BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 42 2282—2286 (1969)

Spectrophotometric Determination of L-Ascorbic Acid*1

Yoshiro OGATA and Yoshio KOSUGI

Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Chikusa-ku, Nagoya

(Received January 16, 1969)

A novel quantitative determination of L-ascorbic acid by UV spectrophotometry in a HCl-KCl buffer has been developed. Ascorbic acid (λ_{max} 244 mμ) of over 2×10^{-5} M can quantitatively be determined by this method. Several oxidizing, reducing, acidic or basic reagents and other organic compounds which may be present together with ascorbic acid in ordinary drugs and drinks do not interfere the determination.

A considerable amount of L-ascorbic acid (Vitamin C) is now consumed as medicine and also as an antioxidant and additive in drinks and foods. A number of estimations of ascorbic acid assay have been developed, *i. e.*, visual titration,[1] spectrophotometry (or colorimetry),[1] polarography,[2] gas chromatography,[3] gravimetry,[4] bioassay method,[5] *etc.* have been postulated. Among them, titration and spectrophotometry are prevailing. The color change of indicators such as Tillman's reagent (2,6-dichlorophenolindophenol), Folin's reagent (phosphomolybdate), methylene blue and iodine are often employed for titration and colorimetry. Although some modifications of these methods have been reported,[6] they have shortcomings; *i.e.*, they are difficult to carry out, when a sample is either colored or contaminated with acidic or reducing substances which may react with the indicators. Fujita *et al.*[7] used a metaphosphoric acid solution and Robertson[8] added KCN to the sample solution, and they determined ascorbic acid content by the estimation of difference of absorbances before and after disappearance of ascorbic acid by oxidase or cupric ion. Reducible substances, however, often react with other reducing agents as well as ascorbic acid. In view of a large absorbance of ascorbic acid in UV region, the spectrophotometry was examined in detial. The present paper is a summary of our data concerning the suitable conditions of this very simple procedure for the spectrophotometric analysis in the presence of a number of additives.

Ascorbic acid (H_2A) is unstable in aqueous solutions, especially in the presence of heavy meta ions such as cupric and ferric ions. Though the rate of autoxidation to dehydroascorbic acid (DA) decreases in less polar organic solvents,[9] the solubility of the acid is generally small in these solvents, but the common organic compounds are very often soluble and their UV absorption bands overlap that of ascorbic acid.

The authors have previously studied kinetics of the copper-salt catalyzed autoxidation of ascorbic acid (H_2A) to dehydroascorbic acid (DA).[10]

$$H_2A + \tfrac{1}{2}O_2 \rightarrow DA + H_2O \qquad (1)$$

According to our mechanism,[10] ascorbic acid should be more stable in more acidic media. However, the cuprous ion formed by the reaction of cupric ion with ascorbic acid may easily be oxidized in acidic media to cupric ion (Eq. (2)), a catalyst for the autoxidation.

$$Cu(I) + \tfrac{1}{4}O_2 + H^+ \rightarrow Cu(II) + \tfrac{1}{2}H_2O \qquad (2)$$

It is known[11] that halide ion, *e. g.*, chloride ion, retards reaction (2) and also inhibits the formation of complex of ascorbic acid and cupric ion. These mechanisms suggest that hydrochloric acid free of metal ion is effective to prevent autoxidation. Even a trace of cupric ion, *e. g.*, 1.5×10^{-9} M,

*1 Contribution No. 120.

1) The Association of Vitamin Chemists, Inc. Ed., "Method of Vitamin Assay," 2nd ed., Interscience Publishers, Inc., New York (1951), Chap. 4, p. 71.

2) H. Woggon, *Ernährungsforschung*, **4**, 35 (1959).

3) T. Uno and H. Okuda, *Yakugaku Zasshi (J. Pharm. Soc. Japan)*, **86**, 1148 (1966).

4) J. H. Roe and M. J. Oesterling, *J. Biol. Chem.*, **152**, 511 (1944).

5) M. E. Reid, "Estimation of the Vitamins, Biological Symposia XII," The Jacques Cattell Press, Lancaster, Pa. (1947), p. 373.

