University of Białystok, Department of Chemistry, Białystok, Poland

L-Ascorbic acid – clinical use, toxicity, properties, methods of determination and application in chemical analysis

E. KLESZCZEWSKA

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1. Introduction – general characteristics of L-ascorbic acid

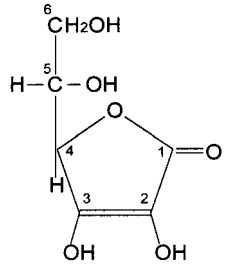
L-Ascorbic acid (vitamin C) is soluble in water and its deficiency in human body causes scurvy [1–3]. Its symptoms in adults are gingivitis, susceptibility of blood vessels to damage and bleeding, changes in bones and cartilages and retarded wound healing [1]. L-Ascorbic acid is necessary in redox processes taking place in cells. It is reversibly oxidized to L-dehydroascorbic acid and partially metabolized to inactive sulphide and oxalic acid, which are expelled in urine. It is well absorbed from the digestive system and easily reaches the tissues. Plasma protein binding is around 25% [4]. L-Ascorbic acid reaches higher concentration in leukocytes and blood platelets than in plasma and erythrocytes. Healthy organism contains 1.5 g of L-ascorbic acid and the daily requirement for L-ascorbic acid is estimated to be 30–100 mg. The sources of vitamin C are fruit and vegetables. Milk and animal products contain little vitamin C. Boiling and long storage inactivate vitamin C. Excess of vitamin C is eliminated by the kidneys.

2. Clinical use

L-Ascorbic acid is administered in scurvy and Moeller-Barlow disease. With a normal diet disease resulting from deficiency of vitamin C rarely occur with full clinical picture and, therefore, relative deficiency of vitamin C plays an important role.

L-Ascorbic acid is used in treatment deficiency of vitamin C caused by improper diet or poor absorption. It is used in large doses in general weakness, infectious diseases [5–6] and during the recovery period. L-Ascorbic acid is also used to treat poisoning with toxins which promote formation of methaemoglobin, retarded wound healing, bone fractures and susceptibility to bleeding. Recent reports have confirmed the positive influence in treating AIDS. Its positive effect on many diseases, especially the infectious ones, has been shown [6].

Positive results have been obtained after therapy of Schoenlein-Henoch disease, Werlhof disease, haemophilia and also in parenchymatous bleeding of internal organs caused by other factors. L-Ascorbic acid is also used in dental decay, paradontosis and allergic diseases. Since the 1980s the antioxidant properties have also been associated with antineoplastic activity [7–9].



L-Ascorbic acid is assumed to act as an antioxidant, although its role in pathological conditions is controversial. Sakagami et al. [10] and Amano et al. [11] showed the influence of sodium ascorbate on the induction of apoptosis. L-Ascorbic acid is sometimes used in eye drops in treating superficial damage of cornea or senile corneal clouding.

Although L-ascorbic acid is administered to minimize the risk of cardiovascular diseases, there is too little information to prove that such treatment is successful.

3. L-Ascorbic acid and toxic substances

L-Ascorbic acid plays a protective role against some toxins. An example can be the prevention of carcinogenic and toxic actions of *N*-nitrosoamines [12–14] and nitrite [15].

L-Ascorbic acid reacts with free radicals of oxygen and transforms them into less toxic or non-toxic compounds. It plays such a role, among others, in the eye, where it protects against oxidative stress due to ozone, and in lung tissue that lacks antioxidative enzymes [16–18].

Jacobsen et al. [4] demonstrated the protective activity of L-ascorbic acid against toxic Cr(VI). Hypothetical mechanism of its activity is explained by the intracellular reduction of Cr(VI) to Cr(III) ions and by the impermeability of cell membrane to Cr(III) ions. The reduction of Cr(VI) ions to Cr(III) with L-ascorbic acid can prevent allergic changes caused by Cr(III) ions.

In the mid 1970s studies of the positive influence of L-ascorbic acid on the levels of selenium, lead, vanadium, cobalt and zinc were published [2, 4, 19–21].

