Improved Method for Vitamin C Analysis in Serum

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BACKGROUND

Vitamin C (VC) is necessary for development of normal connective tissue and human health. It is a labile vitamin that can easily be lost during sample preparation[1] or while on an autosampler prior to injection[2]. VC HPLC separations have remained nearly the same for 25 yrs. Most methods involve the reduction of samples to insure that any dehydroascorbic acid (DHA) is converted to ascorbic acid (AA). This step can be problematic since common reducing agents do not work well at acidic pH.

Recent advances in column technology have permitted some improvements in the separation and we present some beneficial changes to sample preparation methodology.

OBJECTIVES

- To compare the separation of vitamin C on various HPLC columns.
- To improve serum vitamin C selectivity without the use of ion-paring agents or gradients.
- To improve the stability of vitamin C in serum extracts.
- To identify a functional internal standard.

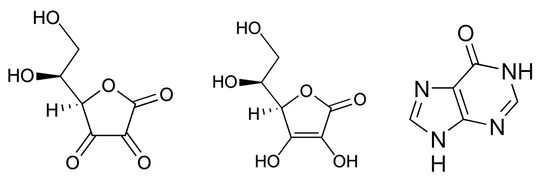
METHODS

- Serum or plasma (250uL) was mixed with 6% metaphosphoric acid (MPA 1000uL) at the time of harvest and frozen.
- Upon thawing samples were mixed and centrifuged at 4°C, 10,000 rpm, 5 min.
- 50uL of supernatant was mixed with 150uL dipotassium phosphate containing tris[2-carboxyethyl]phosphine hydrochloride (TCEP) to reduce any dehydroascorbic acid to ascorbic acid.
- ☐Incubate 1hr at room temp.
- ☐Samples were acidified with 50uL 40% MPA
- 50uL of Hypoxanthine (HYPX) was added as internal standard and mixed prior to transferring to autosampler vials.
- 20uL was injected.

HPLC Conditions

Column: Synergi Hydro RP, 250 x 4.6mm, 4um
Detection: UV 245nm (5cm light-pipe flowcell), EC 200mV, 10nA
Mobile phase: 2% methanol in 25mM Monochloroacetic acid, 2mM EDTA, pH 3.0
Flow rate: 0.8 mL/min
Column temperature: 32°C
Sample temperature: 15°C

Figure 1



Ascorbic Acid | Dehydroascorbic Acid | Hypoxanthine

Figure 1 Illustrates the structures of AA, DHA and HYPX.

Table 1, below, list many of the compounds that were tested as potential internal standards using a Synergi Hydro RP HPLC method.

Potential Internal Standards Tested on Synergi Hydro RP

	MW	Formula	Electroactive	Ultraviolet	Lambda max
Ascorbic acid (for reference)	176.1	C6H8O6	yes	yes	246
Methyl Uric Acid3	182.1	C6H6N4O3	yes	yes	295
Homogentisic Acid4	168.4	C6H8O4	yes	yes	290
Ascorbic Acid Phosphate	289.5		no	yes	
Ascorbic Acid Sulfate	332.3	C6H6K2O9S	yes	yes	
Gentisic Acid	154.1	C6H6O4	no	yes	
Guanine	151.1	C5H5N5O	no	yes	246, 275
Hypoxanthine	136.1	C5H4N4O	no	yes	255
Oxoguanine*			no	yes	
Uric Acid	168.1	C5H4N4O3	yes	yes	247
Cinnamic Acid	148.2	C9H8O2	yes	yes	264
Coumaric Acid	164.2	C9H8O3	yes	yes	223, 286
Ferulic Acid	194.2	C10H10O4	yes	yes	
Gallic Acid	170.1	C7H6O5	no	yes	
Quinic Acid	192.2	C7H12O6	yes	yes	

Other compounds tested include: isoascorbic acid, benzoic acid, caffeic acid, creatinine, lipoic acid, resorcinol, salicylic acid, sinapinic acid, vanillin,.
ref 3 L.F. McCoy et al. Clinical Chemistry 51: 1062-1064, 2005
ref 4 X. Li and A.A. Franke. J Chromatogr B Analyt Technol Biomed Life Sci. 2009
* Donated by Dr. Garry Handelman, U Mass Lowell

