Published in final edited form as:

Anal Biochem. 2007 June 1; 365(1): 31–39.

A Simple and Sensitive Assay for Ascorbate Using a Plate Reader

Jesse M. Vislislis, Freya Q. Schafer, and Garry R. Buettner²

ESR Facility and Free Radical and Radiation Biology The University of Iowa, Iowa City, IA 52242-1101

Abstract

We have developed a rapid, inexpensive, and reliable assay for the determination of ascorbate using a plate reader. In this assay, ascorbic acid is oxidized to dehydroascorbic using Tempol and then reacted with *o*-phenylenediamine to form the condensation product, 3-(dihydroxyethyl)furo[3,4-b]quinoxaline-1-one. The rate of appearance of this product is monitored over time using fluorescence. With this method, it is possible to analyze 96 wells in under 10 min. This permits the analysis of 20 samples with a full set of standards and blanks, all in triplicate. The assay is robust for a variety of samples including orange juice, swine plasma, dog plasma, and cultured cells. To demonstrate the usefulness of the assay for the rapid determination of experimental parameters, we investigated the uptake of ascorbate and two different ascorbate derivatives in U937 cells. We found similar plateau levels of intracellular ascorbate at 24 hours for ascorbate and ascorbate phosphate. However, the intracellular accumulation of ascorbate *via* the phosphate ester had an initial rate that was 3–5 times slower than the palmitate ester. Only lower concentrations of the palmitate ester could be examined because the ethanol needed as solvent decreased cell viability; it behaved similarly to the other two compounds at lower concentrations. To come to these conclusions only nine plates needed to be analyzed to provide us with the end result after only seven hours of analysis. This clearly demonstrates the strength of the plate reader assay which allows the analysis of large sample sets in a fraction of the time required for the methods that are most commonly used today. The assay is quick, very economical, and provides results with uncertainties on the order of only 5%.

Keywords

Ascorbic acid; vitamin C; Tempol

Introduction

Since the discovery of ascorbic acid by Szent-Györgyi in 1928 [1], its importance in human health has been recognized and its antioxidant and pro-oxidant characteristics have been studied. Currently, vitamin C is the leading commercially produced vitamin, with annual global consumption of about 100 million kg [2]. Over the years, many different spectrophotometric and chromatographic assays have been developed to measure ascorbic acid in biological samples such as plasma, urine, cells, and food. These methods vary in sensitivity, specificity, stability, substance interference, speed, simplicity, sample range, and cost [3].

* To whom correspondence should be addressed: Dr. Garry R. Buettner, Free Radical and Radiation Biology, EMRB 68, The University of Iowa, Iowa City, IA 52242-1101, Tel: 319-335-6749, Fax: 319-335-9112, Email: garry-buettner@uiowa.edu.

²Reprint requests to: Garry R. Buettner, ESR Facility, EMRB 68, The University of Iowa, Iowa City, IA 52242-1101, Email: garry-buettner@uiowa.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We have developed a high throughput, simple, and inexpensive assay for ascorbate using a plate reader. Our fluorescence assay is an adaptation of a long used assay that has recently been modified by Ihara *et al.* [4,5]. In this assay ascorbic acid is oxidized to dehydroascorbic, which then reacts with *o*-phenylenediamine (OPDA) to form the condensation product, 3-(dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DHA-OPDA). Appearance of this product is followed over time. Traditionally the enzyme ascorbate oxidase (EC 1.10.3.3) has been used to accomplish the oxidation of AscH⁻ to DHA. However, here we employ the nitroxide, Tempol, as the oxidizing agent. With this, the reagents used in the routine application of our assay are inexpensive and relatively stable, making the assay efficient and affordable. Use of a plate reader in conjunction with our modified approach allows the determination of ascorbate in large sample sets to be accomplished in a fraction of the time required for the methods that are most commonly used today.

Materials and Methods

Chemicals

Ascorbic acid was purchased from EMD Chemical Inc. (Gibbstown, NJ). Ascorbate oxidase, sodium acetate dihydrate, diethylenetriaminepentaacetic acid (DETAPAC or DTPA), *o*-phenylenediamine dihydrochloride (OPDA), and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol; CAS No: 2226-96-2) were from Sigma-Aldrich (St. Louis, MO); methanol was from Fisher Scientific (Fair Lawn, NJ). L-ascorbic acid 6-palmitate was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and L-ascorbic acid phosphate magnesium salt *n*-hydrate was acquired from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

The human promyelocytic leukemia cell line U937 was obtained from ATCC (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and penicillin (100 U/mL), streptomycin (100 µg/mL), all from GIBCO® (Grand Island, NY). Cells were incubated at 37°C in a 95% humidity atmosphere under 5% CO₂. Cells from passages 5–21 were used for the experiments.

