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Assessment of the Stability of Supraphysiological Ascorbate in Human Blood: Appropriate Handling of Samples from Clinical Trials for Measurements of Pharmacological Ascorbate

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Abstract

Based on encouraging results from several early-phase clinical trials, there is renewed interest in the use of pharmacological ascorbate (i.e., intravenous administration resulting in $> \approx 10$ mM plasma ascorbate concentrations) in combination with standard-of-care cancer treatments including radiation and/or chemotherapy. Under normal, healthy physiological conditions, humans maintain plasma ascorbate concentrations in the range of 40–80 μ M. However, *in vivo* antitumor activity requires supraphysiological plasma concentrations on the order of ≈ 20 mM. The stability of ascorbate in whole blood has been well studied. The goal of this work was to determine the appropriate handling methods of blood samples, after treatment with pharmacological ascorbate, which allow for the optimal measurement of ascorbate in plasma for dosing verification. Our findings indicate that ascorbate concentrations (mM) are relatively stable in whole blood collected in sodium heparin tubes and stored on ice (or at 4°C) for up to 24 h. After 24 h, ascorbate levels in plasma are relatively stable at 4°C for up to 72 h. At –20°C, plasma concentrations are relatively stable for 2–3 weeks, while at –80°C, ascorbate concentrations in plasma are stable for at least one month. In contrast, patient samples showed better stability when stored as whole blood compared to plasma at 4°C but increasing hemolysis over time may significantly skew ascorbate measurements. Additionally, patient samples can be reliably stored as plasma at –20°C for up to three weeks in either a frost-containing or frost-free environment. This information can guide the collection, processing and storage of clinical samples after pharmacological ascorbate infusions amenable to multi-center clinical trials.

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SUPPLEMENTARY INFORMATION

Fig. S1. Increasing hemolysis over time in patient whole blood samples. Patient samples that were collected from phase II ascorbate lung and glioblastoma clinical trials were stored at 4°C as whole blood ($n = 11$) and centrifuged at 2,000g for 14 min immediately prior to analysis. Absorbance at 415 nm (panel A) and [HbO₂] (panel B) were measured every 24 h for up to 96 h using a microvolume UV-Vis spectrometer.

INTRODUCTION

Several recently published clinical trials for the treatment in ovarian cancer, pancreatic cancer, non-small cell lung cancer and glioblastoma multiforme have combined standard-of-care cancer therapy with pharmacological ascorbate (P-AscH⁻; i.e., ascorbate treatments resulting in $>\approx 10$ mM concentrations of ascorbate) as an adjuvant to anti-cancer therapy (1–6). As interest in the use of P-AscH⁻ combined with traditional anti-cancer modalities continues to increase, there is a greater need to standardize the approach for handling clinical samples after treatment with pharmacological ascorbate in multi-center trials.

With recommended dietary intake, humans generally maintain steady-state concentrations of ascorbate, as measured in plasma, in the range of 40–80 μ M. Treatment with pharmacological ascorbate (typically a 50–100 g dose of ascorbate given intravenously) has a goal to achieve a concentration in plasma of ≈ 20 mM upon completion of the infusion (1–3, 7).

As the number of clinical trials using P-AscH⁻ increases and enrollment expands due to the involvement of multiple treatment centers, there will be an increased need to assess plasma levels of ascorbate, economically, reliably and reproducibly. Unfortunately, ascorbate can easily oxidize to dehydroascorbic acid (DHA) if not collected or stored appropriately. Both sodium heparin and EDTA blood collection tubes are commonly used in obtaining and storing clinical blood samples. Recently published studies have shown that, in general, blood samples drawn and stored in sodium heparin tubes result in greater stability of ascorbate compared to blood samples collected in EDTA tubes (8). This is because EDTA chelates, such as with iron, are redox active and can facilitate the oxidation of ascorbate to DHA (9, 10). Hemolysis of red blood cells during or after blood collection can lead to the oxidation of ascorbate due to its reaction with the released heme (8, 10, 11).

