

# HPLC-UV method for the simultaneous determinations of ascorbic acid and dehydroascorbic acid in human plasma

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Received 3 Nov 2015

Revised 16 Dec 2015

Accepted 21 Jan 2016

## Keywords

Ascorbic acid,  
Dehydroascorbic acid,  
Dithiothreitol,  
HPLC,  
Method validation

pISSN: 2289-0882

eISSN: 2383-5427

A high performance liquid chromatography (HPLC) paired with UV-vis detection method to determine ascorbic acid and its oxidation product, dehydroascorbic acid, in human plasma was developed. Ascorbic acid in human plasma was extracted and stabilized using 10% metaphosphoric acid, and was analyzed by a Symmetry C18 column with 5 mM Hexadecyltrimethylammonium bromide and 50 mM  $\text{KH}_2\text{PO}_4$  solution as the mobile phase (1.0 mL/min flow rate). Isoascorbic acid served as the internal standard and ultraviolet detector wavelength was 254 nm and 265 nm. Dehydroascorbic acid concentration was calculated from the differences in ascorbic acid concentration before and after reduction by dithiothreitol reagent. Quantification for ascorbic acid in human plasma was linear from 1–100  $\mu\text{g/mL}$ . The inter- and intra-day precisions and accuracy were determined and the results were found to be within  $\pm 15\%$ . This method was successfully applied to a human pharmacokinetic study of ascorbic acid as well as dehydroascorbic acid after oral administration of 4,000 mg vitamin C tablets to healthy Korean volunteers.

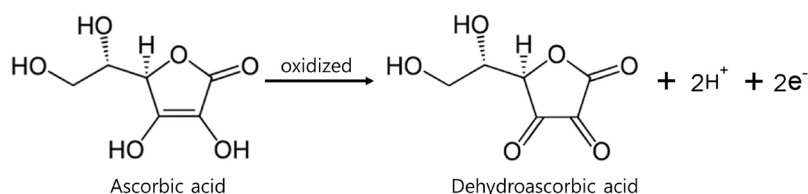
## Introduction

Vitamin C, a naturally occurring compound, plays an essential role in the human body and possesses a variety of biological, pharmaceutical, and dermatological functions. Vitamin C is present in human blood at an average concentration of 50–100  $\mu\text{M}$ , and at least 95% of that is typically in the reduced ascorbic acid (AA) form while the remaining 5% is in the oxidized dehydroascorbic acid (DHAA) form (Fig. 1).[1]

AA is a small and water-soluble antioxidant found in human plasma and is of major importance for protection against diseases caused by oxidative stress.[2] DHAA is also a compound of interest for its role in normal cellular homeostasis.[3] Furthermore, DHAA is well known to exhibit an antiscorbutic effect (as does AA).[4] DHAA concentrations in plasma are low, which is known to be related to rapid tissue uptake and the activity of DHAA reductase.[1] This enzyme activity in cells is dependent on both AA and glutathione, the dominant intracellular reductant.[5] Low plasma AA concentrations could possibly

lead to the impaired transport of DHAA, impaired reduction of DHAA back to AA, or both.[6] Moreover, the ratio of DHAA to AA concentrations is known to be a marker of oxidative stress.[7] The detection of AA and DHAA at low concentrations in human blood is very important for the determination of the vitamin's biochemical functions. However, the accurate detection of AA is not easy because it is very unstable in the presence of air, moisture, light, heat, metal ions, oxygen, or basic media, and it easily decomposes into biologically inactive compounds, such as 2,3-diketo-L-gulonic acid, oxalic acid, L-threonic acid, and L-xylonic acid.

AA is typically detected using a reversed-phase high performance liquid chromatography and ultraviolet detector (HPLC-UV) system at the wavelengths of 265 nm or 240 nm. Although several indirect methods are available for the measurement of AA, they generally have low specificity. Although the direct measurement of DHAA has been demonstrated, the described, indirect methods use highly sensitive HPLC electrochemical detector (ECD) equipment.[8–11] The assessment of DHAA concentration is based upon the difference between AA and total AA concentrations in human plasma.[12] For these reported methods,[8,10,11,13] total AA is estimated after the reduction of DHAA to AA, and DHAA is then calculated from the dif-



**Figure 1.** Chemical structure of ascorbic acid and dehydroascorbic acid as depicted through a redox reaction

ference between total AA and free AA (reduced form) that was originally present in the sample.