Solutions

The stock solution for ascorbic acid standards (100 mM) was made in Nanopure® water using a volumetric flask that was conditioned with ascorbic acid. The concentration of the stock solution was accurate to at least three significant digits. Standards were prepared from this solution by dilution with MeOH/H₂O containing DETAPAC. The exact MeOH/H₂O ratio (v/v) varied to match the ratio used in the samples to be analyzed. We found that it is extremely important that the methanol content of the standards and samples be the same.

Because ascorbate is easily oxidized in the presence of catalytic metals, adventitious metals were removed from all buffer solutions with chelating resin (Sigma-Aldrich, St. Louis, MO) using the batch method [6]. Sodium acetate buffer (2 M) was treated with chelating resin and adjusted with acetic acid to pH 5.5. Tempol (2.32 mM) was dissolved in sodium acetate buffer and stored at 4°C for up to a month. OPDA (5.5 mM) was dissolved in sodium acetate buffer. OPDA can only be stored for 1–2 days at 4°C in the dark. As an additional measure to stabilize the ascorbate extracted from samples, DETAPAC (250 µM) was added to the aqueous portion of the MeOH/H₂O mixture used to precipitate protein or condition the samples [7].

Incubating cells with vitamin C

Cells (10×10^6) were placed into 40 mL of fresh medium. Ascorbic acid or ascorbate phosphate stock solutions were prepared in medium and different amounts were added to the cells. Cells were then incubated for 24 h at 37°C, 5% CO₂. Cells were washed twice with HBSS and cell pellets (6×10^6 cells/vial) were frozen at -80°C to rupture the membranes. After freezing (30 min at -80°C), cell pellets were thawed and extracted with 300 µL MeOH/H₂O (60/40, v/v) containing DETAPAC, incubated on ice for 10 min, and then centrifuged at 12,000 g for 10 min. Supernatant was stored at -80°C until analysis.

Plasma preparation

Whole blood (2 mL) from swine and dog was aliquoted into centrifuge tubes, then centrifuged at 4°C, 300 g, for 10 min. The upper plasma layer was transferred to a new centrifuge tube and 1 part plasma was mixed with 4 parts MeOH/H₂O (90/10, v/v) containing DETAPAC. The mixture was incubated on ice for 10 min to precipitate the protein. The samples were centrifuged at 4°C, 12,000 g, for 10 min. Supernatants were stored at -80°C until analysis.

Orange juice preparation

Orange juice (Minute Maid, Coca-Cola Company, Atlanta, GA) was aliquoted into centrifuge tubes. Juice was centrifuged at 16,000 g for 30 s and supernatant was transferred to a new centrifuge tube. Supernatant (10 µL) was dissolved in 1000 µL MeOH/H₂O (75/25, v/v) containing DETAPAC. The mixture was incubated on ice for 10 min to precipitate the protein. The samples were centrifuged at 4°C, 12,000 g, for 10 min. Supernatants were stored at -80°C until analysis.

Spiking samples

In development of the assay we used the method of standard additions to determine if the various matrices of our samples influenced the assay results. To prepare these standard samples, individual samples were pooled to achieve a total volume of 450 µL. Then 10 µL of an appropriately diluted ascorbate stock solution was added to achieve the desired increase in concentration. These spiked samples were pipetted into the plate and subjected to the same treatments as the non-spiked samples.

Plate reader assay

Samples (cells, plasma, or juice) stored at -80°C were centrifuged at 16,000 g for 30 s. The sample supernatant (100 µL/well) or ascorbate-standard (100 µL/well) were transferred into the wells of a 96-well plate. Next, 100 µL of the Tempol stock (2.32 mM Tempol in acetate buffer) were added to each well and samples were incubated for 10 min at room temperature. In the dark, 42 µL freshly prepared OPDA solution (5.5 mM OPDA in acetate buffer) were added using a 12 channel pipette. To synchronize the reaction with the reading pattern of the plate reader, OPDA was deposited in ≈3-second intervals starting in row A and ending in row H. The plate was then inserted into a TECAN *SPECTRAFLUOR PLUS* plate reader (TECAN, Research Triangle Park, NC). Fluorescence was measured using a 345 nm band pass filter for excitation and a 425 nm band pass filter for emission. The kinetic program (version 3.4x) was set as fast as the instrument allowed (22 scans; approximately every 26 s for 9.5 min).

Statistical analyses

Data are expressed as means ± S.E. Statistical significance of differences between paired data was determined using two sample students' t-tests. Differences among means were considered to be significant at $p < 0.05$. The lower limit of detection (LLD) was determined using the calibration plot method described by Anderson [8].

Results

Overview of assay

The assay we describe is a kinetic assay that follows the formation of DHA-OPDA over time, Rxn 4 below. Because OPDA is in excess, the initial rate of this reaction is proportional to the initial [DHA], Eqn 6. The initial rates from standards are used to construct a standard curve. This curve is defined by a linear function from which the concentrations of unknown samples are easily deduced.