While there are many published clinical studies in which ascorbate levels in blood have been measured (12–18), the handling and processing of blood samples having pharmacological levels of ascorbate has not been well described. The goal of this study was to determine appropriate collection and handling approaches of blood samples with high levels of ascorbate that allow reliable and reproducible measurements of ascorbate concentrations when samples are generated at many different centers that treat patients in clinical trials with P-AscH⁻.

MATERIALS AND METHODS

Blood Collection and Sample Preparation

Healthy volunteers.—Blood was collected by an experienced certified phlebotomist from 12 healthy male and female adults in accordance with an Institutional Review Boards (IRB) protocol at The University of Iowa (Iowa City, IA), with all volunteers giving informed consent (IRB no. 201801820). Blood was collected in sodium heparin coated vacutainers and then the whole blood was immediately spiked with AscH⁻ to achieve a concentration of 20 mM. Once samples were dosed with ascorbate, they were stored at either room temperature or 4°C. Note that blood sample tubes needed to be opened for the addition of

ascorbate and therefore, samples were briefly exposed to air. To produce plasma, whole blood samples were centrifuged at 2,000g for 14 min.

Patients in clinical trials.—Blood samples were collected from patients enrolled in clinical trials at The University of Iowa (NCT02344355 and NCT02905591) and stored as either whole blood or platelet-depleted plasma (centrifuged at 2,000g for 14 min) at 4°C.

[AscH⁻] Measurements

Supraphysiological levels of ascorbate were quantified by direct ultraviolet (UV) spectroscopy using an Implen P330 UV/Visible NanoPhotometer® (Westlake Village, CA) with a CCD array detector using a 0.04 mm cap (dilution factor = 250) as described by Witmer *et al.* (19). All samples were measured in triplicate. Using Beer's law, the concentration of AscH⁻ in the plasma can be determined directly without any dilution using Eq. (1):

$$[\text{AscH}^-] = (A_{265}^{\text{experimental}} - 0.23) / (\epsilon_{265} \cdot L), \quad (1)$$

where the molar extinction coefficient of AscH⁻ in undiluted plasma at 265 nm is $\epsilon_{265} = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$ (19) and path length, $L = 4.00 \times 10^{-3} \text{ cm}$. Samples were quantified without a pre-ascorbate sample; therefore, the concentration of AscH⁻ was calculated by subtracting an average blank absorbance of 0.23 (path length of $4.00 \times 10^{-3} \text{ cm}$) (19). For [AscH⁻] determination in buffer solutions, the molar extinction coefficient at 265 nm is $14,500 \text{ M}^{-1} \text{ cm}^{-1}$ was used (20).

For patient samples stored as whole blood exhibiting significant hemolysis, the absorption of ascorbate at 265 nm was corrected for the contribution due to oxy-hemoglobin. The absorbance of ascorbate at 265 nm was corrected by subtracting the ratio of the respective molar extinction coefficients multiplied by the absorption for oxy-hemoglobin measured at 415 nm, as shown in Eq. (2).

$$A_{265}^{\text{corrected}} = A_{265}^{\text{experimental}} - 0.23 - (\epsilon_{265}^{\text{HbO}_2} / \epsilon_{415}^{\text{HbO}_2}) \cdot A_{415}^{\text{experimental}}, \quad (2)$$

where $\epsilon_{265}^{\text{HbO}_2} = 126,500 \text{ cm}^{-1}$ and $\epsilon_{415}^{\text{HbO}_2} = 523,000 \text{ M}^{-1} \text{ cm}^{-1}$.² If dilutions or a different path length are employed, then Eq. (2) will need to be modified accordingly.

