A PHOTOELECTRIC COLORIMETRIC METHOD FOR THE ESTIMATION OF ASCORBIC ACID

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Received July 16, 1951

Chemically, the outstanding property of ascorbic acid is its ease of oxidation. The oxidation-reduction properties of ascorbic acid are widely used as the fundamental reaction in the measurement of vitamin C. In such methods, acid extracts of the materials being assayed are prepared and the reducing capacity of the extract is measured by treatment with a suitable oxidising agent such as 2 : 6-dichlorophenolindophenol, iodine, ferricyanide, methylene blue, etc. Of these, oxidation with the dye 2 : 6-dichlorophenolindophenol[1,2,3] has found extensive use as the basis of techniques for determining ascorbic acid and has generally been found to be the most satisfactory.

In some materials, the value of the 2 : 6-dichlorophenolindophenol reagent for measuring ascorbic acid is limited by the presence of other reducing substances such as reductones, reductic acid and ferrous iron.[4,5] Ferrous ion reduces the dye in the presence of metaphosphoric acid, so that pharmaceutical preparations containing reduced iron should be titrated in 8 per cent. acetic acid solution, free from metaphosphoric acid. On the other hand, ferric ion interferes with the end-point in the absence of metaphosphoric acid, so that the metaphosphoric-acetic acid mixture should be employed as the titration medium when testing pharmaceutical preparations containing oxidised iron.[6]

Reaction of derivatives of ascorbic acid with 2 : 4-dinitrophenylhydrazine permits the determination of ascorbic acid by methods not based upon oxidation-reduction properties. Dehydroascorbic acid couples with 2 : 4-dinitrophenylhydrazine to produce a compound which, when treated with strong sulphuric acid, yields a red colour. These reactions have been employed[7,8] for the direct determination of dehydroascorbic acid, as well as for the measurement of the reduced form of the vitamin after oxidation by treatment with activated charcoal.

A serious objection to this method is the fact that diketogulonic acid, a biologically inactive oxidation product of vitamin C, reacts like the vitamin with dinitrophenylhydrazine.[9,10]

The Photoelectric Colorimetric Method

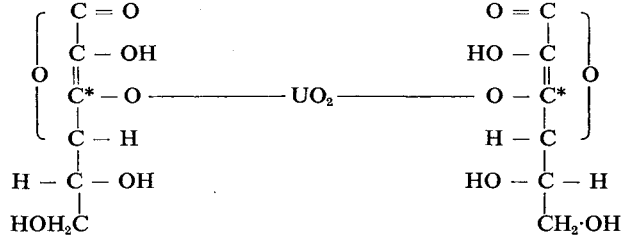
A new, simple and rapid method for the determination of ascorbic acid in pure solutions and in pharmaceutical products, which overcomes the disadvantages previously mentioned is advanced. This method is based on the colour reaction of aqueous 30 per cent. uranium nitrate solution with ascorbic acid (red in concentrated solutions and orange-red in dilute solutions). The colour is immediately obtained by mixing ascorbic acid

solution with the reagent at room temperature and the intensity is directly proportional to the vitamin content. The colour is stable in neutral solutions as well as in slightly acid and alkaline media, and is not affected by the action of heat. In a strongly acid medium the colour is discharged and in strongly alkaline solution a deep brown colour is obtained (on the gradual addition of the alkali) followed by precipitation of sodium uranate.[11,12] Since ascorbic acid is unstable in solution, especially in the presence of air or traces of metals such as copper and iron, and in light,[13,14] the estimation should not be delayed more than half an hour after mixing the ascorbic acid solution with the reagent.

Nature of the Colour Test. In solution, vitamin C exhibits acidic properties, the dissociation constants being $pK_1 = 4{\cdot}17$ and $pK_2 = 11{\cdot}57$. Ascorbic acid has the empirical formula $C_6H_8O_6$. It is a monobasic acid, giving well-defined salts of the type $C_6H_7O_6M$ (M = monovalent metal). The acidic properties of the vitamin are due to the hydroxy (enolic) group attached to the third carbon atom (asterisk in the formula given).[15,16,17,18,19]

A number of salts, for example, the sodium, copper, manganese and iron salts of ascorbic acid have already been isolated and found to possess antiscorbutic activity.[20]

Accordingly it is believed that this colour test is due to the formation of the corresponding metallic derivative, uranium ascorbate.



Dehydroascorbic acid and diketogulonic acid give no colour with the reagent.

This colour test is not based on the strong reducing ability of ascorbic acid, since it is not produced when a current of hydrogen is passed into 30 per cent. aqueous uranium nitrate.

A similar orange-red colour is given by the reagent with phenolic compounds such as an alcoholic solution of 2 : 4-dihydroxybenzaldehyde or salicylic acid and an aqueous solution of sodium salicylate or resorcinol.