Kinetic considerations of assay

This method of analysis for total ascorbate is based on the well-known condensation reaction of dehydroascorbic (DHA) with *o*-phenylenediamine (OPDA) to form a highly colored product that can be detected by fluorescence [4]. In the assay, ascorbate must be oxidized to DHA. This traditionally has been done with ascorbate oxidase [4]. In the plate reader assay we describe here, the oxidation of ascorbate is accomplished using Tempol [5,9]. This oxidation reaction is proposed to consist of two consecutive one-electron reactions,

where Tempol-H is the hydroxylamine [10,11]. With the high level of Tempol used in our protocol, Rxn 2 is rapid. Thus, the rate-controlling step is the initial reaction of AscH^- with Tempol, Rxn 1. The rate constant for the slow step above agrees well with reports that measured the overall rate constant for these reactions: $k_3 = 7.0 \text{M}^{-1} \text{s}^{-1}$ as determined in [12]; we have estimated $12 \text{M}^{-1} \text{s}^{-1}$ from data in [9]. Our measurement of an observed rate constant for this reaction under the conditions of our assay yields an observed rate constant $k_3 = 3.5 \pm 0.6 \text{M}^{-1} \text{s}^{-1}$ [unpublished].

The assay for total ascorbate is a kinetic assay that follows the formation of the condensation product, DHA-OPDA.

The rate constant for this reaction under our conditions is $k_4 = 4.6 \pm 1.8 \text{ M}^{-1} \text{ s}^{-1}$ ($n = 27$). OPDA (1.0 mM final concentration; 60% MeOH) is in great excess compared to DHA; therefore, the reaction will behave as a pseudo first-order reaction with a rate proportional to [DHA].

$$-d[\text{DHA}]/dt = k_4' [\text{DHA}] \text{ where } k_4' = 4.6 \text{ M}^{-1} \text{ s}^{-1} \cdot 1.0 \times 10^{-3} \text{ M} = 0.0046 \text{ s}^{-1}. \quad (5)$$

Thus, the half-life of DHA in Rxn 5 is $\approx 150 \text{ s}$. Because $[\text{DHA}] \ll [\text{OPDA}]$, the appearance of condensation product will follow the function

$$+d[\text{DHA} - \text{OPDA}]/dt = k_4' [\text{DHA}], \quad (6)$$

with the [DHA-OPDA] at any time t being

$$[\text{DHA} - \text{OPDA}]_t = [\text{DHA}]_0 (1 - e^{-k_4' t}). \quad (7)$$

This means we can estimate $[\text{DHA}]_0$, *i.e.* $[\text{AscH}^-]_0$, from the initial slope because these slopes will be directly proportional to $[\text{DHA}]_0$.

The TECAN *SPECTRAFLUOR PLUS* plate reader analyzes each well on the 96-well plate individually because it is equipped with a single set of optics and one detector. The instrument measures the wells beginning at well A1, and proceeding across the row to the right. After reaching the end of the row, it shifts down a row to well B1. It continues in this fashion until it finally reaches well H12. The plate reader requires slightly more than 3 s to read a row; to synchronize the reaction with the instrument's reading pattern, the OPDA is added in ≈ 3 -s intervals with a 12-channel pipette beginning with row A and ending with row H.

Initial slope via a polynomial fit

As seen in Eqn 7, the formation of DHA-OPDA will always be an exponential function that, in principle, would have to be followed for infinite time to determine $[\text{DHA-OPDA}]_\infty$ and thus $[\text{DHA}]_0$. To simplify this we collected fluorescence data for only 4.0 half-lives (570 s), gathering 22 time points that were used to determine the initial slope. Using Microsoft[®] Excel 2003, these data were fit to a second-order polynomial. We then used the equation to determine the y-intercept ($t = 0 \text{ s}$) and the value of the polynomial function at $t = 90 \text{ s}$. The slope of the line between these two points provided a good estimate of the initial slope, Figure 1(A). These slopes have a direct linear relationship to $[\text{AscH}^-]$. The concentration of AscH^- in any sample can be determined from an appropriate standard curve.

We investigated taking measurements over longer periods of time to see if this would increase accuracy and reproducibility. However, this caused the polynomial fit to emphasize the later time points in the development of chromophore rather than the initial slope. Because we make our determinations based on the initial slope (first 90 s) of the polynomial fit, adding more points by extending the time of measurement reduced the quality of the results.

Standard curve

To generate a standard curve 11 different standard solutions (7.5, 10, 20, 30, 40, 50, 70, 90, 110, 130, and 150 μM) that spanned the functional range of the assay were analyzed as described above. When the initial slopes of the standards were plotted against concentration, a linear relationship was observed, Figure 1 (B), allowing us to confidently determine concentrations in unknown samples. Each of the 11 points on the standard curve represents the mean from three individual wells. After dedicating 33 wells to standards and 3 wells to blanks, the 96-well plate has 60 remaining wells that can be devoted to samples.