Numerous analytical methods, including spectrophotometry,[8] amperometry[10,13] and voltammetry[14] have been developed for the determination of AA in foods, pharmaceuticals, and biological samples. AA is an electroactive species that has been easily detected using amperometric and coulometric systems. However, DHAA is difficult to detect by electrochemistry because of its low electroactivity. Moreover, it has a weak absorbance in the UV region. Therefore, electrochemical detection of DHAA is usually carried out by first reducing DHAA to AA with dithiothreitol (DTT), in order to calculate the difference of AA concentrations before and after the reduction of DHAA. [12,15,16]

The aim of this study was to develop and validate an HPLC method for the simultaneous determinations of AA and DHAA in human plasma with rapid and simple sample preparation. This method should be applicable to the determination of AA in human pharmacokinetic studies.

## Methods

### Chemicals and Reagents

L-ascorbic acid was purchased from Shinyo Pure Chemical Co. (Osaka, Japan). D-isoascorbic acid, metaphosphoric acid ( $\text{HPO}_3$ ), potassium dihydrogenphosphate ( $\text{KH}_2\text{PO}_4$ ), hexadecyl trimethyl ammonium bromide (HTAB), and dithiothreitol reagent (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and water were purchased at the highest grade available from Fisher Scientific (Fair Lawn, NJ, USA). All other reagents were of analytical grade and were purchased from Sigma-Aldrich.

### HPLC conditions

Chromatographic analysis was performed using an HPLC system (GILSON, Wisconsin, USA) with 254 nm and 265 nm wavelengths. Chromatographic separations were performed using a C18 (symmetry® 4.6  $\mu\text{m} \times 280$  mm, particle size 5  $\mu\text{m}$ ) (Waters, Milford, MA, USA). The mobile phase consisted of 5 mM HTAB + 50 mM  $\text{KH}_2\text{PO}_4$ . The flow rate was kept constant at 1.2 mL/min.

### Preparation of standard solution and determination of AA

Standard stock solutions of AA and D-isoascorbic acid were prepared as internal standards (IS) in 50% methanol at 1 mg/mL each. The working solutions of AA were diluted with 50% methanol to prepare standards of 10, 20, 40, 100, 500, 800, and 1,000  $\mu\text{g/mL}$ . Human plasma was depleted of AA by leaving it on the bench for 96 h as described in a study by

Karlsen et al.[17] The plasma calibration curves were prepared at concentrations of 1, 2, 4, 10, 50, 80, and 100  $\mu\text{g/mL}$  by spiking 400  $\mu\text{L}$  of blank plasma (AA free status) with 50  $\mu\text{L}$  of each working standard, and 50  $\mu\text{L}$  of the IS (100  $\mu\text{g/mL}$ ) of each analyte was added at the appropriate concentration. The stability of AA in blood is a very critical issue because it rapidly degrades to DHAA, thus metaphosphoric acid was employed as a very efficient stabilizer and protein precipitator for the AA analysis.[18] Five hundred microliter of 10% metaphosphoric acid ( $\text{HPO}_3$ ) was carefully added to each sample. Then, samples were vortexed for 1 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were transferred to injection vials, and 10  $\mu\text{L}$  were injected into an HPLC system for analysis.

### Determination of total AA

The blank plasma (400  $\mu\text{L}$ ) was subjected to reduction with the additions of 50  $\mu\text{L}$  of working standard solution (addition of 2 mM DTT at pH 7.8) and 50  $\mu\text{L}$  of IS. The solution reacted for 20 min at room temperature in the dark. Five hundred microliter of 10%  $\text{HPO}_3$  was carefully added to each sample. Then, the samples were vortexed for 1 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were transferred to injection vials, and 10  $\mu\text{L}$  was used for chromatographic analysis. The concentration of DHAA was determined by subtracting the concentration of AA from the total AA concentration.

