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Development and implementation of an HPLC-ECD method for analysis of vitamin C in plasma using single column and automatic alternating dual column regeneration ☆

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ABSTRACT

Objectives: Vitamin C (L-ascorbic acid) is a water-soluble micronutrient necessary for human life. Inadequate intake can lead to the fatal disease scurvy. Measurement of vitamin C is used to assess nutritional status and to monitor supplementation. The goal of this study was to develop a chromatographic method for the quantitation of vitamin C in human plasma.

Design and methods: Samples were prepared by protein precipitation, addition of internal standard, and reduction with dithiothreitol. Separation of ascorbic acid was accomplished by isocratic elution on a reverse-phase column; concentration was determined by coulometry. The method was validated through studies of assay linearity, sensitivity, imprecision, accuracy, analytical specificity, and carryover.

Results: The new assay was developed using a single pump/single analytical column HPLC system. Results correlated well with our previously used spectrophotometric method. The analytical measurement range was 1.0–2500 μmol/L. The injection-to-injection time was 13 min. Subsequently, to increase method throughput and shorten turnaround time, a dual LC pump system with a 2-position/10-port switching valve capable of performing automatic alternating column regeneration was validated and implemented. The injection-to-injection time was reduced 2-fold to 6 min. The method was linear to 5000 μmol/L; limit of quantification was 1.9 μmol/L. Total imprecision was less than 5%.

Conclusions: We have developed a robust method suitable for routine clinical measurement of vitamin C in plasma specimens. The method incorporates a simplified sample preparation and a stable, non-endogenous internal standard to specifically quantify vitamin C. Faster throughput was achieved by employing an automatic alternating column regeneration system.

Abbreviations: ; AA, ascorbic acid; AMR, analytical measurement range; CDC, Centers for Disease Control and Prevention; CLSI, Clinical and Laboratory Standards Institute; CV, coefficient of variation; DHAA, dehydroascorbic acid; DHBA, 3,4-dihydroxybenzylamine; DTT, dithiothreitol; ECD, electrochemical detection; EDTA, ethylenediamine tetraacetic acid; HDV, hydrodynamic voltammetry; HPLC, high performance liquid chromatography; IAA, isoascorbic acid; IS, internal standard; IV, intravenous; LC, liquid chromatography; LOQ, limit of quantitation; MPA, *meta*-phosphoric acid; NHANES, National Health and Nutrition Examination Survey; NIST, National Institute of Standards and Technology; OxA, oxalic acid; PST, plasma separator tube; SD, standard deviation; SRM, standard reference material; SST, serum separator tube; TSP, trisodium phosphate; UV, ultraviolet

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1. Introduction

Vitamin C (L-ascorbic acid, AA) is a water-soluble micronutrient that is required for human health [1]. Deficiency of vitamin C causes scurvy, a fatal disease first described by Hippocrates in 500 BCE. Chemically, vitamin C is an electron donor. The vitamin C redox system consists of ascorbic acid; its oxidized form, dehydroascorbic acid (DHAA); and the free radical intermediate, semi-dehydroascorbic acid [2]. Physiologically, vitamin C functions as a reducing agent and an enzyme co-factor for oxygenase enzymes responsible for the biosynthesis of collagen, carnitine, norepinephrine, and some peptide hormones, and for the metabolism of tyrosine and pyrimidines. The vitamin promotes iron absorption in the small intestine by reducing iron to the ferrous ion (Fe^{2+}). The potential role of vitamin C as an antioxidant capable of protecting cells from free radical damage and preventing cancer and other chronic diseases, as well as its ability to act as a pro-oxidant with redox active metals and produce harmful reactive oxygen species, makes AA a frequent subject of research studies [3]. Intravenous (IV) infusion of vitamin C is used in complementary and alternative medicine as treatment for cancer [4]. Although concentrations correlate with recent intake and the half-life is relatively short (10–20 d), measurement of plasma vitamin C is used to assess nutritional status and to monitor supplementation [2].

Laboratory measurement of vitamin C is complicated by instability of the analyte. Specimens must be protected from light and stored frozen; preservatives may be used to stabilize vitamin C prior to analysis [5]. For many years, vitamin C has been measured by reaction with compounds such as 2,4-dinitrophenylhydrazine to form colored derivatives that are detected by spectrophotometry [6]. These methods are subject to lack of specificity, limited sensitivity, and interference from other compounds. Newer, more specific techniques utilize high performance liquid chromatography (HPLC) with fluorescence, ultraviolet, or electrochemical detection (ECD) [5,7]. A spectrophotometric method developed in our laboratory had been used for plasma vitamin C analysis for decades [6]. The procedure employed a cumbersome, time-consuming sample preparation, used hazardous concentrated sulfuric acid, and generated large amounts of biohazardous waste. Additionally, as interferences in plasma may form reaction products similar to those of AA, indistinguishable from the analyte using a spectrophotometric method, this assay lacked the specificity afforded by separation techniques [5]. In order to accommodate increasing vitamin C test volume and to improve assay specificity, the decision was made to transfer the assay to an HPLC platform.

This study describes the development and validation of a selective and sensitive HPLC-ECD method for the measurement of vitamin C in plasma using a standard single liquid chromatography (LC) pump as well as a high-throughput option utilizing a dual LC pump system with automatic alternating column regeneration. The method reduces total analysis time to accommodate high test volume and extends assay linearity to facilitate measurement of elevated vitamin C concentrations found in specimens from patients receiving intravenous ascorbic acid therapy.

2. Materials and methods

2.1. Chemicals and reagents

L-Ascorbic Acid (AA), disodium ethylenediamine tetraacetic acid (Na_2EDTA) dihydrate, DL-dithiothreitol (DTT), *meta*-phosphoric acid (MPA), monochloroacetic acid, trisodium phosphate dodecahydrate (TSP), and the internal standard 3,4-dihydroxybenzylamine (DHBA) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide (Macron brand) was purchased from VWR (Radnor, PA). HPLC-grade methanol was purchased from JT-Baker (Center Valley, PA). Lyophilized vitamin C plasma controls Levels I and II were purchased from Chromsystems (Gräfelfing, Germany). NANOpure water, obtained from a Barnstead water system, was used throughout the study.

2.2. Preparation of calibrators, internal standard, and controls

AA stock solution, 5000 μmol/L, was prepared in 5% (w/v) MPA in water and used immediately for preparation of calibration standards at 5, 25, 100 and 500 μmol/L in 5% MPA. Internal standard (IS) working solution, 50 μmol/L, was prepared by dissolving DHBA in 5% MPA. Calibration standards and IS working solution were aliquoted and stored at −70 °C. Calibration curves were constructed using four calibration standards in the single-LC pump method; a fifth standard (5000 μmol/L stock AA solution) was added in the alternating column regeneration method. Control materials were prepared by reconstitution of Chromsystems lyophilized vitamin C plasma controls Level I and II in water to provide concentrations at or near the minimum (23 μmol/L) and maximum (114 μmol/L) of the reference interval. Aliquots of the control solutions were stored frozen at −70 °C.

