4 weeks when stored at -20°C.

Figure 2 illustrates a typical chromatogram obtained from injecting MPA extracts from serum onto the lichrosorb column. Total ascorbate measurements obtained by addition to ascorbate and dehydroascorbate levels confirmed that total ascorbate levels are significantly decreased in rheumatoid sera ($p < 0.001$).

The mean and range (±1SD) quoted for total ascorbate 69.6 ± 20.6 μmol/l (Table 3), in normal sera, was broadly in agreement with previous published findings based on using several methods for measuring total ascorbate[10,11]. However, in contrast to previous findings[11], dehydroascorbate was detectable in normal serum and represented, on average, approximately 17% of the total ascorbate present in normal serum. The levels of dehydroascorbate found in the sera from patients with rheumatoid arthritis was proportionately far greater, on average, than in normal sera. Almost all of the total ascorbate measured in rheumatoid serum was in the form of dehydroascorbic acid (80%).

TABLE III
The measurement of ascorbic acid and dehydrascorbic acid in RA

	Total ascorbate (μmol/l)	Dehydroascorbate (μmol/l)	Ascorbate (μmol/l)
Normals (n = 20)	69.6 ± 20.6	12.0 ± 3.7 *	57.7 ± 19.6 ***
RA sera (n = 13)	27.0 ± 12.5	21.8 ± 8.6 **	5.1 ± 5.0
RA synovial fluid (n = 13)	38.7 ± 9.7	32.8 ± 9.7	7.8 ± 7.9

* $p < 0.01$
** $p < 0.001$
*** $p < 0.001$
A paired t test was used for statistical analysis of the results. All results are expressed as the mean ± 1 standard deviation.

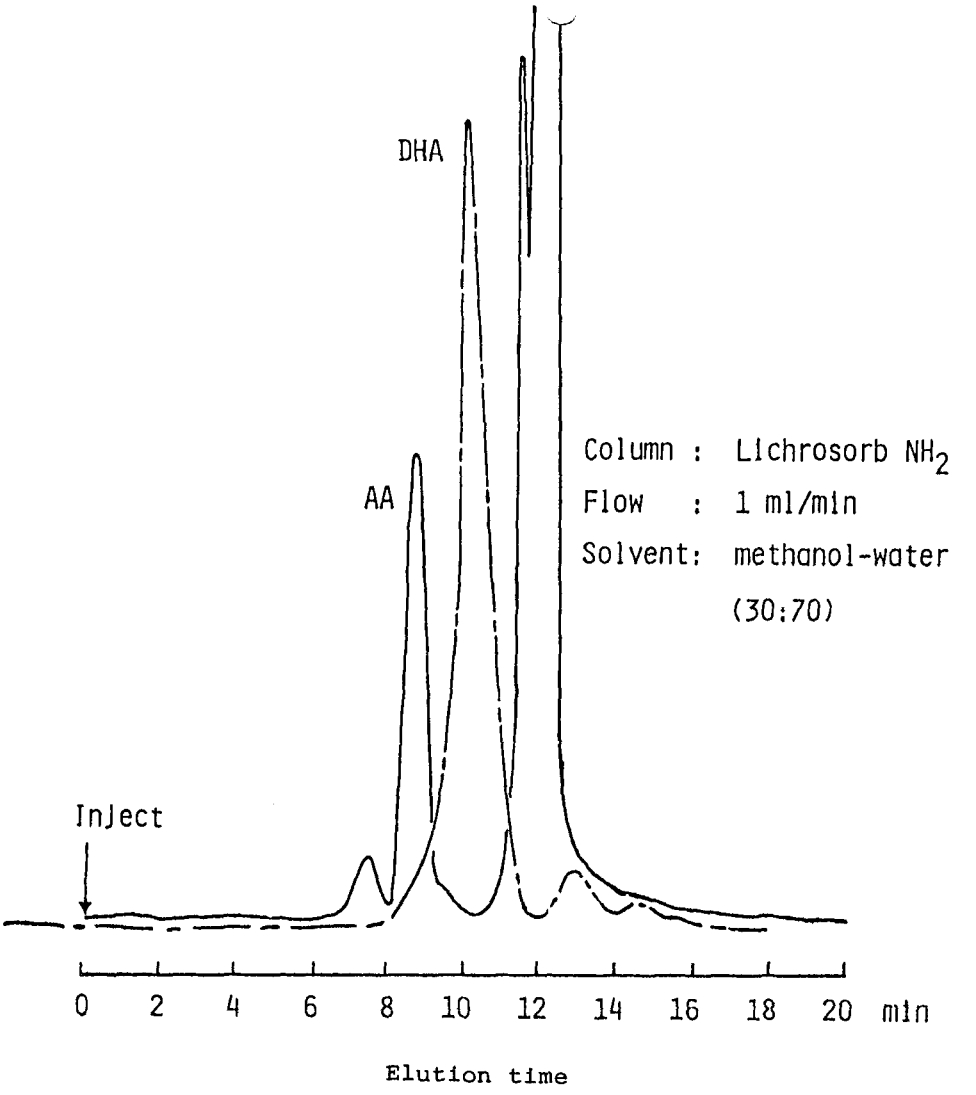


FIGURE 2 HPLC separation of a typical MPA extract of normal human plasma after derivatisation with 1,2 phenylene diamine. Ascorbic acid elution was monitored at 265 nm (——) and the quinoxaline adduct formed between dehydroascorbic acid and 1,2 phenylene diamine was detected by its fluorescence at 525 nm when excited at 340 nm (—- —- —-). This fluorescence elution profile is shown displaced by 2 minutes in this figure for clarity.