### Method Validation

The method for the determination of AA in human plasma was validated by using spiked solutions of AA in AA-depleted human heparin plasma. The method validation parameters studied were specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, and recovery. The specificity of the method was investigated by comparing chromatograms of extracted blank plasma obtained from six different human plasma samples spiked with AA and IS to ensure that they were free of interference around the retention time of AA. The LOD was determined by diluting solutions of known concentration until the response was three times the noise (S/N 3), and the LOQ was defined as the lowest concentration that can be calculated on the basis of minimal accepted value of S/N 10. Linearity of the method was determined with a calibration curve constructed using seven concentration points.

Calibration curves were constructed by plotting the response ratios (ratios of peak areas of analytes to IS) versus concentration of each analyte using a linear least squares regression. The precision study was carried out based on injection repeatability and analysis repeatability of spiked plasma samples. Injection repeatability was determined by repeated injections of plasma samples spiked with standard mixtures equivalent to each analyte into the HPLC system. The accuracy of the method was defined by replicate analysis of samples containing known amounts of the analyte. The deviation of the mean from the true value serves as the measure of accuracy. The recovery of the method was determined as the ratio of the peak area of extracted samples after a full assay to that of a direct injection of equivalent concentration (in 50% methanol). The intra-day precision and accuracy were determined within one day by analyzing five replicate samples. The inter-day precision and accuracy were determined on five separate days at identical concentrations.

### Applications in pharmacokinetic studies

The pharmacokinetic study followed the principles of the Declaration of Helsinki, and was authorized by the Institution Ethics Committee of Chonbuk National University Hospital (IRB No. 2014-09-022). Healthy male volunteers aged  $\geq 19$  and  $\leq 55$  years at screening were included in the study. Normal haematology, blood chemistry, and urinalysis results were required at screening. Subjects with evidence of drug abuse, alcoholism, or any cardiac condition or illness putting the subject at risk were not allowed to be enrolled. The bioanalytical method was applied to study the pharmacokinetics of AA and DHAA in three healthy subjects after administration of 4000 mg of vitamin C tablet. Blood samples (4 mL) were collected into heparinized tubes at pre-dose (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, and 8 h post-dosing. Then plasma was immediately separated by centrifuging at 3,000 rpm for 10 min. The obtained plasma was stored frozen at  $-80^{\circ}\text{C}$  until analysis.