L-Ascorbic acid is known to reduce the toxic effect of nickel in rats [2].

The reductive properties of L-ascorbic acid are necessary for biosynthesis of tetrahydrofolic acid and to maintain the active forms of Cu(II) and Fe(II) ions. It increases iron

absorption by reducing iron to well soluble ferrous salts. As a result, it contributes to the production of haemoglobin in erythrocytes and aids in the treatment of anaemia caused by iron deficiency. Many studies, confirming autooxidation of vitamin C against metals, have been published [2, 4, 22].

Lewin et al. [2] investigated the stimulation of iron absorption from food after L-ascorbic acid had been administered. Iron is present in food in two forms: one form is chemically bond with haem and the other is not (non haem iron). Pauling et al. [2] showed that L-ascorbic acid stimulates increased absorption of non haem iron.

Both, Fe(III) and Cu(II) ions oxidizing L-ascorbic acid with a free radical intermediate; the reaction can be written as follows [22, 23]:

$$H_2Asc + Cu(II) \rightarrow H_2Asc^{\cdot} + Cu(I)$$

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2 \doteq$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^{\cdot} + OH^{-}$$

Many authors [2, 4, 9, 22–25] cite this reaction but its mechanism remains unsolved.

Substantial deficiency was observed after exposition to cadmium. Its concentration in the serum of people exposed to cadmium is much lower than in those who were not exposed to that metal.

Fox et al. [26, 27] proved better complementation of cadmium (II) ions with iron (II) ions in the presence of L-ascorbic acid.

Individuals with L-ascorbic acid deficiencies are more prone to the toxic effects of oxidants, cadmium and lead. It has recently been shown that L-ascorbic acid decreases the toxicity of selenium and vanadium [4].

4. Interactions, contraindications, side effects

In the literature one finds that solutions of L-ascorbic acid should not be mixed with salts of metals, especially salts of copper and iron and oxidizers because L-ascorbic acid is chemically incompatible with those substances [1]. High serum levels can falsify the results of some clinical chemical tests based on redox methods (determination of glucose and creatinine in blood and urine). L-Ascorbic acid increased the rate of elimination of amphetamine and tricycle antidepressants [1]. It destroys vitamin B_{12} and so both should not be administered simultaneously. It also increases the concentration of Na ions and uric acid in blood.

High doses of L-ascorbic acid are contraindicated in the following conditions:

- pregnancy (by causing disturbances in fetus growth),
- diabetes (by falsifying the results of glucose determination in urine and blood),
- deficiency of glucose-6-phosphate dehydrogenase (by intensifying erythrocyte haemolysis and causing anaemia),
- nephrolithiasis (by increasing the risk of forming calculus in urinary tracts and increasing the elimination of oxalates in urine).

Higher doses than 1 g daily may cause disturbances of the alimentary tract (heartburn, diarrhoea) or urinary (facilitated formation of urate, cystine and oxalate calculus). Further administration may increase intolerance to L-ascorbic acid and even addiction to the vitamin in patients with migraine [1].

5. Physical properties

L-Ascorbic acid is a sour, odourless solid and becomes dark when exposed to light. It has good solubility in water, methanol and ethanol, is less soluble in acetone and is insoluble in ether, benzene and chloroform [25, 28]. Some properties are shown in Table 1.

Table 1: Physical properties of L-ascorbic acid

Molecular formula	$C_6H_8O_6$
Molar mass	176.13
Crystal form	monocrystals
Melting point (°C)	190–192
Optical activity (c = 1%, 1 cm in H_2O)	20.5°–21.5°
pH (5 mg/ml)	3
pH (50 mg/ml)	2
pK_1	4.17
pK_2	11.57
Redox potential	$E^{\circ} = 0.39$ V
Solubility (g/ml) in:	
Water	0.33
Ethanol	0.02
Maximum absorption for pure substance dissolved in water	265 nm

Vitamin C occurs in three biologically active forms:

- L-ascorbic acid (reduced form)
- semidehydro- and L-dehydroascorbic acid (oxidized form)
- ascorbic gene (chemically bonded form).