2.3. Specimens

Venous blood was collected from self-reported healthy, non-smoking adults, who were not taking medications or vitamin supplements and who had fasted for 12 h. Discarded heparinized, oxalic acid-preserved, plasma specimens submitted for testing were selected from the routine workload for use in validation experiments. Specimens were protected from light and stored frozen at −20 °C until analysis. All specimens were de-identified and handled according to guidelines approved

by the Institutional Review Board of the University of Utah (IRB #7275).

2.4. Sample preparation

Ice-cold 10% (w/v) MPA in water (200 μL) was added to 200 μL aliquots of plasma specimens and controls in 1.5-mL opaque centrifuge tubes to precipitate proteins. Tubes were vortexed for 30 s and then centrifuged at 23,000*g* for 5 min at 4 °C. In a second set of tubes, 50 μL of ice-cold IS working solution and 300 μL of ice-cold 2.5*g*/L DTT in aqueous 0.1 M TSP were added to 100 μL of calibration standards, controls, and specimen supernatants. Samples were incubated for 30 min at 4 °C in an ice bath. After addition of 50 μL of 10% MPA, tubes were again vortexed and centrifuged at 23,000*g* for 5 min at 4 °C to pellet debris. Sample aliquots were transferred to autosampler vials. All solutions and thawed samples were kept on ice.

2.5. Instrumentation and conditions

Instrumental analysis during method development and original validation was performed on an Agilent 1200 series HPLC system, consisting of one binary pump, a degasser, an autosampler and a temperature controlled column compartment, connected to an electrochemical detection system, consisting of a model 5020 guard cell, model 5011A high sensitivity dual electrode analytical cell and Coulochem® III electrochemical detector (Thermo Scientific, Waltham, MA). Instrument control and data analysis and quantitation were performed using Agilent Chemstation software, revision B.04.03-54 (Agilent Technologies, Santa Clara, CA).

Chromatographic separation was achieved using a Synergi Hydro-RP column (100 mm × 3.0 mm, 2.5 μm particles, 100 Å pores) with a Security Guard cartridge holder containing two C-18 4 mm × 3.0 mm cartridges (Phenomenex, Torrance, CA). The compounds were eluted isocratically using 98.5% mobile phase A (150 mM monochloroacetic acid, 2 mM Na_2EDTA, pH 3.00 ± 0.05) and 1.5% mobile phase B (100% methanol) from 0 to 1.8 min followed by a step gradient to 20% methanol (1.81–3.2 min) and re-equilibration at 1.5% methanol to 12 min. The mobile phase flow rate was 350 μL/min; the column temperature, 30 °C; and the injection volume, 5 μL. The autosampler was held at 4 °C.

For the automatic alternating column regeneration method, the HPLC system was plumbed with two binary pumps, two degassers, an autosampler with a 40-μL sample loop, and a temperature controlled column compartment with a 2-position/10-port switching valve. The instrument was operated in alternating column regeneration mode, i.e. as a sample was injected and separated on the first column, a regeneration gradient was running on the second column. With the next injection, the flow path was changed using the 10-port switching valve, and the separation proceeded on the second column while the first column was regenerated. Chromatographic conditions were modified to elute the compounds isocratically (pump 1) using 98.5% mobile phase A and 1.5% mobile phase B in 5 min. The column was regenerated (pump 2) using a step gradient to 90% methanol (0.1–1.1 min) followed by re-equilibration at 1.5% methanol to 5 min

The Coulochem® III detector settings were as follows: the guard cell was set at a potential of 300 mV, the analytical cell was set at −250 mV for channel 1 and 250 mV for channel 2. The full scale gain/range was 10 μA for channel 2. For both channels, the filter time constant was 5 s, signal output voltage was 1 V, datarate was 20/s and autozero was performed at 0.5 min after the beginning of run. ESA ChemStation Driver, version 1.0.1, was used for control of the detector.

2.6. Method validation

2.6.1. Linearity, limit of quantification, and imprecision

Method linearity was evaluated by analyzing samples prepared by spiking light (ultraviolet, UV)-depleted, unpreserved heparinized, pooled plasma with AA at 1.0, 2.5, 5.0, 10, 25, 100, 250, 500, 625, and 2500 μmol/L. Each standard was analyzed 10 times in three different runs on three separate days. The acceptance criterion for linearity was ± 10% of expected concentration.

Limit of quantification (LOQ) was evaluated by analyzing samples prepared by spiking UV-depleted, unpreserved heparinized plasma pool with AA at 0.625, 1.0, 2.5, 5.0, and 10 μmol/L. Each pool was analyzed a total of 10 times in three different runs on three separate days. A curve was fitted to obtain an estimate of the coefficient of variation (CV) as a function of the mean. The LOQ was defined as the lowest concentration for which the CV was within 20% and the concentration for 80% of the replicates was within 20% of the target value.

Method imprecision was evaluated by analyzing two replicates per run (beginning and end of the sequence), daily, of Level I and Level II controls for 21 days.

2.6.2. Accuracy

Accuracy was evaluated by analyzing a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) [8] and by method comparison. Two concentrations of NIST SRM 970, preserved with MPA, were analyzed on three separate days.

The HPLC-ECD method was compared with the vitamin C in plasma spectrophotometric assay [6] used at the time of the new method validation. In this method, developed in-house, a protein-free filtrate of the patient specimen was prepared using trichloroacetic acid and mixed with acid-washed charcoal, then treated with 2,4-dinitrophenylhydrazine and sulfuric

acid to yield a red complex, which was detected at 515 nm. Two groups of specimens were used for the method comparison: (1) remnant specimens preserved with oxalic acid (OxA) that had been submitted for routine vitamin C testing were analyzed by the new method after the discard date; and (2) light-protected, unpreserved specimens collected from self-reported healthy donors were analyzed after frozen storage at −70 °C for at least 24 h to simulate processing and transport of patient specimens for the newly developed method.

2.6.3. Analytical specificity

To assess analytical specificity, the following conditions and substances were analyzed for interfering properties: hemolysis (hemoglobin from hemolyzed EDTA whole blood), icterus (unconjugated bilirubin), lipemia (Intralipid® 20% fat emulsion), dopamine, epinephrine, glucose, glutamic acid, isoascorbic acid (IAA), metanephrine, 3-methoxytyramine, nicotinamide, nicotinic acid, nicotinuric acid, norepinephrine, normetanephrine, pyridoxal, pyridoxal-5′-phosphate, riboflavin, serine, serotonin, thiamine, thiamine diphosphate, thiamine monophosphate, urea, and uric acid. Medications evaluated as potential inferences included acetaminophen, caffeine, diphenhydramine, ibuprofen, naproxen, nicotine and its metabolites (cotinine and *trans*-3-hydroxycotinine), salicylic acid, and theobromine.

All compounds were spiked into aliquots of unpreserved, heparinized plasma containing 55 μmol/L of AA. The in-sample potential interferent concentration matched or exceeded values recommended in CLSI EP7-A2, Interference Testing in Clinical Chemistry [9], where given. In a separate experiment, a heparinized plasma pool with a lower AA concentration (15 μmol/L) was spiked with hemoglobin at several concentrations to determine the amount of interference for varying amounts of hemolysis. Appropriate baseline samples were generated by spiking the plasma pool with solvents to match the spike solutions solvents and volumes. All samples were analyzed in triplicate. Deviation from baseline (%) was calculated as the difference between the interference sample mean measured concentration and the respective baseline sample mean concentration divided by the baseline sample mean concentration.

