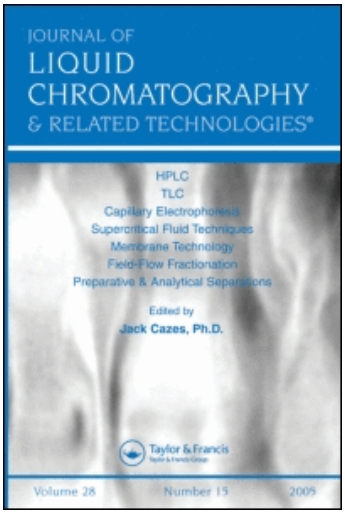

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Simultaneous Determination of Ascorbic Acid, Isoascorbic Acid (Erythorbic Acid) and Uric Acid in Human Plasma by High-Performance Liquid Chromatography with Amperometric Detection

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SIMULTANEOUS DETERMINATION OF ASCORBIC ACID, ISOASCORBIC ACID (ERYTHORBIC ACID) AND URIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION*

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ABSTRACT

A procedure is presented for the direct and simultaneous determination of ascorbic acid (AA), isoascorbic acid (IA), and uric acid (UA) in human plasma by paired-ion reversed-phase high-performance liquid chromatography. An Ultrasphere ODS (C18) column is used with a pH 5.25 mobile phase containing 0.04M sodium acetate, 0.005M tetrabutylammonium phosphate, and 0.2 mg/mL disodium EDTA. Plasma samples preserved with an equal volume of 10% metaphosphoric acid are diluted 10-fold with mobile phase and filtered through 0.2 micron filters. The injection volume is 10 uL. Detection of AA, IA, and UA is by amperometry using a glassy

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carbon electrode and Ag/AgCl reference electrode. The applied potential is +0.6 volt and the sensitivity setting is 100 nAmps. As little as 0.25 ng of each component can be detected at this setting and the electrode response is linear over the AA, IA, and UA ranges encountered in human plasma.

INTRODUCTION

A number of high-performance liquid chromatography (HPLC) methods have been developed for the direct measurement of ascorbic acid (AA) in foods, vitamin preparations, and biological tissues. They use strong anion-exchange (1,2), weak anion-exchange (3,4), reversed-phase (5,6), or paired-ion reversed-phase (7-11) chromatography coupled with ultraviolet (1,3,5-7) or amperometric (2,4,8-11) detection.

In general, methods employing amperometric detection are the most sensitive and well suited for quantifying plasma AA. Because amperometric detectors tend to require a predominantly aqueous mobile phase with at least 0.01M electrolyte concentration (12), they are frequently used in conjunction with a reversed-phase or paired-ion reversed-phase method for AA determinations (8-11).

The reversed-phase chromatographic separation of AA from D-isoascorbic acid (IA), also known as erythorbic acid and D-araboascorbic acid, is usually achieved by the addition of an ion-pairing agent to the mobile phase (7,13,14). Chemically, IA and AA differ only in the spatial orientation of the C-5 hydrogen and hydroxyl group. However IA, which is used as a food preservative, has only 5% of the antiscorbutic potency of AA (15). Its effect on AA retention and elimination has been studied in the mouse (16) and guinea pig (17), but its effect on AA absorption and utilization in man is not well established.

We report a paired-ion reversed-phase HPLC procedure with amperometric detection which resolves and quantifies AA and IA. Uric acid (UA) is also well resolved and, because its electrochemical properties resemble those of the ascorbic acid isomers (18-20), can be assayed simultaneously. Its molar concentration in human plasma is about five times that of AA. While the role of UA in the etiology of gout is well known (21), it has recently been hypothesized that UA provides an antioxidant defense against oxidant- and radical-caused aging and cancer in humans (22).

A procedure which allows direct, simultaneous measurement of AA, IA, and UA is desirable for the study of these biological antioxidants.