6. Chemical properties

L-Ascorbic acid is an endiol form of γ-lactone of 3-keto-L-gulonic acid.

Contrary to its common name and some chemical properties, L-ascorbic acid is not a carboxylic acid but a compound related to sugars (hexoses). Its biosynthesis is one of pathways of glucose changes leading to formation of the compound with the structure shown above.

For many mammals this compound is not a vitamin because they are able to synthetize it themselves. Humans and primates are exceptions to this; in their organisms there is no L-gulone oxidase, which catalyzes the last stage of vitamin C synthesis.

As it has already been mentioned, vitamin C is an enol form of dehydrated lactone of L-gulonic acid. Thus, it contains lactone arrangement combining atoms C1 and C4 from the particle of water, which gives it the form characteristic for hexoses with a furane ring. Because it occurs in the enol form, it consists of two –OH groups in position 2 and 3 and double bond between C2 and C3. The structure described here justifies its systematic name: 2,3-didehydro-L-threo-hexone-1,4-lactone. There is also a stereoisomer with the configuration of type D (erythrobic acid).

The acidic nature of L-ascorbic acid is determined by the presence of two dissociable hydrogen atoms in the endiol moiety. In aqueous solutions L-ascorbic acid is a weak dibasic acid, which dissociates in two stages. In the first step one hydrogen ion is released:

$$H_2Asc \rightleftharpoons H^+ + HAsc^-$$

In the second step the other hydrogen ion is released:

$$HAsc^- \rightleftharpoons H^+ + Asc^{2-}$$

The disociation constants and pK_a values determined by Birch and Harris are the following [2]:

$$K_1 = 6.77 \cdot 10^{-5}, \qquad pK_1 = 4.17$$

$$K_2 = 2.69 \cdot 10^{-12}, \qquad pK_2 = 11.57$$

The second stage of dissociation is insignificant under physiological conditions. Therefore, H_2Asc can be considered a weak monoprotone acid with a strength similiar to acetic acid. The di-negative ion of L-ascorbic acid (Asc^{2-}) is present only in a strong alkaline medium. In this medium H_2Asc quickly oxidizes. Therefore, the use of L-ascorbic acid in titrations is possible only in solutions with pH < 8.5.

The presence of the endiol unit results in the strong reductive properties of L-ascorbic acid. Therefore, in water solutions this acid is easily oxidized to L-dehydroascorbic acid. L-Ascorbic acid is more stable in acid medium than in alkaline medium. In alkaline medium oxidation of the acid leads to L-dehydroascorbic acid and finally oxalic acid and L-threone acid.

L-Ascorbic acid in its crystalline form is resistant to oxidation. In oxygen–free conditions L-ascorbic acid is stable even at high temperature. L-Dehydroascorbic acid is less stable to hent and that is how its loss during heating, is explained. In the presence of oxygen, both forms are irreversibly oxidized to biologically inactive products, especially when ions of some metals, particularly copper and iron, are present. In biological systems oxidation of L-ascorbic acid is catalysed by ascorbic oxidase, peroxidase and various other enzymes. In oxygen-free environment, e.g. when storing canned products, loss of amount of the vitamine by indirect influence of oxygeny, is observed.

Water solutions of L-ascorbic acid are unstable [28, 29] and their stability is related to the purity of water and stabilisers used. The water used to prepare the solution of L-ascorbic acid should not contain dissolved oxygen and ions of heavy metals, e.g. copper. The stability of water solutions of this acid decrease with their dilution. The increase of pH influences the rate as well.

The chemistry of the free radicals of L-ascorbic acid (Asc$\dot{-}$, Asc$\cdot$) was firstdescribed in 1932 when Michaelis [30] suggested the reaction to be a two-step oxidation-reduction process with a free radical intermediate:

$$H_2Asc \rightleftharpoons Asc\dot{-} + 2\ H^+ + e^-$$

$$Asc\dot{-} \rightleftharpoons Asc + e^-$$

where: H_2Asc is L-ascorbic acid, Asc$\dot{-}$ is a free radical anion and Asc is L-dehydroascorbic acid.