Data Analysis

Each sample was measured in triplicate with the average [AscH⁻] being calculated. The average percentage of initial ascorbate concentration is reported for each cohort ($n = 12$) with all error bars representing the standard deviation. Each cohort was evaluated for statistical significance using a paired, two-tailed, Student's t test where $P < 0.05$ was

²From Prahl, S. Tabulated molar extinction coefficient for hemoglobin in water. Accessed on December 1, 2018. (<https://bit.ly/2JLnadv>)

considered significant, indicated in the figures with an asterisk (*). In addition, rates of oxidation were analyzed using twoway analysis of variance (ANOVA). Significant main effects or interactions ($P < 0.05$) were followed by post hoc analyses (Tukey's test) and significant differences were indicated graphically with an asterisk (*).

RESULTS

Stability of Ascorbate Stored as Whole Blood

Immediately after collection, blood samples were spiked with supraphysiological levels of ascorbate and then stored as whole blood. Oxidation of ascorbate occurred more rapidly when samples were held at room temperature compared to samples stored at 4°C (Fig. 1). After 24 h, whole blood samples at 4°C lost only $\approx 7\%$ of the AscH^- , as measured in plasma, a rate of $-0.28\% \text{ h}^{-1}$. Conversely, samples stored at room temperature lost approximately 14% of the initial AscH^- , a rate of approximately $-0.53\% \text{ h}^{-1}$. The oxidation of AscH^- at room temperature reached significance within 24 h and was oxidized at a significantly greater rate compared to samples held at 4°C ($P < 0.05$). These data suggest that whole blood samples from patients treated with pharmacological ascorbate can be stored at 4°C for up to ≈ 36 h before the loss of ascorbate is greater than $\approx 10\%$.

Ascorbate is more Stable when Stored as Plasma

Blood samples dosed with P- AscH^- and then processed immediately by centrifugation to separate the red blood cells (RBCs) and plasma showed a delay in AscH^- oxidation to DHA. When the plasma sample was held at 4°C, ascorbate was significantly more stable than ascorbate in plasma stored at room temperature; two-way ANOVA indicated a main effect of storage condition ($P < 0.05$) (Fig. 2). When held at 4°C, approximately 10% of the AscH^- was oxidized after 72 h, a rate of $-0.15\% \text{ h}^{-1}$. At room temperature, 29% of AscH^- became oxidized to DHA ($P < 0.001$) over a course of 72 h, a rate of $-0.37\% \text{ h}^{-1}$. The oxidation of ascorbate in plasma samples occurred at a significantly higher rate than those held at 4°C. These data suggest that clinical blood samples treated with pharmacological can be processed and plasma stored at 4°C for no longer than 72 h to reliably assess mM concentrations of AscH^- .

Measured ascorbate concentrations in blood samples were consistent among both male and female cohorts with no significant differences in average plasma concentrations between the two cohorts (Table 1). Both male and female cohorts showed the same patterns of AscH^- stability when stored at 4°C. Similarly, male and female cohorts both demonstrated that AscH^- was more stable in plasma held at 4°C, as stored AscH^- concentrations were not significantly different from baseline in either group until >72 h. From these data, we propose that both male and female blood samples may be processed and assessed for blood ascorbate concentration in the same manner.

Long-Term Stability of Ascorbate in Plasma Stored at -80°C

Ascorbate-treated plasma samples stored at -80°C assessed 14 and 28 days after blood draw showed minimal ascorbate oxidation to DHA (Fig. 3), as approximately 2% of AscH^- was lost ($P = 0.54$), a rate of $-0.07\% \text{ day}^{-1}$. These data are similar to previously reported studies

in which it was found that AscH⁻ remains stable for up to five years at -80°C (21). From these data, we propose that plasma collected from patients treated with pharmacological ascorbate may be stored at -80°C for long durations.

