

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

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High-performance liquid chromatography on a Zorbax NH₂ analytical column, with acetonitrile: 0.05 M KH₂PO₄ (75:25, w/w) used as eluant, has allowed the separation, in less than 14 min, of ascorbic acid, erythorbic acid, dehydroascorbic acid, dehydroerythorbic acid, diketogulonic acid, and diketogluconic acid. Ultraviolet monitoring at 268 nm allows ascorbic acid and erythorbic acid to be detected at the 25-ng level, while refractive index detection monitors the elution of all six compounds. Tyrosine is a good internal standard, being well separated from the other compounds and having an adequate ultraviolet absorption at 268 nm. We have found dithiothreitol to be effective in rapidly reducing dehydroascorbic acid to ascorbic acid, providing the basis for indirectly determining dehydroascorbic acid after its reduction. The potential of this high-performance liquid chromatographic procedure for evaluating the levels of these compounds in orange juice and urine is demonstrated.

The traditional chemical methods for ascorbic acid (AA)² determination are time consuming, and interfering substances present in complex matrices such as foods and biological fluids affect the specificity and accuracy of the measurements.

Reports have described successful applications of high-performance liquid chromatography (hplc) to AA determination by reversed-phase (1-3) and strong (4,5) and weak (1,6,7) anion-exchange modes of separation. In chromatographic systems, AA has been detected electrochemically (4,7) or, more frequently, from its characteristic ultraviolet (uv) absorption. While hplc offers advantages over chemical methods for AA

determination, the latter methods have been extended to include compounds directly related to AA, such as dehydroascorbic acid (DHAA) and 2,3-diketogulonic acid (DKGulA) (8,9). In this respect, these methods have continued to offer some advantages over hplc.

A recent report (10) described a separation of AA from its C-5 epimer erythorbic acid (EA, i.e., isoascorbic acid) by weak anion-exchange hplc on aminopropyl-substituted silica packings. This was the first example of the hplc separation of AA from this closely related compound.

We describe here the utility of weak anion-exchange hplc in separating not only AA from its oxidation products DHAA and DKGulA, but, simultaneously, EA from its analogous oxidation products dehydroerythorbic acid (DHEA) and diketogluconic acid (DKGluA).

Reasons why convenient and accurate measurements of these compounds are

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² Abbreviations used: AA, ascorbic acid; hplc, high-performance liquid chromatography; DHAA, dehydroascorbic acid; DKGulA, 2,3-diketogulonic acid; EA, erythorbic acid (isoascorbic acid); DHEA, dehydroerythorbic acid; DKGluA, diketogluconic acid; DTT, dithiothreitol; RI, refractive index.

needed have been noted, many in a recent critical review on ascorbic acid (11). The equilibrium between AA and DHAA is controlled by a number of factors, as is the conversion of DHAA to DKGulA, which possesses no vitamin C activity. Also, EA costs less than AA and is sometimes added to foods as an antioxidant in place of AA, even though it possesses just 1/20th the vitamin C activity of AA. A need exists for readily distinguishing EA from AA in processed foods.

The method described here permits ready determinations of the presence of AA and EA and their oxidation products, thus providing a basis for evaluating effects of processing and storage abuse on their levels in foods. Also, the potential for determining the levels of AA and DHAA in biological fluids such as orange juice and urine is demonstrated.

MATERIALS AND METHODS

Reagents. Ascorbic acid (AA), erythorbic acid (EA), tyrosine, and dithiothreitol (DTT) were obtained from Sigma,³ and acetonitrile (nanograde) from Mallinckrodt. AA and EA were converted to dehydroascorbic acid (DHAA) and dehydroerythorbic acid (DHEA), respectively, by a slight modification (12) of an earlier procedure (13). AA (10.0 g) was stirred for 5 h at ambient temperature in ethanol (450 ml) containing activated charcoal (12.0 g), and air was continuously bubbled through the solution. EA (5.0 g) was stirred for 20 h at 40°C in ethanol (300 ml) containing activated charcoal (10.0 g), again while bubbling air through the solution. Upon removal from the reaction mixtures of charcoal by filtration and ethanol under vacuum, DHAA and DHEA were isolated as pure syrups, as revealed by hplc. Having pure products, no

³ 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.

attempts were made to optimize the reaction conditions. Dimeric DHAA and DHEA were prepared from the monomers in nitromethane according to a recent procedure (14).