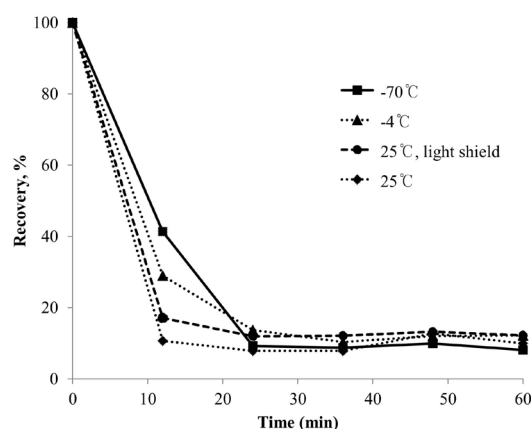
## Results

### Sample preparation

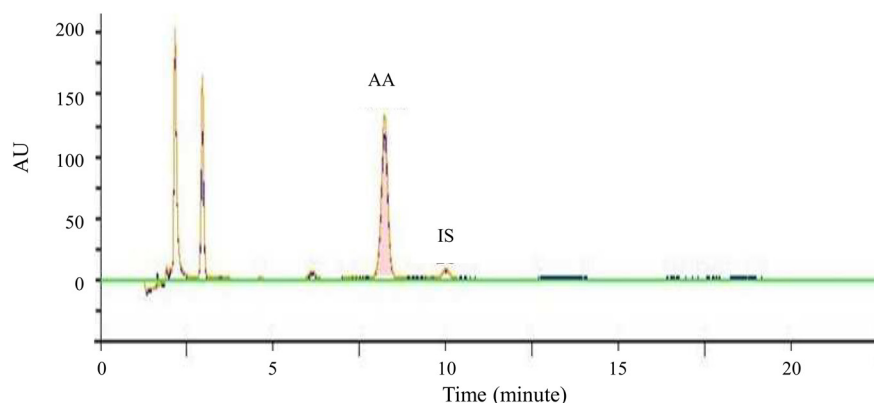
AA stability tests were conducted for the use of human plasma. Plasma samples were stored at  $-70^{\circ}\text{C}$ ,  $-4^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ , and  $25^{\circ}\text{C}$  while masked from light masked during 60 min periods. The results showed that at  $-70^{\circ}\text{C}$ , the highest relative recovery was achieved after around 10 min (Fig. 2). However, all trends indicated the instability of AA in plasma with heat and light over time; therefore, we attempted the sample preparation of AA in plasma after removing from storage at  $-70^{\circ}\text{C}$  in each case.

To prevent oxidation of AA in plasma samples, the addition of a stabilizer like  $\text{HPO}_3$  is crucial, and it should be added immediately after blood sampling (especially for clinical studies).

Several concentrations of DTT (reduction agent) have been employed: 2 mM, 4 mM, and 8 mM. Since similar total AA concentrations were determined for each DTT concentration,



**Figure 2.** Recovered ascorbic acid contents in standard solutions stored at  $-70^{\circ}\text{C}$  (■),  $-4^{\circ}\text{C}$  (▲),  $25^{\circ}\text{C}$  (◆) and light shielded at  $25^{\circ}\text{C}$  (●)



**Figure 3.** Chromatogram of ascorbic acid and internal standards in human plasma. HPLC conditions: mobile phase, 5 mM HTAB + 50 mM  $\text{KH}_2\text{PO}_4$ ; column, C18 column (symmetry<sup>®</sup> 4.6  $\mu\text{m}$   $\times$  280 mm, 5  $\mu\text{m}$  particle size, Waters, USA); flow rate, 1.2 mL/min; UV detection wavelength, 254 and 265 nm.

2 mM of DTT was used for all analyses. The amount of DHAA was calculated from the following difference: Total AA minus reduced AA.

### Chromatographic separation

A representative HPLC chromatogram obtained from a human plasma sample is shown in Figure 3. The identification of the corresponding peaks was performed by comparing the retention times in the samples with those of the separately run pure standards under the same analytical conditions.

### System suitability

The lowest limit of quantification for each validated batch (1 µg/mL, LLOQ) of the samples was measured at least three times, and the system suitability was established as being less than 20% for the measured concentration deviation to average concentration ratio (coefficient variance, CV).

### Linearity

The calibration curve of the blank, zero-blank (internal standard added), and seven standards ranging with 1–100 µg/mL of concentration had a correlation coefficient ( $r^2$ ) of 0.999, which indicated excellent linearity (Fig. 4).

### Specificity

The retention times (RT) of AA and IS were about 9 min and 12 min, respectively. Blank plasma samples of different origins (six different blank samples, one zero-blank sample, and a LLOQ sample) were analyzed, and no interference was introduced by human plasma around the retention time of AA and IS.

### Carryover

We investigated the possibility of carryover (order effects) between evaluations of the LLOQ, blank, and upper limit of quantification (ULOQ = 100 µg/mL) samples. No carryover effects

were observed.

### Accuracy, precision, and limit of quantification for concentration

Accuracy and precision were determined using four concentrations (1, 2, 50, 80 µg/mL) of the quality control (QC) samples for the intra-day and inter-day; measurements were performed five times for each. The accuracy (average % and its CV) was defined as the average and CV of the ratios of measured concentrations (calculated using the calibration curve) divided by the nominal concentrations. Precisions within  $\pm 15\%$  and accuracies within 85–115% were established as qualifying conditions.[19] At the limit of quantification concentration (1 µg/mL), a separate set of qualifying conditions were established: a precision within  $\pm 20\%$  and accuracy within 80–120%. The accuracy and precision of intra-day measurements of the analytical method were 86.43–103.52% and 1.33–15.47%, respectively. The accuracy and precision of inter-day measurements were 85.86–101.65% and 0.65–12.89%, respectively (Table 1).

