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A simple and sensitive assay for ascorbate using a plate reader

Jesse M. Vislisel, Freya Q. Schafer, Garry R. Buettner *

ESR Facility and Free Radical and Radiation Biology, University of Iowa, Iowa City, IA 52242, USA

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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 acid using Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy) 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 less than 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 h for ascorbate and ascorbate phosphate. However, the intracellular accumulation of ascorbate via the phosphate ester had an initial rate that was three to five times slower than that via 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 7 h 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, is very economical, and provides results with uncertainties on the order of only 5%.
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Keywords: Ascorbate; Ascorbic acid; Vitamin C; Tempol; Assay; Plate reader; Kinetics; *o*-Phenylenediamine

Since the discovery of ascorbic acid by Szent-Györgyi in 1928 [1], its importance in human health has been recognized and its antioxidant and prooxidant characteristics have been studied. Currently, vitamin C is the leading commercially produced vitamin, with annual global consumption of approximately 100,000,000 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].

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 was modified recently [4,5]. In this assay, ascorbic acid is oxidized to dehydroascorbic acid (DHA),[1] which then reacts with *o*-phenylenediamine (OPDA) to form the condensation product 3-(dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DHA–OPDA). The appearance of this product is followed over time. Traditionally, the enzyme

* Corresponding author. Fax: +1 319 335 9112.
E-mail address: garry-buettner@uiowa.edu (G.R. Buettner).

[1] *Abbreviations used:* DHA, dehydroascorbic acid; OPDA or OPD, *o*-phenylenediamine; DHA–OPDA, 3-(dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one; AO, ascorbate oxidase; $AscH^-$, ascorbate monoanion; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy; DETAPAC or DTPA, diethylenetriaminepentaacetic acid; MeOH, methanol; ATCC, American Type Tissue Collection; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; LLD, lower limit of detection; Tempol-H, Tempol one-electron reduced form, a hydroxylamine; ULD, upper limit of detection; EDTA, ethylenediaminetetraacetic acid; RDA, recommended daily allowance; EtOH, ethanol; MPA, metaphosphoric acid.

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ascorbate oxidase (AO, EC 1.10.3.3) has been used to accomplish the oxidation of ascorbate monoanion ($AscH^-$) to DHA. However, here we employ the nitroxide Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy) 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. The 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 (Gibbstown, NJ, USA). AO, sodium acetate dihydrate, diethylenetriaminepentaacetic acid (DETAPAC or DTPA), OPDA, and Tempol (CAS 2226-96-2) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and methanol (MeOH) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). L-Ascorbic acid 6-palmitate was purchased from Aldrich Chemical (Milwaukee, WI, USA), 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 American Type Tissue Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), all obtained from Gibco (Grand Island, NY, USA). Cells were incubated at 37 °C in a 95% humidity atmosphere under 5% CO_2. Cells from passages 5 to 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/H_2O containing DETAPAC. The exact MeOH/H_2O ratio (v/v) varied to match the ratio used in the samples to be analyzed. We found it to be extremely important that the MeOH 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) 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 1 month. OPDA (5.5 mM) was dissolved in sodium acetate buffer. OPDA can be stored for only 1 to 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/H_2O mixture used to precipitate protein or condition the samples [7].

Incubating cells with vitamin C

Cells (10×10^6) were placed in 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 under 5% CO_2. Cells were washed twice with Hank's balanced salt solution (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/H_2O (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 and then centrifuged at 4 °C and 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/H_2O (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 and 12,000*g* for 10 min. Supernatants were stored at −80 °C until analysis.

Orange juice preparation

Orange juice (Minute Maid, Coca-Cola, Atlanta, GA, USA) 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/H_2O (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 and 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 whether 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 nonspiked 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) was transferred into the wells of a 96-well plate. Then 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 approximately 3-s intervals starting in row A and ending in row H. Then the plate was inserted into a Tecan SpectraFluor Plus plate reader (Tecan, Research Triangle Park, NC). Fluorescence was measured using a 345-nm bandpass filter for excitation and a 425-nm bandpass 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 ± standard errors. Statistical significance of differences between paired data was determined using two-sample Student's *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].