MATERIALS AND METHODS

Samples and Controls

Plasma samples were obtained from normal healthy adult female subjects admitted to the Human Nutrition Unit of this center and placed on an AA- and IA-free formula diet for 12 days. Prior to blood sampling, the subjects received with the AA-free diet 600 mg IA daily for 12 days or 600 mg IA with 90 mg AA daily for 10 days. Blood samples were drawn in ethylenediaminetetraacetic acid (EDTA) tubes and the cells removed by centrifugation. Plasma was mixed with an equal volume of cold metaphosphoric acid solution (HPO_3, 10% w/v; MCB, Norwood, OH) to precipitate proteins and prevent oxidation of AA and IA. After centrifugation, supernatants were stored at -70 degrees C until analyzed.

Lyophilized normal human control serum, AA- and IA-free (Fisher Diagnostics, Orangeburg, NY) was reconstituted according to the manufacturer's instructions using an aqueous solution containing 0.005 mg/mL AA and IA instead of water. Proteins were precipitated by addition of an equal volume of cold 10% HPO_3(w/v). After centrifugation, the supernatant was aliquoted, stored at -70 degrees C, and used for a daily control and in reproducibility studies.

Immediately prior to analysis, the preserved samples and controls were thawed and diluted 10-fold with cold HPLC mobile phase (see below for composition). After vortexing, each diluted sample and control was filtered through a 0.2 micron disposable filter assembly (Gelman Sciences, Ann Arbor, MI) into a glass vial for HPLC analysis. The injection volume was 10 microliters.

Standards

Stock solutions of AA (Hoffman-LaRoche, Inc., Nutley, NJ) and IA (ICN Nutritional Biochemicals, Cleveland, OH) were prepared fresh daily by dissolving 10 mg of either isomer in 100 mL cold mobile phase (see below for composition) containing HPO_3, 0.5% (w/v). The UA stock solution was made by mixing 10 mg UA (Fisher Scientific, Pittsburgh, PA) in a few mL water, adding 2N NaOH dropwise until the UA dissolved and bringing the volume to 100 mL with water.

The three stock solutions were further diluted with the cold mobile phase-HPO_3 solution to make four levels of working

standards such that 10 microliters of each contained 0.5, 1.0, 2.5, or 5.0 ng of both isomers and 10, 25, 50, or 60 ng UA. These correspond to AA and IA plasma levels of 0.1, 0.2, 0.5, and 1.0 mg/dL; and UA plasma levels of 2,5, 10, and 12 mg/dL. The working standards were filtered through 0.2 micron filters prior to HPLC analysis. The volume injected was 10 microliters.

HPLC Analysis

A Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Santa Clara, CA) was fitted with a 25 cm by 4.6 mm Altex Ultrasphere ODS (C18) reversed-phase analytical column and a 3 cm Brownlee Spheri-5 RP-18 guard column (both columns obtained from Rainin Instrument Company, Woburn, MA). The particle size of both column packings was five microns.

The mobile phase was made by dissolving 0.200g disodium EDTA (Sigma Chemical Company, St. Louis, MO), 5.44 g sodium acetate trihydrate (Mallinckrodt, Inc., Paris, KY), and one vial tetrabutylammonium phosphate (TBAP, Paired Ion Chromatography Reagent A; Waters Associates, Milford, MA) in 950 mL HPLC-grade water (J. T. Baker, Phillipsburg, NJ). Glacial acetic acid was added dropwise with continuous stirring until pH 5.25 was reached. The solution was then brought to a final volume of one liter with the addition of more HPLC-grade water. The final molarities of acetate and TBAP in the mobile phase were 0.04 and 0.005, respectively. After vacuum-filtering through a 0.2 micron nylon filter (Rainin Instrument Company), the mobile phase was

introduced into the chromatograph. No additional degassing was necessary. The mobile phase was run isocratically at ambient temperature and a 0.4 mL/minute flow rate, with continuous recycling of the detector cell effluent back into the mobile phase reservoir. The column temperature was 30 degrees C.

