CHROM. 6300

Resolution of ascorbic, dehydroascorbic, and diketogulonic acids by anion-exchange column chromatography

To investigate the chelating properties of ascorbate and its oxidation products and hydrolytic derivatives, we required a rapid qualitative and quantitative analysis of mixtures of ascorbic, dehydroascorbic, and diketogulonic acids[1,2]. Overlapping specificities in the chemical assays[3-5] for the individual components made prior fractionation of the mixtures necessary. We therefore developed a chromatographic system for unambiguously separating mixtures on a column of anion-exchange resin using continuous elution with a single eluent. The qualitative specificity of this system takes advantage of differentials in acidity to resolve the components; the separated components are then assayed quantitatively by colorimetric determination of their 2,4-dinitrophenylosazones.

Experimental

Ascorbic acid and derivatives. Crystalline USP L-ascorbic acid was obtained from Merck and Co., Rahway, N.J. L-Dehydroascorbic acid was prepared by the method of KENYON AND MUNRO[6]. 2,3-Diketo-L-gulonate was isolated as the barium salt[7] from a mutarotated solution of dehydroascorbic acid prepared by bromine oxidation of ascorbic acid. To obtain crystalline barium bis(2,3-diketo-L-gulonate) octahydrate, we dissolved 6.6 g of the barium salt in 10 ml of 1 *N* HCl and raised the pH to 2.5 with 1 *N* NaOH; the turbid solution was brought to 50 ml with water, filtered, purged with nitrogen, and kept in the cold for two days; the large spherulites were washed with cold water, ethanol, and acetone, and dried *in vacuo*.

Chromatography. Dowex 1-X2 (200–400 mesh; total capacity 3.5 mequiv. per dry gram; control No. 10186) was obtained as the "Analytical Grade" from Bio-Rad Laboratories, Richmond, Calif. Resin was converted first to the hydroxide form by washing with 100 volumes of 1 *N* NaOH and water, then to the phosphate form by washing with 100 volumes of 0.625 *M* H_3PO_4. Columns, 0.9 × 56 cm, were equilibrated with at least 400 ml of 0.05 *M* H_3PO_4 immediately before use. A sample containing 25–50 μmoles of each desired component was applied to the column in 1–2 ml of 0.05 *M* H_3PO_4. Eluent was 0.05 *M* H_3PO_4 at room temperature (21–23°), and the flow-rate was 30–35 ml/h.

Analysis of ascorbic acid. The strong ultraviolet absorbance of ascorbic acid[1] served as a sensitive, semi-quantitative assay and was preferred to the reduction of 2,6-dichlorophenolindophenol[8]. The absorptivity of ascorbic acid in acidic solution is maximal at about 245 nm[1] and is attributable to an enediol structure which is absent in the oxidized forms. Because dehydroascorbic and diketogulonic acids also absorb slightly at this wavelength[1], we chose to measure ascorbic acid by its absorbance at 260 nm in order to achieve somewhat greater specificity.

Analysis of dehydroascorbic acid. We are not aware of a specific assay for dehydroascorbic acid, other than the reaction of its hydrolytic product, diketogulonic acid, with 2,4-dinitrophenylhydrazine (2,4-DNPH)[2]. Dehydroascorbic acid can to some extent be distinguished from ascorbic acid by its more rapid reaction with 2,4-DNPH[2,4] and from diketogulonic acid by reducing it to ascorbic acid with H_2S[4].

These reactions, however, are not practicable for a large number of column fractions.

Analysis of diketogulonic acid. This compound couples more rapidly than either dehydroascorbic or ascorbic acid with 2,4-DNPH. The reaction can be exploited for quantitative assay[5] but is only relatively specific. A 1.0-ml aliquot of the column fractions (containing up to 3 μmoles diketogulonic acid per ml), 3.5 ml of water, and 0.5 ml of 0.01 *M* 2,4-DNPH/2 *M* HCl were thoroughly mixed and incubated at 25° for 1 h; 2.0 ml of 1 *N* NaOH were then added and mixed immediately. The dark brown-red reaction product faded after about 30 min at room temperature to a fairly stable red color which was measured by absorbance at 520 nm. Absorbance was not linearly proportional to concentration.