Detection limits

The 96-well plates we use can hold a maximum volume of approximately 350 μL . However, a practical limit to allow handling of the plate is less. In our assay, the total volume of solution in each well is 242 μL . We found the lower limit of detection to be approximately 6.2 μM in the sample; the upper limit was about 150 μM . Because of dilution with the reagents of the assay, these concentrations correspond to final concentrations of 1.1 and 56 μM in the wells of the 96-well plate.

These detection limits were established using standard solutions covering a range of AscH^- ascorbate concentrations from 0.1 to 1000 μM . The lower limit of detection (LLD) was determined using the calibration plot method [8]. We estimated the LLD to be a sample concentration of 6.2 μM (1.1 μM in the well). As expected, this value was highly dependent on the R^2 -value of the 11-point standard curve. Using mean data from 15 plates, the LLD was found to be 5.4 μM when $R^2=0.99$. When the R^2 -value dropped to 0.98, the LLD increased to 9.4 μM in the sample. Thus, careful measuring and pipetting is critical when analyzing samples with low $[\text{AscH}^-]$; R^2 can be used as an indicator for a reasonable LLD.

While the standard curve is linear in the span employed by the assay, there is a loss in linearity at higher $[\text{AscH}^-]$. After examining several extended standard curves, we determined that the break in linearity occurs at a sample concentration of $\approx 150 \mu\text{M}$ (56 μM in the well). This concentration was established as the upper limit of the assay. This upper limit does not pose a significant problem as very concentrated samples can easily be diluted to fall within the linear range of the assay.

Methanol slows the OPDA+DHA reaction

Methanol is used in the preparation of samples both to precipitate protein and to stabilize the DHA. To determine if methanol influences the rate of the reaction of dehydroascorbic with OPDA we determined the rate of chromophore formation of an ascorbate standard (25 μM) prepared with different ratios of $\text{MeOH}/\text{H}_2\text{O}$. We observed that higher concentrations of MeOH in the sample resulted in a lower rate of reaction between OPDA and DHA, Figure 2. Thus, if the methanol concentration of standard solutions differs from that of the samples, the ascorbate concentration in samples will be over- or underestimated. In accordance with Levine *et al.* we use 60% MeOH to extract cell pellets [3]. However, plasma, serum, and blood samples require 90% MeOH to precipitate the protein, due to their high water content. After extraction, the final $[\text{MeOH}]$ in these samples is about 75%. The $[\text{MeOH}]$ of the standard solutions has to be the same as the final $[\text{MeOH}]$ in the sample.

In addition, we replaced the ethylenediaminetetraacetic acid (EDTA) used by Levine *et al.* with the metal chelator diethylenetriaminepentaacetic acid (DETAPAC). DETAPAC is a cousin of EDTA, but the Fe(III)DETAPAC complex catalyzes the oxidation of ascorbate much more slowly than Fe(III)EDTA thereby stabilizing AsC⁻ more effectively [7].

There are two ways to maintain a consistent methanol concentration in the assay. The [MeOH] of the standard solutions may be adjusted to that of the final [MeOH] of the samples, or the samples may be diluted with H₂O/DETAPAC to match the [MeOH] of the standard solutions. Both approaches are valid. As our data show, it is extremely important that the methanol content of the standards and samples be the same.

Ascorbate can be measured in a variety of materials

Ascorbate determinations are required for a wide range of materials. The concentrations can vary from nM to mM and the matrix of the samples range from solid tablets to colorful foods and drinks to complex biological fluids and tissues. Few assays can measure such a variety of samples. To test the versatility of our assay we measured the ascorbic acid content of orange juice, swine plasma, dog plasma, and cultured cells. All samples were prepared with appropriate dilution to lie within the limits of the assay and then spiked with two different known concentrations of ascorbic acid to determine accuracy.

The assay was able to consistently measure the amount of AsC⁻ in all samples tested. The orange juice, at 2.7 ± 0.08 mM, was highly concentrated, Table 1. This concentration translates to 169 mg/serving, slightly higher than the 130% RDA stated on the bottle. The RDA for vitamin C for adults is 75 – 90 mg [13]. The swine and dog plasma samples yielded results within the published expected ranges of 11.4 – 68.1 μ M and 11.4 – 119.3 μ M, respectively [14]. In our samples we determined the [AsC⁻] of swine plasma to be 44.8 ± 3.0 μ M. The dog plasma had a higher [AsC⁻] of 93.2 ± 4.8 μ M. The U937 cells grown in standard cell culture media supplemented with ascorbate had 6.5 ± 0.3 mM as an intracellular concentration.

The samples were spiked with two different concentrations of ascorbate standards to test for effects of matrix interference. The concentration of ascorbate added to each sample type was dependent on the amount of ascorbate present before the spike. In general, the first spike was designed to raise the concentration of AsC⁻ to about 150% of the amount present in the original samples. The second spike roughly doubled the amount of AsC⁻ present in the samples.