2.6.4. Carryover

Carryover was evaluated as described in the EP Evaluator software. A set of High and Low samples was assayed in the following sequence: 3 Low, 2 High, 1 Low, 2 High, 4 Low, 2 High, 1 Low, 2 High, 1 Low, 2 High, 1 Low. A 14,200 μmol/L plasma specimen was used as the High sample. A 10 μmol/L standard solution in 5% MPA was used as the Low sample.

2.6.5. Injection reproducibility and autosampler stability

Injection reproducibility and stability of the analyte and the IS in the autosampler during the time required for full batch (10 calibration and control samples; 48 specimens) analysis (> 12.5 h) were tested by injecting a set of test samples consisting of 5 and 500 μmol/L calibration standards and two concentrations of quality control materials 9 times in the span of 15.4 h.

2.6.6. Collection tube type and specimen preservation study

Venous blood from 15 self-reported healthy, non-smoking adults, who were not taking medications or vitamin supplements and who had fasted for 12 h, was collected into 5 types of collection containers: no additive, serum separator (SST), sodium heparin, plasma separator (PST), and K_2EDTA; and centrifuged at 2500*g* for 10 min within 2 h. The resulting serum or plasma specimens were aliquoted immediately, placed on dry ice, and stored at −70 °C. Two types of aliquots were prepared for all tube types: light-protected and otherwise unpreserved (LP) and light-protected, preserved 1:1 (v/v) with 10% MPA (LP-MPA). Additionally, for serum and heparinized plasma specimens, 2-mL aliquots were placed into clear tubes to which 40 mg of oxalic acid had been added as a preservative. These tubes were used for the preservation, storage, and transport of samples submitted for routine vitamin C testing at the time of this validation. Specimens from 10 (2 males, 8 females; aged 21–55 years) of the donors were analyzed in duplicate by the HPLC-ECD method after 2.5–3 months of storage at −70 °C.

2.6.7. Reference interval

An established reference interval was verified by analyzing 54 heparinized plasma specimens collected from self-reported healthy non-smoking adult donors, who were not taking medications or vitamin supplements and who had fasted for 12 h prior to blood collection. These specimens were prepared as described previously and stored frozen at −70 °C until analysis.

2.6.8. Data evaluation

Statistical evaluation of the data was performed according to CLSI guidelines using EP Evaluator software, release 10 (David G. Rhoads Associates, Inc.). The results reported for the spectrophotometric method were converted from conventional units (mg/dL) to molar units (μmol/L) and data analysis was performed using Deming regression.

2.6.9. Validation of the dual LC pump system with alternating column regeneration

To validate the newly developed method on the two-LC pump system with alternating column regeneration, an abbreviated set of experiments was performed. Method linearity and limit of quantification were evaluated by analyzing samples prepared by spiking UV-depleted, unpreserved heparinized plasma pool with AA at 1.0, 2.5, 5, 20, 100, 500, and

5000 μmol/L. Each standard was analyzed 6 times in three different runs on three separate days. Method imprecision was evaluated by analyzing two replicates per run of two concentrations of control material in nine runs over five days.

To establish equivalence of the two columns, a total of 96 injections of calibration standards (44), controls (24), and heparinized plasma specimens (28) were performed on each column over 3 days. Column equivalence was assessed as bias (%) between the two columns for AA retention times and IS (DHBA) retention times and peak areas, and using Deming regression for AA peak areas and concentrations.

To assess the performance of the alternating column method, 40 heparinized plasma samples, stored at −70 °C for 4 months, were analyzed and the results were compared to those obtained for separate aliquots of the same samples analyzed on the single LC pump HPLC-ECD system during the original method validation.

A simple carryover check was performed by injecting a concentrated plasma specimen (18,740 μmol/L AA) followed by two injections of reagent blank solution (with IS only) twice.

3. Results

3.1. Chromatography and electrochemical detection

Optimal electrochemical potentials for AA and DHBA were determined using hydrodynamic voltammetry (HDV). Curves were constructed by injecting standard solutions of the compounds in duplicate into the HPLC system and plotting the measured oxidative current (as peak area) while increasing the analytical potential (E_2) in 50 mV increments. The analytical potential E_1 was held constant at −250 mV, a value 50 mV below the potential that produced AA current. The optimal potential was 200 mV for AA and 250 mV for DHBA. As it is not practical to use different potentials for closely eluting compounds because of long electrode equilibration times, the higher of the two optimal potentials, E_2=250 mV, was chosen for this method. The potential for the guard cell was set at 300 mV.

AA eluted in approximately 2 min; cycle time was 13 min for analysis on the single-LC pump system and 6 min using the dual column system. Fig. 1 shows representative chromatograms of plasma specimens analyzed on the single-LC pump system (A) and the two-LC pump system (B, C). Vitamin C concentrations were determined from multi-point calibration curves produced using $1/x^2$ weighted linear regression analysis. Representative calibration curves are shown in Fig. 2.

3.2. Linearity, limit of quantification, and imprecision

The assay was linear from 1 to 2500 μmol/L. Measured AA concentrations were plotted against expected concentrations to give y=0.977x–0.040; observed error 1.9% (Table 1). The LOQ could not be estimated for AA using the EP Evaluator Sensitivity module, because the CV values for all samples were below 20%. However, three of the 10 replicates of the 0.625 μmol/L sample did not meet the ±20% accuracy criterion and therefore, the LOQ was assigned as 1.0 μmol/L. Within-run, between-day, and total imprecision for the two quality control concentrations were between 2.1% and 6.3% (Table 1).

3.3. Accuracy

The analysis of the NIST SRM 970 showed excellent agreement of the results obtained by our method with the target values. The deviations of the mean measured AA concentration were –1.7% and –0.9% compared to NIST Level I and Level II target concentrations, respectively (Table 1).

Comparison of results obtained using the HPLC-ECD method (Table 1) with those obtained by the spectrophotometric method yielded Deming regression equations: y=0.983x–8.93; $S_{y/x}$=7.42; R=0.9901 for discarded specimens (n=44) and y=0.834x–1.08; $S_{y/x}$=6.77; R=0.9167 for healthy donor specimens (n=41). A slight negative bias was observed for the newly developed HPLC-ECD method compared to the spectrophotometric method. Method comparison plots are shown in Fig. 3.

3.4. Analytical specificity

The conditions and substances tested for interfering properties in the analytical specificity experiment are listed in Table 2. Hemolyzed specimens were considered acceptable for hemoglobin concentrations up to 39 μmol/L (250 mg/dL), which exhibited a bias <15%. Only epinephrine, IAA, and uric acid produced peaks within the detection window. Epinephrine and uric acid were both baseline resolved from AA and DHBA. IAA (also called erythorbic acid), an AA stereoisomer sometimes used as an antioxidant in processed foods and frozen vegetables, coeluted with AA. All samples were assessed for potential interferences by evaluating additional peaks in the chromatogram that coeluted with the analyte or the IS and examining results for other chromatographic disturbances.