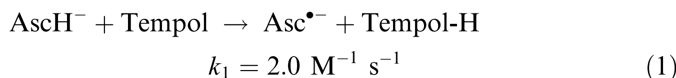
Results

Overview of assay

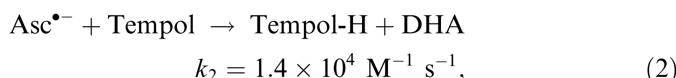
The assay we describe is a kinetic assay that follows the formation of DHA–OPDA over time (see Reaction 4 below). Because OPDA is in excess, the initial rate of this reaction is proportional to the initial [DHA] (see Eq. (6) below). The initial rates from standards are used to construct a standard curve. 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 DHA with OPDA to form a highly colored product that can be detected by fluorescence [4]. In the assay, ascorbate must be oxidized to DHA. Traditionally, this has been done with AO [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 [10]:

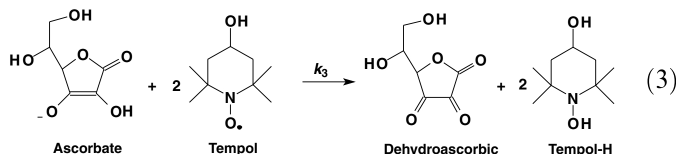


$$\mathrm{AscH^- + Tempol \rightarrow Asc^{\bullet -} + Tempol\text{-}H} \quad k_1 = 2.0\ \mathrm{M^{-1}\ s^{-1}} \tag{1}$$

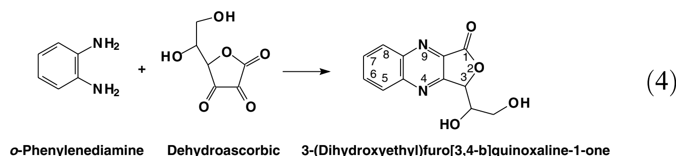


$$\mathrm{Asc^{\bullet -} + Tempol \rightarrow Tempol\text{-}H + DHA} \quad k_2 = 1.4 \times 10^4\ \mathrm{M^{-1}\ s^{-1}}, \tag{2}$$

where Tempol-H is the hydroxylamine [10,11]. With the high level of Tempol used in our protocol, Reaction (2) is rapid. Thus, the rate-controlling step is the initial reaction of $AscH^-$ with Tempol (Reaction 1). The rate constant for the slow step above agrees well with reports that measured the overall rate constant for these reactions: $k_3 = 7.0\ M^{-1}\ s^{-1}$ as determined in Ref. [12]; we have estimated $12\ M^{-1}\ s^{-1}$ from data in Ref. [9]. Our measurement of an observed rate constant for this reaction under the conditions of our assay yields an observed rate constant $k_3 = 3.5 \pm 0.6\ M^{-1}\ s^{-1}$ (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 $k_4 = 4.6 \pm 1.8\ M^{-1}\ s^{-1}$ ($n = 27$). OPDA (1.0 mM final concentration, 60% MeOH) is in great excess compared with DHA; therefore, the reaction will behave as a pseudo first-order reaction with a rate proportional to [DHA]:

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

Thus, the half-life of DHA in Reaction (5) is approximately 150 s. Because [DHA] ≪ [OPDA], the appearance of condensation product will follow the function

$$+d[\mathrm{DHA\text{–}OPDA}]/dt = k_4'[\mathrm{DHA}], \tag{6}$$

with the [DHA–OPDA] at any time *t* being

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

This means that we can estimate $[DHA]_0$ (i.e., $[AscH^-]_0$) from the initial slope because these slopes will be directly proportional to $[DHA]_0$.

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 proceeds

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 approximately 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 Eq. (7), the formation of DHA–OPDA always will be an exponential function that, in principle, would need to be followed for infinite time to determine $[DHA–OPDA]_\infty$ and thus $[DHA]_0$. 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 ($t = 0$ s) and the value of the polynomial function at $t = 90$ s. The slope of the line between these two points provided a good estimate of the initial slope (Fig. 1A). These slopes have a direct linear relationship to $[AscH^-]$. The concentration of $AscH^-$ in any sample can be determined from an appropriate standard curve.