Using two sample students' t-tests, we found the observed concentrations of ascorbate after standard additions were equal to the expected concentrations for all but one set of samples, Table 1. The higher spike into the dog plasma was the only addition found to be significantly different from the expected [AsC⁻] ($p = 0.001$, df (degrees of freedom) = 22). This result may be slightly misleading as the concentration is within 12% of the expected value. Exceptionally low standard errors relative to a higher concentration of ascorbate led to this result. The method of standard additions demonstrated that the assay works well on a variety of samples.

Comparing the uptake of ascorbate and its derivatives palmitate and phosphate in U937 cells—The antioxidant ascorbate is not stable in typical buffer solutions [6,7] or cell culture medium [15,16,17]. Derivatives of ascorbate such as L-ascorbic acid-6-palmitate and L-ascorbic acid phosphate have been developed to eliminate this disadvantage. The palmitate ester is compatible with lipid environments while ascorbate phosphate is water-soluble. We posed two questions in these experiments: 1. What are the best conditions to compare the behavior of the uptake of ascorbate and derivatives of ascorbate? 2. Can our new assay speed up the search for optimal uptake conditions?

Ascorbic acid 6-palmitate is more stable than AscH^- under most conditions [18]. In contrast to the phosphate, this derivative accumulates in the lipid fraction of cell membranes. While ascorbate and ascorbate phosphate are highly water-soluble the palmitate must be dissolved in ethanol. Ethanol can be toxic to cells, thus our final palmitate concentration in the medium could not reach the concentrations of ascorbate phosphate examined because of this toxicity.

Ascorbate phosphate also shows high stability and has been used in a wide range of applications from cosmetics [19] to cell culture experiments [20]. In cell experiments, cells must take up the esterified ascorbate and then remove the phosphate. When this occurs, the ester bond of the phosphate group is hydrolyzed inside the cell resulting in the accumulation of intracellular ascorbate.

We exposed U937 cells to all three forms of ascorbate to compare intracellular ascorbate accumulation. Cells were exposed to different concentrations of ascorbate and its phosphate derivative (10–1000 μM) in full medium for 24 h. After 24 h, intracellular accumulation was comparable for ascorbate and its phosphate derivative, Figure 3. Cells were only exposed to low palmitate concentrations because high $[\text{EtOH}]$ decreased cell viability. At these lower concentrations (10–50 μM) ascorbic acid accumulation was similar to the other two forms. While the maximum ascorbate accumulation is comparable, it takes a longer period of time for the phosphate to reach the maximum ascorbic acid accumulation, Figure 4. Intracellular ascorbate reaches a maximum after 8 h when cells were exposed to ascorbate. Cells exposed to ascorbate phosphate reached a similar maximum $[\text{AscH}^-]$ at 24 h.

This example of testing the differential uptake of ascorbate and ascorbate derivatives is typical of many cell culture experiments on ascorbate in that many samples must be analyzed. A large number of samples requires considerable time for analysis when using HPLC. All of the data for Figures 3 and 4 could have been obtained from 9 plates (although we used 13 due to the timing of sample availability and because we were running other additional samples on the plates). This would require about 7 hours of lab work to prepare harvested samples and measure the plates. If this were done using HPLC, the same set of samples would have taken >350 hours of nonstop measurements (allowing 30 min/sample, accounting for standards and instrument maintenance). Thus, while the HPLC assay is more specific, it pales in comparison when efficiency is taken into consideration.

Discussion

Our ascorbate assay is a high-speed, efficient, and cost-effective alternative to the other current methods of measurement. High performance liquid chromatography (HPLC) may be the most sensitive and specific method for the measurement of ascorbic acid, but it has several disadvantages. HPLC determination is time consuming, the instrumentation may not be available to every lab, and the supplies and maintenance are relatively expensive.

Speed of the assay

The time required for sample determination is one of the primary advantages of our assay. An HPLC measurement requires sequential determination of samples each taking 15–45 minutes, while our plate reader assay can measure 96 wells in under 10 min. Running samples at this rate makes it feasible to measure all standards and samples in replicates of three or more to reduce error. Additionally, with 96 wells available there is enough space to include 3 “blanks” containing all of the reagents for the assay but substituting buffer for the fraction containing ascorbate. These wells allow the investigator to probe the level of background noise of the instrument.

Specificity of the assay and substance interference

The difference in the time required between the two methods is substantial, but the HPLC has the advantage of sensitivity and specificity. In our plate reader assay the condensation product DHA-OPDA is detected *via* fluorescence, which results in high sensitivity and a higher specificity than typical colorimetric measurements. Using the same plate reader, we investigated the detection of the DHA-OPDA product using absorbance. However, we had limited success. The results using the change in absorbance yielded linear standard curves, but samples often provided non-reproducible and unreliable results. This was likely a consequence of the interference of other substances in the samples. By analyzing the samples *via* fluorescence, we were able to avoid much of this interference, creating highly reproducible measurements of samples.

