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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

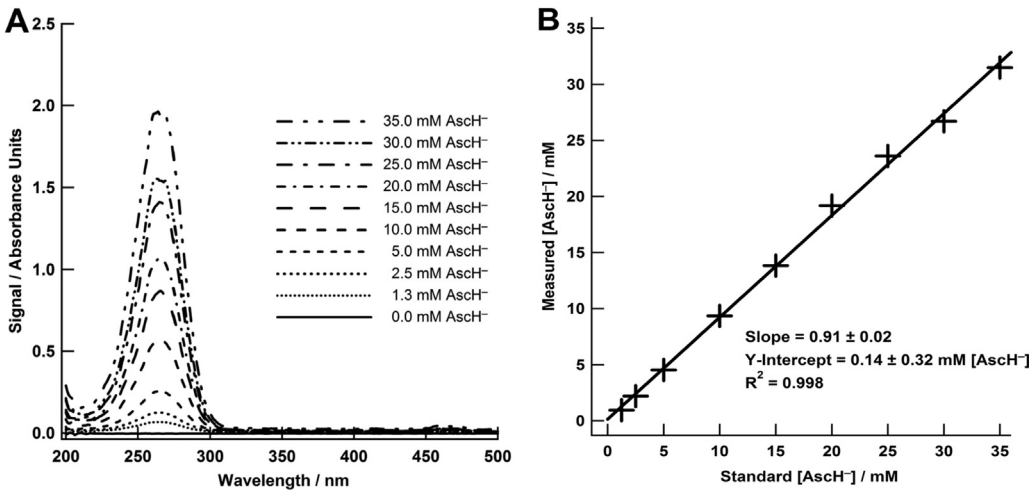

Fig. 1. UV/Vis spectra of known concentrations of ascorbate in pH neutral buffer. Aliquots of an $AscH^-$ standard solution, prepared gravimetrically, were added to Chelexed phosphate buffer (100 mM, pH 7.0) to achieve a series of known concentrations. An Implen Nanophotometer P-330 with 250× dilution lid (path length $= 4.00 \times 10^{-3}$ cm) was used to gather spectra. (A) A plot of one set of spectra of various [$AscH^-$] (0.0, 1.3, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 mM). (B) The measured [$AscH^-$] (ordinate) were determined using $\varepsilon_{265} = 14{,}500\ M^{-1}\ cm^{-1}$ [21,22] are plotted against the concentration of the gravimetrically prepared standard (abscissa); slope $= 0.91 \pm 0.02$, $R^2 = 0.998$. The blank was buffer only.

found, Fig. 2B. Thus, using a microvolume UV/Vis spectrometer, the levels of $AscH^-$ in plasma, 2.9 mM lower limit of detection, up to at least 35 mM can be determined directly with no sample processing.

To estimate the effective molar extinction coefficient for $AscH^-$ in plasma at 265 nm a plot of the absorbance obtained directly from the Implen Nanophotometer P-330 with a 250× dilution lid *vs.* the known standard was made, Fig. 2C**.** The slope of this line will yield the apparent molar extinction coefficient in the matrix of plasma. This line has a slope $= 0.053 \pm 0.002\ mM^{-1}$; in standard units for molar extinction coefficients, this slope yields $\varepsilon_{265} = 13{,}000 \pm 500\ M^{-1}\ cm^{-1}$.

3.3. The direct assay agrees well with results from a fluorescence plate reader assay

In order to determine how the results of the direct assay for $AscH^-$ in plasma using a microvolume UV–vis spectrophotometer agree with another assay, we analyzed a large set of plasma samples ($n = 179$) from subjects that were participating in clinical trials of pharmacological $AscH^-$ as an adjuvant in cancer treatment [4–6]. The plasma samples from the trials were analyzed using a kinetic-based assay [4,15]. We found remarkable agreement between the plasma concentrations determined for $AscH^-$ by these two very different assays, as manifest in the slope of 1.00 when plotting the [$AscH^-$] for each assay, Fig. 3.

