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Research paper

Direct spectrophotometric measurement of supra-physiological levels of ascorbate in plasma

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ABSTRACT

Background: Supra-physiological concentrations of ascorbate, vitamin C, in blood, greater than 1 mM, achieved through intravenous administration (IV), are being tested in clinical trials to treat human disease, *e.g.* cancer. These trials need information on the high levels of ascorbate achieved in blood upon IV administration of pharmacological ascorbate so appropriate clinical decisions can be made.
Methods: Here we demonstrate that in the complex matrix of human blood plasma supra-physiological levels of ascorbate can be quantified by direct UV spectroscopy with use of a microvolume UV–vis spectrophotometer.
Results: Direct quantitation of ascorbate in plasma in the range of 2.9 mM, lower limit of detection, up to at least 35 mM can be achieved without any sample processing, other than centrifugation.
Conclusions: This approach is rapid, economical, and can be used to quantify supraphysiological blood levels of ascorbate associated with the use of IV administration of pharmacological ascorbate to treat disease.

1. Introduction

Since the discovery of vitamin C, ascorbic acid/ascorbate ($AscH_2/AscH^-$) [1], many methods for its analysis in foods, biological fluids, and tissues have been developed. In human blood plasma concentrations of $AscH^-$ in the range of ≈40–80 μM are considered normal and healthy [2]. The highest concentration of $AscH^-$ that can be achieved in blood plasma *via* oral administration is on the order of 200 μM or so [3]. However, supra-physiological concentrations in blood, ≈10,000 to 30,000 μM or more, achieved through intravenous administration (IV), are being tested in clinical trials to treat human disease, *e.g.* cancer [4–9]. These types of trials need information on the high levels of $AscH^-$ achieved in blood upon IV administration of pharmacological $AscH^-$ so appropriate clinical decisions can be made. Thus, rapid and economical assays for these high levels of $AscH^-$ in blood plasma/serum are needed.

A wide variety of assays for the levels of $AscH^-$ in cultured cells, biological fluids, and tissues have been developed: direct measurement *via* HPLC [10–12], colorimetric methods [13], fluorescence of products formed upon reaction with dehydroascorbic acid [14,15], use of the chemistry in a fingerstick blood glucose monitor [16], and by electron paramagnetic spectroscopy [17].

Ascorbic acid is a diprotic acid with $pK_{a1}=4.0$ at an ionic strength of 150 mM and $pK_{a2}=11.5$ [18–20]. Thus, at near-neutral pH, the dominant form will be the $AscH^-$ monoanion. Ascorbic acid and the $AscH^-$ monoanion have distinctly different ultraviolet (UV) spectra. The diprotic acid, $AscH_2$, has a maximum molar absorptivity at 243 nm, $\varepsilon_{243}=9650\ M^{-1}\,cm^{-1}$ [21]. The $AscH^-$ monoanion has an absorption maximum at 265 nm. Many values for its molar absorptivity have been reported, with ε_{265} ranging from 7500 to 20,400 $M^{-1}\,cm^{-1}$ [20,21]. A careful study found $\varepsilon_{265}=14{,}560\pm450\ M^{-1}\,cm^{-1}$ and $\varepsilon_{251}=8250\pm150\ M^{-1}\,cm^{-1}$ at the isobestic point (250.7 nm) for the diacid and the monoanion [21]. When unwanted oxidation is avoided, $\varepsilon_{265}=14{,}500\ M^{-1}\,cm^{-1}$ is the maximum UV absorbance of $AscH^-$ [22]. These values for the molar absorptivity allow quantification of $AscH^-$ in aqueous buffers when $[AscH^-]$ is greater than about 5 μM using a typical UV spectrophotometer (1.00 cm cuvette).

Here we propose that in the complex matrix of human blood plasma supra-physiological $[AscH^-]$ of 3–30 mM, achieved by IV administration [4], can be quantified by direct UV spectroscopy.

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Use of a microvolume UV–vis spectrophotometer will allow quantitation in plasma without any sample processing (*i.e.* no chemistries and no dilutions), other than centrifugation of the whole blood to deplete platelets and remove red blood cells.

2. Materials and methods

2.1. Plasma samples

Blood plasma samples were from subjects of clinical trials being conducted at The University of Iowa that are investigating the use of pharmacological $AscH^-$ as an adjuvant in the treatment of cancer. These trials were approved by The University of Iowa IRB and are listed on https://clinicaltrials.gov [4–6]. Whole blood was collected using BD Vacutainer® green top blood collection tube, 4 mL, NaHeparin 75 USP units. Samples were centrifuged at 2000*g* for 15 min; RBC-free, platelet-depleted plasma was collected for analysis. The plasma was divided into aliquots (650 μL) and analyzed for $AscH^-$ immediately or stored at −80 °C for later analysis. We found that $AscH^-$ in plasma was quite stable for long periods of time at −80 °C, consistent with detailed studies on the stability of $AscH^-$ in plasma [23–25].

