https://doi.org/10.31925/farmacia.2022.1.22 **ORIGINAL ARTICLE**

STUDY REGARDING A NEW EXTENDED-RELEASE CALCIUM ASCORBATE AND HESPERIDIN SOLID ORAL FORMULATION

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Abstract

Vitamin C is indispensable for the normal functioning of the most important biological processes in the human body and plays an essential role in the immune response to stress and viral and bacterial infections. Hesperidin is a bioflavonoid with vascular-protecting and anti-inflammatory properties. Given the benefits of vitamin C and hesperidin and the increased absorption of vitamin C by bioflavonoids, a dietary supplement containing both active ingredients has been developed to improve the functioning of the human immune system. Calcium ascorbate was used for vitamin C intake to reduce the high acid side effects at the gastric level. A slow-release pharmaceutical formulation was chosen to gradually release the active ingredients and a prolonged effect (prolonged-release tablets). The present study presents the quality analysis of this innovative dietary supplement with a formula based on non-acidic vitamin C (containing 605 mg of calcium ascorbate, equivalent to 500 mg of ascorbic acid) and bitter orange (*Citrus aurantium*) bioflavonoid complex (standardised to 80% hesperidin) by testing the following parameters: the neutrality of the product, the vitamin C assay, the hesperidin assay, and *in vitro* vitamin C dissolution. The study revealed suitable active substances contents of the product and a delayed pharmaceutical formulation that extends the duration of beneficial effects on the human body with reduced side effects.

Rezumat

Vitamina C este indispensabilă pentru desfășurarea normală a celor mai importante procese biologice și joacă un rol esențial în modularea răspunsului imun la stres și la infecțiile virale și bacteriene. Hesperidina este un bioflavonoid cu proprietăți vasculoprotectoare și antiinflamatoare. Având în vedere beneficiile vitaminei C și ale hesperidinei, precum și creșterea absorbției vitaminei C în prezența bioflavonoidelor, a fost dezvoltat un supliment alimentar pentru îmbunătățirea funcționării sistemului imunitar, care conține ambele ingrediente active. Pentru aportul de vitamina C a fost utilizat ascorbat de calciu pentru a reduce efectele secundare de hiperaciditate gastrică. A fost aleasă o formulare farmaceutică cu cedare prelungită pentru a asigura o eliberare treptată a ingredientelor active și un efect prelungit (comprimate cu eliberare prelungită). Prezentul studiu prezintă controlul calității acestui supliment alimentar inovator cu o formulă bazată pe vitamina C neacidă (605 mg de ascorbat de calciu, echivalent cu 500 mg de acid ascorbic) și un complex bioflavonoid din portocală amară (*Citrus aurantium*) (standardizat la 80% hesperidină), prin testarea următorilor parametri: neutralitatea produsului, dozarea vitaminei C, dozarea hesperidinei și dizolvarea *in vitro* a vitaminei C. Studiul a demonstrat conținutul adecvat de substanțe active al produsului și formularea farmaceutică care oferă o prelungire a duratei efectelor benefice asupra organismului uman, cu efecte secundare reduse.

Keywords: calcium ascorbate, vitamin C, hesperidin, extended-release, HPLC

Introduction

Vitamin C (L-Ascorbic acid, (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one) is an indispensable substance in the normal development of the most important biological processes, including synthesis of adrenal hormones, the transformation of folic acid into folinic acid and transferrin to ferritin, synthesis of norepinephrine and intracellular substances (e.g., collagen, skeletal and dental bone matrix). Ascorbic acid plays an essential role in the immune response to stress and viral and bacterial infections - cold and flu - as it participates in forming antibodies and interferons activation of enzymes [1, 2]. In addition, ascorbic acid plays an essential role in cellular respiration, accelerates wound healing, protects against cancer - by stopping the formation of nitrosamines, carcinogens that form during the assimilation of amino acids in the stomach [3].

Vitamin C deficiency may be due to an inadequate diet that lacks fruits and vegetables but is rare in adults and appears mainly in children, alcoholics, and the elderly [4, 5]. Stress, exposure to cold, and sports performance led to increased oxidative processes, and a vitamin C supplement is recommended [6]. In addition, the need for vitamin C increases during intense physical activities [7, 8] and in children regularly exposed to cigarette smoke [9, 10].