6) I. Onishi and T. Hara, This Bulletin, **37**, 1314 (1964); R. Charonnat and L. B. Beauquesne, *Ann. Pharm. France*, **16**, 96 (1958); M. Schmall, C. W. Pifer, E. G. Wollish, R. Duschinsky and H. Gainer, *Anal. Chem.*, **26**, 1521 (1954).

7) A. Fujita and T. Sakamoto, *Biochem. Z.*, **297**, 10 (1938).

8) E. B. Robertson, *J. Soc. Chem. Ind.*, **53**, 277 (1934).

9) Y. Ogata and Y. Kosugi, Solvent Effect on the Autoxidation of L-Ascorbic Acid, *Tetrahedron*, **25**, 1055 (1969).

10) Y. Ogata, Y. Kosugi and T. Morimoto, *ibid.*, **24**, 4057 (1968).

11) L. W. Mapson, *Biochem. J.*, **39**, 228 (1945).

however, acts as an effective catalyst[12] and the complete removal of these metallic ions are often difficult. Furthermore, the very low concentration of chloride ion accelerates the autoxidation,[11] but its high concentration retards the overall reaction. Thus, as a source of chloride ion a large amount of alkali chlorides besides HCl should be advantageous for the stabilization of ascorbic acid.

Experimental

Ultraviolet Spectroscopy. An automatic recording spectrophotometer (Type Simadzu SV-50A) equipped with quartz cells (light path of 10 mm) was used.

Materials. Ascorbic acid was of 99.5% pure with decomposition point of 190.9°C [lit. 190—192°C (dec.)[13]] and optical rotation of $[\alpha]_D^{25}$ +20.94° [lit. 20.5—21.5°[13]]. Dehydroascorbic acid was prepared from ascorbic acid by the method of Fujimura *et al.*,[14] and crystallized from acetic acid[15]: decomposition point 218—222°C (dec.) [lit. 196°C (dec.),[14] 225°C (dec.),[16] 237—240°C (dec.)[15]]. Potassium chloride and the following organic compounds were of commercial guaranteed reagent grade; saccharose, oxalic acid, nicotinamide, sodium cyclohexylsulfamate, D-fructose, DL-methionine. Hydrochloric, phosphoric, metaphosphoric acids and other organic compounds were of commercial extra pure grade and were used without further purification. Oxygen and nitrogen gases were of over 99.7% and 99.95% pure, respectively. Distilled water was used. Cupric and ferric chlorides were estimated by iodometry and chelation with EDTA, respectively.

Procedure. Sample solutions of given concentrations in stoppered volumetric flasks were kept standing at constant temperature (20°C) for a known period. There was no appreciable difference in the decreasing rate of ascorbic acid concentration between 2—3 day's storage of samples in a glass flask and that in a polyethylene bottle. After being kept for a certain period, ascorbic acid was diluted to *ca.* 5×10^{-5} M and the absorption curve ranging 238—255 mμ was drawn. The ascorbic acid concentrations in the dilute solutions were obtained by means of a calibration curve.

Titration with the Indophenol Method.[1] Samples were diluted to the concentration of *ca.* 10^{-1} M ascorbic acid and titrated with a 0.012% 2,6-dichlorophenolindophenol solution.

Results and Discussion

Absorption Spectrum and Molar Extinction Coefficient. Ultraviolet absorption peak (λ_{max}) of ascorbic acid varies with solvent, pH and temperature. The variation of λ_{max} may be caused by the change of the degree of solvation, ionization and/or association of ascorbic acid. The peak (λ_{max}) was 244 mμ in these acidic solutions in the present experiment. In a solution of 0.4 N HCl and 0.4 M KCl, the absorbance (E_{244}) at 244 mμ of various concentrations of ascorbic acid had the relation $E_{244}=10^4[H_2A]-0.035$ with the concentration ($[H_2A]$), at least, at 2×10^{-5}—9×10^{-5} M. Molar extinction coefficient (ε_{244} or ε_{max}) was found to be 10000.

Acidic Solutions. Acids can suppress the dissociation of ascorbic acid. The stability of ascorbic acid was examined under various conditions in a mixture of 0.4 N HCl and 0.4 M KCl, 0.1 N HCl, 0.4 N HCl, 0.1 N H_3PO_4 or *ca.* 2% metaphosphoric acid.