The amperometric detection system (BAS, West Lafayette, IN) included an LC4B controller and an electrode flowcell consisting of a glassy carbon electrode, a stainless steel electrode top, and an Ag/AgCl reference electrode. The applied potential was +0.600 volts (oxidative); the sensitivity range was 100 nanoamps; and the baseline offset current about 2 nanoamps. The one volt output jack on the LC4B controller was used for input to the 1084B recorder-integrator. After introducing a new batch of mobile phase, the electrode system was allowed about eight hours to stabilize.

RESULTS

Identification of Peaks

AA, IA, and UA were identified in plasma sample chromatograms by comparison of peak retention times with those of pure standard solutions. The co-elution of added standard AA, IA, or UA with endogenous plasma AA, IA, or UA provided additional evidence of sample peak identities. Stopped flow ultraviolet (UV) scans of AA, IA, and UA standard peaks showed their characteristic absorbance spectra. Scans were not obtained for plasma sample peaks due to the inadequate UV response, especially for AA and IA, at the levels endogenous to human plasma.

Mean retention times for AA, IA, and UA were 10.80, 11.54, and 13.64 minutes, respectively, for four analyses run on the same day with negligible variation. Small day-to-day variations were observed in 40 analyses run four per day over a four-week interval. These results are summarized in Table 1.

Standard Curves

Working standards from freshly prepared stock solutions were assayed daily. Curves were constructed by plotting electrode response expressed in units of peak area versus equivalent plasma levels of AA, IA, and UA in mg/dL for the four working standard points. The following linear regression equations were typical:

AA: $y = -5.859 + 131.99x$ $(r=0.9995)$

IA: $y = -4.307 + 124.54x$ $(r=0.9997)$

UA: $y = 0 + 0.205x$ $(r=1.000)$

TABLE 1

Intra- and Inter-assay Variability in Retention Times for Ascorbic Acid, Isoascorbic Acid, and Uric Acid Standards (Means and Coefficients of Variation (C.V.))

	Intra-assay (4 hours, n=4)		Inter-assay (4 weeks, n=40)	
	Mean Retention Time (Minutes)	C.V. (%)	Mean Retention Time (Minutes)	C.V. (%)
AA	10.80	0.1	10.85	3.7
IA	11.54	0.1	11.59	3.9
UA	13.64	0.2	13.65	3.0

where x is concentration in mg/dL for AA, IA, and UA; y is area units $x10^{-3}$ for AA and IA, and area units $x10^{-6}$ for UA; and r is the correlation coefficient. Linearity extended to at least 2 mg/dL for AA and IA, and 20 mg/dL for UA. The minimum amounts quantifiable at the 100 nanoamp sensitivity setting were about 0.25 nanograms for each substance. Lesser amounts can be detected at higher sensitivity settings, however, 100 nanoamps gives an excellent signal-to-noise ratio and is suited to the ranges encountered in plasma.

The AA- and IA-spiked normal human control serum was assayed daily over a five-day period to obtain the overall inter- and intra-assay variability. For all three substances the intra-assay coefficient of variation was 2% or less. Inter-assay variation was 5.6% or less. These results are summarized in Table 2.

TABLE 2

Intra- and Inter-assay Variability in Concentrations of Ascorbic Acid, Isoascorbic Acid, and Uric Acid in Ascorbic Acid- and Isoascorbic Acid-Spiked Normal Control Serum

	Intra-assay (n=6)		Inter-assay (n=5)	
	Mean (mg/dL)	C.V.(%)	Mean (mg/dL)	C.V.(%)
AA	0.44	1.4	0.44	1.9
IA	0.44	2.0	0.44	5.6
UA	3.96	0.3	3.83	3.3

Recovery Studies

Recovery studies were performed by determining AA, IA, and UA levels in plasma samples with and without the addition of an equal volume of one of two levels of an AA-IA-UA standard to 0.2 mL of each preserved sample during the preparation step. The samples were obtained from four AA- and IA-depleted subjects after repletion with either IA (600 mg daily) or IA with AA (600 mg and 90 mg, respectively). The two standard levels added corresponded to plasma levels of (a) 0.25 mg/dL AA and IA, 5 mg/dL UA; and (b) 0.5 mg/dL AA and IA, 6 mg/dL UA. Recoveries ranged from 102 to 107% for AA, 92 to 101% for IA, and 99 to 101% for UA. The results are summarized in Table 3. Figures 1 and 2 show a typical unspiked plasma sample chromatogram and a standard chromatogram, respectively.