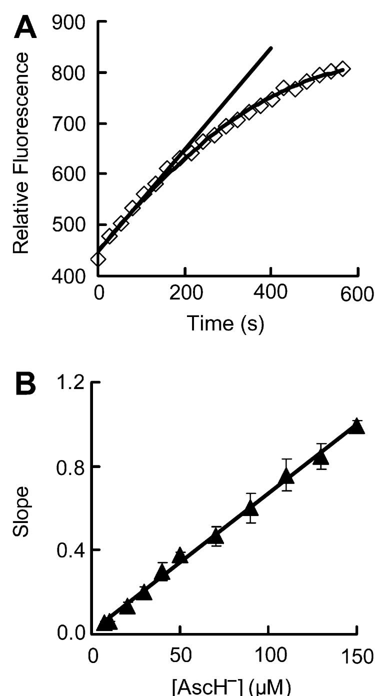


Fig. 1. An overview of the assay is shown. (A) The reaction of DHA with OPDA produces a fluorescent product that is monitored over time, as shown in this 150 μM concentration standard. The initial rate of formation of DHA–OPDA is dependent on the [DHA] in the sample, which is proportional to the total $AscH^-$ 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 versus standard concentration, the $[AscH^-]$ of unknown samples is determined. Data are means ($n = 3$), and error bars represent standard errors.

We investigated taking measurements over longer periods of time to see whether 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 (initial 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 (Fig. 1B), allowing us to confidently determine concentrations in unknown samples. Each of the 11 points on the standard curve represents the mean from 3 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 plates is less. In our assay, the total volume of solution in each well is 242 μl. We found the LLD to be approximately 6.2 μM in the sample, with the upper limit of detection (ULD) being approximately 150 μM. Because of dilution with the reagents of the assay, these concentrations correspond to final concentrations of 1.1 and 56 μM, respectively, in the wells of the 96-well plate.

These detection limits were established using standard solutions covering a range of $AscH^-$ concentrations from 0.1 to 1000 μM. The 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 $[AscH^-]$; R^2 can be used as an indicator for a reasonable LLD.

Although the standard curve is linear in the span employed by the assay, there is a loss in linearity at higher $[AscH^-]$. After examining several extended standard curves, we determined that the break in linearity occurs at a sample concentration of approximately 150 μM (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 because very concentrated samples can easily be diluted to fall within the linear range of the assay.

MeOH slows the OPDA + DHA reaction

MeOH is used in the preparation of samples both to precipitate protein and to stabilize the DHA. To determine whether MeOH influences the rate of the reaction of DHA with OPDA, we determined the rate of chromophore formation of an ascorbate standard (25 μM) prepared with different ratios of MeOH/H_2O. We observed that higher concentrations of MeOH in the sample resulted in a lower rate of reaction between OPDA and DHA (Fig. 2). Thus, if the MeOH concentration of standard solutions differs from that of the samples, the ascorbate concentration in samples will be over- or underestimated. In accordance with Levine and coworkers, 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 [MeOH] in these samples is approximately 75%. The [MeOH] of the standard solutions needs to be the same as the final [MeOH] in the sample.

In addition, we replaced the ethylenediaminetetraacetic acid (EDTA) used by Levine and coworkers [3] with the metal chelator DETAPAC. DETAPAC is a cousin of EDTA, but the Fe(III)DETAPAC complex catalyzes the oxidation of ascorbate much more slowly than does Fe(III)EDTA, thereby stabilizing $AscH^-$ more effectively [7].

There are two ways to maintain a consistent MeOH 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 H_2O/DETAPAC to match the [MeOH] of the standard solutions. Both approaches are valid. As our data show, it is extremely important that the MeOH content of the standards and samples be the same.

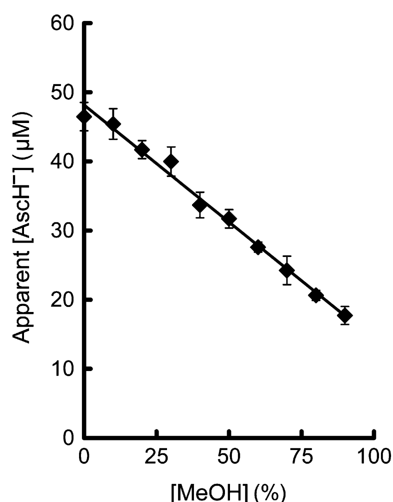


Fig. 2. MeOH slows chromophore formation. Solutions of ascorbate (25 μM) were prepared using different concentrations of MeOH (0–90%, v/v). The apparent concentrations of ascorbate were determined relative to standards containing 60% MeOH. Data are means ($n = 6$), and error bars represent standard errors.

