Analytical Methods

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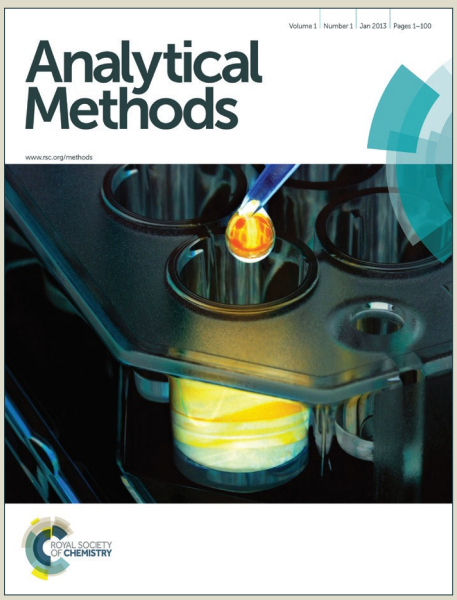

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ARTICLE

Simultaneous determination of L-ascorbic acid and dehydroascorbic acid in human plasma

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A method combining high-performance liquid chromatography with ultraviolet detection (HPLC-UV) was developed and validated for the accurate determination of L-ascorbic acid (AA) and dehydroascorbic acid (DHAA) concentrations in human plasma. In conventional HPLC-UV analysis, DHAA is indirectly measured by subtracting the native AA concentration from the total AA concentration. However, it is important to develop a direct method for the accurate and rapid analysis of AA and DHAA. Analyses were performed on a Primesep SB column (4.6 mm × 250 mm, particle size 5 µm), and the mobile phase consisted of 0.1% formic acid in water, 80%; and 0.08% formic acid in acetonitrile, 20%. The intra- and inter-day accuracies of the AA assay were 95.92-98.18% and 92.22-98.22%, respectively. The intra- and inter-day accuracies of the DHAA assay were 93.34-97.73% and 99.42-97.53%, respectively. The calibration curve was linear within the tested range of 2-100 µg/mL for AA and 10-200 µg/mL for DHAA. This HPLC method is a highly sensitive and reproducible analytical method for the simultaneous determination of ascorbic acid and DHAA in human plasma.

Introduction

Ascorbic acid (AA) is an important antioxidant in humans. However, humans cannot synthesize AA and must consume it in food or supplements [1]. Higher plasma concentrations of AA can reduce oxidative damage at the cellular level, which may extend lifespans by blocking the aging effects of active oxygen [2, 3] and prevent various cancers [4], cardiovascular disease [5], and cataracts [6]. AA and its first oxidation product, dehydroascorbic acid (DHAA), are important biomarkers for the evaluation of oxidative stress, and the accurate and sensitive measurement of AA and DHAA is crucial to the assessment of their roles as biomarkers in disease diagnostics [7, 8]. To improve research on oxidative stress factors and their diagnostic assessment, we intended to establish a fast, accurate, and robust assay to analyze AA and DHAA concentrations.

The unstable nature of AA and its products is a challenge to their accurate quantification, especially considering the relatively low concentrations of AA present in plasma. Various analytical procedures for determining AA concentrations have been reported, including spectrometric assays [9], a high-performance liquid chromatography-ultraviolet (HPLC-UV) method [10, 11], and HPLC-electrochemical detection (ECD) [12, 13]. Among these methods, the HPLC-UV method is the most widely used clinically because of its simplicity and relatively low cost. However, currently, there is no direct and simultaneous UV measurement for AA and DHAA in human plasma because AA and DHAA have different UV absorption maxima: The absorption maximum (λ_{max}) of AA lies in the range 244-265 nm, whereas that of DHAA is 185 nm [14]. In previous studies, UV detection did not prove suitable for DHAA analysis because DHAA exhibits little absorbance above 220 nm [15]. As a result, DHAA concentration is typically calculated by substrating the native AA concentration from the total AA (TAA) concentration, where the latter is generated by reducing native DHAA to AA using dithiothreitol (DTT) as a common reducing agent [16, 17]. However, this method cannot achieve sufficient selectivity for the bioanalysis of complex matrices, such as human plasma, for both AA and DHAA. Moreover, the indirect method commonly used for DHAA analysis is labor intensive and insufficiently accurate. HPLC-UV is frequently used in pharmaceutical and food analyses.