In all of these solutions, λ_{max} and ε_{max} were the same, *i. e.*, 244 mμ and 10000, respectively. Metaphosphoric acid may be advantageous in the presence of cupric chloride of *ca.* 15 ppm in a dilute solution. However, polymerization degree (n) of commercial metaphosphoric acid, $(HPO_3)_n$, and hence the degree of dissociation in solution is not measurable by means of the usual titration method. Further, the solution of metaphosphoric acid for UV measurements should be filtered to remove the turbidity and stored in a refrigerator to prevent depolymerization. Because of this troublesome treatment as well as instability of the solution (safe storage is within a week), metaphosphoric acid was less suitable as a stabilizer of ascorbic acid. A mixture of aqueous HCl and KCl (a modified Clark-Lubs buffer solution) was found to be the most advantageous solution. All experiments described below were carried out in this buffer solution of 0.4 N HCl and 0.4 M KCl.

Effects of Other Factors. Our kinetic study[10] shows that the rate of autoxidation of ascorbic acid decreased at high concentration of ascorbic acid and at lower partial pressure of oxygen. These facts imply that ascorbic acid is more stabilized at its high concentration and in the absence of oxygen. These effects are shown in Table 2 combined with the result in Table 1. Ascorbic acid is more stable in nitrogen than in air or oxygen, but the effect was too low to necessitate determination under nitrogen atmosphere, except for the presence of a large amount of metallic ion.

Ferric ion also catalyzes the autoxidation of ascorbic acid, but the examination of the effect of ferric chloride (Table 3) shows that ferric ion of the concentration below *ca.* 13 ppm in a dilute solution does not affect stabilization.

Absorption of Organic Compounds. For the application of this analytical method to drugs, drinks and foods, it is necessary to know the possibility of the overlap of the absorption band with those of organic compounds which may be present

12) L. W. Mapson, *ibid.*, **35**, 1332 (1941).
13) P. G. Stecher, "The Merck Index," 7th ed., Merck & Co., Inc., Rahway, N. J., U. S. A. (1960), p. 106.
14) K. Fujimura and A. Matsuda, *Memoirs Research Inst. Food Science, Kyoto Univ.*, No. 8 (1954), p. 17.
15) H. Staudinger and W. Weis, *Z. Physiol. Chem.*, **337**, 284 (1964).
16) P. G. Stecher, "The Merck Index," 7th ed., Merck & Co., Inc., Rahway, N. J., U. S. A. (1960), p. 323.

TABLE 1. STABILITY OF ASCORBIC ACID IN VARIOUS ACIDIC SOLUTIONS IN THE ABSENCE OR PRESENCE OF CUPRIC CHLORIDE AT 20°C

Solution	Added $CuCl_2$ (10^{-4} M)	Concentration of ascorbic acid (10^{-5} M)				
		Initial	1 hr	5 hr	1 day	2 day
0.4 N HCl 0.4 M KCl	0	3000 6.01	2980 5.89	2940 5.68	2900 4.49	2620 3.71
	2.3	3000 6.01	2940 5.84	2930 4.22	2500 2.16	2330 —
0.1 N HCl	0	3040 6.00	2930 5.97	2890 5.62	2740 5.32	2550 3.55
	2.3	3040 6.00	3000 4.83	2890 1.22	2590 0.22	2100 —
0.4 N HCl	0	2990 6.03	2910 5.82	2910 5.61	2780 5.51	2550 4.27
	2.3	2990 6.03	2920 5.90	2880 3.06	2610 0.48	2340 —
0.1 N H_3PO_4	0	3000 6.00	3000 5.87	2870 5.78	2750 5.40	2510 4.15
	2.3	3000 6.00	3000 5.93	2760 5.67	2550 5.21	1790 4.92
ca. 2% $(HPO_3)_n$	0	2950 5.83	2920 5.80	2860 5.75	2770 5.46	2770 5.19
	2.3	2950 5.83	2950 5.83	2910 5.58	2870 5.54	2770 4.90
Water	0	3000	2920	2980	2850	2540
	2.3	3.00	2.82	2.80	2.60	2.33

