Quantitative Estimation of Ascorbic Acid and Related Substances in Biological Extracts by Separation on a Paper Chromatogram

By YU-TUAN CHEN, F. A. ISHERWOOD and L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

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In studies on the synthesis of L-ascorbic acid in plants and animals the need arose for a method permitting separation and quantitative estimation of closely related enediol compounds such as D-araboascorbic and L-ascorbic acids. Simple titration methods using 2:6-dichlorophenolindophenol (Harris & Ray, 1935; Birch, Harris & Ray, 1933; Harris & Olliver, 1942) will not differentiate between closely related enediols, and even more specific methods, based on the different rates of condensation of enediols with formaldehyde (Lugg, 1942; Mapson, 1943; Snow & Zilva, 1943), will not distinguish between those with the same ring structure. It seems clear that a preliminary separation must precede the estimation of the individual enediols. Such a separation has been described by Mapson & Partridge (1949). These authors have shown that L-ascorbic acid, D-araboascorbic acid, the enol form of 3-hydroxytetrahydrofuran-2:4-dione (hydroxytetronic acid), reductic acid, and reductone can be separated on a paper chromatogram using *n*-butanol : acetic acid : water, or phenol : acetic acid : water, as solvents. Their experiments were confined to the qualitative separation of made-up mixtures of pure compounds, and no attempts were made to estimate the enediols after separation on the paper chromatograms. The problem of separating L-ascorbic acid from D-araboascorbic acid in extracts from biological material was not touched and it was suggested that the separation might be difficult. This opinion was based on the known instability of the enediols and their low concentration in extracts from biological materials.

The method now described uses paper chromatograms not only to effect the final separation of the enediols but also to achieve a preliminary concentration from the bulk of the other compounds present in the extract. Since enediols are known to be most stable in strongly acid solution and when heavy metals are absent, both the initial extract of the biological material and the two chromatographic solvents used were saturated with oxalic acid. Oxalic acid was used because it readily forms complexes with most heavy metals and because it forms a component of two very stable chromatographic solvents (*n*-butanol : water : oxalic acid, and phenol : water : oxalic acid) which will separate most of the common enediol compounds. The presence of oxalic acid, however, does not prevent a slow decomposition of the enediols, which is particularly noticeable in weak solutions. This occurs even when the solutions are stored in the dark, but is more pronounced when they are exposed to light. The same is true of solutions in metaphosphoric acid, so that it appears to be independent of the compound used to inactivate the heavy metals. This slow breakdown inevitably makes it difficult to devise a really quantitative method. However, the present method, whilst not having a very high accuracy, does give an approximately quantitative estimate of the enediols in extracts of biological material. It can usefully be employed to confirm the reliability of the simple titration methods which are commonly used for the estimation of L-ascorbic acid in extracts of biological materials.

EXPERIMENTAL

Materials. The L-ascorbic and D-araboascorbic acids were purchased from Roche Products Ltd., Welwyn. Titration with 2:6-dichlorophenolindophenol according to the method of Harris & Olliver (1942) gave results within 1% of the theoretical. The hydroxytetronic and reductic acids were a gift from Prof. Reichstein, Basel, and the reductone a gift from Prof. Bergel, Roche Products Ltd. The oxalic acid was an A.R. sample.

The two solvents used for development of the paper chromatograms were as follows. The first was prepared by shaking *n*-butanol (60 ml.) with water (40 ml.) and excess of oxalic acid in a separating funnel and allowing to stand at 20°. The water-poor phase (upper layer) was used as solvent. The second was prepared by dissolving water (10 g.) in phenol (50 g.), filtering the solution through charcoal to remove coloured quinone compounds present, and then adding an excess of oxalic acid. The mixture was stored at 20° and the water-poor phase (lower layer) used as solvent.

A stock solution of 2:6-dichlorophenolindophenol in water was prepared by dissolving 0·8 g. of the dyestuff in 1 l. of water. To this solution was added a slight excess of potassium oxalate. It was allowed to stand overnight and then centrifuged to remove calcium oxalate. For the estimation of ascorbic acid the stock solution was diluted to a concentration of 0·04 g./l. by adding 0·25 M-Na_2HPO_4 in glass-distilled water.

Chromatography. L-Ascorbic acid and related compounds were separated on paper chromatograms before being quantitatively determined. The apparatus and general conditions were similar to those described for the separation of the sugars by Isherwood & Jermyn (1951). *n*-Butanol: water: oxalic acid was used for the general separation of the enediols from accompanying substances in plant or animal extracts and phenol: water: oxalic acid for the final separation of L-ascorbic acid from D-araboascorbic acid. These two solvents are referred to below as the butanol and phenol solvents, respectively.