The objective of this study was to identify a simultaneous determination method that is capable of measuring AA and DHAA concentrations in human plasma with high precision. The HPLC method was evaluated due to its high speed and sensitivity.

Experimental

Reagents

L-AA, DHAA, meta-phosphoric acid (MPA), and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile and water were purchased at the highest grade available from Fisher Scientific (Seoul, Korea). All other reagents used were of analytical grade and were purchased from Sigma-Aldrich.

HPLC conditions

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Chromatographic analysis was performed using an HPLC system (GILSON, Wisconsin, USA) at wavelengths of 190 nm and 230 nm. Chromatographic separations were performed using a Primesep SB column (4.6 mm × 250 mm, particle size 5 µm) (SIELC, Wheeling, IL, USA). The column temperature was maintained at 25 °C and the autosampler tray temperature was maintained at 4 °C. The mobile phase consisted of 0.1% formic acid in water (80%) and 0.08% formic acid in acetonitrile (20%). The flow rate was kept constant at 0.7 mL/min.

Standard preparation and calibration curve

Standard stock solutions of AA and DHAA were prepared with acetonitrile and 0.1% acetic acid in water (50:50, pH 3.7) at 1 mg/mL and 2 mg/mL each. Working solutions of AA were diluted with acetonitrile and 0.1% acetic acid in water (50:50) to prepare standards of 20, 40, 80, 100, 500, 800, and 1,000 µg/mL. DHAA working solutions were prepared at concentrations of 100, 200, 400, 1,000, 1,500, 1,800, and 2,000 µg/mL. Human blank plasma was prepared via the removal of AA and DHAA by incubation at room temperature (21 °C) for 120 hours. This plasma was frozen and stored after the HPLC peak of AA in the plasma was measured. Plasma calibration curves were prepared at concentrations of 2-100 µg/mL for AA and 10-200 µg/mL for DHAA by spiking 400 µL of blank plasma (AA depleted) with 50 µL of each working standard. The stability of AA in blood is an issue because AA rapidly degrades to DHAA. MPA (pH 1.2) was employed as a highly efficient stabilizer and protein precipitator for AA analysis. First, 500 µL of 10% MPA 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 µL was injected into an HPLC system for analysis.

Method validation

The HPLC method was validated for its specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, and recovery parameters. The specificity of the method was investigated by comparing the chromatograms of extracted blank plasma obtained from six different human plasma samples spiked with AA and DHAA to ensure that the samples were free of interference near the retention time of AA and DHAA. The LOD was determined by diluting solutions of known concentrations until the response was three times the noise (S/N ratio: 3), and the LOQ was defined as the lowest concentration that could be calculated based on the minimal accepted S/N ratio of 10. The linearity of the method was verified using a calibration curve constructed with seven concentration points. Calibration curves were constructed by plotting the response ratios versus the concentrations of each analyte using a linear least squares regression. A precision study was conducted based on the repeatability of the injection and analysis of the spiked plasma samples. The injection repeatability was determined by the repeated injection of plasma samples spiked with standard mixtures equivalent to each analyte into the HPLC system. The accuracy of the method was defined by the replicated analysis of samples containing known amounts of analyte. The deviation of the mean from the true value serves as a measure of accuracy. The recovery parameter was determined to be the ratio of the peak area of the extracted samples after the complete assay to the peak area of a direct injection of equivalent concentration (in the mobile phase). The intra-day precision and accuracy were determined within one day by analyzing five replicates. The inter-day precision and accuracy were determined on five separate days using identical concentrations.

Results and discussion

HPLC-UV method development

A representative HPLC chromatogram obtained from a human plasma sample is shown in Figure 1. The identification of corresponding peaks was performed by comparing the retention times of the samples with the retention times of the separately run pure standards under the same analytical conditions. The AA ion is the predominant species at typical biological pH values, with a mild reducing agent, and with an antioxidant. AA is oxidized via the loss of one electron to form a radical cation and by the loss of a second electron to form dehydroascorbic acid. AA typically reacts with oxidants of reactive oxygen species, such as hydroxyl radicals. Both AA and DHAA are highly polar and cannot be retained by a reverse-phase mechanism. In this study, an analytical method for the HPLC separation of AA and DHAA was developed on a Primesep® SB HPLC column. Primesep® SB columns have an anion exchange ligand (quaternary amine) with a C18 carbon chain. Preliminary experiments with C18 columns of various dimensions produced unsatisfactory results. The best analyte separation was obtained using a Primesep® SB column (4.6 × 250 mm, particle size 5 µm). AA and DHAA in human plasma are well separated and produce well resolved peaks; they are eluted at 8.2 min and 3.3 min, respectively, in the HPLC chromatograms. Monitoring the UV wavelength λ_{max} using a DAD (diode-array detector) indicated peaks at 190 nm and 230 nm, respectively.

