

[1] High-Performance Liquid Chromatographic Separation of Ascorbic Acid, Erythorbic Acid, Dehydroascorbic Acid, Dehydroerythorbic Acid, Diketogulonic Acid, and Diketogluconic Acid

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L-Ascorbic acid (AA) and L-dehydroascorbic acid (DHAA) are the principal natural compounds with vitamin C activity, and chemical methods for their determination remain widely used. Interfering compounds, however, are often present in complex matrices such as biological tissues and foods. As a result, traditional approaches are being replaced by high-performance liquid chromatographic (HPLC) methods, which are more selective and sensitive. Recent comprehensive reviews¹⁻⁵ refer to various chemical and chromatographic methods available for determining AA, DHAA, and closely related compounds. In these reviews, their determination by HPLC on reversed-phase and ion-exchange stationary phases are discussed, as well as their detection by combinations of ultraviolet, electrochemical, and refractive index techniques. The importance of selecting appropriate extracting media and stabilizing solutions for these relatively unstable compounds is emphasized.

The primary basis for the physiological roles of vitamin C is the oxidation-reduction system between AA and DHAA. Hydrolysis of DHAA occurs outside a narrow acidic pH range and yields L-diketogulonic acid (DKGulA), with irreversible loss of vitamin C activity. D-Erythorbic acid (EA, i.e., isoascorbic acid) is epimeric to AA at C-5 and possesses little or no vitamin C activity; it may, in fact, be an antagonist of vitamin C.⁶ EA forms an oxidation-reduction couple with D-dehydroerythorbic acid (DHEA), which readily and irreversibly hydrolyzes to D-diketogluconic

¹ J. R. Cooke and R. E. D. Moxon, in "Vitamin C (Ascorbic Acid)" (J. N. Counsell and D. H. Hornig, eds.), p. 167. Applied Science Publishers, London, 1981.

² H. E. Sauberlich, M. D. Green, and S. T. Omaye, in "Ascorbic Acid: Chemistry, Metabolism, and Uses" (P. A. Seib and B. M. Tolbert, eds.), Adv. Chem. Ser., No. 200, p. 199. Am. Chem. Soc., Washington, D.C., 1982.

³ L. W. Doner, in "Trace Analysis" (J. F. Lawrence, ed.), Vol. 3, p. 113. Academic Press, New York, 1984.

⁴ L. A. Pachla, D. L. Reynolds, and P. T. Kissinger, *J. Assoc. Off. Anal. Chem.* **68**, 1 (1985).

⁵ G. M. Jaffe, in "Handbook of Vitamins" (L. J. Machlin, ed.), p. 199. Dekker, New York, 1984.

⁶ D. Hornig, *Acta Vitaminol. Enzymol.* **31**, 9 (1977).

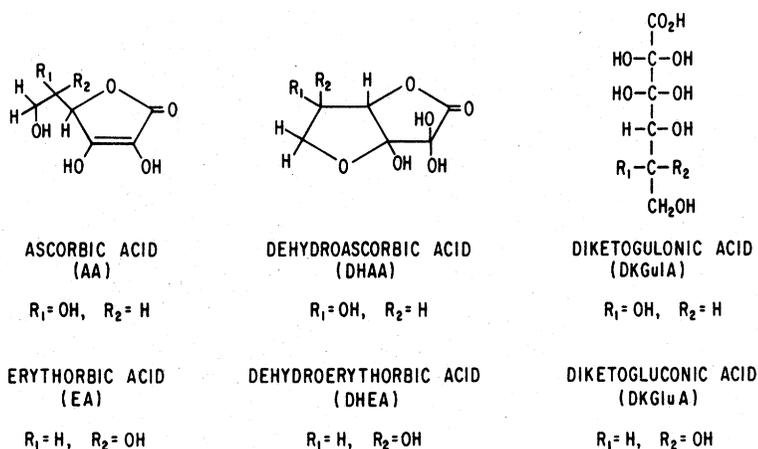


FIG. 1. Predominant structures in aqueous solution of separated compounds; DHAA and DHEA as hydrated bicyclic hemiketals, DKGulA and DKGluA as dihydrates.

acid (DKGluA). EA is widely used as an antioxidant by the food industry, since it is less expensive than AA.