### Recovery

AA recovery tests were performed for three QC concentrations, and the absolute recovery of IS (absolute recovery) was

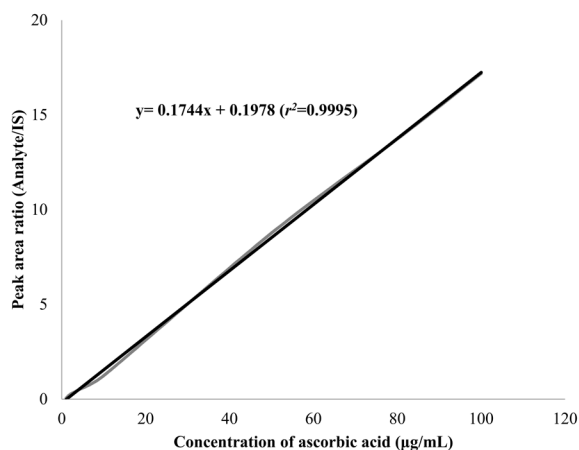


Figure 4. Standard calibration curve of ascorbic acid in human plasma

Table 1. Intra- and inter-day precision and accuracy ( $n=5$ ) of ascorbic acid QC samples in human plasma

Statistical variable	Concentration (µg/mL)			
	1	2	50	80
Intra-day				
Mean	0.86	1.86	51.76	79.18
CV (%)	15.47	5.28	2.77	1.33
Accuracy (%)	86.43	92.82	103.52	98.98
Inter-day				
Mean	0.86	1.81	50.82	79.48
CV (%)	9.55	12.89	1.60	0.65
Accuracy (%)	85.56	90.26	101.65	99.34

Table 2. Intra- and inter-day recovery of ascorbic acid in human blank plasma QC samples ( $n=5$ )

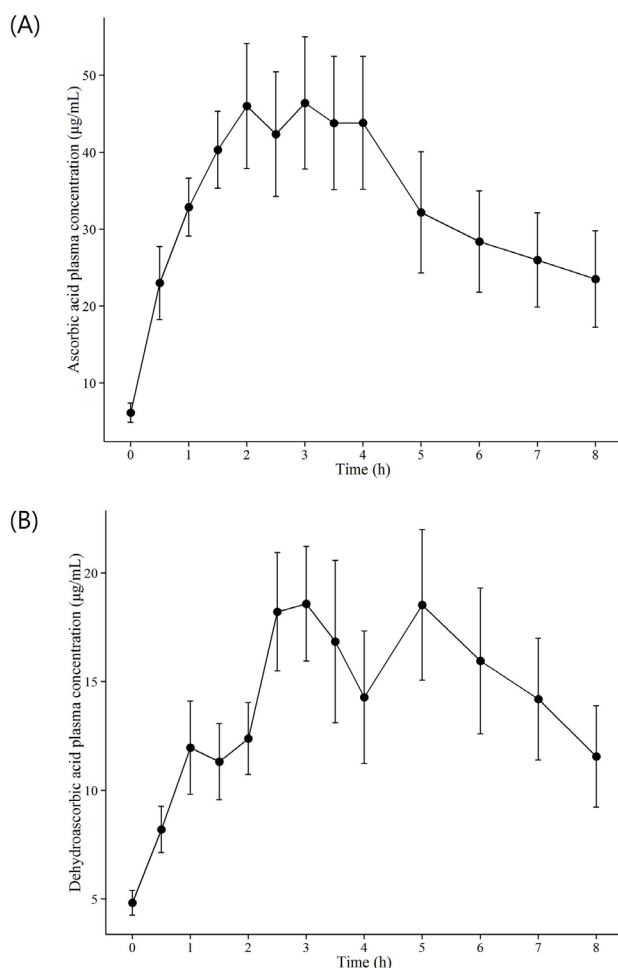
Concentration (µg/mL)	Recovery (%; Mean $\pm$ S.D)	
	Intra-day	Inter-day
1	106.00 $\pm$ 3.20	98.98 $\pm$ 6.52
2	108.30 $\pm$ 4.39	110.05 $\pm$ 12.84
50	118.41 $\pm$ 3.44	109.41 $\pm$ 8.93
80	100.89 $\pm$ 2.69	104.01 $\pm$ 2.72