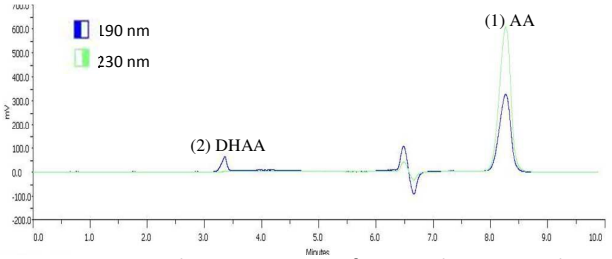

Figure 1. HPLC chromatograms of AA and DHAA in human plasma. Peaks: (1) 2 µg/mL of AA and (2) 10 µg/mL of DHAA. HPLC conditions: isocratic elution (mobile phase, A:B =0.1% formic acid in water, 0.08% formic acid in acetonitrile=80:20). The stationary phase was a Primesep SB analytical column (4.6 × 250 mm, 5 µm particle size, SEILC, USA). The flow rate was kept constant at 0.7 mL/min, and the separation was monitored at 190 and 230 nm.

The instability of AA and DHAA in solution was resolved by decreasing the temperature (i.e., sample extraction on ice and analysis at 4 °C), protecting the samples from light, and adding MPA as a stabilizing agent. Sophisticated sample preparation significantly prevents AA oxidation and DHAA hydrolysis. In this study, to prevent the oxidation of AA in human plasma samples, a stabilizer such as MPA is crucial and should be added immediately after blood sampling (especially for clinical studies).

Method validation

The lowest limit of quantification for each validated batch (2 µg/mL, LLOQ) of the AA samples was measured at least three times, and the system suitability was established by a deviation in measured concentration of less than 20% with respect to the average concentration ratio (coefficient variance, CV). Linearity was assessed by correlation efficiencies and an F-test as suggested by Araujo et al. [18].

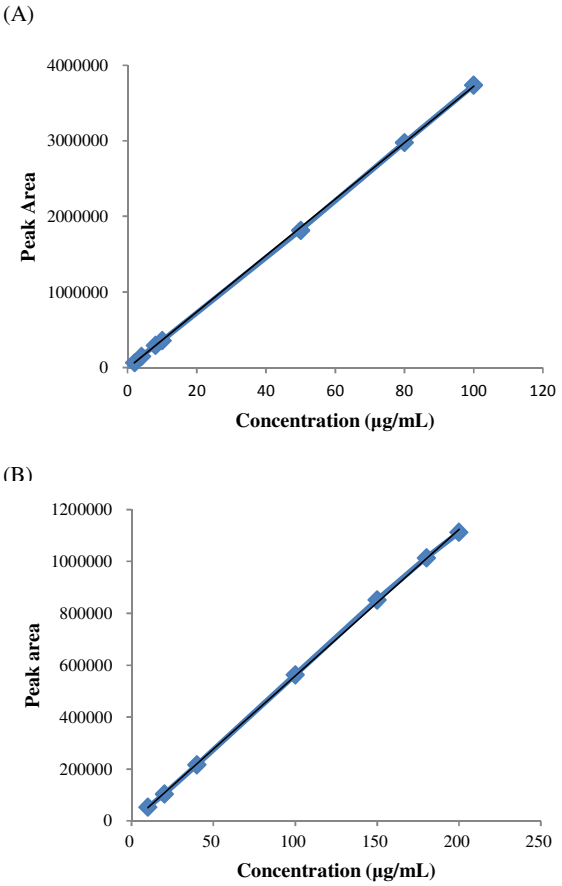

Figure 2. Standard calibration curves for ascorbic acid ((A); concentration range 2 - 100 µg/mL) and dehydroascorbic acid ((B); concentration range 10 - 200 µg/mL) in human plasma