3.5. Carryover

Carryover was not detected at the evaluated AA concentration of 14,200 μmol/L; that is, the calculated carryover value

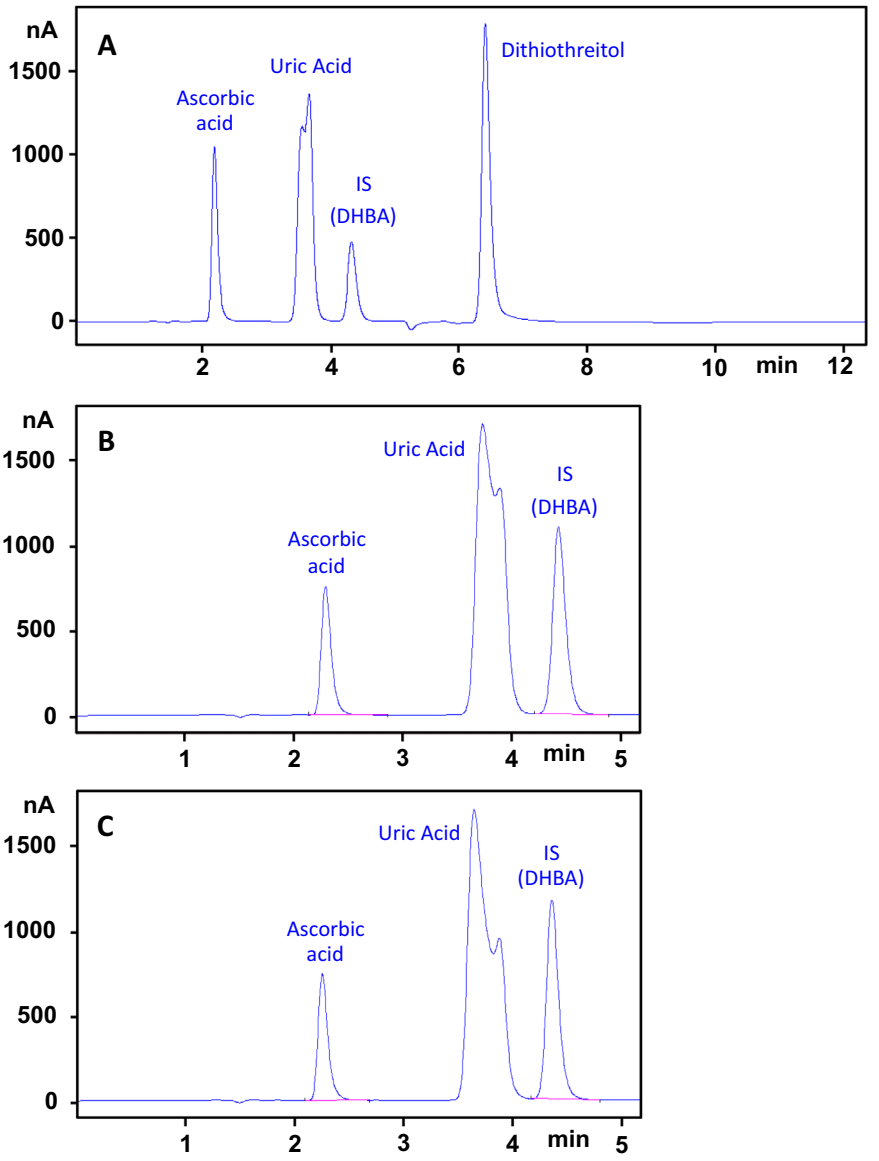

Fig. 1. Representative chromatograms of patient specimens. (A) Specimen 1 on a single LC pump system and specimen 2 on a system with alternating column regeneration: (B) column 1, (C) column 2.

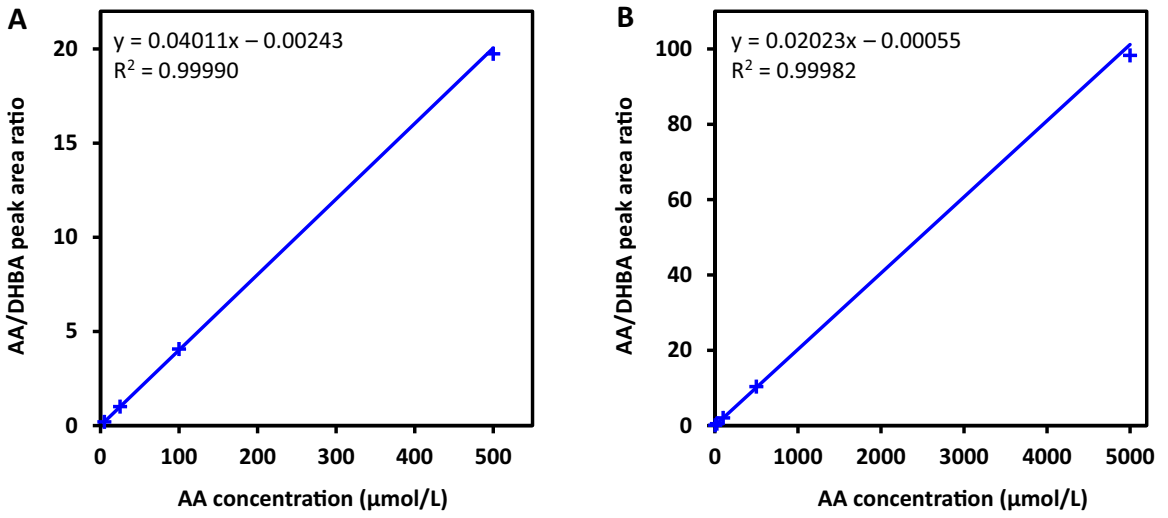

Fig. 2. Representative calibration curves. (A) Single column method, (B) dual column method with extended analytical measurement range (AMR).

Table 1
Validation parameters – single LC pump system.

Linearity

Matrix-matched samples prepared at 1.0, 2.5, 5.0, 10, 25, 100, 250, 500, 625, and 2500 μmol/L. Analyzed 10 aliquots of each concentration over 3 days.
The assay was linear 1–2500 μmol/L.

Deming regression:	y=0.977x–0.04; observed error 1.9%
Means (μmol/L)	1.0, 2.5, 5.0, 9.6, 24.4, 98.9, 246.9, 492.3, 622.3, 2455.0
Accuracy (%)	103.6, 99.7, 100.0, 96.3, 97.7, 98.9, 98.8, 98.5, 99.6, 98.2
Precision (CV, %)	6.9, 4.7, 3.9, 3.5, 1.4, 1.1, 0.7, 0.9, 1.3, 0.8

Limit of Quantification (LOQ)

Matrix-matched samples prepared by spiking UV-depleted heparinized plasma with AA to 0.625, 1.0, 2.5, 5.0, and 10 μmol/L. Analyzed 10 aliquots of each concentration over 3 days.
LOQ= 1.0 μmol/L

Target Concentration (μmol/L)	Mean Measured Concentration (μmol/L)	Fitted CV (%)	Recovery (%)
0.625	0.7	12.2	111
1.0	1.0	8.9	104
2.5	2.5	5.0	100
5.0	5.0	3.5	100
10	9.6	2.9	96

Analytical Measurement Range

1–2500 μmol/L

Imprecision

Controls were assayed in duplicate for 21 days.