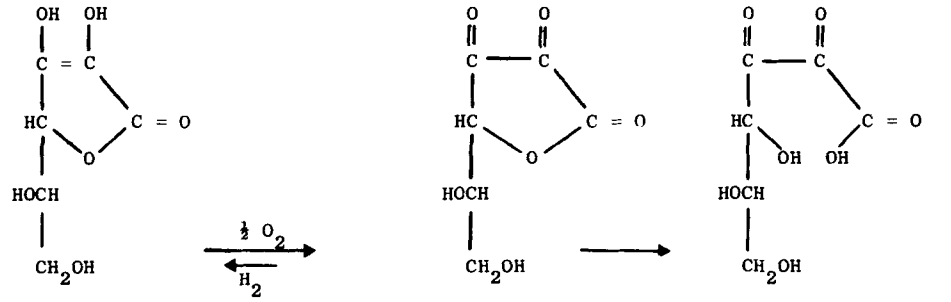
This left approximately 5–10 μmol/l of true ascorbate in rheumatoid sera. This value was close to the limits of detection of ascorbate by this method (2.5 μmol/l). In some instances no ascorbate could be detected in rheumatoid synovial fluid and sera. (This was not found to be an artefact related to storage since serum ascorbate levels remained constant for up to a period of 4 weeks, when extracts were stored at -25°C). Direct comparison of paired synovial fluid and serum from patients with rheumatoid arthritis indicated firstly, that there was significantly more total ascorbic acid in the synovial fluids compared to corresponding sera ($p < 0.01$) and secondly, that this total ascorbate was predominantly in the dehydro form (78%). In addition, of the fluids and sera tested, dehydroascorbate levels were, without exception, higher in synovial fluids that in corresponding sera. Levels of ascorbate were also significantly elevated in synovial fluids compared to corresponding sera.

In rheumatoid sera there was a significant positive correlation between dehydroascorbic acid and ascorbic acid ($r = +0.32$, $p < 0.001$) while in synovial fluids there was a negative correlation ($r = -0.39$, $p < 0.001$).

DISCUSSION

Current evidence indicates that ascorbate is metabolised by a two step, biologically reversible oxidation reaction. Only L-ascorbic acid and its first oxidation product dehydroascorbic acid possess biological activity. The activities are approximately equal.

In man dehydroascorbic acid can be reduced to ascorbic acid by a glutathione-glutathione reductase enzyme system. Since L-ascorbic acid and dehydroascorbic acid are both physiologically active, it is necessary to measure them both. Routine procedures for measuring ascorbate cannot differentiate between L-ascorbic acid,



Ascorbic Acid Dehydroascorbic Acid 2,3 Diketogulonic Acid

dehydroascorbic acid and the physiologically inactive diketogulonic acid form of the vitamin. This may mean that in a healthy population the levels of physiologically active ascorbate may be lower than they appear to be.

The procedure described here, is a simultaneous measure of ascorbic acid and its immediate oxidation product dehydroascorbic acid with O-phenylene diamine. This method is specific for dehydroascorbate since interfering reactants can be removed or distinguished during HPLC separation. This method does not involve errors inherent in measurements made by calculating differences between total ascorbate and dehydroascorbate measures.

We have used this procedure to estimate the relative concentrations of dehydroascorbate and ascorbate in both rheumatoid sera and synovial fluid. In complete contrast to publish findings we were able to detect significant amounts of the oxidation product dehydroascorbic acid in normal sera. This finding reflects the high sensitivity and specificity of this assay compared with conventional procedures.

The abnormally high levels of dehydroascorbate we found in rheumatoid sera could reflect either, increased conversion of ascorbate by aberrant oxidative mechanisms or, a decreased rate of reduction of dehydroascorbate back to ascorbic acid. Free radical oxidation may be important in both instances since the latter pathway is dependent on the maintenance of levels of reduced glutathione. In rheumatoid disease it is known that there are abnormal oxidation (lipid peroxide) reaction products[12,13] generated which could potentially deplete levels of glutathione and hence decrease the rate of conversion of dehydroascorbic acid back to ascorbate.

Once formed dehydroascorbic acid is unstable having a life time of only minutes of physiological temperatures and pH. Thus in the absence of mechanisms available to reconvert it to ascorbate it will rapidly decompose to oxalic acid and be excreted in the urine. If glutathione is depleted therefore, by abnormal free radical oxidation mechanisms, we would expect to see a reduction of both ascorbic acid and dehydroascorbic acid. Our results therefore, are entirely consistent with an increased requirement for the anti-oxidant and free radical scavenging activity of this vitamin in RA.

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