To improve the specificity of our assay, we explored the use of ascorbate oxidase (AO) as an oxidizing agent. This enzyme is specific for L-ascorbic acid and several of its analogs [21]. Using a UV-visible spectrophotometer, we found that in the proportions employed in the assay, AO is able to oxidize the amount of ascorbate found in a typical sample (20 μM) in under a minute. Using this enzyme (1.5 U/mL) in place of Tempol, we obtained similar $[\text{AsCH}^-]$ in both orange juice and U937 cell samples, but not in swine plasma. The plasma yielded irreproducible, wildly fluctuating ascorbate measurements probably due to substance interference between the biological sample and the enzyme. Other drawbacks of AO lie in its instability and price. AO must be prepared fresh each day from stock, as it quickly loses its activity. It is also considerably more expensive than Tempol. Thus, Tempol is a better choice for the assay, saving both time and money.

We also experimented with the oxidizing capabilities of activated charcoal to convert AsCH^- to DHA. However, the proper filtration required to remove the charcoal from each individual sample and standard solution was much too time consuming and an unrealistic additional step to the assay, making it unusable.

OPDA can react with alpha-keto acids to form fluorescent products. Consequently, we investigated pyruvate, a common component in many biological samples, as a potential source of error in our assay. The reaction between OPDA and pyruvate requires heat and highly acidic conditions [22]. Even when these conditions are met, the reaction requires ≈ 1 h to reach 90% of the asymptote fluorescence value. To determine if pyruvate could interfere with our assay for ascorbate, we tested a series of pyruvate standards (10–350 μM) for fluorescence development. This range spans the physiological concentrations of human skeletal muscle, blood, and cerebrospinal fluid [23]. These levels of pyruvate produced no detectable fluorescence (data not shown) under the conditions of our assay. Thus, pyruvate is not an interference; other similar alpha-keto acids also should not interfere.

Stability of the assay

The stability of ascorbate and dehydroascorbic depends on temperature, light, pH, dissolved oxygen, solvent, ionic strength, and the presence of oxidizing enzymes and redox-active metals [1,6,24,25]. The assay reagents are most stable in cold, dark environments. Weakly acidic conditions stabilize the lactone ring of DHA preventing rapid hydrolysis and further oxidation ($\text{pH} \approx 3\text{--}6$) [24]. Precipitation of the protein in samples can be achieved using either MeOH/ H_2O or acids such as meta-phosphoric acid (MPA). These conditions also conveniently enhance the stability of ascorbate by lowering the pH of the solution. Furthermore, to reduce loss of ascorbate due to redox-active metals, we included the metal chelator DETAPAC in the aqueous portion of the MeOH/ H_2O mixture and tested both MeOH/DETAPAC and MPA for their suitability in the assay [7]. Our results comparing the two approaches were in agreement with Levine *et al.* [3,25]; using methanol to precipitate the protein resulted in higher

reproducibility and better stabilization of the ascorbate than could be achieved with meta-phosphoric acid.

Sensitivity of the assay

Our assay is sensitive over a wide range of ascorbate concentrations. The lower limit of the assay is mainly determined by the background noise of the instrument. A practical lower limit is 6.2 μM in the sample being measured. The upper limit, 150 μM in the sample, results from a loss of linearity in the standard curve at higher concentrations. However, this does not pose much of a problem because concentrated samples can easily be diluted to fall within the established range of the assay.

Sample range of the assay

Ascorbic acid measurements are needed for a wide range of samples. Samples range from foods, juices, and vitamin tablets with very high levels of AscH^- to plasma, tissue, and cell culture with sometimes very low AscH^- levels. In addition to the varying ascorbate concentrations, each type of sample brings its own challenges in terms of substance interference and amount of sample available.

We examined orange juice, dog plasma, swine plasma, and U937 cells with our assay. Even with such a wide range of sample types, we had no problems achieving reproducible measurements. Furthermore, as long as the samples contain the same concentration of MeOH as the standards, multiple sample types can be run on the same plate without a problem.

Summary

- This kinetic assay estimates $[\text{AscH}^-]$ using the initial slope of chromophore development.
- It requires little time and has the ability to measure 96 wells in under 10 min.
- The assay is able to determine $[\text{AscH}^-]$ in samples having concentrations between 6.2 and 150 μM .
- A wide variety of samples may be reliably analyzed with little substance interference including food substances, blood plasma, and cells.
- $[\text{MeOH}]$ in the samples must be consistent with $[\text{MeOH}]$ in the concentration standards to achieve accurate determinations.
- In a practical application, ascorbate reached similar maximum intracellular levels when either ascorbate or ascorbate phosphate was provided in the growth medium; however, the maximum level requires more time to accumulate with the phosphate ester.
- The assay is sensitive, rapid, and affordable.