Concentration (μmol/L)	Within-run CV (%)	Between-day CV (%)	Total CV (%)
23.6	5.2	3.6	6.3
117.6	2.1	3.0	3.7

Accuracy

Comparison to reference standard
Both concentrations analyzed three times on three separate days.

	Target Concentration (μmol/L)	Mean Measured Concentration (μmol/L)	Deviation from Target (%)
NIST SRM 970 I	8.4	8.3	−1.7
NIST SRM 970 II	28.1	27.8	−0.9

Method Comparison
Discarded specimens:
Specimens analyzed using current spectrophotometric method and the evaluated method.
Deming regression: $y=0.983x–8.93$; $S_{y/x}=7.42$; $R=0.9901$; $n=44$
Self-reported healthy donor specimens:
Specimens analyzed using current spectrophotometric method and the evaluated method.
Deming regression: $y=0.834x–1.08$; $S_{y/x}=6.77$; $R=0.9167$; $n=41$

Carryover

No carryover observed after plasma specimen at 14,200 μmol/L concentration.

(0.10 μmol/L) was less than the error limit (0.45 μmol/L), defined as three times the standard deviation (SD) of two consecutive low results.

3.6. Injection reproducibility and autosampler stability

Injection reproducibility was calculated as the CV (%) of AA concentrations and peak areas for the 9 injections for each of the four test samples, and of DHBA peak areas in all 36 injections. The CV ranged from 0.5% to 1.4% for AA concentrations and from –0.7% to 1.8% for AA peak areas. The overall injection reproducibility for DHBA was 1.3%.

The autosampler stability was measured as deviation (%) of AA concentrations and peak areas and DHBA peak areas for each injection compared to the first injection of the respective sample. Deviation for AA concentrations ranged from –2.6% to 3.7%, for AA peak areas from –1.7% to 5.5%, and for DHBA peak areas from –0.2% to 3.2%, signifying that both AA and DHBA were stable in the processed samples for at least 15.4 h at 4 °C.

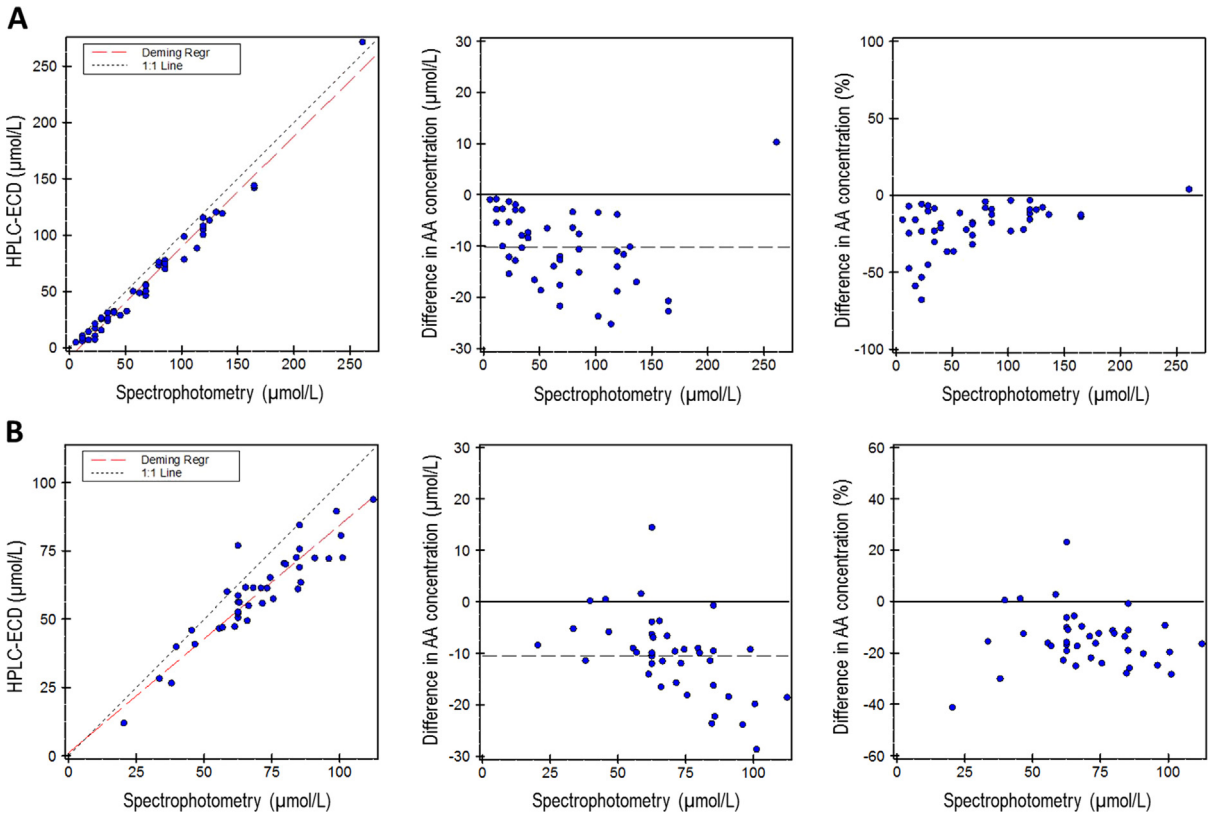

Fig. 3. Method comparison plots – HPLC-ECD vs. spectrophotometry. (A) Discarded specimens, (B) fresh specimens.

3.7. *Collection tube type and specimen preservation study*

Our spectrophotometric method used heparinized plasma preserved with oxalic acid as the specimen of choice. A blood collection tube type and specimen preservation study was conducted to determine which collection tubes were acceptable for the newly developed vitamin C assay and whether preservation additional to light protection and freezing was necessary. Two sets of aliquots from each of the five tube types (serum, SST, heparinized plasma, PST, and EDTA plasma) were prepared: light-protected (LP) and light-protected/MPA-preserved (LP-MPA). Vitamin C concentrations for both sets of specimens were compared to results for corresponding heparinized plasma specimens preserved with oxalic acid as illustrated in Fig. 4. Results for SST and PST were very similar to the corresponding tube types without a separator and were omitted from the graphs for clarity. Similar to other studies [10,11], we found that using light protection without a preservative, only heparinized plasma (including PST) concentrations agreed well (deviation $< 10\%$) with those for OxA-preserved specimens (see Fig. 4A). Using light protection with MPA preservation, good correlation was found for heparinized plasma and EDTA specimens, but not for serum and SST (see Fig. 4B).