2.2. UV/Vis spectroscopy

2.2.1. Standard curve

A 0.100 M [$AscH^-$] standard solution (10.0 mL) was prepared in a phosphate buffer, PBS pH=7.0 with [phosphate]=100 mM; all buffers were treated with chelating resin to remove adventitious catalytic metals [22]. The exact concentration of the stock solution was verified by absorbance at 265 nm, ε_{265}=14,500 M^{-1} cm^{-1} [22]. Absorbance measurements were accomplished using an Implen Nanophotometer P-330 with a 250× dilution lid, path length=4.00×10^{-3} cm, *i.e.* 40 μm. Nine separate dilutions using the 0.100 M [$AscH^-$] stock solution and PBS pH=7.0 buffer were made. The concentrations of these standard solutions (250 μL total volume each) were: 35.0, 30.0, 25.0, 20.0, 15.0, 10.0, 5.0, 2.5, 1.3, and 0.0 mM.

All standard curve samples were examined in triplicate. The standard curve was prepared using the median obtained for each standard [26].

2.2.2. Standard addition

Using standard solutions of $AscH^-$, a standard addition was made to plasma samples obtained from four different subjects. Absorbance measurements at 265 nm were accomplished using an Implen Nanophotometer P-330 with a 250× dilution lid (path length=4.00×10^{-3} cm=40 μm). Nine separate standard additions using the 0.100 M [$AscH^-$] stock solution in plasma samples were made. The concentrations of these standard additions (250 μL total volume each) were: 35.0, 30.0, 25.0, 20.0, 15.0, 10.0, 5.0, 2.5, 1.3, and 0.0 mM. Care must be taken to thoroughly clean the lid and cuvette surfaces as $AscH^-$ quickly crystallizes out of solution leading to incorrect readings of absorbance.

All experimental samples were examined in triplicate. To determine the absorbance due to $AscH^-$ at 265 nm any absorbance from the 0.0 mM sample was subtracted out. The standard addition curve was prepared using the median obtained for each standard [26].

2.2.3. Reduction of dehydroascorbic acid to ascorbate

We confirmed that plasma samples had undetectable amounts of dehydroascorbic acid (DHA), the two-electron oxidation product of $AscH^-$, by addition of dithiothreitol (DTT). A stock solution of DHA was made in 10 μM phosphate buffer, pH 6.5, by adding 10 mg of DHA to 1.00 mL buffer; a working solution was made by diluting 1.5 μL of stock solution into 1.00 mL of buffer yielding 86 μM DHA. A stock DTT solution was prepared with 50 mg of DTT solubilized into 1.00 mL of 10 μM phosphate buffer, pH 6.5. A blank with deionized water was collected using a 1.00 cm path length cuvette in an Implen Nanophotometer. The working solution of DHA was added and a baseline spectrum, 250–900 nm, was obtained. As soon as possible after adding and mixing 4 μL of DTT stock another spectrum was obtained. Then additional spectra were captured at 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240, and 300 s after mixing with DTT.

2.2.4. Statistical approaches

All margins of error displayed in graphs and provided in the text were determined using Igor 5.03 software (WaveMetrics, Inc., Lake Oswego, OR) and represent one standard deviation. The lower limit of detection for [$AscH^-$] in neutral buffer was found to be 2.9 mM as determined using the calibration plot method [27]. All uncertainties presented are standard deviations unless noted otherwise. Precision was addressed by repeated measurements of absorbance of the plasma at 265 nm from a single subject using an Implen Nanophotometer P-330 with a 250× dilution lid. Absorbance of both the pre-infusion and post-infusion plasma was determined ten or more times on 5 different days.

3. Results and discussion

3.1. UV/Vis spectra of known concentrations of ascorbate in pH neutral buffer

To determine the response of the microvolume UV/Vis spectrometer to $AscH^-$ in typical near-neutral buffer, aliquots of an $AscH^-$ standard solution, prepared gravimetrically, were added to Chelex®-treated phosphate buffer (100 mM, pH 7.0) to achieve a series of known concentrations, 0–35 mM. Using a microvolume UV/Vis spectrometer in conjunction with a 250× dilution lid (path length=4.00×10^{-3} cm=40 μm), spectra were captured for the various [$AscH^-$], Fig. 1. Using ε_{265}=14,500 M^{-1} cm^{-1} [22], a plot of the measured concentrations of $AscH^-$ (ordinate) *vs.* the concentration of the gravimetrically prepared standard (abscissa) showed the expected linear response, slope=0.91, R^2=0.998. Concentrations from 2.9 mM, lower limit of detection, up to at least 35 mM can be determined directly with no dilution [27]. A 35 mM solution of $AscH^-$ in a 1.00 cm cuvette would have a theoretical absorbance of 508 at 265 nm. These results indicate that use of a microvolume UV/Vis spectrometer has the potential for use to assess quickly pharmacological [$AscH^-$] in plasma.

3.2. Ascorbate concentrations in blood plasma after standard additions of ascorbate

In order to examine the potential for the direct detection of $AscH^-$ in plasma using a microvolume UV/Vis spectrometer, standard additions of $AscH^-$ were made to blood plasma samples from four different subjects to achieve known concentrations ranging from 0.0 to 35.0 mM. As expected, with no addition of $AscH^-$, there is an absorbance in the same region as the $AscH^-$ absorption due to the protein in the plasma. Addition of known amounts of $AscH^-$ to the samples of plasma resulted in increasing absorbance at 265 nm, Fig. 2. The concentration of $AscH^-$ from the absorbance measurement of the microvolume UV/Vis spectrometer (ordinate) was calculated by subtracting the absorbance of the baseline from each standard addition of $AscH^-$ and applying the path length, 4.00×10^{-3} cm, and molar extinction coefficient ε_{265}=14,500 M^{-1} cm^{-1} [22]. An excellent linear correlation was