**Table 3.** Dehydroascorbic acid concentrations

Free AA (μg/mL)	Total AA (μg/mL)	DHAA
AA 50 μg/mL	AA 50 μg/mL + 2 mM DTT	Total AA – free AA
37.06	44.24	7.18

**Table 4.** Pharmacokinetic parameters for ascorbic acid and dehydroascorbic acid in human plasma (mean±SD) obtained after administration of vitamin C 4,000 mg

Parameters	AA	DHAA
C <sub>max</sub> (ng/mL)	49.57±23.93	20.49±8.32
T <sub>max</sub> (h)	2.67±0.58	3.83±1.04
T <sub>1/2</sub> (h)	7.12±3.79	4.65±1.27
AUC <sub>last</sub> (h*ng/mL)	270.02±159.71	115.11±61.42
AUC <sub>inf</sub> (h*ng/mL)	575.18±527.74	187.57±99.99



**Figure 5.** Mean plasma concentration-time profiles of ascorbic acid (A) and dehydroascorbic acid (B)

also carried out. The recovery of AA and internal standard were 98.98-118.41% and 99.99%, respectively (Table 2).

### DHAA concentration

The concentration of DHAA was calculated by subtracting the initial AA concentration from the total AA concentration after reductive treatment of 50 μg/mL of AA (Table 3). These results demonstrate that it is possible to utilize the described HPLC-UV method to accurately determine the amount of AA and DHAA in plasma samples, which contradicts assertions made by Li and Franke that HPLC-UV is not suitable for AA determination in plasma.[10] Since the total time required for the analysis of one sample (including the sample preparation) was less than 15 minutes, further improvement in the analytical reliability is expected in an AA assay.

### Pharmacokinetic study

The validated HPLC method has been successfully applied to a pharmacokinetic study of AA and DHAA after oral administration of 4,000 mg AA tablet to three healthy Korean volunteers with age of (27.67±8.96) years, weight of (61.17±16.86) kg, height of (164.00±12.88) cm, and BMI of (22.37±2.45) kg/m<sup>2</sup>. The mean plasma concentration-time profiles of AA and DHAA are shown in Figure 5 and the related pharmacokinetic parameters calculated using Phoenix WinNonlin 6.3 software (Pharsight Corporation, Mountain View, CA, USA) are listed in Table 4. As a results, T<sub>max</sub> (2.67±0.58 h) of the AA was consistent with those reported before (T<sub>max</sub>: 3 h).[20] Unlike previous reports on the assays of DHAA using HPLC-UV or HPLC-ECD,[13,21,22] the HPLC-UV method in this report was applied for the human pharmacokinetic study of AA and DHAA.

### Discussion

A validated and rapid isocratic HPLC-UV method was successfully developed for the simultaneous determination of AA and DHAA levels in human plasma. The method offers good precision, accuracy, and reproducibility for the determination of AA within a 10 min window and is well suited to routine measurements and/or high-throughput clinical analysis. The LOQ for AA was 1 μg/mL. The use of a standard reversed-phase column enabled good performance for the elution and separation of AA. The method involves a simple and cheap preparation step. The validated method was successfully applied to the pharmacokinetic study of AA and DHAA in healthy subjects after administration of 4,000 mg of vitamin C.



## Acknowledgements

This paper was supported by funding from the Global Center of Excellence in Early Clinical Trials of Chonbuk National University Hospital.

## Conflict of interest

The authors have no conflicts of interest to disclose.

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