Vitamin C is also recommended in pregnancy and lactation [11], during the growth period of children [12], in diseases such as periodontitis, gingivitis, stomatitis, fractures, osteoporosis [13]. It is also supplemented in haemorrhage due to capillary fragility [14], iron deficiency anaemia (with iron or folic acid), overwork, neurasthenia, prolonged physical effort, serious illness, surgical interventions [15, 16]. It also favours iron absorption and, together with folic acid and vitamin B12, stimulates the development of red blood cells.

At the intestinal level, the absorption of vitamin C is achieved against the concentration gradient through a specific transport system (active transport). When administering high doses of vitamin C, intestinal absorption is achieved by passive transport (diffusion). The maximum intestinal absorption capacity is limited to 1200 mg in 24 hours at a dose of 3 g/day. The physiological plasma concentration is 0.7 - 1 µg/100 mL. Vitamin C has the advantage of not accumulating in the body. Therefore, it does not present any danger of overdose or toxicity (LD_{50} for rats is 11900 mg/kg b.w.), thus allowing unrestricted use. However, because the body does not store vitamin C, a constant intake through diet or food supplements is required.

Ascorbic acid (AA) causes an acidic pH in the body, which can maintain or trigger gastrointestinal sensitivity when administered in large quantities to people with gastric sensitivity. The mineral salts of ascorbic acid (ascorbates) are buffered and thus less acidic than ascorbic acid. Therefore, ascorbates (mineral salts) are recommended for people with gastrointestinal problems (abdominal pain or diarrhoea) caused by ascorbic acid. Vitamin C absorption in the body is also acquired when using ascorbates.

Sodium, potassium, magnesium, and calcium salts of ascorbic acid are used in the food industry. The ingested ascorbic acid salts are hydrolysed at the gastric level and release the higher bioavailability ascorbate ion and the corresponding mineral that is also absorbed. The Recommended Dietary Allowance (RDA) for sodium is 1500 mg; additional unadjusted intake may cause changes in blood pressure. An increased intake of potassium (4700 mg RDA) may cause hyperkalaemia with cardiac arrhythmia in patients with renal impairment (or with potassium-sparing diuretic treatment as angiotensin inhibitors) [17]. The daily requirement of magnesium is 400 mg RDA.

To increase AA's bioavailability in the proposed innovative formula, we replaced AA with its' mineral calcium salt - calcium ascorbate (CAA), to decrease its acidity. Also, CaA provides an intake of 1/2 molecule of calcium for each molecule of Vitamin C, necessary to compensate for the renal elimination of calcium caused by Vitamin C.

Bioflavonoids are a class of secondary metabolites corresponding to polyphenols. Flavonoids have a 15 carbons bone chemical structure constituted by a standard skeleton of phenyl-benzo-γ-pyran (C6–C3–C6), also known as *nucleus flava*, composed of two phenyl rings (A and B) and a heterocyclic ring (pyran) C. Flavonoids include flavonols, flavones, flavonoids, flavanones, anthocyanidins, and isoflavones [18].

Citrus flavonoids contain hesperidin (Figure 1), rutin, quercitrin and tangerine. Water-soluble antioxidants protect cells from damage caused by free radicals at different levels of the body. Natural sources rich in bioflavonoids are citrus fruits (especially grapefruit), grapes, apples, plums, cherries, blackberries, sour cherries, raspberries, blueberries, blackcurrants, onions, spinach [19]. The number of flavonoids is higher in the peel of fruits or vegetables, except for citrus fruits, where flavonoids are more present in the white cuticles surrounding these fruits' slices.

The *Citrus* bioflavonoids provide better absorption and higher bioavailability of vitamin C in the body and potentiate its effect. Albert Szentgyorgy, the discoverer of vitamin C, pointed this synergistic action in the first clinical trials on the compound, when he administered pure and “impure” vitamin C to his patients; he found that the “impure” form had an increased efficiency, due to the presence of bioflavonoids [20, 21].

At the cardiovascular level, flavonoids protect the structure and maintain the elasticity of blood vessels, regulate their permeability, improve circulation, have vasodilation and antithrombotic effects. Flavonoids prevent the oxidation of fatty acids reduce the risk of atherosclerosis, hypertension, hypercholesterolemia, myocardial infarction, stroke, varicose veins and haemorrhoids. At the respiratory system level, bioflavonoids protect the membranes of the airways against the harmful effects of air pollution and smoking, increase resistance to respiratory infections, and are effective in asthma. At the nervous system level, the bioflavonoids have an intense neuroprotective action, have a direct effect on the regeneration of nerve cells, and prevent mental decline and degenerative diseases such as Alzheimer's, Parkinson and dementia. Bioflavonoids also have a beneficial effect on tissues: they promote regeneration, reduce inflammation, and increase the body's energy metabolism.