The AA calibration curve of the blank and eight standards with concentrations in the 2-100 µg/mL range had a correlation coefficient (r^2) of 0.9999. The LLOQ of DHAA was 10 µg/mL, the CV was less than 20%, and the calibration curve with concentrations in the 10-200 µg/mL range had a correlation coefficient (r^2) of 0.9997, which indicated linearity (Figure 2). The results of the F-test indicated that the linearity of the calibration curves of the seven analytes was acceptable at 95% confidence level as $F_{calculated}$ (2.19 for AA and 2.35 for DHAA) was below 2.56 (tabulated at the 95% level with 5 and 28 degrees of freedom). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the calibration curve. The LOD and LOQ of AA were 0.55 µg/mL and 1.66 µg/mL and those of DHAA were 3.19 µg/mL and 9.67 µg/mL, respectively.

In the specificity test, the retention times of AA and DHAA were approximately 8.2 min and 3.3 min, respectively. Blank plasma samples of different origins (six different blank samples and an LLOQ sample) were analyzed, and no interference was introduced by human plasma around the retention times of AA and DHAA. We investigated the possibility of carryover (order effects) between evaluations of the LLOQ, blank, and upper limit of quantification (ULOQ = 100 µg/mL and 200 µg/mL) samples, and no carryover effects were observed. The accuracy and precision of the assay were determined using four concentrations (2, 8, 50, and 80 µg/mL for AA and 10, 20, 100, and 180 for DHAA) of quality control (QC) samples for the intra-day and inter-day values; measurements were performed five times for each. The average measured concentrations (calculated using the calibration curve) divided by the known concentration denotes the accuracy and is expressed as a percentage of the ratio. The standard deviation of the measured concentration divided by the average percentage is expressed as a percentage of that ratio (CV %). Precisions within ±15% and accuracies within 85-115% were established as qualifying conditions according to international guidelines [19]. At the limit of quantification concentration (1 µg/mL), a separate set of qualifying conditions was established: precision within ±20% and accuracy within 80-120%. The accuracy and precision of the intra-day measurements of AA were 95.92-98.18% and 0.77-3.32%, respectively. The accuracy and precision of the inter-day measurements of AA were 92.22-98.22% and 0.39-3.02%, respectively (Table 1).

Table 1. Method validation parameters for the determination of AA in human plasma

Statistical variable	Concentration (µg/mL)			
	2	4	50	80
Intra-day				
Mean±SD	1.82±0.05	3.81 ±0.16	48.36 ±0.75	81.49 ±5.15
CV (%)	3.04	4.34	1.57	6.33
Accuracy (%)	91.09	95.20	96.72	100.61
Inter-day				
Mean±SD	1.86±0.05	3.80± 0.08	48.12 ±0.70	77.91 ±0.88
CV (%)	3.10	2.31	1.47	1.13
Accuracy (%)	93.73	95.11	96.23	97.38

Table 2. Method validation parameters for the determination of DHAA in human plasma

Statistical variable	Concentration (μg/mL)			
	10	20	100	180
Intra-day				
Mean±SD	9.39±0.20	18.13 ±0.53	96.27 ±1.54	174.95 ±2.30
CV (%)	2.15	2.93	1.60	1.32
Accuracy (%)	93.87	90.64	96.27	97.19
Inter-day				
Mean±SD	9.40±0.34	18.10 ±0.66	96.78 ±1.29	175.60 ±1.43
CV (%)	3.65	3.65	1.34	0.82
Accuracy (%)	93.97	90.48	96.78	97.56

The accuracy and precision of the intra-day measurements of DHAA were 93.34-97.73% and 0.19-3.34%, respectively. The accuracy and precision of the inter-day measurements of DHAA were 99.42-97.53% and 0.76-3.57%, respectively (Table 2). AA and DHAA recovery tests were performed for three QC concentrations. The extraction of the analyte was followed by its detection, revealing that 95.92-97.18% of AA was recovered (Table 3), and 93.34-97.73% of DHAA was recovered (Table 4).