Ascorbate can be measured in a variety of materials

Ascorbate determinations are required for a wide range of materials. The concentrations can vary from nanomolar to millimolar, and the matrix of the samples can 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 were spiked with two different known concentrations of ascorbic acid to determine accuracy.

The assay was able to consistently measure the amount of $AscH^-$ 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 (130%) the recommended daily allowance (RDA) stated on the bottle. The RDA of vitamin C for adults is 75 to 90 mg [13]. The swine and dog plasma samples yielded results within the published expected ranges of 11.4 to 68.1 μM and 11.4 to 119.3 μM, respectively [14]. In our samples, we determined the [$AscH^-$] of swine plasma to be 44.8 ± 3.0 μM. The dog plasma had a higher [$AscH^-$] of 93.2 ± 4.8 μM. 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 $AscH^-$ to approximately 150% of the amount present in the original samples. The second spike roughly doubled the amount of $AscH^-$ present in the samples.

Using two-sample Student's t tests, we found that 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 [$AscH^-$] ($P = 0.001$, $df = 22$). This result may be slightly misleading because 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.

Table 1
Verification of [$AscH^-$] by method of standard additions

	Swine plasma (μM)	Dog plasma (μM)	Orange juice (μM)	U937 cells (μM)
[$AscH^-$]	44.8 ± 3.0	93.2 ± 4.8	2700 ± 81	6500 ± 320
[$AscH^-$] in well[a]	3.7 ± 0.25	7.7 ± 0.4	11.0 ± 0.33	36.9 ± 1.8
Expected spike 1[b]	5.2[c] ± 0.25	12.7[d] ± 0.4	16.0[d] ± 0.33	56.9[e] ± 1.8
Observed spike 1[b]	5.6[c] ± 0.35	13.9[d] ± 0.5	16.4[d] ± 0.49	57.8[e] ± 1.5
Expected spike 2[b]	6.7[f] ± 0.25	17.7[g] ± 0.4	21.0[g] ± 0.33	76.9[h] ± 1.8
Observed spike 2[b]	7.6[f] ± 1.1	19.8[g] ± 0.4	21.4[g] ± 0.68	81.3[h] ± 3.1

[a] These numbers are the final concentrations in the nonspiked wells of the plate after all additions and dilutions.
[b] These numbers are the final expected and observed concentrations in the spiked wells of the plate after all additions and dilutions. Using two-sample Student's t tests, the following P values were found: for the swine plasma, $P = 0.358$ ($df = 37$) for spike 1 and $P = 0.434$ ($df = 20$) for spike 2; for the dog plasma, $P = 0.080$ ($df = 19$) for spike 1 and $P = 0.001$ ($df = 22$) for spike 2; for the orange juice, $P = 0.501$ ($df = 52$) for spike 1 and $P = 0.600$ ($df = 40$) for spike 2; for the U937 cells, $P = 0.706$ ($df = 15$) for spike 1 and $P = 0.243$ ($df = 12$) for spike 2.
[c–h] Ascorbate was added to increase the final concentration in the well by 1.5 μM (c), 5.0 μM (d), 20 μM (e), 3.0 μM (f), 10 μM (g), and 40 μM (h).

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 [15–17]. 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, whereas ascorbate phosphate is water soluble. We posed two questions in these experiments. First, what are the best conditions to compare the behavior of the uptake of ascorbate and derivatives of ascorbate? Second, 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. Whereas ascorbate and ascorbate phosphate are highly water soluble, the palmitate must be dissolved in ethanol (EtOH). EtOH 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 (Fig. 3). Cells were exposed only to low palmitate concentrations because high [EtOH] decreased cell viability. At these lower concentrations (10–50 μM), ascorbic acid accumulation was similar to the other two forms. Although the maximum ascorbate accumulation is comparable, it takes a longer period of time for the phosphate to reach the maximum ascorbic acid accumulation (Fig. 4). Intracellular ascorbate reached a maximum after 8 h when cells were exposed to ascorbate. Cells exposed to ascorbate phosphate reached a similar maximum [$AscH^-$] at 24 h.