Many assays for [$AscH^-$] in plasma precipitate the protein [12,15]. This results in a decease in the effective volume of the plasma sample; as a result the [$AscH^-$] in the plasma will be overestimated. The partial specific volume of protein is approximately 0.73 $cm^3\ g^{-1}$ [28]. Plasma is 7.5% protein, or 75 $g\ L^{-1}$; thus, (75 g/1000 mL) × (0.73 cm^3/g) × 100% = 5.5% of the volume of plasma is protein. If this protein is removed, then the [$AscH^-$] will be concentrated by ≈ 5.8% (100/94.5). In the direct assay using a microvolume UV/Vis spectrometer the protein is not removed while in the kinetic fluorescence assay it is removed. If the correction for protein volume is not made, then the slope through the origin in Fig. 3 is 0.95, exactly as predicted. This demonstrates the importance of considering the volume of protein, if removed in any type of quantitative assay.

3.4. Ascorbate concentrations determined in plasma using an average blank

In Fig. 3 the blank for each sample was the plasma from a blood sample drawn just before infusion of pharmacological $AscH^-$. However, such a sample may not always be available nor needed to minimize discomfort and risk. To determine if an average blank for the absorbance at 265 nm can be used, 170 samples were analyzed for the concentration of $AscH^-$. A comparison was made between the results using the average absorbance at 265 nm of all 170 samples as a blank compared to using the pre-infusion blood sample as a blank for each subject, Fig. 4. For the values of the abscissa the blank was the corresponding plasma of each subject taken prior to infusion of pharmacological $AscH^-$. For the ordinate the blank was the average of the absorbance at 265 nm (0.23 ± 0.04, path length $= 4.00 \times 10^{-3}$ cm) of the pre-infusion samples for all 170 subjects. A slope of 0.98 demonstrates that an average blank can be used to estimate the supra-physiological [$AscH^-$]. Thus, a pre-infusion sample of plasma is not necessary.

To estimate the precision of the assay, the absorbance at 265 nm from a single sample of plasma was determined multiple times ($n \geq 10$) on five different days. The absorbance values at 265 nm for the pre-infusion samples were, in order of day 1-day 5, mean, medium, (standard deviation): 0.16, 0.15, (0.01); 0.21, 0.20, (0,03); 0.22, 0.22, (0.02); 0.23, 0.23, (0.02); and 0.19, 0.19, (0.01); respectfully. The absorbance at 265 nm for the post-infusion samples were, in order of day 1 to day 5, mean, medium, (standard deviation): 1.01, 0.98, (0.08); 1.11, 1.07, (0.12); 1.03, 1.02, (0.05); 1.06, 1.03, (0.11); and 1.00, 0.99, (0.04); respectfully. The coefficient of variation for each day was determined and the average for the pre-infusion was found to be 9.4% and for the post-infusion, 7.8%.

Using these absorbance data, path length $= 4.00 \times 10^{-3}$ cm, and $\varepsilon_{265} = 13{,}000\ M^{-1}\ cm^{-1}$, the estimated concentration of $AscH^-$ from each the five days would be: 16.0, 16.7, 15.4, 15.4, 15.4 mM; median = 15.4 mM. If one uses the average absorbance of 0.23 ± 0.04 for the blank, the estimated concentration of $AscH^-$ from each the five days would be: 14.4, 16.2, 15.2, 15.4, 14.6; median = 15.2 mM, excellent agreement between the two approaches.

