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Direct Measurement of Supra-Physiological Levels of Ascorbate in Plasma using a Nanophotometer

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Goal

To provide a fast, efficient, and cost effective assay to determine supra-physiological vitamin C (ascorbate) concentrations in blood.

Background

- High-dose ascorbate is currently being tested in clinical trails as an adjuvant to current standard of care therapies for cancer;
- Supra-physiological concentrations of ascorbate in blood, 300x to 500x normal concentrations, is the goal;
- Previous methods to determine the level of ascorbate require extensive preparation, time, and materials;
- This new assay requires: minimal sample preparation, less time, and fewer materials;
- The new assay has been deployed to support ongoing clinical trials.

Materials and Methods

Plasma Samples

Blood plasma samples were from subjects of clinical trails being conducted at The University of Iowa. Trials were approved by The University of Iowa IRB and are listed on https://clinicaltrials.gov. Whole blood was collected and centrifuged at 2000 ***g*** for 15 min. Plasma was than collected and immediately analyzed or stored at -80 ºC for future analyses.

UV/Vis spectroscopy

Standard Curve

A 0.100 M ascorbate standard stock solution (10.0 mL) was prepared in a phosphate buffer. The exact concentration of the stock solution was verified by absorbance at 265 nm, with an extinction coefficient of 14,500 M^{-1} cm^{-1}. Absorbance measurements were collected on a Implen Nanophotometer P-330 with path length of 40 µm.

Dilutions were made to make standard solutions concentrations of 35.0, 30.0, 25.0, 20.0, 15.0, 10.0, 5.0, 2.5, 1.3, and 0.0 mM. A standard curve was made with 0.0, 1.3, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 mM ascorbate that was prepared from the dilutions of the stock solution.

Measurements

To determine the absorbance due to ascorbate any absorbance from 0.0 mM was subtracted out. Samples were compared against a standard curve to determine ascorbate concentration in blood plasma. Samples were measured in triplicate.

Results

Use of UV/Vis Nanophotometer yields reliable concentrations of ascorbate.

A plot of the measured concentrations of ascorbate *vs*. the concentration of gravimetrically prepared standards showed linear correlation, **Figure 1 A-B**.

- Addition of ascorbate to plasma samples showed direct detection of ascorbate in plasma is possible, **Figure 2**.
- Nanophotometer assay agrees with older technique, **Figure 3**.
- A pre-infusion sample is not required to estimate plasma ascorbate levels, **Figure 4.**

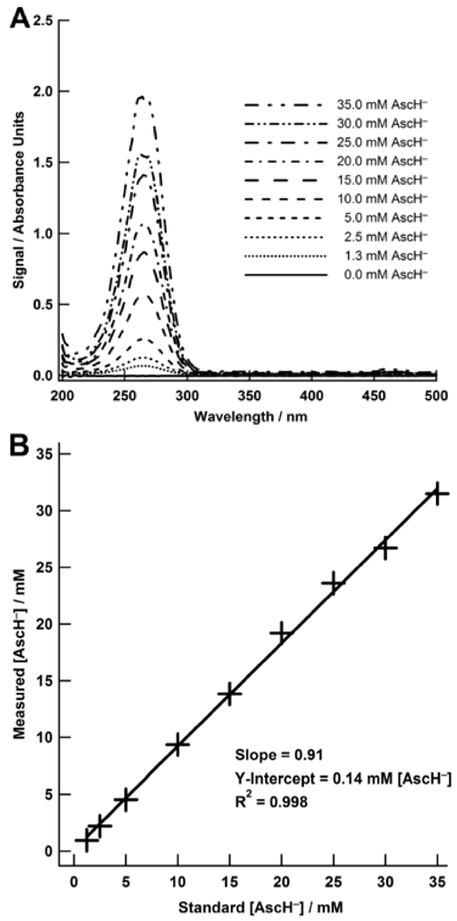

Figure 1. A robust standard curve demonstrates the potential of this direct approach to determine ascorbate in blood.

(A) UV/Vis spectra of known standard solution ascorbate in buffer. Standard solutions prepared gravimetrically and spectra for 0.0, 1.3, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 mM observed on Implen Nanophotometer P-330 with path length of 40 µm**.**

(B) Plot of measured concentrations of ascorbate using extinction coefficient of 14,500 M^{-1} cm^{-1} (ordinate) *vs*. gravimetrically prepared standards (abscissa). Slope = 0.91 ± 0.02, R^2 = 0.998.