Stability of AscH⁻ in Plasma Stored at -20°C

Plasma from ascorbate-treated blood samples stored for five days at -20°C demonstrated similar AscH⁻ stability to samples stored at a -80°C (Fig. 4). No significant changes in ascorbate concentrations were noted in samples stored at -20°C for up to 14 days. These data suggest that plasma samples may be stored at -20°C reliably for up to 14 days without appreciable AscH⁻ oxidation to DHA. This is consistent with data previously reported by Karlsen et al. demonstrating that significant ascorbate oxidation did not begin to occur at -20°C until after approximately 20 days (11).

Ascorbate Stability in Patient Samples

Ascorbate stability in patient samples stored at 4°C.—When patient blood samples were stored on ice and analyzed as either whole blood or plasma, ascorbate appeared to be more stable when stored as whole blood (Fig. 5). When stored as plasma, ascorbate was lost at a rate of 0.26% h⁻¹ (6.2% day⁻¹). Meanwhile, samples that were stored as whole blood lost ascorbate at a rate of 0.06% h⁻¹ (1.4% day⁻¹). This number appears to be an underestimation of the rate of ascorbate lost because of the accumulation of oxyhemoglobin (HbO₂), which presents as an artifact in the spectrum and alters spectral analysis (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR15328.1.S1>).

Ascorbate stability in patient samples stored at -20°C.—Due to the significant stability exhibited in plasma samples stored at -20°C, five patient samples were saved as plasma and stored in either a frost-free or a frost-allowing freezer. The patient samples were measured every 5–7 days for 33 days (Fig. 6). It appeared that ascorbate was stable in both a frost-allowing and frost-free environment for up to three weeks, as after three weeks we were able to measure 107% and 103% of the initial ascorbate concentration in the frost-free and frost-containing environments, respectively. Yet, after three weeks there was a significant decrease in ascorbate concentrations, resulting in the measurement of 84% and 76% of the initial ascorbate concentration in the frost-free and frost-containing environments. The downward trend continued at day 33 as the mean percentage of initial ascorbate concentration measured as 81% (frost-free) and 75% (frost-containing). This indicates that ascorbate measurements may only be reliable for plasma samples held at -2°C for up to three weeks.

DISCUSSION

We have attempted to generate a concise set of standard conditions for which blood samples from patients treated with P-AscH⁻ may be processed and handled in a way that analyses of AscH⁻ yield accurate and reliable results, facilitating multi-site clinical trials utilizing P-AscH⁻. A high level of reproducibility is important when comparing data from clinical trials utilizing P-AscH⁻ to ensure that patient ascorbate levels can be evaluated in an accurate and reliable manner.

Previously published literature suggests the use of sodium heparin coated collection tubes as a first-line method to reduce the oxidation of ascorbate in blood (8). When collecting samples that are able to be processed and measured within 24 h, it would be appropriate to keep samples as whole blood stored at 4°C (or on ice), an expected rate of loss of AscH⁻ of 0.28% h⁻¹. Our data are consistent with the data of Pullar *et al.* (8) who showed no significant loss of plasma ascorbate when samples are held on ice for up to 24 h in a sodium heparin coated tube. We have shown that significant loss of ascorbate occurs within 24 h when the blood sample was kept at room temperature. If a blood sample needs to be stored for greater than 24 h, the sample should be processed to isolate the plasma and stored at 4°C. When plasma is stored at 4°C, oxidation of AscH⁻ to DHA appears to occur at a rate of 0.15% per hour. The sample may be stored as plasma at 4°C for up to 72 h, after which time significant oxidation would be expected to occur.

To prevent significant ascorbate loss in patient blood samples that must be stored for greater than 72 h, the plasma sample may be stored at -20°C or -80°C. At -20°C or -80°C, the rate of AscH⁻ oxidation in plasma is much slower, occurring at rates of 0.18% and 0.07% ascorbate loss per day, respectively. When plasma samples were held at -20°C, we found that the ascorbate remained stable for 14 days, while Karlsen *et al.* showed that a significant loss of ascorbate did not occur until after approximately 20 days (11). As for patient samples that need to be stored for an extended period of time, we show that P-AscH⁻ treated plasma samples are stable after being held at -80°C for up to 28 days.