Based on these results, heparinized plasma (including PST) was selected as the specimen of choice. Next, results for LP aliquots were compared to those for LP-MPA aliquots of heparinized plasma and PST specimens to determine whether preservation with MPA could be omitted. Results for the unpreserved specimens were slightly lower for both heparinized plasma (–0.2% to –8.1%, mean –4.6%) and for PST (–0.8% to –6.0%, mean –4.2%). It should be noted that these samples were analyzed 2.5–3 months after collection and therefore the AA degradation in the LP specimens was expected to be greater than that in specimens analyzed within a few days, the time frame for actual patient samples. This assumption was confirmed by comparing results from LP heparinized plasma aliquots to those of LP-MPA aliquots for the reference interval samples analyzed within 1–3 days of collection. The deviation ranged from −4.2% to 2.9%, with mean deviation –1.3%. Consequently, light protection of specimens without a preservative was deemed adequate for the new assay. A comparison graph is shown in Fig. 5.

3.8. *Reference interval*

A published reference interval in use for our spectrophotometric method (23–114 µmol/L) was verified [12]. Values for specimens from 54 self-reported healthy adults (26 F, 28 M), aged 20–57 years, ranged from 12.0 to 93.9 µmol/L; the mean

Table 2
Potential interferents, molecular weights, recommended and actual test concentrations in plasma, and % deviations from baseline samples.

Substance	M.W. (g/mol)	Recommended Test Concentration		Actually Tested Concentration		% Dev from Baseline
Sample conditions						
Hemolysis–hemoglobin[a]	64,458	31	μmol/L	166	μmol/L	−12.3%
Hemolysis–hemoglobin[b]				16	μmol/L	−4.8%
				39	μmol/L	−12.5%
				78	μmol/L	−16.7%
Icterus–bilirubin (unconjugated)	584.66	342	μmol/L	248	μmol/L	1.6%
Lipemia–Intralipid®, 20% emulsion	~275	Not specified		~72	mmol/L	2.1%
Endogenous compounds						
Dopamine	153.18	5.87	μmol/L	131	μmol/L	−0.1%
Epinephrine	183.21	0.5	nmol/L	109	μmol/L	0.4%
Glucose	180.16	55	mmol/L	56	mmol/L	−0.4%
Glutamic acid	147.13	600	μmol/L	1359	μmol/L	−1.4%
Isoascorbic acid	176.13	Not specified		284	μmol/L	566.4%
Metanephrine	197.23	1.5	nmol/L	1	μmol/L	0.0%
3-Methoxytyramine	167	Not specified		299	μmol/L	−0.2%
Norepinephrine	169.18	4.1	nmol/L	118	μmol/L	0.0%
Normetanephrine	183.2	2.7	nmol/L	1	μmol/L	−1.0%
Serine	105.09	750	μmol/L	952	μmol/L	0.3%
Serotonin	176	3.8	μmol/L	284	μmol/L	−2.1%
Urea	60.06	43	mmol/L	83	mmol/L	−0.9%
Uric acid	168.11	1400	μmol/L	1487	μmol/L	3.6%
Vit B_1 (Thiamine)	300.81	90	mmol/L	20	μmol/L	−1.2%
Vit B_1 (Thiamine monophosphate)	345.34	Not specified		20	μmol/L	−1.9%
Vit B_1 (Thiamine diphosphate)	425.31	Not specified		20	μmol/L	−1.4%
Vit B_2 (Riboflavin)	376.36	150	nmol/L	2	μmol/L	−2.6%
Vit B_3 (Nicotinic acid)	123.11	217	μmol/L	406	μmol/L	0.2%
Vit B_3 (Nicotinamide)	122.12	Not specified		409	μmol/L	0.8%
Vit B_3 (Nicotinuric acid)	180.16	Not specified		278	μmol/L	−0.3%
Vit B_6 (Pyridoxal)	167.16	375	nmol/L	40	μmol/L	−2.7%
Vit B_6 (Pyridoxal-5′-phosphate)	247.14	Not specified		40	μmol/L	−2.3%
Stimulants and pharmaceuticals						
Acetaminophen	151.16	1324	μmol/L	331	μmol/L	−3.0%
Caffeine	194.19	308	μmol/L	257	μmol/L	0.1%
Cotinine	176.22	10.8	μmol/L	567	μmol/L	0.8%
trans-3-Hydroxycotinine	192.22	Not specified		520	μmol/L	0.1%
Diphenhydramine	255.36	19.6	μmol/L	196	μmol/L	−0.7%
Ibuprofen	206.29	2425	μmol/L	485	μmol/L	−0.2%
Naproxen	230.26	2170	μmol/L	1563	μmol/L	1.0%
Nicotine	162.12	6.2	μmol/L	617	μmol/L	1.0%
Salicylic acid	138.12	4.34	mmol/L	362	μmol/L	−0.5%
Theobromine	180.16	278	μmol/L	278	μmol/L	−0.4%

[a] AA concentration in plasma pool 55 μmol/L.
[b] AA concentration in plasma pool 15 μmol/L.

and median were 59.1 μmol/L and 59.3 μmol/L, respectively. One specimen concentration (2% of the results) fell outside of the reference interval.

3.9. Method validation results for dual LC column system with alternating column regeneration

In addition to verification of assay performance using the dual column system, linearity was extended to an upper limit of 5000 μmol/L. Results for validation of the assay using the dual column system are summarized in Table 3.

4. Discussion

In the development of the HPLC-ECD method, we followed the HPLC protocol published by Li and Franke [13], a modification of the Centers for Disease Control and Prevention (CDC) Laboratory Procedure for vitamin C using HPLC with electrochemical detection [14], but implemented changes in the selection of analytical column and internal standard. The