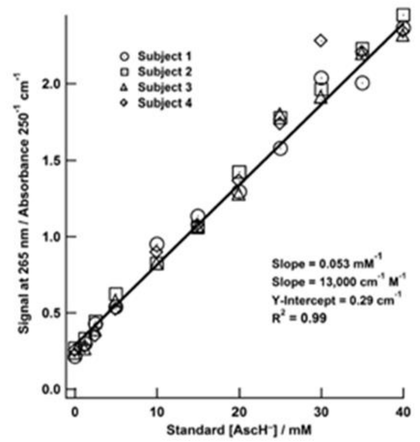

Figure 2: The method permits accurate determination of ascorbate in the complex matrix of human blood plasma. The absorbances obtained directly from the Implen Nanophotometer P-330 *vs*. the known gravimetric standard show a liner relationship. **NOTE. The extinction coefficient of ascorbate in plasma is different than in phosphate buffer, 13,000 *vs*. 14,500 M^{-1} cm^{-1}.**

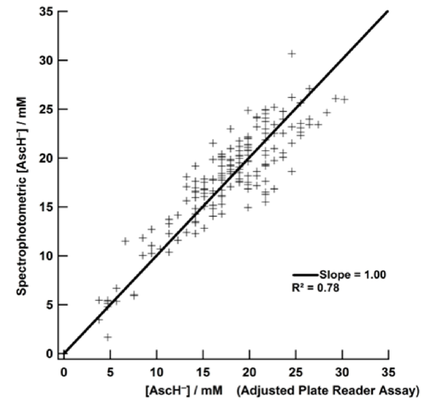

Figure 3: Direct assay using Implen Nanophotometer P-330 agrees with results from previous assays. 179 samples were analyzed. With a linear regression set to go through the origin a slope of 1.00 ± 0.01 and R^2 = 0.78.

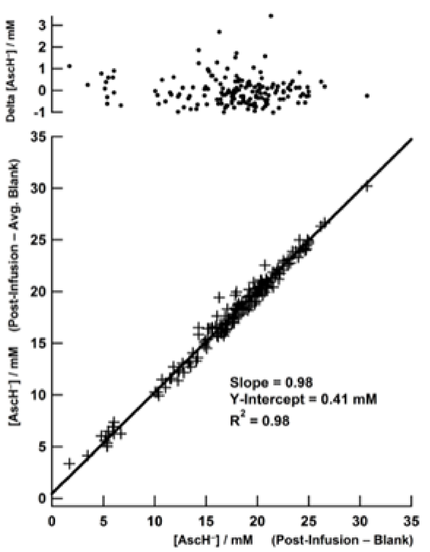

Figure 4. Ascorbate concentrations determined with "post-infusion – average blank" vs "post-infusion – pre-infusion" with differences plotted as residual in top plot. 169 samples were analyzed for ascorbate concentrations. A slope of 0.96 ± 0.01 shows the ability of assay to estimate plasma ascorbate concentrations, even if no pre-infusion plasma sample is available using average blank instead.

Conclusions

Direct measurement of ascorbate is possible

- A microvolume UV/Vis spectrometer, such as the Implen Nanophotometer P-330, can be used to determine supra-physiological levels of ascorbate in blood plasma directly without any sample processing.

High ascorbate specificity achieved

- High specificity for ascorbate was observed. A coefficient of variation of about 13% was observed if a pre-infusion blank was available as a blank. If the average blank is used the estimated coefficient of variation was about 20%. Variation comes from the variation in the absorbance of the plasma at the same wavelength as ascorbate (265 nm).

Easy clinical implementation

- A microvolume UV/Vis spectrometer takes up little space.
- The Implen Nanophotometer P-330 requires no external computer and can print data directly from the instrument.
- The protocol is easy to follow.
- A quick assessments of ascorbate plasma concentrations within minutes enables rapid decision making in a clinical setting.

Current Use

- The method has been deployed currently supporting two clinical trials addressing cancer treatment with high-dose ascorbate.

Reference

Witmer JR, **Wetherell BJ**, Wagner BA, Du J, Cullen JJ, Buettner GR**.** (2016) Direct spectrophotometric measurement of supra-physiological levels of ascorbate in plasma. *Redox Biology.* **8:**298-304. PMID: 26928133 http://dx.doi.org/10.1016/j.redox.2016.02.004 ***Open Access*** PMCID: PMC4776628

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