Nowadays spectrophotometric methods, electron magnetic resonance, nuclear magnetic resonance, pulse methods and kinetic enzymatic methods (with particular consideration of pH effect) are used to identify and characterize such free radicals [2, 4, 7, 8, 12, 13, 18, 24, 31–34].

The most frequently described compounds in literature are L-ascorbic acid, ascorbate, free radical of L-ascorbic acid and L-dehydroascorbic acid. This configuration confirms that redox process has two stages with a free radical intermediate.

It is commonly assumed that ascorbate is an active reducing agent, whereas the free radical of L-ascorbic acid is relatively inactive. The reaction of disproportionation can be showed the following way [4]:

$$2Asc\dot{-} + H^+ \rightleftharpoons HAsc^- + Asc$$

$$2Asc\dot{-} + 2\ H^+ \rightleftharpoons H_2Asc + Asc$$

$$2\ HAsc\cdot \rightleftharpoons H_2Asc + Asc$$

One of the studies concerned with the property of L-ascorbic acid as a scavenger of free radicals analyzed the following reaction:

$H_2O \rightsquigarrow$ OH (2.74), eag(2.76), H(0.55), H_2O_2(0.72), H_2(0.55),

where H_2Asc reduces the energy of breaking and forming molecules.

Squadrito et al. [15] studied the kinetics of the reaction of L-ascorbic acid with nitric oxides. In water solutions the nitric oxides shifted the electron to a free radical OH$\dot{-}$ with simultaneous formation of Asc$\dot{-}$:

$$N_2O + H_2O + e^-_{aq} \rightarrow N_2 + OH\dot{-} + OH^-$$

$$HAsc^- + OH\dot{-} \rightarrow Asc\dot{-} + H_2O$$

$$HAsc^- + OH\dot{-} \rightarrow [HAsc^-\ (OH)\text{-substract}] \rightarrow Asc\dot{-} + H_2O + \text{product}$$

The spectrophotometric examinations of the free radical showed that its spectrum is a symmetric Gauss curve with absorption maximum at wavelength $\lambda = 360$ nm. Both, the L-ascorbic acid and the free radical of hydroxyl have similar spectra with an absorption maximum at $\lambda = 360$ nm and that is the source of difficulties in the interpretation of their spectra. The spectrophotometric assay seems quite complicated. Pulse analysis can be of some help. In pulse analysis, the relationship between absorption and pH was determined and a new description of the acid-base chemistry of Asc$\dot{-}$ was suggested:

$$2\ Asc\dot{-} \rightarrow (Asc\dot{-}\ \ Asc\dot{-})$$

$$(Asc\dot{-}\ \ Asc\dot{-}) \rightarrow 2Asc^-$$

$$(Asc^-\ Asc\dot{-}) + H^+ \rightarrow HAsc^- + Asc$$

$$(Asc\dot{-}\ \ Asc\dot{-}) + H_2O \rightarrow HAsc^- + Asc + OH^-$$

(Asc$\dot{-}$ Asc$\dot{-}$) complex of the reaction is relatively stable at pH = 3.3–9.0.

As it has already been mentioned, kinetic techniques [35] allowed the formation of free radicals of L-ascorbic acid to be followed.