In this report we emphasize the separation by weak anion-exchange HPLC of AA and EA, and these from their respective oxidation and hydrolysis products, extending an earlier study.⁷

Principle

The HPLC separation of AA, EA, DKGulA, and DKGluA on amino-propyl bonded-phase silica was accomplished by weak anion-exchange chromatography with acetonitrile-0.05 M KH_2PO_4 eluant. DHAA and DHEA are not truly organic acids, so their separation presumably results from partitioning between the mobile phase and the relatively water-enriched hydrated stationary phase. The conjugated enediol chromophore in AA and EA allows their detection at 270 nm; reduction of DHAA and DHEA with dithiothreitol (DTT) allows their indirect determination from the increase in AA and EA absorption. In aqueous solution, DHAA exists as a hydrated bicyclic hemiketal, and in DKGulA both ketone functions are hydrated. Analogous structures (Fig. 1) probably exist for DHEA and DKGluA. Although some carbonyl character is masked in these structures, they do exhibit some ultraviolet absorption. For many purposes, refractive index detection is the method of choice for the six compounds

of interest in this study, especially with the recent development of highly sensitive detectors.

Preparation of Standard Compounds

DHAA and DHEA were readily prepared from AA and EA (both commercially available) as described earlier,^{7,8} by air oxidation of ethanolic solutions containing activated charcoal. After filtration and removal of ethanol, pure syrups of DHAA and DHEA were obtained, as revealed by HPLC. These compounds are known only as syrups, but are conveniently stored as crystalline dimers, prepared^{7,9} after dissolution of the monomers in nitromethane. The dimeric forms readily convert to DHAA and DHEA upon dissolving in acetonitrile-water (50:50, v/v).

Dimeric DHAA and DHEA were converted to DKGulA and DKGluA, respectively, by gradually titrating (in an ice bath over a period of 1 hr) aqueous solutions of 10 mg/ml with 0.5 N NaOH until the pH remained constant at 7.0. Solid samples of the sodium salts are obtained by lyophilization of these saponified solutions.

Preparation of Samples for HPLC

Standard mixtures of the title compounds were prepared just prior to analysis by HPLC (refractive index detection) by dissolving 32 mg each of AA, EA, and DHAA dimer, 64 mg of DHEA dimer, and 128 mg each of DKGulA and DKGluA (sodium salts) in 2 ml water. This solution was diluted with 2 ml acetonitrile and 20 μ l injected.

For analysis of AA and EA by ultraviolet detection, 10 μ g/ml solutions in acetonitrile-water (50:50, v/v) give high absorbances. The addition (1.0 mg/ml) of dithiothreitol (DTT) to one of duplicate samples at a final sample concentration of 1 mg/ml allows the indirect determination of DHAA and DHEA by increase in AA and EA absorption, since DTT quantitatively reduces these to AA and EA, respectively, within 15 min. To prevent reoxidation, DTT was also added to the mobile phase (1.0 mg/ml). Tyrosine serves well as internal standard for quantitation,⁷ being well separated from the title compounds and having adequate ultraviolet absorption at 270 nm; it can be added to the sample diluent at a level of 30 mg/100 ml.

Orange juice samples were analyzed for AA by injecting after filtration (0.45- μ m filter). Aqueous diluent containing tyrosine can be used for AA

⁸ M. Ohmori and M. Takage, *Agric. Biol. Chem.* **42**, 173 (1978).

⁹ H. Dietz, *Justus Liebigs Ann. Chem.* **738**, 208 (1970).

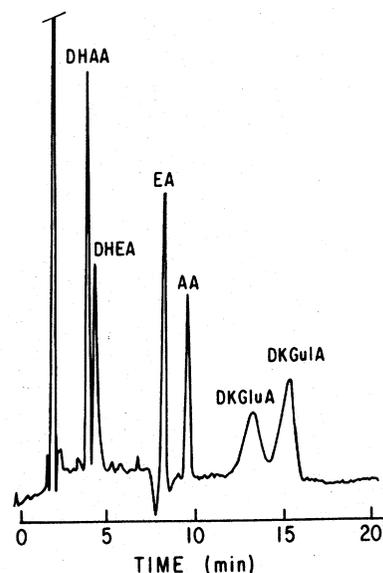


FIG. 2. Chromatography of standard mixture (20 μ l injected) of dehydroascorbic acid (DHAA), dehydroerythorbic acid (DHEA), ascorbic acid (AA), erythorbic acid (EA), diketogluonic acid (DKGluA), and diketogulonic acid (DKGuIA). Operating conditions: column, Zorbax NH₂; mobile phase, 3:1 acetonitrile-0.05 M KH₂PO₄; flow rate, 1.5 ml/min; refractive index detection, 16 \times attenuation; recorder chart speed, 0.25 cm/min.

quantitation, and dilution of a duplicate sample with both tyrosine and DTT (50 and 1.0 mg/ml, respectively) allows DHAA determination from the increase in AA absorbance. Likewise, urine samples (after adding an equal volume of acetonitrile and filtering) can be analyzed for AA and DHAA.