TABLE 2. EFFECTS OF CONCENTRATION OF ASCORBIC ACID AND NITROGEN OR OXYGEN ON THE STABILITY IN A BUFFER SOLUTION OF 0.4 N HCl - 0.4 M KCl

Atmosphere	Added $CuCl_2$ (10^{-4} M)	Concentration of ascorbic acid (10^{-2} M)				
		Initial	1 hr	5 hr	1 day	2 day
Air	0	12.0	12.0	11.9	11.6	10.8
	2.3	12.0	12.0	11.7	10.7	—
N_2	0	3.00	2.96	2.95	2.94	2.74
	2.3	3.00	2.95	2.84	2.83	2.67
O_2	0	3.00	2.95	2.95	2.70	2.64
	2.3	3.00	2.87	2.74	2.60	2.21

TABLE 3. EFFECT OF FERRIC CHLORIDE ON THE STABILITY OF ASCORBIC ACID

Added $FeCl_3$ (10^{-4} M)	Concentration of ascorbic acid (10^{-2} M)				
	Initial	1 hr	5 hr	1 day	2 day
2.3	3.00	2.99	2.98	2.78	2.47
2300	3.00	1.76	1.69	1.49	1.13

together with ascorbic acid.

Results are shown in Table 4 as ε_{244}, ε_{max} and ε_{275}, where ε_{275} of ascorbic acid is only 400. Common vitamins are usually slightly soluble in the buffer solution and hence they are not examined. Extraction of ascorbic acid from solid samples with a small amount of the buffer solution can avoid contamination of such less soluble substances. If a sample is liquid, extraction with diethyl ether is recommended to remove vitamins A, D, E, *etc.*, and other many organic compounds.

A given amount of ascorbic acid was mixed with organic compounds whose ε's at suitable wavelengths were known (*e. g.*, see Table 4) and analyzed

TABLE 4. MOLAR EXTINCTION COEFFICIENTS AT λ_{244}, λ_{275} AND λ_{max} OF SOME ORGANIC COMPOUNDS IN A BUFFER OF 0.4 N HCl - 0.4 M KCl

Compound	ε_{244}	ε_{275}	λ_{max}	ε_{max}
Dehydroascorbic acid	9.4×10	1.1×10^2	302	1.3×10^2
Nicotinamide	2.05×10^3	8.3×10^2	261.5	5.6×10^3
Aspartic acid	0.1>	0.1>	—	—
Pantothenic acid	1.4×10	4.6	—	—
DL-Methionine	1.7×10	0.1>	—	—
Taurine	0.1>	0.1>	—	—
Glycine	0.1>	0.1>	—	—
Dextrose	0.1	0.1	—	—
D-Fructose	0.8	2.7	280.5	2.8
Saccharose	1.2	1.1	258	1.3
D-Sorbitol	0.6	0.7	264	0.1
Saccharin	1.8×10^3	7.5×10^2	278.5	7.7×10^2
Sodium cyclohexylsulfamate	3.1	0.1>	—	—
Citric acid	4.7	0.1>	—	—
DL-Malic acid	1.6×10	1.6	—	—
Tartaric acid	6.5	0.1>	—	—
Oxalic acid	3.7×10	1.1×10	244	3.7×10
Succinic acid	6.5	1.2	—	—
Tannic acid	2.5×10^3	6.9×10^3	275	6.9×10^3
Caffeine	2.8×10^3	7.5×10^3	269.5	8.0×10^3
Tartrazine	1.3×10^4	1.3×10^4	(Visible region)	

TABLE 5. MODELS OF APPLICATION

Sample	Composition	Concentration prepared (10^{-5} M)	$E_{244}^{calc.}$	$E_{244}^{obs.}$	$E_{275}^{calc.}$	$E_{275}^{obs.}$
A	ascorbic acid	6.435	0.608	—	0.026	—
	nicotinamide	5.70	0.117	—	0.045	—
	total absorption		0.725	0.730	0.071	0.073
B	ascorbic acid	4.52	0.417	—	0.018	—
	tannic acid	5.64	0.141	—	0.378	—
	caffeine	3.61	0.101	—	0.274	—
	total absorption		0.659	0.671	0.670	0.669

spectrophotometrically.