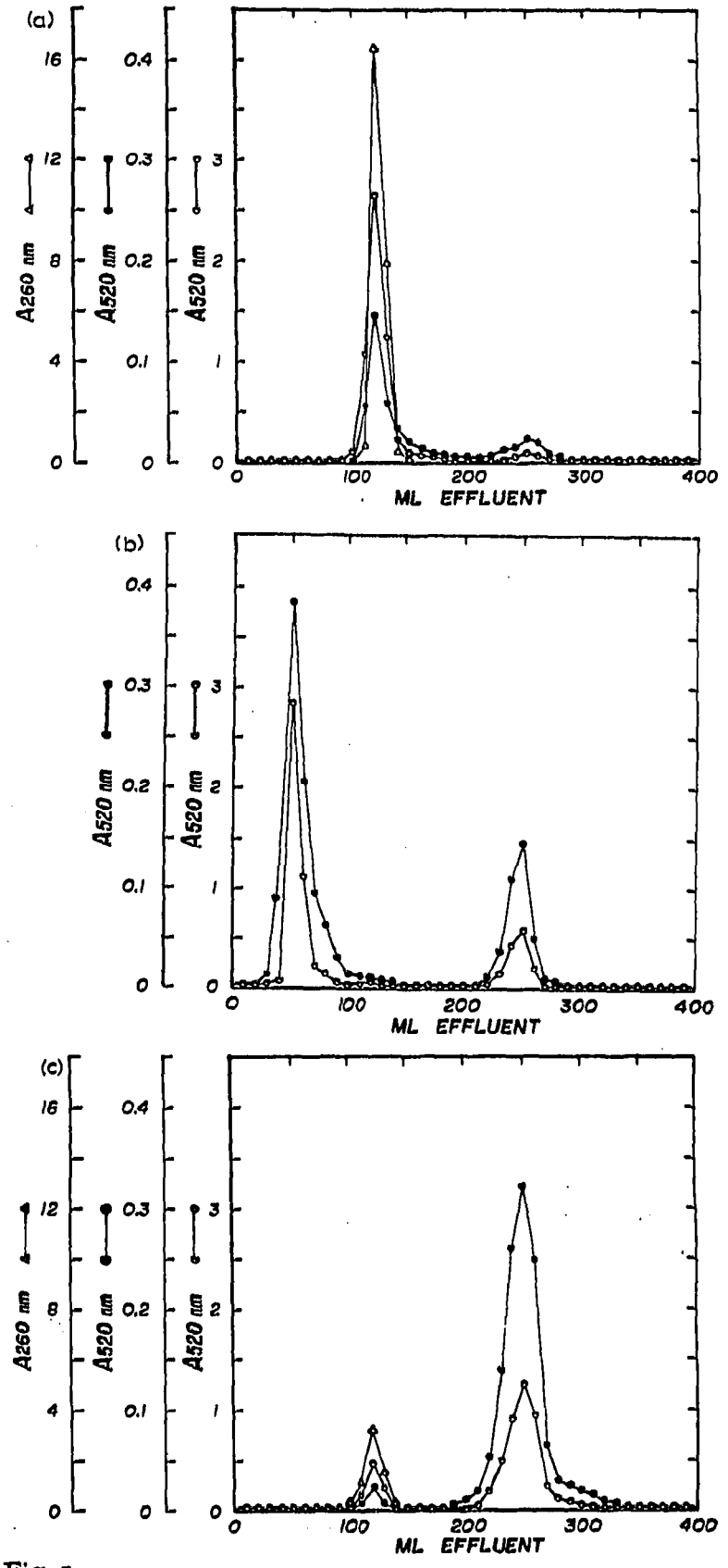
Analysis of ascorbic, dehydroascorbic, and diketogulonic acids. The 2,4-dinitrophenylosazones of ascorbic, dehydroascorbic, and diketogulonic acids are identical[2,9], since 2,4-DNPH ultimately condenses with diketogulonic acid in the case of all three compounds. The reaction, if it goes to completion, can be utilized as a specific and quantitative colorimetric assay for any of the three components. A 1.0-ml aliquot of the column fractions (containing up to about 2 μmoles ascorbic, dehydroascorbic, or diketogulonic acid per ml), 4.0 ml of 0.625 *M* H_3PO_4, and 1.0 ml of 0.1 *M* 2,4-DNPH/4.5 *M* H_2SO_4 were thoroughly mixed and incubated either at 37° for 16 h (ref. 4) or at 57° for 45 min[10]. The tubes were chilled to 2° in an ice-bath, and the red osazone precipitate was dissolved by adding 5.0 ml of 16 *M* H_2SO_4 with continuous mixing. Absorbance at 520 nm was measured after the tubes had cooled. Molar absorptivities for the osazones of ascorbic acid and dehydroascorbic acid (freshly prepared by bromine oxidation of ascorbic acid) were identical; we assumed that diketogulonic acid would behave similarly.