The filter paper used was Whatman no. 1 for qualitative work and no. 3 for preparative work. In all cases the papers were dipped into 2% (w/v) aqueous oxalic acid and then dried in air.

In qualitative examinations the positions of the enediols on the paper chromatograms were located by spraying with 0·08% (w/v) aqueous 2:6-dichlorophenolindophenol. The enediols appear as white spots against a pink background. The limit of detection is 1–2 μg./cm.^{2} on Whatman no. 1 paper. In quantitative analyses a 'guide' chromatogram was run alongside the main chromatogram and only this guide chromatogram was sprayed. The enediols on the main chromatogram were located by comparison.

Analytical procedure. This is described for the quantitative estimation of L-ascorbic acid in urine and in cress seedlings. A qualitative examination is similar except that less care need be taken to ensure a complete recovery of L-ascorbic acid during the preliminary isolation from the plant or animal source. The description is given in terms of L-ascorbic acid but applies equally to D-araboascorbic acid and related compounds.

With urine the procedure varied somewhat, depending on the amount of L-ascorbic acid present. For samples containing less than 1 mg./ml. it was necessary to isolate the L-ascorbic acid from the bulk of the other substances present before attempting to estimate it by the standard method.

The rats were housed in standard metabolism cages, and the urine collected in a bottle containing sufficient solid oxalic acid to ensure that it was saturated with oxalic acid throughout the period of collection. All the subsequent operations were carried out in the dark or in a dim light. Even exposure to diffused daylight was avoided as much as possible. This urine (0·4 ml.) was applied to the starting line of an oxalic acid-treated Whatman no. 3 paper as a streak about 20 cm. long. The paper was then irrigated with the butanol solvent until the solvent front had moved about 30 cm. (16 hr.). After development it was removed from the jar and dried in a current of air at room temperature. A transverse strip of paper (30 × 3 cm.), corresponding to the L-ascorbic acid on the guide chromatogram, was cut out of the main piece of paper, and extracted chromatographically with glass-distilled water until about 2 ml. of extract had been obtained. In cases where the concentration of L-ascorbic acid in the urine was very low, a number of chromatograms were run and the extracts of L-ascorbic acid from each combined until about 100 μg. had been obtained. Since the urine from normal rats contained about 50 μg./ml., six chromatograms carrying 2·5 ml. of the urine gave sufficient for the present purpose. The combined extracts were then saturated with H_2S and treated with 10% (w/v) aqueous calcium acetate until most of the oxalic acid had been removed. Adding calcium acetate until the pH was about 2·0 left just sufficient oxalic acid to stabilize the L-ascorbic acid through subsequent operations. The solution was then centrifuged to remove calcium oxalate. The supernatant was evaporated to dryness *in vacuo* over P_2O_5. Leaving the solution in a small beaker overnight in a vacuum desiccator was sufficient. The residue was dissolved in 0·1 ml. of glass-distilled water. 10 μl. of this solution from a micropipette were applied to the starting line of an oxalic acid-treated Whatman no. 1 paper and the paper was developed with the phenol solvent for 20 hr. (the solvent front has moved about 35 cm.). The appropriate piece of paper was cut out and the L-ascorbic acid estimated.

For samples that contain amounts of L-ascorbic acid larger than 1 mg./ml., the urine was applied directly to the starting line of an oxalic acid-treated Whatman no. 1 paper and developed with the phenol solvent as described above.

For cress seedlings the procedure was similar to that devised for urine except that the extract to be applied to the starting line was obtained from 100 cress seedlings (wet wt. 2 g. after 120 hr. germination in the dark) which were ground with 0·2 g. of crystalline oxalic acid in a mortar at 0° and then centrifuged.

Colorimetric estimation. The paper (3 × 3 cm.) containing the L-ascorbic acid (about 15 μg.) was folded twice in one direction and once in the other, using glass rods. Metal forceps were avoided because contamination with heavy metals would be likely to cause oxidation of the L-ascorbic acid. The folded paper was inserted into a clean, dry colorimeter tube and 3 ml. of a 3% (w/v) aqueous solution of oxalic acid added. An EEL single-photocell colorimeter was used (Evans Electroselenium Ltd., Harlow, Essex). This was very convenient because the whole estimation could be carried out in one of the standard colorimeter test tubes (8 × 1·5 cm.). The solution was agitated by passing a slow stream of N_2 for 2 min. The paper was then removed with a glass rod. To the solution was added 4 ml. of the freshly prepared 0·004% solution of 2:6-dichlorophenolindophenol. The solution in the colorimeter tube was mixed by rocking gently and then the colour measured within 30 sec. of adding the dye. A green-blue filter was used (Ilford 623 with maximum transmission at 490 mμ.). 15 μg. of L-ascorbic acid gave a reading of about twelve divisions (blank 62, actual reading 50). The change in colour was proportional to the amount of L-ascorbic acid present. The dye was standardized against a freshly prepared solution of L-ascorbic acid.