Figure 2

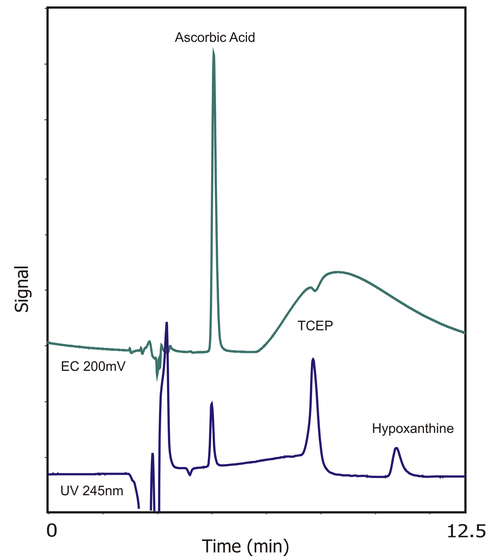
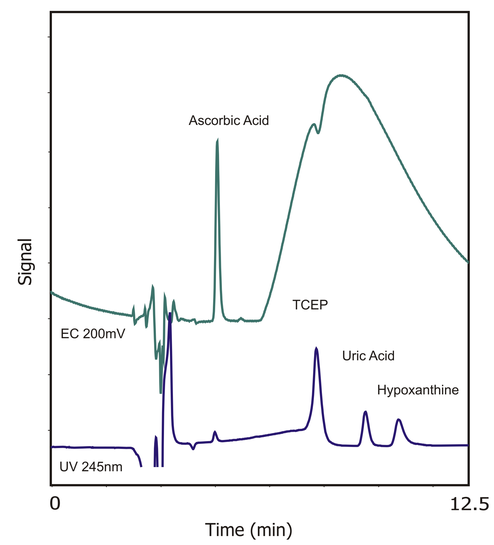


Figure 2, above, illustrates the separation of standards of AA, HYPX and reagents used in the sample preparation using a Synergi Hydro RP HPLC method.

Figure 3, below, represents the separation of a serum extract using the method described.

Figure 3



Why trade a flatter baseline for the humps in Figure 2?

- TCEP increases the stability of AA in extracts for at least 72 hrs[5].
- TCEP is an effective reducing agent at lower pH[6].
- The cost of TCEP is lower than DTT.
- TCEP elutes at a shorter retention time than DTT thereby decreasing analysis time or "dodging" the DTT in the next injection.
- TCEP elutes faster so gradients can be avoided.

CONCLUSIONS

- Several HPLC columns, possible internal standards and two buffers at different concentrations were examined.
- Both UV and electrochemical (EC) detection was used to provide greater flexibility.
- Most columns offered poor retention and peak shape of VC.
- Phenomenex Synergi Hydro-RP column provided good retention (k=1.3) symmetry (A=1.1) and tailing (T=1.15).
- Tris(2-carboxyethyl)phosphine (TCEP) was used as an alternate reducing agent to dithiothreito (DTT)l. It serves as a cost effective and superior reducing agent providing longer sample stability.
- Hypoxanthine, selected as an internal standard, eluted isocratically at k'=3.2 with UV absorbance at 245nm. Unfortunately it provided no EC response at typical voltage potentials.

REFERENCES

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3 McCoy LF, Bowen MB, Xu M, Chen H, Schleicher RL. Improved HPLC assay for measuring serum vitamin C with 1-methyluric acid used as an electrochemically active internal standard. Clin Chem. 2005, 51(6):1062-4.

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5 Lykkesfeldt J. Determination of ascorbic acid and dehydroascorbic acid in biological samples by high-performance liquid chromatography using subtraction methods: reliable reduction with tris[2-carboxyethyl]phosphine hydrochloride. Anal Biochem. 2000, 282(1):89-93.

6 Wechtersbach L, Cigić B. Reduction of dehydroascorbic acid at low pH. J. Biochem. Biophys. Methods. 2007, 7: 767772.