When considering a diverse patient population, we observed no difference in ascorbate metabolism between male and female volunteers. From these data, we suggest that blood samples from both the male and female cohorts may be handled and processed under the same conditions.

In patient samples stored as either whole blood or plasma, an increased stability was noted in the whole blood at 4°C compared to plasma. This finding is contrary to our results in ascorbate-dosed blood samples from healthy subjects, which indicated that ascorbate held as plasma at 4°C was more stable. One possible reason for this discrepancy is that the initial samples from healthy volunteers were artificially dosed with ascorbate, resulting in concentrations beyond those that have been observed in human patients. The mean initial concentration of ascorbate in the plasma of spiked samples was approximately 34.5 ± 1 mM, while the average initial concentration in plasma observed in the patient samples was 17 ± 3.4 mM. In this context, patient blood samples that have been stored as whole blood at 4°C may, in fact, be more stable for short-term storage than samples stored as plasma because the ascorbate that is already in blood circulation is able to be recycled in the erythrocytes by glutathione reserves (22–24). Although recycling of ascorbate by glutathione enables greater stability of ascorbate in whole blood samples, one issue that can lead to a lack of reliability in high throughput blood sample analysis is hemolysis. If a sample must be stored for more than 24 h, it is recommended that the samples be stored as frozen plasma (i.e., -20°C or -80°C).

However, it has been shown that although plasma samples may be slightly less stable at 4°C, they may be stored at -20°C for up to 3 weeks with relatively high stability in both frost-

containing and frost-free environments. We considered that there may be a difference in the stability of samples held in a frost-containing compared to frost-free environment because of the periodic warming of freezer contents when a frost-free freezer cycles to remove accumulated frost. However, there did not appear to be any significant difference in the stability of ascorbate despite the slight divergence at 14 days. But after three weeks there was a large decrease in measured ascorbate ($>10\%$ [P-AscH⁻]) for samples held in both frost-containing and frost-free environments, indicating that plasma samples held at -20°C are stable in either a frost-containing or frost-free environment for up to three weeks.

From our study we are able to draw the following conclusions to aid in appropriate sample collection, processing and storage and ensure repeatable and reliable measurements from centers participating in clinical trials investigating pharmacological ascorbate:

1. Ideal processing and handling should utilize sodium heparin coated blood collection tubes rather than EDTA tubes;
2. Samples may be held at 4°C as whole blood for <24 h, with loss of ascorbate occurring at a rate of $0.28\% \text{ h}^{-1}$;
3. Samples being held >24 h prior to processing for the analysis of ascorbate should be centrifuged initially to separate plasma from whole blood and held at 4°C for no longer than 72 h, with loss of ascorbate occurring at a rate of $0.15\% \text{ h}^{-1}$;
4. Samples that have been centrifuged and are stored as plasma are stable in -20°C for 14–21 days, with ascorbate being oxidized at a rate of 0.18% ascorbate loss day^{-1} ;
5. For long-term storage, samples should be centrifuged and stored as plasma at -80°C , with an expected ascorbate loss of 0.07% ascorbate loss day^{-1} ;
6. There appears to be no need to handle blood samples from male and female subjects differently;
7. Analysis of samples from clinical trial patients revealed greater stability of ascorbate in samples held as whole blood at 4°C compared to plasma;
8. For storage of patient samples longer than 24 h, significant hemolysis can occur, resulting in loss of reliability in measurements of ascorbate concentration;
9. Patient plasma samples may be reliably stored at -20°C for 2–3 weeks in either a frost-containing or frost-free environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Ascorbate stability in whole blood. Blood samples from healthy human subjects were dosed with ascorbate (20 mM) and stored at either room temperature (RT) or 4°C. At the time of analysis, samples (n = 12) were centrifuged at 2,000g for 14 min to separate RBCs and plasma. [AsCH⁻] was determined in the plasma using a microvolume UV-Vis spectrometer; values are reported as a fraction of the initial concentration ± SD. **P* < 0.05 based on two-way ANOVA comparing the interaction between storage conditions.