It is worth noting here that the reaction of ascorbates and free radicals of L-ascorbic with oxidizing agents or oxidizing solutions is significant for biological reasons because it occurs in the cells of living organisms. Studies of auto-oxidation of L-ascorbic acid suggest the mechanism to be a charge transfer. EPR studies showed that at pH = 4.8 the reaction with hydroxyl group is dominant, while at pH between 6.6–9.6 the reaction with $O_2\dot{-}$ group becomes dominant. Auto-oxidation of ascorbates at pH.7.8 is inhibited by superoxide dismutase, the enzyme catalysing the reaction according to the following equation:

$$O_2\dot{-} + O_2\dot{-} + 2H^+ \rightarrow H_2O_2 + O_2$$

and then

$$HAsc^- + O_2 \rightarrow Asc\dot{-} + O_2\dot{-} + H^+.$$

The presence of radicals in neutral and alkaline solutions suggests the following mechanism of oxidation:

$HAsc^- + O_2 + H^+ \rightarrow Asc + H_2O_2$ and then reactions

$$Asc + HAsc^- \rightleftharpoons 2Asc\dot{-} + H^+$$

NMR and EPR examinations showed that the free radical of L-ascorbic acid is formed when L-ascorbic acid and L-dehydroascorbic are in an equilibrium. Studies conducted by Coudert et al. [31] were concerned with the oxidation of L-ascorbic acid and its analogues in the free-radical stage. A different interpretation of the occurrence of free radicals in a solution has been suggested by Nadezhidin

and Dunford [4]:

$H_2O_2 + hv \rightarrow 2OH\dot{-}$

$OH\dot{-} + H_2O_2 \rightarrow HO_2\dot{-} + H_2O$

$HO_2\dot{-} \rightleftharpoons O_2\dot{-} + H^+$

where: $pK = 4.7$ for stoichiometric equation

$2(HO_2) : 1(H_2Asc)$.

Pelizzetti et al. [36] investigated the reaction of charge transfer from L-ascorbic acid to a free radical of phenothiazine derivatives. The investigations with the use of electron pulse radiolysis were conducted on phenothiazine, promazine, chlorpromazine and promethazine. They showed the complexity of reactions and their multidirectional character resulting from the initial condition of the reaction.

L-Ascorbic acid is characterized by a great complexing power partly due to the presence of an endiol unit. It is also a relatively good ligand forming complex compounds, mostly on the second level of oxidation [4, 19, 20, 25, 37–47].

The possibilities of using L-ascorbic acid as ligand were investigated with X-ray, crystallographic, IR and NMR methods. The following possibilities of complex configuration were shown: ML^{n-2}, $ML_2{}^{n-4}$ or ML^{n-2}, $ML_2{}^{n-4}$, $ML_3{}^{n-6}$ [4], predicting constant values to be 10^5–10^{10}.

A rich body of work has been compiled on complexes with Cu(II), Fe(II), Mn(II), Be(II), Mo(II), Pb(II), Ba(II), Mg(II) and Ag(I), Al(III) [37–47]. UV-VIS spectrophotometric, potentiometric, ions exchange and voltametric methods are the most common methods used to examine the complexes. Masłowska and Owczarek investigated $Fe(II)-H_2Asc-H_2O$ complexes with spectrophotometric methods and $[Zn(HAsc)]^-$, $[Ni(HAsc)]^-$, $[Co(HAsc)]^-$ complexes with a potentiometric surface method [38, 39]. Seib et al. [4] made up a list of constant values of complexes and discovered that their values lie within the range of 1.6–4.03 for ion strength = 0.1 m/l and a temperature of 25 °C. The studies showed that L-ascorbic acid forms the most stable combination when the coordination numbers are 4 and 6. The pH of the solutions has a great influence on the type of complex. The complexes formed at pH = 2.2–7 show greater stability. At the end of the 1980s complexes of metal ions such as Ni(II), Co(II), Zn(II), Fe(II), Be(II), Mg(II), Ba(II), Ca(II), Sr(II) were examined with pulse polarography [40] and their constant values and structures of combination were established. The final stage of investigation of complexes is usually a quantitative assay of the reaction.