The results in Table 5 indicate that this method is satisfactory, when two absorbances at 244 mμ and at an appropriate wavelength of additives are known.

Applications. A commercial vitamin pill (Sample I) contains many organic compounds. For example, the indicated composition was as follows: vitamin A palmitate 2500 I. U., calciferol 250 I. U., thiamine tetrahydrofurfuryl disulfite 2 mg, vitamin B_2 2.5 mg, nicotinamide 10 mg, vitamin B_6 2.5 mg, folic acid 0.25 mg, calcium pantothenate 5 mg, vitamin B_{12} 0.001 mg, vitamin C 37.5 mg, DL-α-tocopherol acetate 1 mg in one tablet. The pill (0.5506 g) was powdered and extracted with the KCl-HCl buffer solution of less than 20 m*l*. The filtrate was further extracted twice with diethyl ether (each *ca.* 50 m*l*). The residual aqueous solution was diluted to 1 tablet/6250 m*l* buffer solution and its absorbance at 244 mμ was measured to be 0.357. Accordingly, the content of ascorbic acid in the pill was calculated to be 37.7 mg/tablet using a calibration curve. The observed content agreed with the indicated content (37.5 mg/tablet) of ascorbic acid and also with the content estimated by indophenol method (37.0 mg/tablet).

Besides ascorbic acid, tannic acid and caffeine are main components of green tea (Sample II). The sum of these two components was estimated to be 2.68×10^{-4} mol/g using $E_{275}=0.495$, assuming that they have the same ε_{275} and ε_{244} values of 7.2×10^3 and 2.65×10^3, respectively (*cf.* Table 4). Thus the ascorbic acid content was calculated from $E_{244}-0.316$ and dilution degree of 1 g/4 *l*.

E_{244}(obs.) $-E_{244}$(tannic acid + caffeine) $=0.316-0.177=0.139$

This value of 0.139 corresponds to 1.67×10^{-5} M ascorbic acid solution and the concentration gives an approximate content of the acid in Sample II

to be *ca.* 11.8 mg/g. The sample was diluted to 1 g/100 m*l* and titrated with indophenol method. The content of ascorbic acid was determined to be 9.6—13.1 mg/g. This uncertainty may be due to the coloration of the sample.

Ascorbic acid content in an orange juice (Sample III) or a purified drink (Sample IV) was also difficult to be estimated exactly by the indophenol method even by employing the xylene extraction technique.[10] But the UV method is not interfered by coloring materials; moreover the sample is so diluted (*ca.* 5×10^{-5} M of ascorbic acid) that it is almost colorless. In addition, the additive property of the UV absorbance enables the accurate determination of the acid content. In the present experiments, a known amount of ascorbic acid (0, 1.25×10^{-5}, 2.5×10^{-5} or 3.5×10^{-5} M) was added to a sample and a straight line, obtained from plots of *E vs.* concentration, afforded an exact value of the acid content.

In these two cases of drinks (Samples III and IV), the values found were higher than the labeled values. This may be caused by the fact that the makers added the acid in a greater amount than that indicated on the label taking the consumption of ascorbic acid during storage into consideration.

Conclusion

The strong UV absorption band of ascorbic acid (λ_{max} 244, ε_{max} 10000 in an acidic solution) enables its facile quantitative determination with a very simple procedure, even if a sample contains a small amount of the acid (10^{-4} M). Oxidizing agents (*e. g.*, some metallic ions) and reducing agents (*e. g.*, oxalic acid and dextrose) which may react with ordinary titration indicators do not interfere with this direct spectophotometry. Also, colored materials which may sometimes be contained, do not interfere with it. Even when their compositions are unknown, the following circumstances may take place: (i) some components of drugs, drinks or foods have only small absorbance at 244 mμ on high dilution of the sample, (ii) some components of the sample are slightly soluble in a buffer solution consisting of 0.4 N HCl and 0.4 M KCl, and (iii) the total absorbance of a sample is known, as is often the case, before adding ascorbic acid.

The authors would like to thank Takeda Pharmaceutical Ind. Co., Sankyo Pharmaceutical Co. and The Aichi Food Research Institute for their gifts of some of the materials, and also Mr. M. Gotoh for his helpful assistance.