Acknowledgements

Acknowledgements: This work was supported by NIH grant CA66081. We thank Dr. Brian Smith for his insights and advise on the statistical analyses.

References

1. Szent-Györgyi A. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex: Description of a new carbohydrate derivative. *Biochem J* 1928;22:1387–1409. [PubMed: 16744155]

2. McCoy M. Vitamins. Chem Eng News 2005;83(25):136.<http://pubs.acs.org/cen/coverstory/83/8325/8325vitamins.html>
3. Levine M, Wang Y, Rumsey SC. Analysis of ascorbic acid and dehydroascorbic acid in biological samples. Methods Enzymol 1999;299:65–77. [PubMed: 9916197]
4. Brubacher, G.; Vuilleumier, JP. Vitamin C. In: Curtius, HC.; Roth, M., editors. Clinical Biochem. Principles and Methods. 2. de Gruyter; Berlin: 1974. p. 989-997.
5. Ihara H, Matsumoto N, Shino Y, Aoki Y, Hashizume N, Nanva S, Urayama T. An automated assay for measuring serum ascorbic acid with use of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, free radical and o-phenylenediamine. Clin Chim Acta 2000;301:193–204. [PubMed: 11020473]
6. Buettner GR. In the absence of catalytic metals, ascorbate does not autoxidize at pH 7: Ascorbate as a test for catalytic metals. J Biochem Biophys Meth 1988;16:20–40.
7. Buettner GR. Ascorbate autoxidation in the presence of iron and copper chelates. Free Rad Res Commun 1986;1:349–353.
8. Anderson DJ. Determination of the lower limit of detection. Clin Chem 1989;35:2152–2153. [PubMed: 2619804]
9. Kocherginsky, N.; Swartz, HM. Nitroxide Spin Labels. CRC Press Inc; Boca Raton: 1995.
10. Liu YC, Wu LM, Liu ZL, Han ZX. Studies on Nitroxides XII: A kinetic ESR study on the oxidation of ascorbic acid by nitroxide. Acta Chim Sinica 1985;43:669–674.
11. Shiga M, Miyazono Y, Ishiyama M, Sasamoto K, Ueno K. 4-Hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy free radical as a novel ascorbic acid quencher. Anal Commun 1997;34:115–117.
12. Lin Y, Liu W, Ohno H, Ogata T. Determination of ascorbate concentration in a raw leaf with electron spin resonance spectroscopy. Anal Sci 1999;15:973–977.
13. Institute of Medicine (U.S.). Panel on dietary antioxidants and related compounds: Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. National Academy Press: Food and Nutrition Board; Washington, DC: 2000.
14. Dittmer, DS., editor. Blood and Other Body Fluids. Federation of American Societies for Experimental Biology; Washington, DC: 1961.
15. Clement MV, Ramalingam J, Long LH, Halliwell B. The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. Antioxid Redox Signal 2001;3:157–163. [PubMed: 11291594]
16. Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner GR, Shacter E, Levine M. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci USA 2005;102:13604–13609. [PubMed: 16157892]
17. Kramarenko GG, Wilke WW, Dayal D, Buettner GR, Schafer FQ. Ascorbate enhances the toxicity of the photodynamic action of Verteporfin in HL-60 cells. Free Radic Biol Med 2006;40:1615–1627. [PubMed: 16632121]
18. Hata R, Senoo H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J Cell Physiol 1989;138:8–16. [PubMed: 2910890]
19. Elmore AR. Final report of the safety assessment of L-ascorbic acid, calcium ascorbate, magnesium ascorbate, magnesium ascorbyl phosphate, sodium ascorbate, and sodium ascorbyl phosphate as used in cosmetics. Int J Toxicol 2005;24(Suppl 2):51–111. [PubMed: 16154915]
20. May JM, Qu ZC, Li X. Ascorbic acid blunts oxidant stress due to menadione in endothelial cells. Arch Biochem Biophys 2003;411:136–44. [PubMed: 12590932]
21. Liu TZ, Chin N, Kiser MD, Bigler WN. Specific spectrophotometry of ascorbic acid in serum or plasma by use of ascorbate oxidase. Clin Chem 1988;28:2225–2228. [PubMed: 7127767]
22. Verity MA, Gallagher R, Brown WJ. Fluorimetric Determination of D-Amino Acid Oxidase. Biochem J 1967;103:375–381. [PubMed: 4382256]
23. Sattler UGA, Walenta S, Mueller-Klieser W. A bioluminescence technique for quantitative and structure-associated imaging of pyruvate. Lab Invest 2007;87:84–92. [PubMed: 17170741]
24. Bogdanski KA, Bogdanska H. Sur la stabilité de l'acide l-déhydroascorbique dans les solutions aqueuses. Bulletin de l'Académie Polonaise des Sciences, Classe 2e 1955;3:41–44.