DHAA and DHEA were saponified to diketogulonic acid (DKGulA) and diketogluconic acid (DKGluA), respectively, by titrating solutions (10 meq/1.0 ml H₂O) of each with an equimolar quantity of 0.5 N NaOH. The sodium salt of DKGulA was converted to the free acid before hplc by being stirred with Amberlite IR-120(H+), then filtered and evaporated to syrup. The sodium salt of DKGulA was evaporated to a syrup and used directly for hplc.

Instrumentation. The modular hplc system consisted of an Instrumentation Specialties Company (ISCO, Omaha, Nebr.) metering pump 314, Series 1240-003, a pressure monitor (ISCO Model 1590), a Waters Associates (Milford, Mass.) Model R401 refractive index (RI) detector, a Perkin-Elmer (Norwalk, Conn.) LC-55 variable wavelength uv detector, a DuPont (Wilmington, Del.) Zorbax NH₂ analytical column (4.6 × 250 mm), and an Alltech Scientific (Deerfield, Ill.) 20- μ l loop injector.

Separation procedures. Optimal separations were achieved by use of the Zorbax NH₂ column in the weak anion-exchange mode, with a mobile phase of acetonitrile:0.05 M KH₂PO₄ (75:25, w/w), at a flow rate of 1.5 ml/min. It was essential that the 0.05 M KH₂PO₄ solution be heated and maintained at about 40°C during the addition of acetonitrile; otherwise KH₂PO₄ precipitates because of the marked cooling when acetonitrile is mixed with aqueous solutions. Separations of DHAA and DHEA also could be achieved with the column used in the normal-phase mode by elution with acetonitrile:water (75:25, w/w). AA and EA were detected by uv (268 nm) and RI, while the remaining compounds, DHAA, DHEA, DKGulA, and DKGluA, were detected by RI. In some cases, DHAA and DHEA were

determined by uv after their reduction to AA and EA, respectively, by reaction with DTT.

Sample preparation. For hplc, standard mixtures as well as orange juice and urine samples were diluted to appropriate levels with mobile phase containing tyrosine (50 mg/100 ml). The standard mixtures had to be prepared daily. In cases where AA and EA alone were determined by uv detection at 268 nm, no DTT was added to the diluent. DTT was added to the mobile phase at a level of 100 mg/100 ml for determinations of AA, DHAA, EA, and DHEA. DTT effectively reduced the dehydro forms to AA and EA in 15 min (8), so that the level of each form could be determined by hplc before and after DTT addition.

RESULTS AND DISCUSSION

The structures of AA and EA differ only in the configuration at carbon five, as do their respective oxidation products. Figure 1 shows the hplc separation (refractive index detection) of these six compounds with the Zorbax NH₂ column used in the weak anion-exchange mode. As indicated, AA is baseline separated from EA, and the separation of all six compounds requires less than 14 min. Table 1 summarizes the chromatographic behavior of the standard compounds and the internal standard tyrosine. The ratio of capacity factors (k'), referred to as α , is a reproducible way to identify the peaks, as this ratio is fairly constant regardless of slight changes in flow rates or eluant composition.

The column had to be used in the anion-exchange mode (i.e., with KH₂PO₄ in the mobile phase) to separate the compounds with ionizable protons, AA, EA, DKGulA, and DKGluA. In the normal phase, these compounds are not eluted, as they presumably form salts with the amine functionality in the column packing. DHAA and DHEA, however, are not truly acids, and separation of this pair is readily achieved by normal-

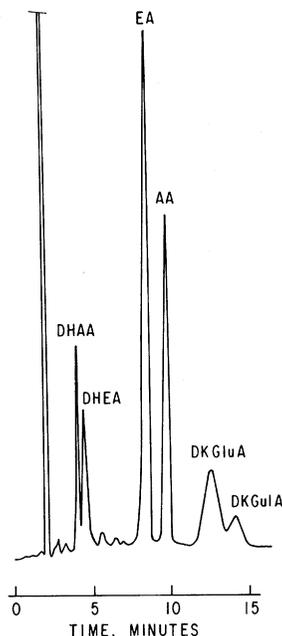


FIG. 1. High-performance liquid chromatogram; the separation of dehydroascorbic acid (DHAA), dehydroerythorbic acid (DHEA), erythorbic acid (EA), ascorbic acid (AA), diketogluconic acid (DKGluA), and diketogulonic acid (DKGulA). Conditions: column, Zorbax NH₂, 4.6 mm × 25 cm; mobile phase, CH₃CN:0.05 M KH₂PO₄ (75:25, w/w); refractive index detection (8× attenuation); flow rate, 1.5 ml/min; chart speed, 8 in./h.