RESULTS

Table 1 shows the R_F values obtained with the two solvent mixtures for different enediol compounds.

The phenol solvent gives a clear-cut separation of L-ascorbic acid from D-araboascorbic acid.

Table 1. *Separation of compounds related to L-ascorbic acid on paper chromatograms*

Substance	R_F value on Whatman no. 1 paper at 20°: *n*-Butanol sat. with water and oxalic acid	Phenol sat. with water and oxalic acid
L-Ascorbic acid	0·29	0·43
D-Araboascorbic acid	0·30	0·50
Hydroxytetronic acid	0·61	0·68
Reductic acid	0·60	0·81
Reductone	0·59 (trailing)	0·68

Table 2. *The effect of light on the recovery of* L-*ascorbic acid from paper chromatograms developed with the phenol solvent* (*see text*)

Amount added (μg.)	Amount found (μg.)	Recovery (%)
	In daylight	
12·0	10·1	83
24·5	20·6	84
36·5	30·3	83
	In the dark	
12·0	10·8	90
20·0	18·4	92
32·5	30·2	93
40·5	39·0	94

The effect of light on the destruction of ascorbic acid added to paper chromatograms, and then extracted and estimated as described previously, is illustrated in Table 2. Parallel experiments were carried out in which, during the normal handling necessary, the papers were exposed close to the light from a north window during March–April, and also in the absence of light. The papers were not exposed to light during the development of the chromatogram, nor during any extraction procedures. The results indicate that exposure to diffuse daylight caused an extra loss of about 10 %.

The complete procedure for the quantitative estimation of the enediols in extracts of biological materials was tested by adding known amounts of L-ascorbic acid and D-araboascorbic acid to rat's urine, and to an extract of cress seedlings. In some experiments, where the amount of enediol was greater than 1 mg./ml., the extract was examined, without any preliminary concentration, on a paper chromatogram using the phenol solvent. The enediols were then estimated from this chromatogram. The results obtained are given in Table 3.

The results given in Table 3 indicate that the loss of enediol varies between 10 and 20 % depending on the concentration in the original extract. A similar loss occurs when pure solutions of the enediols are kept in the dark for the same period (total time 3 days) so that the observed recovery is regarded as satisfactory. In practice, an estimate of the loss was obtained by carrying out a parallel determination on a pure solution containing similar amounts of the enediol and then a correction applied to the figures observed for the extract from the biological material. The figures in Table 3 were not corrected in this manner but are given to illustrate errors inherent in the method.

SUMMARY

1. L-Ascorbic acid and related compounds have been separated on a paper chromatogram using two new solvent mixtures, *n*-butanol saturated with water and oxalic acid, and phenol saturated with water and oxalic acid. The phenolic solvent will give a clear-cut separation of L-ascorbic acid from D-araboascorbic acid.

2. A quantitative procedure is described for the estimation of enediols from plant and animal tissues. Enzymes are inactivated by saturating the extract with oxalic acid and the enediols are stabilized throughout subsequent operations by maintaining a high concentration of oxalic acid. The enediols are estimated after separation on a paper chromatogram by a colorimetric method using a solution of 2:6-dichlorophenolindophenol in 0·25 M-Na_2HPO_4. Recovery of L-ascorbic acid and D-araboascorbic acid added to rat's urine, and extracts of cress seedlings was 85 % even at the lowest concentration examined (0·5 mg./ml. of urine or cress-seedling extract).

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Table 3. *The recovery of* L-*ascorbic acid and* D-*araboascorbic acid added to rat urine and to an extract of cress seedlings* (*uncorrected*)

Biological material	Procedure	L-Ascorbic acid present initially (mg./ml.)	Amount added: L-Ascorbic (mg./ml.)	Amount added: D-Arabo-ascorbic (mg./ml.)	Amount found: L-Ascorbic (mg./ml.)	Amount found: D-Arabo-ascorbic (mg./ml.)
Rat's urine	Full	0	0·6	0·6	0·51	0·52
Rat's urine	Preliminary concentration omitted	0	2·00	2·10	1·86	1·95
Cress seedlings	Full	0·30	0·53	0·74	0·72	0·65
Cress seedlings	Preliminary concentration omitted	0·39	2·23	2·50	2·49	2·37

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