RESULTS AND DISCUSSION

Calibration of ion-exchange column. Ascorbic (I), dehydroascorbic (II), and diketogulonic (III) acids were chromatographed individually to establish references for elution volume (Fig. 1). The order in which these compounds were eluted (II, I, III) is consistent with their ionizability as acids (II < I < III): Diketogulonic acid (III) is a relatively strong carboxylic acid[1], although its p*K* has not yet been established. The acidity of ascorbic acid (I) is attributable to ionization of the enolic hydroxyl on carbon 3 and is lost on oxidation to dehydroascorbic acid (II)[1]. The heterogeneity of our materials, evident from Fig. 1, probably reflects their difficult preparation and lability on storage, rather than degradation by the chromatographic resin. Inspection of Fig. 1 also points out the ambiguities associated with determination of these three compounds by assays having overlapping specificities.

Resolution and quantitation of mixtures. Fig. 2 illustrates the separation of a mixture of 50 μmoles each of ascorbic acid, dehydroascorbic acid, and diketogulonic acid. Individual components were well separated and symmetrically eluted.

Fig. 1. Chromatography of 50 μmoles of (a) ascorbic acid, (b) dehydroascorbic acid, and (c) diketogulonic acid on a column of Dowex 1-X2 (0.9 × 56 cm), eluted with 0.05 *M* H_3PO_4. Ascorbic acid in the effluent was "assayed" by absorbance at 260 nm (triangles). Dehydroascorbic acid was determined colorimetrically (as the 2,4-dinitrophenylosazone) at 520 nm (open circles). Diketogulonic acid was determined colorimetrically (as a derivative of its 2,4-dinitrophenylosazone) at 520 nm (filled circles). Light path was 1 cm.


(a)
A260 nm
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(b)
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(c)
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A520 nm
ML EFFLUENT

Fig. 1.

Their detection colorimetrically as 2,4-dinitrophenylosazones permitted a sensitive and quantitative estimation. The total areas under each curve of Fig. 2 are practically equal, if allowance is made for the presence of "impurities" in each

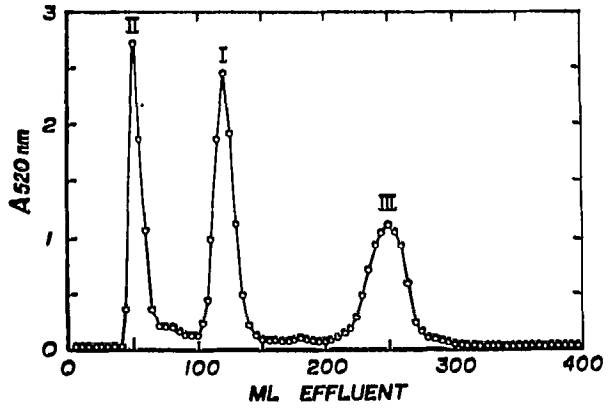

Fig. 2. Chromatography of a mixture of ascorbic, dehydroascorbic, and diketogulonic acids (50 μmoles each) on a column of Dowex 1-X2 (0.9 × 56 cm), eluted with 0.05 *M* H_3PO_4. The three acids were determined colorimetrically (as their 2,4-dinitrophenylosazones) at 520 nm. Light path was 1 cm.

of the reference compounds. Recoveries from the column were substantially complete, as judged by quantitative comparison with an external standard of ascorbic acid.

Applications. The analytical system, we describe here, permits direct identification and simultaneous quantitation of the oxidative and hydrolytic reaction products of ascorbic acid. We have used this method successfully to study the complex interactions of ascorbic acid and its derivatives with iron salts under various conditions. Some of the kinetic parameters of this interaction have been followed previously[11] only by laborious measurement of the disappearance of one of the components — with inconclusive results. The practical difficulty of differentiating these compounds by chemical assay alone underscores the advantage of chromatographic separation.

We were assisted by MARCIA MELTZER and DEBORAH SHEPPARD RHEAD. The U.S. Public Health Service supported this study through Research Grant AM12386 to P.S. and Postdoctoral Fellowship AM 48724 to J.H.

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First received June 30th, 1972; revised manuscript received August 10th, 1972