FIG. 2.

Ascorbate stability in plasma. Blood samples from healthy human subjects were dosed with ascorbate (20 mM). Samples ($n = 12$) were centrifuged at 2,000g for 14 min to separate RBCs and plasma. After centrifugation, plasma samples were stored at either room temperature (RT) or 4°C. [AscH⁻] was determined in the plasma using a microvolume UV-Vis spectrometer; values are reported as a fraction of the initial concentration $\pm$ SD. * $P < 0.05$ based on two-way ANOVA comparing interaction between storage conditions.

FIG. 3.

Ascorbate stability in plasma at -80°C . Blood samples from healthy human subjects were dosed with ascorbate (20 mM). After addition of ascorbate, samples were centrifuged at 2,000g for 14 min to separate RBCs and plasma. Plasma samples were stored at -80°C for up to 28 days. $[\text{AscH}^-]$ measurements were done using a microvolume UV-Vis spectrometer at 14-day intervals for up to 28 days and are reported as a fraction of the initial concentration $\pm$ SD.

FIG. 4.

Ascorbate stability in plasma at -20°C . Blood samples from healthy human subjects were dosed with ascorbate (20 mM). After addition of ascorbate, samples were centrifuged at 2,000g for 14 min to separate RBCs and plasma. Samples ($n = 12$) were stored at -20°C for 5 days. $[\text{AscH}^-]$ measurements were done every 5–7 days for 14 days using a microvolume UV-Vis spectrometer and are reported as a fraction of the initial concentration $\pm$ SD

FIG. 5.

Ascorbate stability in patient samples stored at 4°C. Patient samples collected from phase II ascorbate lung and glioblastoma clinical trials were stored at 4°C as either plasma ($n = 13$) or whole blood ($n = 11$). Samples were centrifuged at 2,000g for 14 min to separate RBCs and plasma prior to analysis. $[\text{AscH}^-]$ measurements were done every 24 h for up to 96 h using a microvolume UV-Vis spectrometer and are reported as a fraction of the initial concentration. * $P < 0.05$ based on two-way ANOVA comparing interaction between storage and analysis conditions.

FIG. 6.

Ascorbate stability in patient samples stored at -20°C . Patient samples ($n = 5$) were stored as plasma for 33 days in either a frost-containing or frost-free environment, with ascorbate plasma concentrations being measured every 5–7 days. $*P < 0.05$ based on two-way ANOVA comparing interaction between storage conditions.

TABLE 1

Average AscH⁻ Concentrations when Samples are Stored at 4°C

[AscH ⁻] (mM)	0 h	24 h	48 h	72 h
Whole blood				
Total (<i>n</i> = 12)	33.6 ± 4.6	30.5 ± 3.8	28.1 ± 4.2	26.4 ± 3.1
Male (<i>n</i> = 8)	33.2 ± 4.7	31.1 ± 3.9	28.5 ± 4.4	27.2 ± 3.2
Female (<i>n</i> = 4)	34.5 ± 5.2	29.1 ± 3.7	27.1 ± 4.2	24.8 ± 2.6
		<i>P</i> > 0.05	<i>P</i> > 0.05	<i>P</i> > 0.001
Plasma				
Total (<i>n</i> = 12)	32.3 ± 4.2	30.64 ± 4.2	29.8 ± 5.1	28.7 ± 3.8
Male (<i>n</i> = 8)	31.9 ± 4.6	30.9 ± 4.3	29.5 ± 5.8	29.2 ± 4.4
Female (<i>n</i> = 4)	32.9 ± 4.1	30.1 ± 4.6	30.3 ± 4.2	27.8 ± 2.4
		<i>P</i> > 0.05	<i>P</i> > 0.05	<i>P</i> > 0.001