TABLE 1
HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC
EVALUATION OF SEPARATED COMPOUNDS
ON ZORBAX NH₂

Compound	Retention time (min)	Capacity factor (k')	α^a
DHAA	3.69	0.97	0.24
DHEA	4.17	1.22	0.31
Tyrosine	7.51	3.02	0.76
EA	8.17	3.36	0.84
AA	9.37	3.99	1.00
DKGluA	12.31	5.58	1.40
DKGulA	13.76	6.33	1.58

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

phase hplc with acetonitrile:water (86:14, w/w) as eluant.

The resolution of the weak anion-exchange separation of AA from EA, and DKGluA from DKGluA is quite surprising, since there is no significant difference in pK_a values of the ionizable protons between these pairs. Presumably, factors in addition to degree of ionization affect interactions with the functionalities in the packing. Hydrogen bonding of hydroxyl protons in the compounds with the neutral amino groups in the packing also may play a role. While 268-nm uv detection will monitor AA, EA, and the internal standard tyrosine, the other four compounds are transparent at this wavelength; refractive index detection was used to determine these compounds. Table 2 demonstrates the reproducibility of uv absorption of AA, with tyrosine used as internal standard. Similar results are obtained with EA, which also separates well from tyrosine, as shown in Fig. 2. To achieve stable results, it is necessary that dithiothreitol (DTT) be present and that the solutions be stored in the dark. The response of both AA and EA to uv detection is linear over a wide range, and each can be detected at the 25-ng level.

TABLE 2

REPRODUCIBILITY OF TYROSINE AND ASCORBIC ACID ABSORPTIONS		
Run	$A_{268 \text{ nm}}$	
	Tyrosine	Ascorbic acid
1	0.052	0.223
2	0.052	0.234
3	0.051	0.235
4	0.051	0.239
5	0.053	0.233
6	0.050	0.231
7	0.049	0.232
8	0.048	0.231
Mean	0.051	0.232
SD	0.0017	0.0045
CV (%)	3.33	1.94

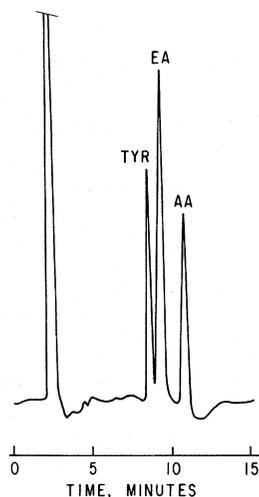


FIG. 2. Chromatogram of tyrosine (Tyr), EA, and AA separation. Ultraviolet detection, 268 nm (0.100 AUFS); other conditions as in Fig. 1.

The linearity of response for AA and tyrosine is shown in Fig. 3.

Preliminary results demonstrate that with uv detection, this hplc method is useful for determining levels of AA and EA in biological materials and with refractive index (RI) detection, the related compounds also may be determined. Figure 4 shows chromato-

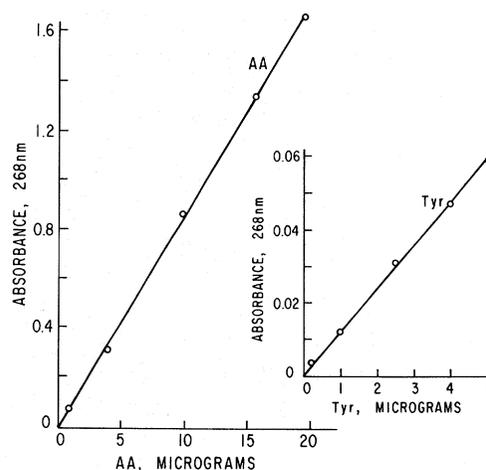


FIG. 3. Linearity plot for uv (268 nm) absorptions of AA and tyrosine (Tyr) eluting from hplc columns.