On the other hand, a much paler colour is obtained with an alcoholic solution of phenol, and no colour is developed with an alcoholic solution of benzoic acid or an aqueous solution of sodium benzoate. This observation supports the idea that the enolic group of ascorbic acid is responsible for the development of the colour. Salicylates, if present in pharmaceutical preparations, can be eliminated in the form of salicylic acid by rendering the solution just acid.

This colour test may be applied for the estimation of ascorbic acid in food materials since the following substances which might interfere have

no influence on the colour; amino-acids such as glycine, alanine, valine, arginine and isoleucine; thiamine hydrochloride and riboflavine, carbohydrates such as glucose, lactose, sucrose and starch; diketogulonic acid, urea and uric acid, acetone, ethyl acetate and aceto-acetic ester. This colour test is not affected by the presence of other reducing substances such as ferrous salts, tartaric acid, oxalates, tartrates and citrates, formaldehyde, sodium sulphite, bisulphite and thiosulphate, thio-urea, hydrogen sulphide and pyridinium compounds. The presence of ferric salts does not interfere with the test; inorganic and organic ferrous and ferric compounds also interfere with the determination by the dye 2 : 6-dichlorophenolindophenol.[21]

This method avoids interference by reducing substances that may be present in association with ascorbic acid in pharmaceutical products and food materials. It includes only the determination of ascorbic acid, not of dehydroascorbic acid; the latter can be determined after reduction with hydrogen sulphide.[22]

The sensitivity of the test is 1 in 17,000.

Colour Tests for Ascorbic Acid. The following colour tests are based on the strong reducing property of ascorbic acid:

(1) To a solution of 10 mg. of ascorbic acid in 1 ml. of distilled water add 2 ml. of 8 per cent. aqueous ammonium molybdate solution; a yellow, green and then a deep blue colour, "molybdenum blue," develops in the cold.

(2) (*a*) When a solution of 10 mg. of ascorbic acid in 1 ml. of distilled water is treated with 2 ml. of sodium tungstate solution (10 per cent.) and the mixture warmed for 1 minute and treated with 3 drops of 20 per cent. sulphuric acid, a blue colour, "tungstic blue," is obtained.

(*b*) To 10 mg. of ascorbic acid in 1 ml. of distilled water, add 2 ml. of 10 per cent. sodium tungstate solution and 3 drops of 20 per cent. sulphuric acid in the cold; an orange-red colour develops which darkens on standing.

(3) To 10 mg. of ascorbic acid in 1 ml. of distilled water, add 2 ml. of potassium ferricyanide solution (5 per cent.) and heat for 1 minute; a blue colour develops which turns green on standing and deposits a blue precipitate.

(4) To 10 mg. of ascorbic acid in 1 ml. of distilled water, add 2 ml. of potassium chromate solution (10 per cent.); a green colour develops in the cold which turns to wine-red on adding 3 drops of 20 per cent. sulphuric acid.

(5) To 20 mg. of ascorbic acid in 1 ml. of distilled water, add 1 ml. of 10 per cent. platinic chloride solution and heat for 1 minute; a deep red colour is obtained.

Experimental

Preparation of the Metallic Derivative. To a solution of 1 g. of pure ascorbic acid (2 mol.) in 5 ml. of ethanol in a small crucible, 1·43 g. of uranium nitrate (1 mol.) in 5 ml. ethanol is added; on mixing well an

intensely dark red solution is obtained. This solution is concentrated on the electric plate to about 3 ml. and allowed to stand at room temperature, after which reddish-brown crystals separate out; these are filtered off and dried; m.pt. 178° to 180° C. with decomposition. The salt dissolves in distilled water giving a red colour, which is discharged on adding strong mineral acids; the colour becomes deep brown on adding 30 per cent. sodium hydroxide solution with the final precipitation of sodium uranate. The reddish, crystalline solid salt decomposes at room temperature when exposed to the atmosphere, being changed gradually to a yellow, somewhat elastic, non-crystalline mass.

Equipment and Reagents. (1) Lumetron photoelectric colorimeter using a yellow-green filter, 530 against water as the blank, set at 100 per cent. transmission.

(2) Two micro-pipettes (5 ml.), one of which is graduated.

(3) Uranium nitrate reagent, prepared by dissolving 30 g. of uranium nitrate in 100 ml. of distilled water and filtering if necessary.

(4) Standard stock solution of ascorbic acid prepared by dissolving 0·4 g. of pure ascorbic acid (previously dried over concentrated sulphuric acid in a desiccator to constant weight) in distilled water and completed to 200 ml. in a standard flask.