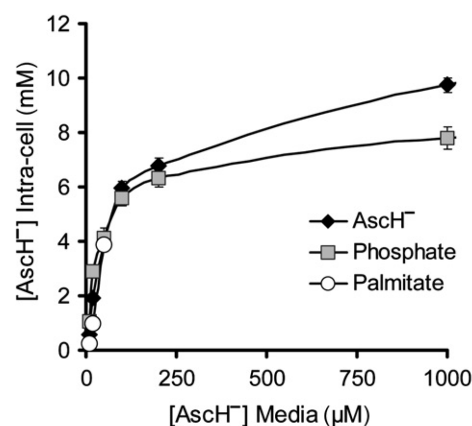


Fig. 3. Ascorbate and ascorbate phosphate uptake are comparable in U937 cells. U937 cells were exposed for 24 h to different concentrations of ascorbate (10–1000 μM), ascorbate phosphate (10–1000 μM), or ascorbate palmitate (10–50 μM) in full medium. Palmitate was dissolved in 100% EtOH. Concentrations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

This example of testing the differential uptake of ascorbate and ascorbate derivatives is typical of many cell culture experiments with ascorbate in that many samples must be analyzed. Using a large number of samples requires considerable time for analysis when using HPLC. All of the data for Figs. 3 and 4 could have been obtained from 9 plates, although we used 13 plates due to the timing of sample availability and because we were running other additional samples on the plates. This would require approximately 7 h 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 more than 350 h of nonstop measurements (allowing 30 min/sample and

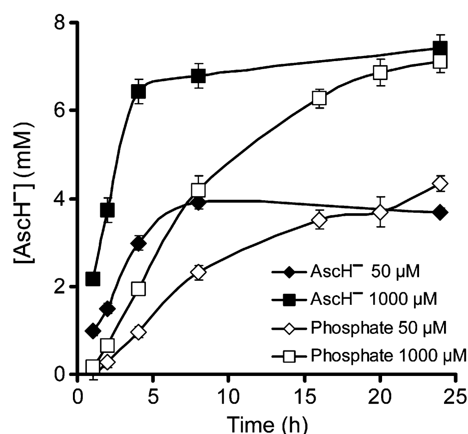


Fig. 4. Ascorbate phosphate results in a slower intracellular accumulation of ascorbate compared with nonderivatized ascorbate. U937 cells were incubated in full media and exposed to ascorbate (50 or 1000 μM) or ascorbate phosphate (50 or 1000 μM). Cells were harvested at different time points (1, 2, 4, 8, 16, 20, and 24 h). All experiments were done at least three times. In each experiment, each point is the result of triplicate samples. Data are means, and error bars represent standard errors.

accounting for standards and instrument maintenance). Thus, although 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. 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 might 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, with each determination taking 15 to 45 min, whereas our plate reader assay can measure 96 wells in less than 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. In addition, 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 method has the advantage of sensitivity and specificity. In our plate reader assay, the condensation product DHA–OPDA is detected via fluorescence, resulting 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 nonreproducible 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 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 less than 1 min. Using this enzyme (1.5 U/ml) in place of Tempol, we obtained similar [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 freshly each day from stock because it loses its activity quickly. 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 α-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 approximately 1 h to reach 90% of the asymptote fluorescence value. To determine whether 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, and other similar α-keto acids also should not interfere.

Stability of the assay

The stability of ascorbate acid and DHA 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 (pH ~ 3–6) [24]. Precipitation of the protein in samples can be achieved using either MeOH/H_2O or acids such as metaphosphoric 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 those of Levine and coworkers [3,25]; using methanol to precipitate the protein resulted in higher reproducibility and better stabilization of the ascorbate than could be achieved with MPA.

Sensitivity of the assay

Our assay is sensitive over a wide range of ascorbate concentrations. The lower limit of the assay is determined mainly 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 in achieving reproducible measurements. Furthermore, so 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 [$AscH^-$] using the initial slope of chromophore development. It requires little time and has the ability to measure 96 wells in less than 10 min. The assay is able to determine [$AscH^-$] in samples having concentrations between 6.2 and 150 μM. A wide variety of samples, including food substances, blood plasma, and cells, may be reliably analyzed with little substance interference. [MeOH] in the samples *must* be consistent with [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.

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