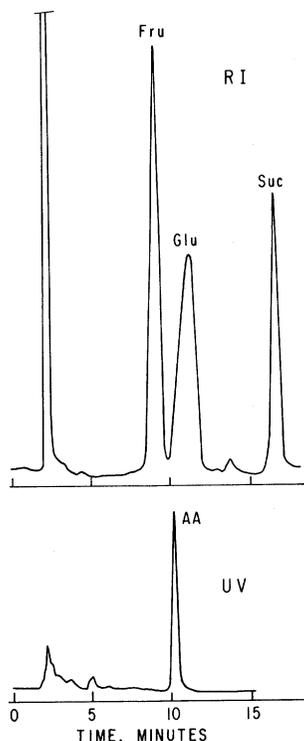


FIG. 4. Chromatogram of orange juice monitored by ultraviolet (uv, 268 nm) and refractive index (RI) tandem detection. Ultraviolet displays AA, and RI the sugars fructose (Fru), glucose (Glu), and sucrose (Suc); other conditions as in Fig. 1.

grams obtained when an orange juice sample (filtered before injection) was chromatographed with uv and RI detectors used in tandem. Little other than AA was detected in the uv at 268 nm, while the RI detector revealed the major constituents of orange juice, the sugars fructose, glucose, and sucrose. RI detection is therefore too insensitive to monitor AA at the level it occurs in orange juice.

We also tested this hplc procedure for its ability to determine AA and DHAA in urine. Figure 5 is a chromatogram of a urine sample directly injected onto the column, the eluant detected by RI. The major peak, at about 3.75 min, includes both DHAA and urea, and AA is indicated at about 9.14 min.

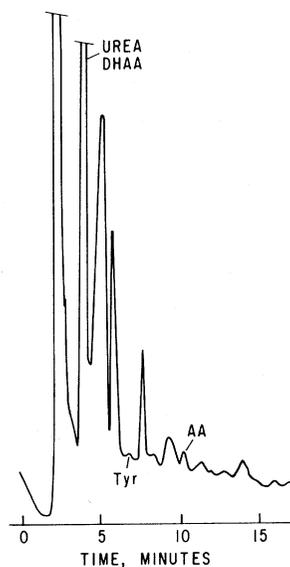


FIG. 5. Chromatogram of urine sample by RI detection. Conditions as in Fig. 1.

Figure 6 demonstrates that both DHAA and AA can be determined in urine by uv (268 nm) detection. Chromatogram A represents

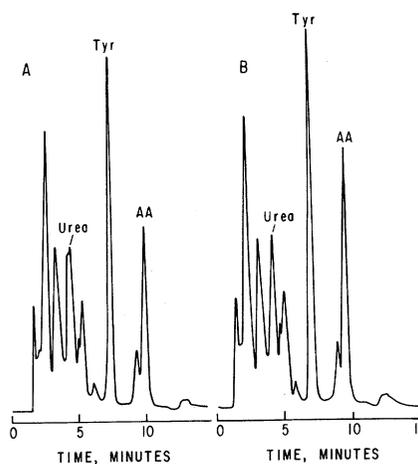


FIG. 6. (A) Urine sample diluted with equal volume of mobile phase containing tyrosine (Tyr) and detected by uv (268 nm). (B) Obtained 15 min after same urine sample was diluted with an equal volume of mobile phase containing both tyrosine and dithiothreitol. Other conditions for both chromatograms A and B as in Fig. 1.

a urine sample diluted with mobile phase containing tyrosine; from this profile, AA can be quantified, assuming that no coeluting uv-absorbing compounds are present. We are currently examining the possibility of such a presence. Chromatogram B represents the same except that DTT was added to the diluant 15 min before this sample was run. DTT effectively reduced DHAA to AA in this time, making it possible to determine DHAA from the increase in absorption of the AA peak after reduction.

Extension of this method to the determination of other compounds related to or derived from vitamin C may lead to a better understanding of the fate of this vitamin in processed foods. Also, other uv-absorbing materials present in both orange juice and urine are resolved under the chromatographic conditions described here. Most of these are as yet unknown, but their quantitation should be possible once they have been characterized.

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