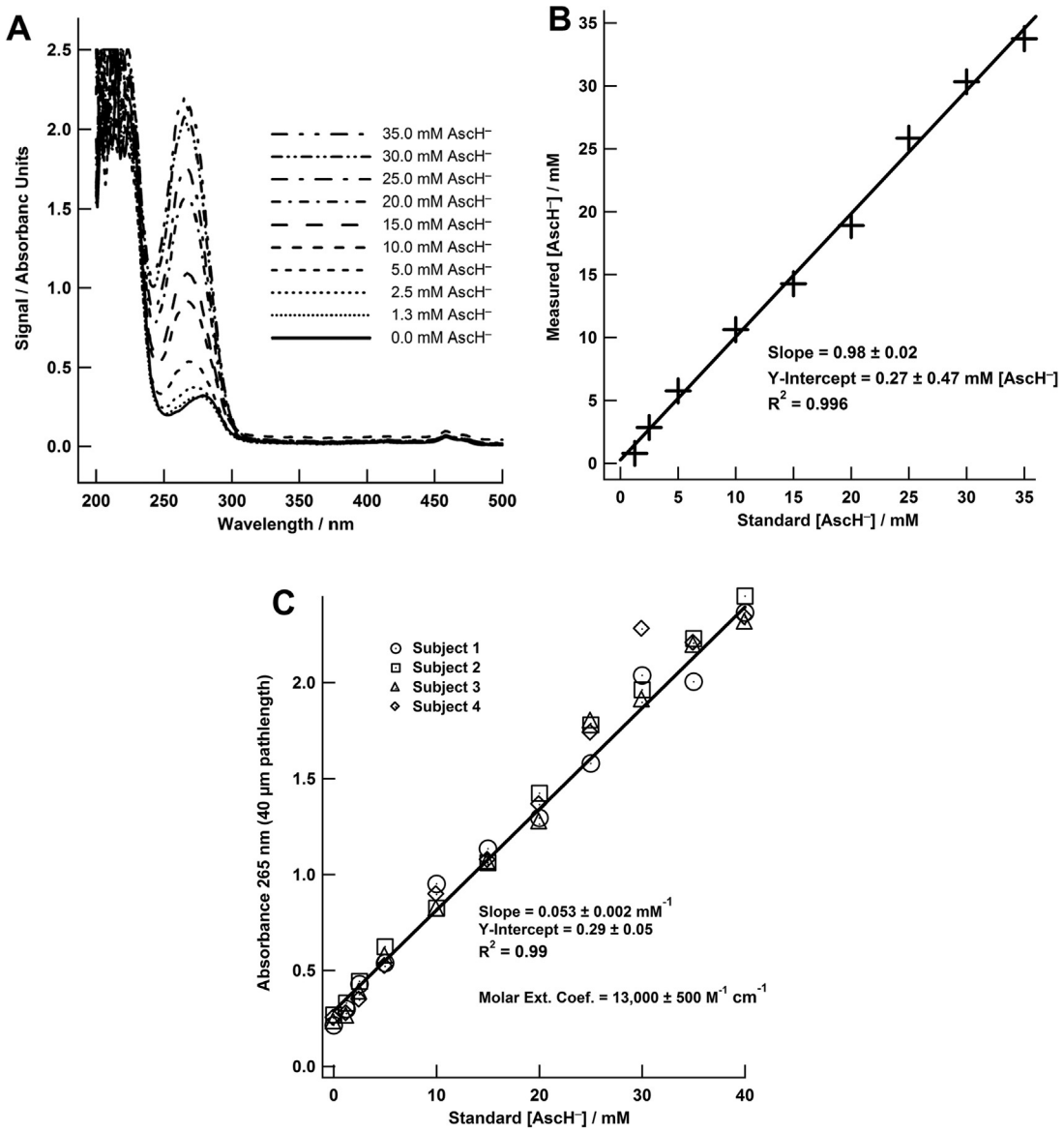

Fig. 2. Spectrophotometric determination of ascorbate concentrations in blood plasma after standard additions of ascorbate. Standard additions of $AscH^-$ were made to blood plasma samples from four different subjects to achieve known concentrations of 0.0, 1.3, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 mM. Absorbance was determined using an Implen Nanophotometer P-330 with 250 × dilution lid, *i.e.* path length = 4.00×10^{-3} cm. (A) One set of example spectra obtained after the standard additions to the plasma of one subject. (B) The measured $AscH^-$ concentration (ordinate) was calculated by taking the difference between absorbance of the baseline, 0.0 mM $AscH^-$, from each standard addition of $AscH^-$. The difference was then used to determine the measured [$AscH^-$] using $\varepsilon_{265} = 14{,}500\ M^{-1}\ cm^{-1}$ [21]. From these data a plot was generated using the median values obtained from the measured [$AscH^-$] and plotted against the known gravimetric standard; slope = 0.98 ± 0.02, $R^2 = 0.996$. (C) A plot of the absorbance obtained directly from the Implen Nanophotometer P-330 with a 250 × dilution lid is plotted against the known gravimetric standard; slope = 0.053 ± 0.002 mM^{-1} 250^{-1} cm^{-1}. Converting this to standard units for the molar extinction coefficient and accounting for the dilution, $\varepsilon_{265\ nm} = 13{,}000 \pm 500\ M^{-1}\ cm^{-1}$. This is the apparent molar extinction coefficient for $AscH^-$ in plasma. All measurements were done in triplicate.