25. Washko PW, Welch RW, Dhariwal KR, Wang Y, Levine M. Ascorbic acid and dehydroascorbic acid analyses. *Anal Biochem* 1992;204:1–14. [PubMed: 1514674]

Abbreviations

AO	ascorbate oxidase
AscH₂	ascorbic acid
AscH⁻	ascorbate monoanion
DETAPAC or DTPA	diethylenetriaminepentaacetic acid
DHA	dehydroascorbic
DHA-OPDA	3-(dihydroxyethyl)furo[3,4-b]quinoxaline-1-one
HBSS	Hank's Balanced Salt Solution
MeOH	methanol
MPA	meta-phosphoric acid
OPDA or OPD	<i>ortho</i> -phenylenediamine, Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy
Tempol-H	the one-electron reduced form of Tempol, a hydroxylamine

Figure 1. Overview of assay

A. The reaction of DHA with OPDA produces a fluorescent product that is monitored over time, as shown in this 150 μM concentration standard. The initial rate of formation of DHA-OPDA is dependent on the [DHA] in the sample, which is proportional to the total AscH^- in the original sample.

B. The initial rates from a set of concentration standards are used to plot a standard curve. From this linear plot of slope vs. standard concentration, the $[\text{AscH}^-]$ of unknown samples is determined. Data are means, $n=3$; error bars represent S.E.

Figure 2. Methanol slows chromophore formation

Solutions of ascorbate (25 μM) were prepared using different concentrations of MeOH (0–90% v/v). The apparent concentrations of ascorbate were determined relative to standards containing 60% MeOH. Data are means, n=6; error bars represent S.E.

Figure 3. Ascorbate and ascorbate phosphate uptake are comparable in U937 cells

U937 cells were exposed for 24 hours to different concentrations of ascorbate (10–1000 μM), ascorbate phosphate (10–1000 μM), or ascorbate palmitate (10–50 μM) in full medium. Palmitate was dissolved in 100% ethanol. Concentrations of ascorbate palmitate higher than 50 μM decreased cell viability thus higher concentrations were not studied. All experiments were done at least 3 times. In each experiment each point is the result of triplicate samples. Data are means; error bars represent S.E.

Figure 4. Ascorbate phosphate results in a slower intracellular accumulation of ascorbate compared to non-derivatized ascorbate

U937 cells were incubated in full media and exposed to ascorbate (50 or 1000 μ M) or ascorbate phosphate (50 or 1000 μ M). Cells were harvested at different time points (1, 2, 4, 8, 16, 20, and 24 h). All experiments were done at least 3 times. In each experiment each point is the result of triplicate samples. Data are means; error bars represent S.E.

Table 1Verification of [AsCH⁻] by the method of standard additions.

	Swine Plasma	Dog Plasma	Orange Juice	U937 Cells
[AsCH ⁻] / μ M	44.8 $\pm$ 3.0	93.2 $\pm$ 4.8	2700 $\pm$ 81	6500 $\pm$ 320
[AsCH ⁻] in well / μ M ^a	3.7 $\pm$ 0.25	7.7 $\pm$ 0.4	11.0 $\pm$ 0.33	36.9 $\pm$ 1.8
Expected Spike 1 / μ M ^b	5.2 $\pm$ 0.25 ^c	12.7 ^d $\pm$ 0.4	16.0 ^d $\pm$ 0.33	56.9 ^e $\pm$ 1.8
Observed Spike 1 / μ M ^b	5.6 ^c $\pm$ 0.35	13.9 ^d $\pm$ 0.5	16.4 ^d $\pm$ 0.49	57.8 ^e $\pm$ 1.5
Expected Spike 2 / μ M ^b	6.7 ^f $\pm$ 0.25	17.7 ^g $\pm$ 0.4	21.0 ^g $\pm$ 0.33	76.9 ^h $\pm$ 1.8
Observed Spike 2 / μ M ^b	7.6 ^f $\pm$ 1.1	19.8 ^g $\pm$ 0.4	21.4 ^g $\pm$ 0.68	81.3 ^h $\pm$ 3.1

^aThese numbers are the final concentrations in the non-spiked wells of the plate after all additions and dilutions.

^bThese numbers are the final expected and observed concentrations in the spiked wells of the plate after all additions and dilutions. Using two sample students' t-tests, the p-values were found to be: for the swine plasma, p = 0.358, df = 37 for the first spike and p = 0.434, df = 20 for the second; for the dog plasma p = 0.080, df = 19 for the first spike and p = 0.001, df = 22 for the second; for the orange juice p = 0.501, df = 52 for the first and p = 0.600, df = 40 for the second; for the cells p = 0.706, df = 15 for the first spike and p = 0.243, df = 12 for the second.

^{c-h}Ascorbate was added to increase the final concentration in the well by: (c) 1.5 μ M; (d) 5.0 μ M; (e) 20 μ M; (f) 3.0 μ M; (g) 10 μ M; and (h) 40 μ M.