TABLE 3

Recovery of Added Ascorbic Acid, Isoascorbic Acid, and Uric Acid from the Plasma of Four Ascorbic Acid- and Isoascorbic Acid-Repleted Subjects

Spike Level	Subject	Ascorbic Acid mg/dL Plasma Before Spike	Ascorbic Acid % Recovered After Spike	Isoascorbic Acid mg/dL Plasma Before Spike	Isoascorbic Acid % Recovered After Spike	Uric Acid mg/dL Plasma Before Spike	Uric Acid % Recovered After Spike
a	1	0.28	102	0.27	102	2.49	101
a	2	0.19	102	0.26	92	2.84	99
b	3	0.87	104	0.22	101	2.28	100
b	4	0.82	107	0.27	101	2.69	99

Subjects 1,2: AA-, IA-depleted; IA-repleted (600 mg/day)
Subjects 3,4: AA-, IA-depleted; AA-and IA-repleted (90 mg/day and 600 mg/day, respectively)

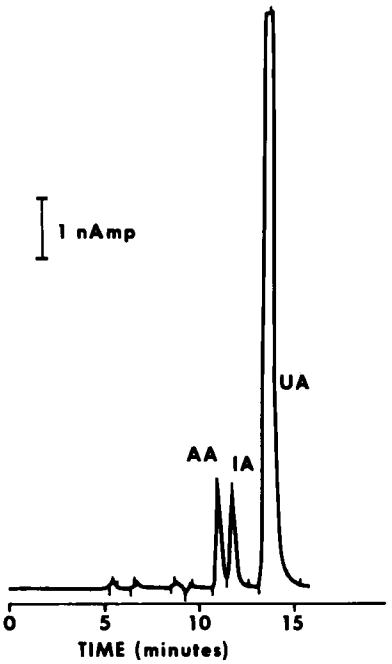

FIGURE 1. Chromatogram of unspiked human plasma (Subject 1) containing 0.28, 0.27, and 2.49 mg/dL AA, IA, and UA, respectively. The plasma is preserved with an equal volume of 10% HPO_3 and diluted 10-fold with mobile phase prior to analysis. Injection volume is 10 uL. The detector-integrator response is within the 100 nAmp sensitivity setting for all 3 components. A higher attenuation will bring the pen deflection on scale for the UA peak so that AA, IA, and UA can be determined by peak heights if desired.

Potential Interfering Substances

Several substances known to be electrochemically active in the oxidative mode were analyzed to see if they interfered with the determination of AA, IA, or UA by this method. The substances and their observed retention times are given in Table 4. None of those tested showed interference.

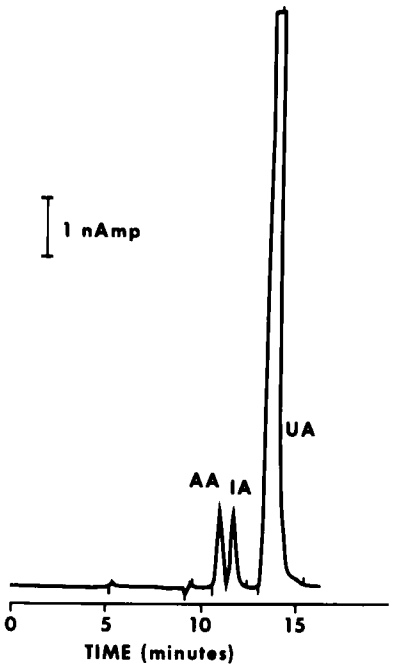

FIGURE 2. Chromatogram of 1 ng AA and IA, and 25 ng UA standards in mobile phase containing 0.5% HPO_3. Volume injected is 10 uL. Levels correspond to undiluted plasma concentrations of 0.2 mg/dL AA and IA, and 5 mg/dL UA. The detector-integrator response is within the 100 nAmp sensitivity setting for all 3 components. A higher attenuation will bring the pen deflection on scale for the UA peak so that AA, IA, and UA can be determined by peak heights if desired.