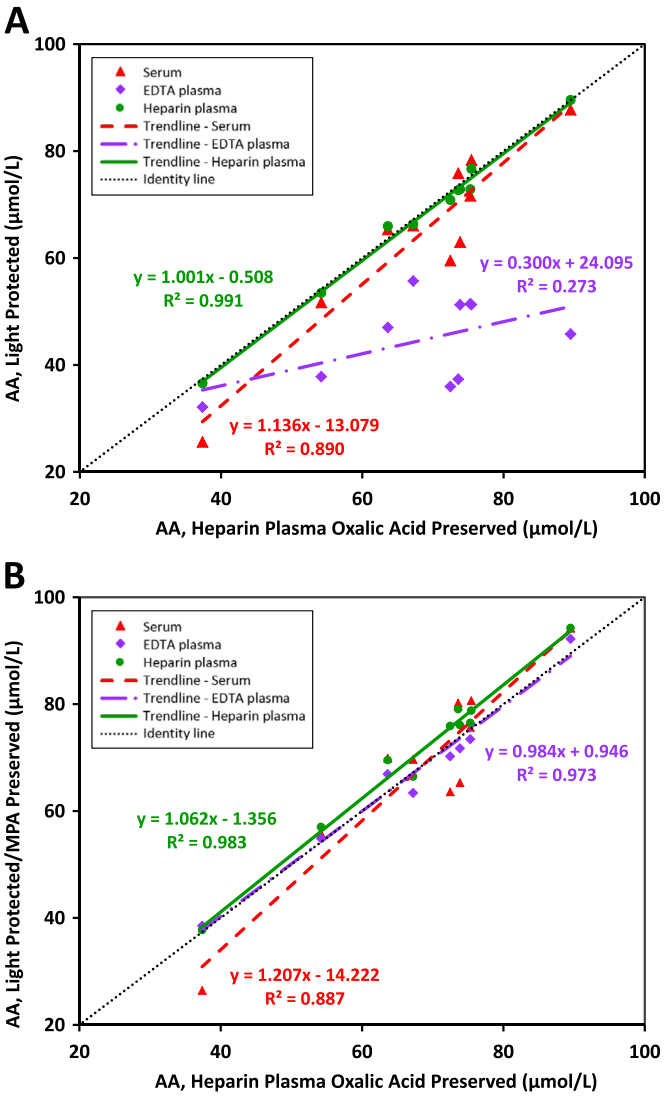

Fig. 4. Collection tube type and preservation comparison. (A) Light protected serum, EDTA plasma, and heparinized plasma concentrations compared with concentrations for heparinized plasma specimens preserved with oxalic acid; (B) Light protected/MPA preserved serum, EDTA plasma, and heparinized plasma concentrations compared with concentrations for heparinized plasma specimens preserved with oxalic acid. Legend: Serum – red triangles and dashed trendline, EDTA plasma – lavender diamonds and dash-and-dot trendline, heparinized plasma – green circles and solid trendline, 1:1 line – dotted. The regression equation and coefficient of determination are listed for each set of data.

analytical measurement range of the assay was extended and a two pump HPLC system was employed to shorten analysis time.

The YMC ODS-AQ HPLC column (120 Å, 2.0 mm × 150 mm) used in the CDC method [14] was replaced with a Phenomenex Synergi Hydro-RP 100 Å, 3.0 mm × 100 mm, 2.5 μm particle column. As the sample cleanup in our method is minimal (protein precipitation and 5-fold dilution of the supernatant), we opted for an LC column with conventional particle size, which we find more robust for biological specimens. For IS, Li and Franke [13] used homogentisic acid, which can be present in plasma at significant concentrations in alcaptonuria, a genetic disorder of phenylalanine and tyrosine metabolism, and the CDC method employed 1-methyluric acid, which elutes long after AA and DTT. We evaluated several compounds, including 3-O-ethyl-ascorbic acid and 4-hydroxy-3-methoxybenzylamine (HMBA), 5-fluorocytosine and 5-fluorouracil, and selected 3,4-dihydroxybenzylamine (DHBA) as an IS. DHBA is not endogenous and elutes significantly closer to AA and before the DTT peak. Unlike homogentisic acid, DHBA is stable under the strong basic conditions required for reduction of DHAA to AA (results not shown), and could be added at the beginning of the sample preparation procedure instead of after the final re-acidification step, thus providing good compensation for experimental variability.

The calibration range was extended to 5000 μmol/L to allow measurement of the high concentrations of vitamin C found in specimens from individuals on IV ascorbic acid therapy. This treatment produces plasma concentrations above 1 mmol/L, much greater than concentrations expected in routine specimens or for individuals on conventional vitamin supplementation.

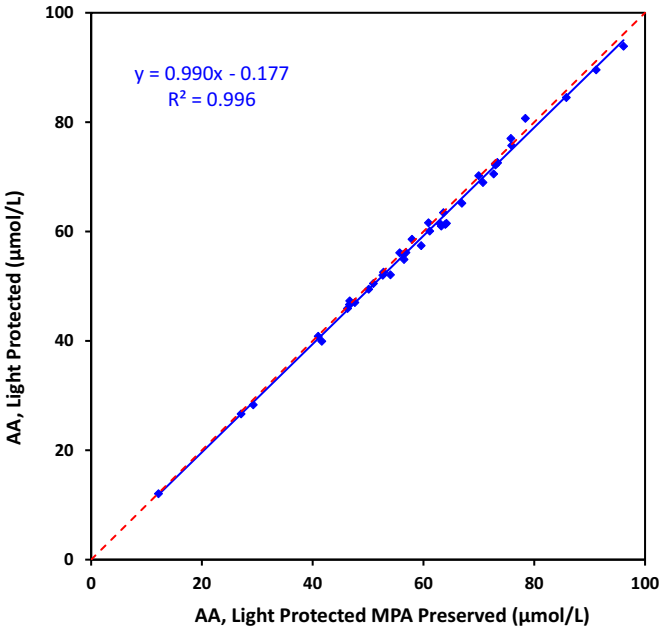

Fig. 5. Comparison of results for light-protected and light-protected/MPA preserved heparinized plasma specimens.

The injection-to-injection time of the newly-developed assay using a single LC pump was 13 min, similar to previously developed HPLC methods [13–15]. To increase method throughput and shorten turnaround time, we implemented a dual LC pump system with an automatic alternating column regeneration. The injection-to-injection time was reduced to 6 min. In addition, the use of the dual pump system allowed diversion of excess reducing reagent to waste and washing of the LC column "off-line" with a significantly higher percent methanol than is possible in the single LC pump setup. The modification facilitated faster equilibration of the current in the analytical cell and increased analytical column lifetime.

The new method affords shorter sample preparation time (from 7.5 h to 2.5 h) using less specimen volume (from 1200 μL to 200 μL). While the analysis time increased from 0.5 to 6 h (based on a batch of 60 samples), the technologist involvement was approximately 0.5 h for both methods: manual reading of individual cuvettes in the spectrophotometric method versus data review in the HPLC-based method. Hence the hands-on time was reduced from 8 to 3 h. The use of concentrated sulfuric acid was eliminated and the amount of biohazardous waste was reduced significantly. Frozen, light-protected unpreserved heparinized plasma or light-protected PST specimens are suitable for analysis. Vitamin C in specimens collected and stored using these conditions provides concentrations well within expected limits of biological variation (intraindividual biological CV 20%) [16]. The AA stereoisomer, isoascorbic acid, co-elutes with the vitamin in this method. IAA has not been detected in human plasma or serum [15,17,18] and is not expected to occur in appreciable concentration in specimens submitted for vitamin C analysis.

Evaluation of results for several thousand specimens submitted for testing over one year showed adequate plasma vitamin C in 66.2% of specimens tested and deficient concentrations (< 23 μmol/L) in 31.3%. Approximately 17% of these specimens contained less than 11.4 μmol/L, the AA concentration associated with risk of clinical symptoms of scurvy. In contrast, 7% of the U.S. population sampled as part of the 2003–2004 National Health and Nutrition Examination Survey (NHANES), were significantly deficient [19]. The overall age-adjusted mean of the NHANES subjects, 51.4 μmol/L, compared well with the mean of our small study of healthy, non-smoking adults (59.1 μmol/L), and indicates adequate intake of vitamin C [20]. Concentrations consistent with oral (> 115 μmol/L) and intravenous (> 1 mmol/L) supplementation were detected in 2% and 0.5%, respectively, of specimens tested.

5. Conclusions

In conclusion, we have developed, thoroughly characterized, and validated a robust method for the determination of ascorbic acid in plasma using 3,4-dihydroxybenzylamine as a stable, non-endogenous internal standard. The assay can be performed in a standard single-LC pump/single column configuration, or in a high-throughput mode, utilizing a two-LC pump HPLC-ECD system with automatic alternating column regeneration.