Uncertainties will add in quadrature (square root of the sum of the squares of the coefficients of variation) yielding a coefficient of variation of about 13%, if a pre-infusion sample is available as a blank. If the average blank is used, then the estimated coefficient of variation would be about 20%, the largest contribution coming from the biological variation in the absorbance of the plasma at 265 nm. Normal levels of $AscH^-$ in plasma range from ≈40–80 µM; these levels of $AscH^-$ in plasma would contribute 0.002–0.004 absorbance units to the blank (4.00×10^{-3} cm path length), *i.e.* at the noise level. Thus, if an average blank is used, the variation observed is, as expected, from the biological variation in the protein content of plasma [29].

3.5. The reduction of dehydroascorbic acid to ascorbate by dithiothreitol is rapid

Ascorbate can readily undergo oxidation, resulting in formation of DHA. It has long been known that thiols such as glutathione will reduce DHA back to $AscH^-$ [30]. To determine if a significant amount of the $AscH^-$ had oxidized to DHA we used dithiothreitol (DTT) as a reducing agent [31]. When an excess of DTT was added to DHA in near-neutral buffer, the reduction to $AscH^-$ was rapid, within 3 min, Fig. 5. Analysis of the plot of absorbance at 265 nm *vs.* time, assuming a pseudo first-order reaction (in DTT) and fitting to a single exponential, yields a pseudo first-order rate

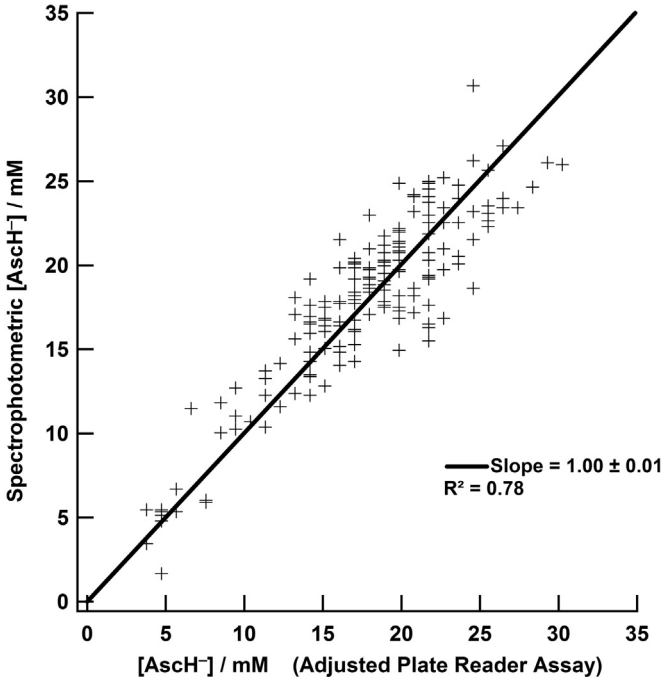

Fig. 3. The direct assay using an Implen Nanophotometer agrees well with results from the fluorescence plate reader assay. Using the [AscH$^-$] from the fluorescence plate ready assay [15] adjusted for protein precipitation [28] and the [AscH$^-$] from the direct UV/Vis Implen Nanophotometer assay, 179 different samples from subjects were analyzed for AscH$^-$. For each sample, the blank used was the plasma from a blood sample drawn just before infusion of pharmacological AscH$^-$ in a treatment session [4–6]. The linear regression that is set to go through the origin yields a slope of 1.00 ± 0.01 and $R^2 = 0.78$.