Procedure. Into a 25-ml. volumetric flask x ml. of the standard stock solution of ascorbic acid (1 ml. represents 2 mg. of ascorbic acid) is introduced. The volume is then completed to 25 ml. with distilled water, well mixed, and left to stand for about 5 minutes. Then 5 ml. of this solution is transferred to a dry colorimeter tube and 3 ml. of the uranium nitrate reagent added. The mixture is well mixed and its percentage transmission is read after 3 minutes in a Lumetron photoelectric colorimeter, Model 400–A using a yellow green filter, 530 against water as the blank, set at 100 per cent. transmission. $x = 25$ ml. of the standard stock solution of ascorbic acid (0·4 g. per 200 ml. w/v), representing a concentration of 10 mg. of ascorbic acid per 5 ml. of solution or 22·5, 20, 17·5, 15, 12·5, 10, 7·5, 5, 2·5 and 1·25 ml. of the standard stock solution of ascorbic acid representing concentrations of 9, 8, 7, 6, 5, 4, 3, 2, 1 and 0·5 mg. of ascorbic acid per 5 ml. w/v respectively.

From the results obtained it was found that:—(*a*) transmission readings should be spread out sufficiently to allow a determination of ascorbic acid to be made within concentrations ranging from 0·5 to 10 mg. per 5 ml. w/v of ascorbic acid; (*b*) since the graph shows that within these concentrations there is a slight deviation from Beer's Law, a calibration table can replace the graph and give more accurate results, provided that the estimations are carried out at the same (room) temperature and under the same conditions.

Calibration Table. From the standard stock solution of ascorbic acid (0·4 g. per 200 ml.) standard solutions are prepared, so that 5 ml. of each dilution contains an amount of ascorbic acid ranging from 1 mg. to 10 mg., increasing in the order of 1 mg.; 5 ml. of each dilution is accurately measured in a dry, colorimeter tube; 3 ml. of uranium nitrate reagent is

added, well mixed and left to stand for about 3 minutes. The percentage transmission of the solution is then read. The results obtained at room temperature (28° C.) are shown in Table I.

TABLE I

CALIBRATION TABLE

Ascorbic acid mg.	Percentage transmission	Ascorbic acid mg.	Percentage transmission
10	22	5	45
9	25	4	52
8	28	3	61
7	32	2	72
6	38	1	84
		½	94

METHOD OF ASSAY

(1) *Test Solutions.* A dilution of the test solution is made so that 5 ml. contains an amount of ascorbic acid ranging between 0·5 and 10 mg. 5 ml. of this dilution is accurately measured into a dry colorimeter tube, 3 ml. of the uranium nitrate reagent is added, mixed well and left to stand for about 3 minutes. The percentage transmission of the solution is measured and the amount of ascorbic acid corresponding to this transmission is obtained from the calibration table, and the amount in the original test solution calculated. On applying this method to accurately weighed amounts of pure ascorbic acid the results obtained did not differ by more than ± 1 per cent.

(2) *Injections.* For the estimation of ascorbic acid in solutions prepared for injection the following method is recommended.

A known volume of the solution is diluted with distilled water in a standard flask so that 5 ml. contains 0·5 to 10 mg. of ascorbic acid. 5 ml. of this dilution is accurately measured and introduced into a dry colorimeter tube; 3 ml. of the uranium nitrate reagent is added and mixed well. The percentage transmission of the solution is measured and the concentration read from the calibration table. The figure obtained is the amount in mg. contained in 5 ml. of the diluted solution.

This method has been successfully applied to several kinds of injections obtainable in Egypt.

(3) *Tablets.* 10 tablets are weighed and powdered and an accurately weighed quantity of the powder, equivalent to 50 mg. of ascorbic acid, is introduced into a 50-ml. standard flask. Successive small quantities of distilled water (10 ml.) are added with continuous and vigorous shaking and the volume made up to the mark with distilled water. The mixture is well shaken for 15 minutes and filtered. The assay is carried out using 5 ml. of the filtrate, as described for injections. The result obtained multiplied by 10, gives the amount of ascorbic acid in the original weight taken. This method has been applied to two kinds of tablets common in Egypt giving very satisfactory results. The assay can be carried out even on one tablet giving reproducible results.

SUMMARY

1. A new colour test for ascorbic acid is described. The colour is shown to be due to the coloured uranium derivative.

2. A photoelectric colorimetric method for the assay of ascorbic acid is described. This method is recommended for the assay of pharmaceutical preparations such as injections and tablets. The assay is carried out within the limits of 0·5 to 10 mg. of ascorbic acid. Work is proceeding on the application of this method for citrus fruits.

3. New colour tests based on the strong reducing property of ascorbic acid are mentioned.

The authors wish to express their thanks to the Memphis Chemical Co. for the help extended to them in carrying out this work and for the supply of ascorbic acid preparations and instruments required.

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