Table 3
Validation parameters – dual LC pump method with alternating column regeneration.

Linearity

Matrix-matched samples prepared at 5.0, 10, 100, 500, and 5000 μmol/L. Analyzed 6 aliquots of each concentration over 3 days on both columns.
The assay was linear 5–5000 μmol/L.

	Column 1	Column 2
Deming regression:	y = 1.040x–0.48; observed error 2.8%	y = 1.041x–0.56; observed error 2.5%
Means (μmol/L)	4.8, 19.8, 106.5, 517.6, 5310.1	5.1, 20.0, 106.5, 517.2, 5305.7
Accuracy (%)	96.9, 98.8, 106.5, 103.5, 106.2	101.4, 100.2, 106.5, 103.4, 106.1
Precision (CV, %)	3.0, 5.2, 3.5, 2.6, 1.6	4.2, 4.6, 3.7, 2.4, 1.8

Limit of Quantification (LOQ)

Matrix-matched samples prepared by spiking UV-depleted heparinized plasma with ascorbic acid to 0.625, 1.0, 2.5, 5.0, and 10 μmol/L. Analyzed 10 aliquots of each concentration over 3 days on both columns.

Column 1 LOQ = 1.9 μmol/L (EP Evaluator estimate)

Target Concentration (μmol/L)	Mean Measured Concentration (μmol/L)	SD (μmol/L)	Fitted CV (%)	Accuracy (%)
1.0	1.0	0.2	24.7	105
2.5	2.3	0.4	12.7	93
5.0	4.8	0.1	7.8	97
20.0	19.8	1.0	4.3	99
100.0	106.5	3.7	3.4	107

Column 2 LOQ = 1.0 μmol/L (assigned)

Target Concentration (μmol/L)	Mean Measured Concentration (μmol/L)	SD (μmol/L)	Fitted CV (%)	Accuracy (%)
1.0	1.1	0.1	17.6	108
2.5	2.3	0.3	10.4	92
5.0	4.8	0.2	7.0	95
20.0	19.8	1.0	4.7	99
100.0	106.2	4.0	4.1	106

System LOQ = 1.9 μmol/L (assigned)

Analytical Measurement Range

5–5000 μmol/L

Imprecision

Controls were assayed in duplicate in nine runs over 5 days.

Column 1		Column 2	
Concentration (μmol/L)	Total CV (%)	Concentration (μmol/L)	Total CV (%)
22.9	3.0	23.0	3.2
108.6	3.3	109.4	4.3

Accuracy/Method Comparison

Self-reported healthy donor specimens
Reference interval specimens analyzed by the single LC pump method and the evaluated method (dual LC pump).
Deming regression: y = 0.992x − 2.19; Sy/x = 2.37; R = 0.9898; n = 40

Carryover

No carryover observed after plasma specimen at 18,740 μmol/L concentration.

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References

[1] M. Levine, A. Katz, S.J. Padayatty, Vitamin C, in: M.E. Shils, M. Shike, A.C. Ross, B. Caballero, R.J. Cousins (Eds.), Modern Nutrition in Health and Disease, 10th ed. Lippincott Williams & Wilkins, Philadelphia, 2006, pp. 507–524.
[2] A. Shenkin, N.B. Roberts, Vitamins and trace elements, in: C.A. Burtis, E.R. Ashwood, D.E. Bruns (Eds.), Tietz Textbook of Clinical Chemistry and Molecular diagnostics, 5th ed. Elsevier Saunders, St. Louis, 2012, pp. 895–983.
[3] J. Mandl, A. Szarka, G. Banhegyi, Vitamin C: update on physiology and pharmacology, Br. J. Pharmacol. 157 (2009) 1097–1110.

[4] J. Du, J.J. Cullen, G.R. Buettner, Ascorbic acid: chemistry, biology and the treatment of cancer, Biochim. Biophys. Acta 2012 (1826) 443–457.
[5] P.W. Washko, R.W. Welch, K.R. Dhariwal, Y. Wang, M. Levine, Ascorbic and dehydroascorbic acid analyses in biological samples, Anal. Biochem. 204 (1992) 1–14.
[6] R.J. Henry, D.C. Cannon, J.W. Winkelman, Clinical Chemistry Principles and Techniques, 2nd ed. Harper and Row, Hugerstown (1974), pp. , 1974, 1395–1398.
[7] L. Novakova, P. Solich, D. Solichova, HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids, Trends Anal. Chem. 27 (2008) 942–958.
[8] S.A. Margolis, M. Vangel, D.L. Duewer, Certification of standard reference material 970, ascorbic acid in serum, and analysis of associated inter-laboratory bias in the measurement process, Clin. Chem. 49 (2003) 463–469.
[9] CLSI, Interference Testing in Clinical Chemistry; Approved Guideline–Second Edition, CLSI document EP7-A2, Clinical and Laboratory Standards Institute: Wayne, PA, 2005.
[10] A. Karlsen, R. Blomhoff, T.E. Gundersen, High-throughput analysis of Vitamin C in human plasma with the use of HPLC with monolithic column and UV-detection, J. Chromatogr. B 824 (2005) 132–138.
[11] A. Karlsen, R. Blomhoff, T.E. Gundersen, Stability of whole blood and plasma ascorbic acid, Eur. J. Clin. Nutr. 61 (2007) 1233–1236.
[12] A.H.B. Wu (Ed.), Tietz Clinical Guide to Laboratory Tests, 4th ed. Saunders Elsevier, St. Louis, 2006.
[13] X. Li, A.A. Franke, Fast HPLC-ECD analysis of ascorbic acid, dehydroascorbic acid and uric acid, J. Chromatogr. B 877 (2009) 853–856.
[14] E.J. Sampson, Vitamin C in Serum by HPLC with Electrochemical Detection, CDC Environmental Health Laboratory Procedure Manual, May 2006.
[15] J. Lykkesfeldt, 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. 282 (2000) 89–93.
[16] D.K. Talwar, M.K. Azharuddin, C. Williamson, Y.P. Teoh, D.C. McMillan, D.S.J. O'Reilly, Biological variation of vitamins in blood of healthy individuals, Clin. Chem., 51, , 2005, 2145–2150.
[17] M.A. Kall, C. Andresen, Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples, J. Chromatogr. B 730 (1999) 101–111.
[18] S.A. Margolis, R.M. Schapira, Liquid chromatographic measurement of L-ascorbic acid and D-ascorbic acid in biological samples, J. Chromatogr. B 690 (1997) 25–33.
[19] R.L. Schleicher, M.D. Carroll, E.S. Ford, D.A. Lacher, Serum vitamin C and the prevalence of vitamin C deficiency in the United States: 2003-2004 National Health and Nutrition Examination Survey (NHANES), Am. J. Clin. Nutr. 90 (2009) 1252–1263.
[20] M. Levine, S.C. Rumsey, R. Daruwala, J.B. Park, Y. Wang, Criteria and recommendations for vitamin C intake, J. Am. Med. Assoc. 281 (1999) 1415–1423.