Instrument and Separation Procedure

The HPLC system consisted of a Hewlett-Packard HP 190 instrument equipped with a diode-array ultraviolet detector, 5- or 20- μ l loop injectors, and an HP 85B computer. A Waters Associates¹⁰ Model R401 refractive index detector also was used, sometimes in tandem with the ultraviolet detector. Separations were achieved by isocratic elution of a Zorbax NH₂ column (4.6 \times 250 mm) with acetonitrile-0.05 M KH₂PO₄ (75:25,

¹⁰ Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

CHROMATOGRAPHIC EVALUATION OF SEPARATED
COMPOUNDS ON ZORBAX NH₂^a

Compound	Retention time (min)	Capacity factor (<i>k'</i>)	α^b	<i>R_s</i> ^c
DHAA	3.6	0.91	0.22	1.04
DHEA	4.1	1.19	0.29	5.00
EA	8.1	3.32	0.82	1.80
AA	9.5	4.05	1.00	3.50
DKGluA	13.4	6.12	1.51	1.23
DKGulA	15.5	7.24	1.79	

^a Chromatographic conditions as in Fig. 2.

^b $\alpha = k'/k'_{AA}$.

^c Resolution between adjacent peaks.

v/v) at a flow rate of 1.5 ml/min. Acetonitrile was added gradually with stirring to warmed 0.05 M KH₂PO₄, in order to avoid precipitate formation.

Discussion

The HPLC separation of the six compounds with a Zorbax NH₂ column is shown in Fig. 2, and the chromatographic characteristics are listed in the table. The resolution (*R_s*) between all adjacent pairs is >1.0, so the six compounds can be accurately quantified. If AA and EA are the analytes of interest, the flow rate (or polarity of eluant) can be increased; they remain efficiently separated at retention times of less than 5 min. Quantities injected (Fig. 2) were 160 μg AA, EA, and DHAA; 320 μg DHEA; and 640 μg DKGluA and DKGulA. The signals were quite attenuated (16×), and with highly sensitive refractive index detectors that are now available, detection of these compounds at 10–20% of these levels should be practical.

Figure 3 demonstrates the ability of tandem ultraviolet–refractive index detection to determine simultaneously AA and the various sugars in orange juice. The sugars are transparent to detection at 268 nm, while the level of AA is too low to be revealed by refractive index detection. Using tandem detection, all can be efficiently quantified, even though AA is not well separated from glucose, which is present in 50-fold excess of AA.

Measurements of DHAA have been made by others using ultraviolet detection; we find that the hydrated forms of DHAA and DHEA, as well as DKGulA and DKGluA, possess low extinction coefficients. Also, the

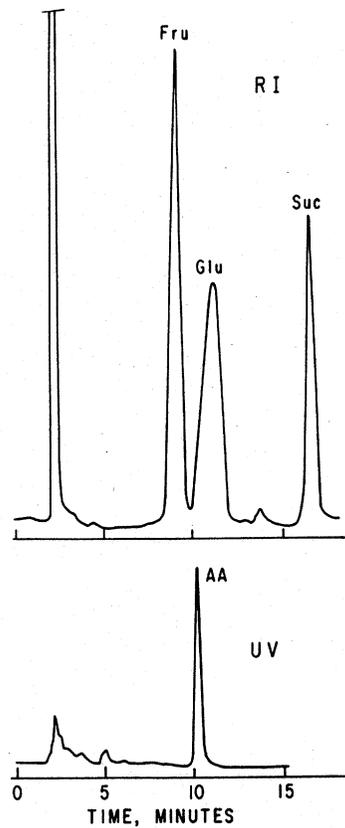


FIG. 3. Chromatogram of orange juice monitored by (below) tandem ultraviolet (UV, 268 nm) and (above) refractive index (RI, 8× attenuation) detection. Conditions as in Fig. 2. Fru, fructose; Glu, glucose; Suc, sucrose. (Reprinted with permission from Academic Press and Doner and Hicks.⁷)

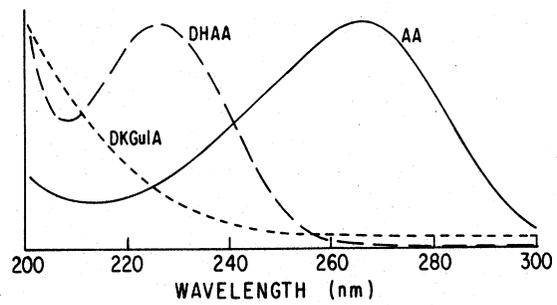


FIG. 4. Ultraviolet absorption spectra of AA, DHAA, and DKGulA.