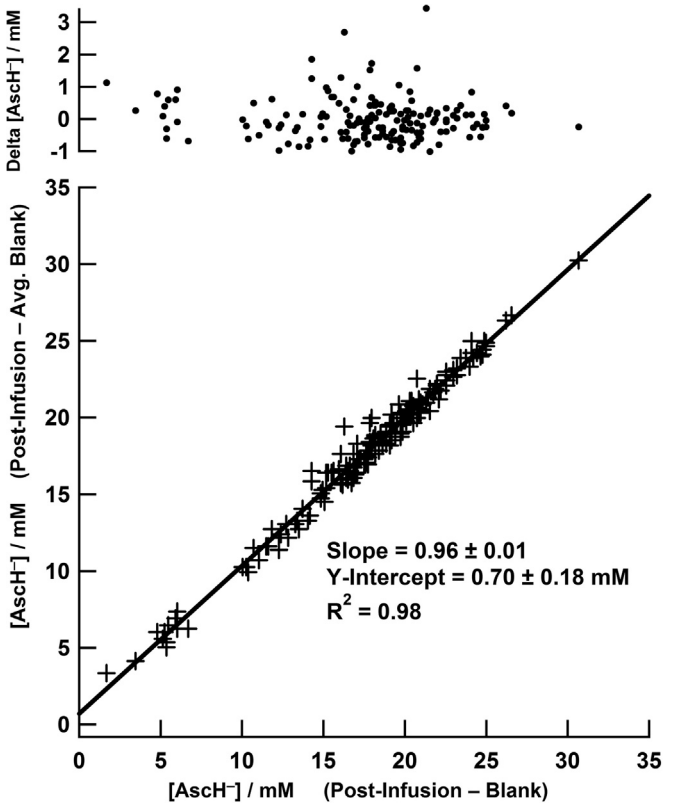

Fig. 4. Ascorbate concentrations determined with "post-infusion – average blank" *vs.* "post-infusion – pre-infusion" with differences plotted as residual. Using the absorbance at 265 nm, 169 samples were analyzed for the concentration of AscH$^-$. For the values of the abscissa the blank was the corresponding plasma of each subject taken prior to infusion, *i.e.* pre-infusion, of pharmacological AscH$^-$. For the ordinate the blank was the average of the absorbance at 265 nm (0.23 ± 0.04), path length $= 4.00 \times 10^{-3}$ cm, of the pre-infusion samples for all 169 subjects. The upper plot shows the residuals (ordinate value – abscissa value) between the two measurements. The slope of 0.96 ± 0.01 demonstrates the ability to estimate supra-physiological AscH$^-$ concentrations even if a pre-infusion plasma sample is not available.

constant of $k' = 3.0 \times 10^{-2}\ s^{-1}$, and $k = 9.4\ M^{-1}\ s^{-1}$ as a second-order rate constant for the reaction DHA+DTT → AscH$^-$ +DTT$_{oxidized}$ under these experimental conditions. This is consistent with the observed rate constant $k_{obs} = 0.3\ M^{-1}\ s^{-1}$ at pH 7.4 for the reaction of glutathione with DHA, as determined from the data presented in [32].

When we added DTT to plasma samples, such as those of Fig. 5, and took an absorbance reading 10 min later, no changes in absorbances were observed, other than dilution, indicating our sample handling protocols resulted in no significant oxidation of AscH$^-$.

3.6. Ascorbate specificity

As many biological compounds absorb light in the UV, a simple approach to confirm that AscH$^-$ is the analyte being detected at 265 nm is to collect the spectrum ranging from around 200 nm to at least 350 nm. The peak absorbance of AscH$^-$ at 265 nm will be the peak of the spectrum; it should not be on the shoulder of the absorption spectrum; the absorbance should begin around 230 nm and end around 300 nm, Fig. 2A. Because the concentration of AscH$^-$ can be on the order of 20 mM, the only interference expected would be from a xenobiotic, not from endogenous proteins or other natural components of plasma.

3.7. Easy clinical implementation

This technique can be easily implemented in a clinical setting. The spectrophotometer used here has a small footprint, ≈ 25 cm $\times \approx 50$ cm, does not require an external computer, and can independently print data as a record. The protocol is