7. Use in chemical analysis

Ascorbimetry is a field of quantitative chemical analysis, which uses the reductive properties of L-ascorbic acid. The use of L-ascorbic acid as a titration agent is limited due to its instability in aqueous solutions. By direct titration with L-ascorbic acid the following ions were determined: Fe(III), Ag(I), Hg(II), Ti(III), Cu(II), Ce(IV), $I_2[Fe(CN)_6]^{3-}$, ClO_3^-, BrO_3^-, IO_3^- and VO_3^-. Many reduction reactions with L-ascorbic acid are slow. As a result, many assays are done as indirect methods, i.e. an excess of L-ascorbic acid is added and then the remaining excess is back-titrated with an other titrant (usually iodine solution). Fe(III) ions can be determined in this way. Using the method of back titration, Hg(II), Cr(VI), V(II), Cu(II), Cd(II) ions and chloramine T, chlorine, iodine, bromine, hypochlorite ions, persulphate ions, nitrate ions, chlorates, dichromates, sulphides, cyanides, rhodanates, iodides, hydrazine and formaldehyde were determined. Erdey and Svehla [48] described titration with L-ascorbic acid in non-aqueous solution. With this method iodine, Au(III), Hg(II), Fe(III) and Pb(II) ions were determined.

8. Methods of determination

According to the International Pharmacopoeia [49], the standard determination method of L-ascorbic acid is the titration with solutions of iodine, potassium periodate or 2,3-dichlorophenolindophenol (Tillmans's reagent). The disadvantage of this methods is their low selectivity because many other oxidants may react with both, iodine and 2,3-dichlorophenolindophenol. Such methods are unusable in coloured solutions because the use of Tillmans' s reagent is based on the reductive properties of L-ascorbic acid and dying pink in acidic medium and blue in neutral and alkaline solution. Titration with iodine and the method that uses 2,6-dichlorophenolindophenol are also recommended by the Polish Standards Association (ZN-79 MDCh/F-412) for quantitative determination of L-ascorbic acid in chemical mass**,** dragees and injection fluids.

Krayannis [50] used 2,6-dichlorophenolindophenol in determination of L-ascorbic acid in tablets. Gupta et al. [51] assayed uranium using L-ascorbic acid. Arya et al. [52] used calorimetric method to determine L-ascorbic acid in pharmaceuticals. Puzanowska-Tarasiewicz et al. [53] used bromometrical determination and empolyed phenothiazine derivatives (chlorpromazine, promazine, diethazine, perazine, fluphenazine, propercyazine and thiorydazine) as redox indicators. Puzanowska-Tarasiewicz et al. [53] were concerned with the use of phenothiazine derivatives in ceriometric determination. Iodatometric determination was developed by Puzanowska-Tarasiewicz et al. [53]. Phenothiazines were used as redox indicators.

Another group of quantitative assays of L-ascorbic acid are spectrophotometric methods. The spectrophotometric methods usually based on the measurement of absorbance of:

- L-ascorbic acid solutions at wavelength $\lambda = 265$ nm in neutral and acidic medium,
- a coloured product which is formed in the reaction of L-ascorbic acid with an oxidizing reagent in acidic medium.

Seib and Tolbert [4] developed a spectrophotometric assay which used dinitrophenylhydrazine. Dinitrophenylhydrazine reacts with the 2,3-ketogulone group to give a red product. This method allows a one-test assay for L-ascorbic acid and L-dehydroascorbic acid. Spectrophotometric methods also include a method based on reaction with o-phenycodiamine and its 4,5-dimethyl derivatives.

In the mid 1970s, Hansen and Ruzicka [54–57] introduced a FIA method. One of the first studies with the FIA method with spectrophotometric detection was done by Burns et al. [54]. They determined L-ascorbic acid as a pure substance and in tablets. They used the reaction with dodecanophosphoric acid.

Sultan [55] used Ce(IV) ions for the determination in pharmaceuticals. In another work, Sultan et al. [56] used tris-1-10 phenanthroline for determination. Kojło and Kleszczewska [57] used reaction of iron (III) ions with 2,2-pyridyl for quantitative assay in substance, dragees and blood.

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Dr. Ewa Kleszczewska
University of Białystok
Institute of Chemistry
Al. J. Pilsudskiego 11/4
15-443 Białystok
Poland