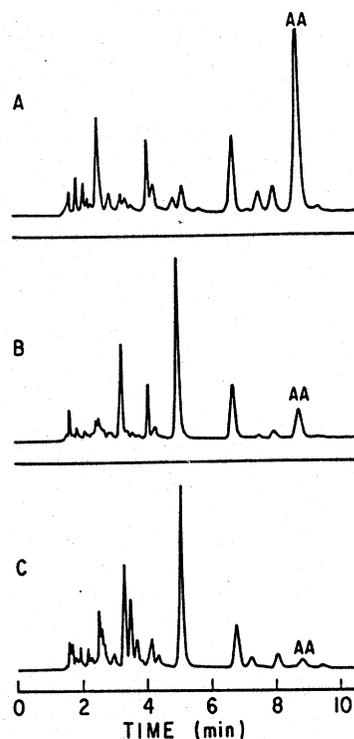


FIG. 5. Chromatogram of urine sample, monitored at (A) 270 nm, (B) 230 nm, and (C) 200 nm. Other conditions as in Fig. 2. Relative attenuations for (A), (B), and (C) are 4.7, 2.0, and 1.0, respectively.

maximum absorbance for DHAA and DHEA occurs at a wavelength of 227 nm, and for DKGulA and DKGluA <200 nm; other compounds which frequently occur in biological samples, however, also absorb at these low wavelengths. We, therefore, determined the dehydro forms indirectly, after reduction to their reduced forms, which possess high molar extinction coefficients (16,500) at 270 nm. Sensitive refractive index detection is suitable, in many cases, for the oxidized and hydrolyzed derivatives of AA and EA. Ultraviolet absorption spectra of AA, DHAA, and DKGulA are given in Fig. 4. Levels of AA and EA less than 5 ng can be detected, with good linearity for quantitation over a wide range.

Figure 5 shows that the chromatographic conditions described here can resolve many components of human urine and that monitoring the chromatogram at various wavelengths reveals significant differences. The AA peak corresponds to 26 mg/100 ml urine, and an ultraviolet scan of

this peak verified its identity. DHAA can be indirectly quantified after reduction of a duplicate sample with DTT, which is added to the urine diluent along with tyrosine, the internal standard.⁷

Acknowledgment

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[2] Glutathione Dehydrogenase (Ascorbate)

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Glutathione dehydrogenase (ascorbate), i.e., dehydroascorbate reductase (glutathione:dehydroascorbate oxidoreductase, EC 1.8.5.1), catalyzes the glutathione (GSH)-dependent reduction of dehydroascorbic acid to ascorbic acid. The enzyme is widely distributed among plant tissues, where its presence was originally established using a dichlorophenol-indophenol titration technique.^{1,2} Dehydroascorbate reductase has been detected in various animal cells using a coupled assay which monitors the oxidation of NADPH by glutathione reductase.³ Some limitations of this method have been indicated.⁴ Recently, the direct spectrophotometric assay for dehydroascorbate reductase described below was developed.⁵

Assay Method

Principle. The spectrum of ascorbic acid has a peak at 265 nm, while dehydroascorbic acid has no absorbance at that wavelength. The activity of dehydroascorbate reductase is assayed spectrophotometrically by measuring the change in absorbance at 265 nm associated with the generation of ascorbic acid.⁵

Reagents. Dehydroascorbic acid is made as follows by the oxidation of ascorbic acid according to Staudinger and Weis.⁶ From a solution of

¹ B. M. Crook, *Biochem. J.* **35**, 226 (1941).

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³ E. I. Anderson and A. Spector, *Invest. Ophthalmol.* **10**, 41 (1971).

⁴ R. L. Stahl, L. F. Liebes, and R. Silber, *Biochim. Biophys. Acta* **839**, 119 (1985).

⁵ R. L. Stahl, L. F. Liebes, C. M. Farber, and R. Silber, *Anal. Biochem.* **131**, 341 (1983).

⁶ H. Staudinger and W. Weis, *Hoppe-Seyler's Z. Physiol. Chem.* **337**, 284 (1964).