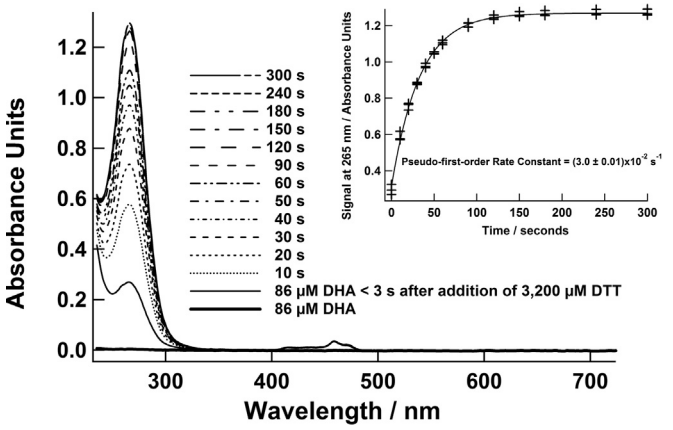

Fig. 5. The reduction of dehydroascorbate to ascorbate by dithiothreitol is rapid. Dehydroascorbic acid (DHA; 86 μM) in Chelex®-treated phosphate buffer (10 μM, pH 6.5) was dispensed into a 1.00 cm path length cuvette and the UV–vis absorbance was monitored using an Implen Nanophotometer P-330. The Nanophotometer was blanked with a 10 μM phosphate buffer, pH 6.5. The solid line with an absorbance near 0.0 AU at 265 nm is the spectrum for 86 μM DHA. Upon addition of 3200 μM dithiothreitol (DTT) the spectra show the time-dependent increase in the concentration of AscH$^-$ due to the reduction of DHA by DTT to AscH$^-$. (Inset) The plot shows the change in the absorbance at 265 nm *vs.* time. Assuming a pseudo first-order reaction (in DTT) and fitting to a single exponential a pseudo first-order rate constant yields $k' = (3.0 \pm 0.01) \times 10^{-2}\ s^{-1}$, and $k = 9.4\ M^{-1}\ s^{-1}$ as a second-order rate constant for the reaction DHA+DTT → AscH$^-$ +DTT$_{oxidized}$ under these experimental conditions ($n = 3$).

straightforward and requires only to blank the instrument with water as preparation when subtracting with the average plasma background, Fig. 4. The absorbance of the plasma can be read directly with minimal training; no dilutions, and no buffers make it possible to obtain data within a minute and immediately determine [AscH$^-$] to make rapid clinical decisions.

4. Conclusions

Here we demonstrate:

1. A microvolume UV/Vis spectrometer can be used to determine supra-physiological levels of AscH$^-$ in blood plasma directly (2.9 mM, lower limit of detection, to at least 35 mM), *i.e.* without the need for any sample processing;
2. With appropriate sample handling, oxidation of AscH$^-$ to DHA in the plasma is not a concern;
3. The effective molar extinction coefficient for the AscH$^-$ monoanion in the matrix of blood plasma appears to be $\varepsilon_{265} = 13{,}000\ M^{-1}\ cm^{-1}$.
4. An average blank absorbance of 0.23 (4.00×10^{-3} cm path length) at 265 nm can be used to determine the [AscH$^-$] in plasma using a microvolume UV/Vis spectrometer;
5. For an optical path length of 4.00×10^{-3} cm ($250\times$ dilution lid of an Implen Nanophotometer P-330) the concentration of AscH$^-$ in plasma (no dilution or processing) can be found using Eq. (1) when no pre-infusion sample is available, or using Eq. (2) when a pre-infusion sample is available:

$$[AscH^-] = (Absorbance_{265} - 0.23) / (13{,}000\ M^{-1}\ cm^{-1} \times 4.00 \times 10^{-3}\ cm) \quad (1)$$

$$[AscH^-] = (Absorbance_{265} - Pre - infusion\ Absorbance_{265}) / (13{,}000\ M^{-1}\ cm^{-1} \times 4.00 \times 10^{-3}\ cm) \quad (2)$$

These equations can be adjusted for other path lengths; the blank of Eq. (1) will need to be scaled appropriately. Here the goal was to assay for AscH$^-$ in plasma without employing any chemistries or dilutions. This basic assay can be employed upon dilution of the plasma sample to accommodate a standard 1.00 cm path length cuvette; attention must then be given to all aspects of the determination and the analysis.
6. Although the determination of the absorbance of a plasma sample at 265 nm has good precision, the resulting uncertainties in the concentration of AscH$^-$ in plasma have a coefficient of variation $\approx$13% if a pre-infusion sample is available to be use for the blank. If an average blank is used, then the coefficient of variation is $\approx$20%, because of the biological variation in the absorbance of plasma.

Acknowledgments

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