TABLE 4

Retention Times of Potentially Interfering Substances

Substance	Nanograms Injected	Retention Time (Minutes)
Epinephrine	2	5.80
Norepinephrine	1.37	5.80
Gentisic Acid	10,000	Not Detected
Acetylsalicylic Acid (extracted from aspirin)	3,250	Not Detected
Acetaminophen (extracted from Tylenol)	30	38.3

DISCUSSION

The procedure reported here has been used to determine AA, IA, and UA in plasma samples which were preserved in 5% HPO_3, a substance widely used to extract AA from foods and biological samples (23). We observed no loss of AA, IA, or UA from our preserved plasma samples between two freezing-thawing cycles 5 weeks apart as long as they were maintained at -70 degrees C. Specimens were thawed at room temperature for approximately 10 minutes each time, and were stored in a refrigerator 6-8 hours and 2-3 hours during the two thaw periods until refrozen. The addition of EDTA to the mobile phase further stabilized AA and IA during chromatography, presumably by chelating copper ions.

Besides TBAP, two other ion-pairing agents, n-octylamine and n-decylamine, were tested at concentrations of one to five mM in the mobile phase. Both proved unsatisfactory because they appeared to form turbid complexes with HPO_3 when the mobile phase was used to dilute the preserved samples prior to analysis. This may explain in part the significantly improved electrode response when TBAP was substituted for the alkylamines. The minimal organic content of the mobile phase also contributes to improved electrochemical detector response (24).

The retention time of UA showed a mobile phase pH-dependency much stronger than that of the ascorbic acid isomers. When the pH of the mobile phase was ≤ 4, UA was eluted before AA and IA. When it was ≥ 5, UA was eluted after AA and IA. A mobile phase pH of 5.25 (closer to the UA pKa of 5.8) was chosen to avoid any

tendency for the ascorbic acid isomers to be eluted on the trailing slope of the much larger UA peak.

The same analytical and guard columns have been used to analyze over 200 plasma samples and standards in a three-month interval with no significant loss of performance or efficiency. System maintenance included weekly flushing with 200 mL water followed by 200 mL methanol. The glassy carbon electrode surface did not require polishing during this period as indicated by consistently low background currents of two to four nanoamps.

One analysis can be completed in 14 minutes by synchronizing sample injections so that a small HPO_3 peak with a retention time of 22 minutes is eluted within the first six minutes of the next chromatogram. AA, IA, and UA are eluted without interference.

For analysis of samples known to contain only AA and/or UA, the flow rate can be increased to at least 0.8 mL/minute. AA and UA will be eluted after 6.0 minutes and 7.5 minutes, respectively. This effectively halves analysis time, allowing the simultaneous determination of these components to be completed in about eight minutes.

The procedure has been successfully applied to the determination of AA in fruit juices. Some showed trace amounts of a substance with a retention time identical to UA, suggesting animal contamination. Work is in progress to apply the procedure to the determination of AA and UA in other biological fluids and tissues.

NOTE ADDED IN PROOF

Shortly after submission of this manuscript, a paper appeared describing the HPLC measurement of UA, AA, and related metabolites in biological fluids (25). The procedure involves sample extraction by ion-exchange chromatography, followed by HPLC on a weak anion-exchange column using a 75% acetonitrile mobile phase and UV detection. This high acetonitrile concentration may be incompatible with electrochemical detection techniques; however, UV detection allows measurement of dehydroascorbic acid and other resolved, UV-absorbing metabolites of AA (and UA) if present in high enough concentration. The chromatographic behavior of IA in this system is not discussed.

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