Table 3. Intra- and inter-day recoveries of standard AA added to human blank plasma

Added (μg/mL)	Intra-day		Inter-day	
	Observed[1)] (μg/mL)	Recovery (%)	Observed (μg/mL)	Recovery (%)
2	1.82±0.07	91.09	1.88±0.06	93.97
4	3.81±0.08	95.20	3.80±0.05	95.07
50	48.36±0.50	96.72	48.01±0.58	96.02
80	78.49±0.90	98.11	77.97±1.99	97.46

[1)] The mean of quintuplicate assays is recorded (mean±SD).

Table 4. Intra- and inter-day recoveries of standard DHAA added to human blank plasma

Added (μg/mL)	Intra-day		Inter-day	
	Observed[1)] (μg/mL)	Recovery (%)	Observed (μg/mL)	Recovery (%)
10	9.39±0.53	93.87	9.30±0.77	93.03
20	18.13±0.55	90.64	18.11±0.94	90.54
100	96.27±0.56	96.27	95.70±3.39	95.69
180	174.97±2.72	97.21	175.55±4.23	97.53

[1)] The mean of quintuplicate assays is recorded (mean±SD).

These results demonstrate that it is possible to use the described HPLC-UV method to accurately determine the concentrations of AA and DHAA in plasma samples, although Li et al. reported that HPLC-UV is not suitable for the determination of AA and DHAA concentrations in plasma [20]. Because the total time required for the analysis of one sample (including sample preparation) was less than 15 minutes, further improvements in the analytical reliability of the AA assay are expected.

A post-preparative stability test was performed after sample preparation using standard solutions. The stabilities of AA and DHAA in spiked human plasma after 24 hours at 4 °C were determined. Triplicate samples spiked at concentrations of 4 μg/mL and 80 μg/mL AA and 20 μg/mL and 180 μg/mL DHAA were maintained at 4 °C for 24 hours before extraction. The samples were then extracted and injected. The mean concentrations of the stability samples were compared to the theoretical concentrations (Table 5). AA exhibited a -9.83% change in the low concentration sample (4 μg/mL) and a -15.64% change in the high concentration sample (80 μg/mL). DHAA exhibited a -7.22% change in the low concentration sample (20 μg/mL) and a -4.26% change in the high concentration sample (180 μg/mL). AA and DHAA were stable at 4 °C for approximately 24 hours.

The simultaneous HPLC analysis of AA and DHAA in human plasma was therefore confirmed to exhibit sufficient linearity, accuracy and precision for use as a quantitative analysis in pharmacokinetic studies.

Table 5. Post-preparative stability of AA and DHAA in human plasma

(A) Ascorbic acid

Post-Preparative 4 °C	Measured Concentration (μg/mL)			
	Low (4 μg/mL)		High (80 μg/mL)	
	Initial	24 hour	Initial	24 hour
1	3.946	3.215	78.945	70.546
2	3.849	3.546	77.542	71.549
3	3.718	2.952	78.154	69.482
Mean	3.838	3.238	78.214	70.526
SD	0.114	0.298	0.703	1.034
CV (%)	2.98	9.20	0.90	1.47
Difference (%)	-15.64		-9.83	

(B) Dehydroascorbic acid

Post-Preparative 4 °C	Measured Concentration (μg/mL)			
	Low (20 μg/mL)		High (180 μg/mL)	
	Initial	24 hour	Initial	24 hour
1	19.846	18.165	178.546	170.549
2	18.946	17.940	177.565	171.549
3	19.463	17.946	178.591	169.846
Mean	19.418	18.017	178.234	170.648
SD	0.452	0.128	0.580	0.856
CV (%)	2.33	0.71	0.33	0.50
Difference (%)	-7.22		-4.26	

Conclusions

The analytical method described in this paper is suitable for the simultaneous analysis of AA and DHAA concentrations in human plasma. This method was simultaneously analyzed using Primesep SB columns, which increased the accuracy of

the assay and reduced the duration of analysis to 3.3 min for DHAA and 8.2 min for AA. This method exhibited satisfactory precision and accuracy. The validation results for this assay prove that this method is robust, sensitive, specific, and accurate. The calibration curve extends from 2 μg/mL to 100 μg/mL for AA and from 10 μg/mL to 200 μg/mL for DHAA, with 400 μL of human plasma. This method is suitable for research on biomarkers for disease diagnostics and can be easily adapted for clinical trials.

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