Flavonoids are easily destroyed by heat, acidity, and food processing, so they must be supplemented with natural products rich in these elements. Unfortunately, no non-acidic vitamin C slow-release formula is

available to our best knowledge. Given the benefits of both vitamin C and hesperidin and increased the absorption of vitamin C by bioflavonoids, a dietary supplement containing both active ingredients has been developed to improve the functioning of the human immune system.

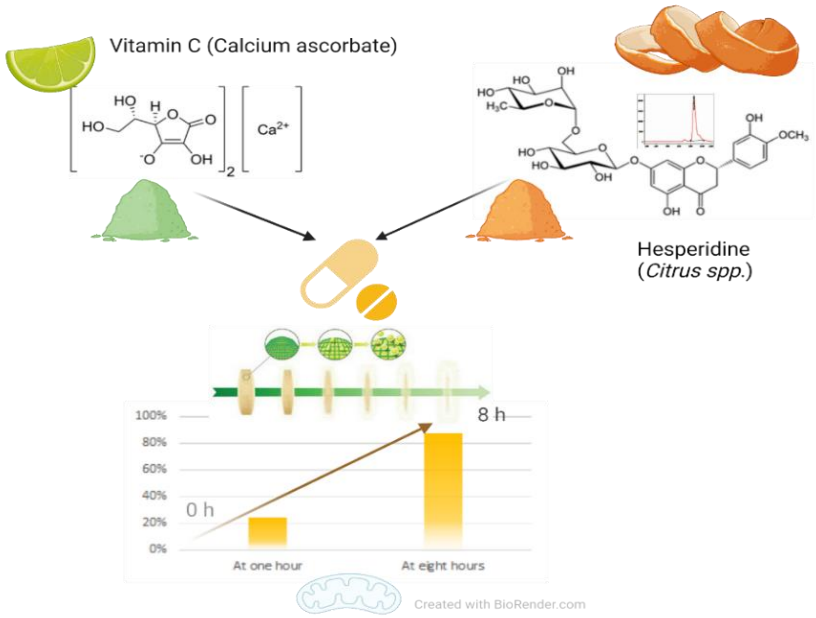

Figure 1.
Graphical representation of the research plan

Calcium ascorbate was used for vitamin C intake to reduce the high acid side effects at the gastric level [22]. In addition, a slow-release pharmaceutical formulation was chosen to ensure a gradual release of the active ingredients and a prolonged effect (prolonged-release tablets) [23]. The present study presents the quality analysis of this innovative dietary supplement with a formula based on non-acidic vitamin C (containing 605 mg of calcium ascorbate, equivalent to 500 mg of ascorbic acid) and bitter orange (*Citrus aurantium*) bioflavonoid complex (standardised to 80% hesperidin).

Materials and Methods

Samples

We tested an innovative dietary supplement formula (IDS) (batches S350515; S460615, and S550715) compared to three reference products on the market (Product T1/S150341ROAA, Product T2/L4150511 and Product T3/L4450640 – containing the same amount of ascorbic acid with slow-release dosage form).

Non-acidic character testing (Neutrality testing)

The neutrality of the product was tested by pH measurement of solutions resulting from the dissolution of food supplement retard tablets (after 8 hours) and comparative products. The testing was performed for the solutions obtained by progressive dissolution within 8 hours in 900 mL of distilled water. It was considered an acidic pH in the range 1 - 6, neutral pH in the range 6 - 7, and alkaline medium over 7. Three batches of samples were assessed for each formula. The mean value and standard deviation were determined.

There were used: the innovative dietary supplement (IDS) and three comparative prolonged-release products containing 600 mg Vitamin C and Zinc (Product 1); 500 mg Vitamin C, Zinc, Selenium and Vitamin D3 (Product 2) and 300 mg Vitamin C and Zinc (Product 3), a pH meter CONSORT C831, calibration solutions (Buffer Solution pH: 4.01, 7.01 and 10.01), distilled water. Acceptability criteria: a pH value in the range 6 - 7.

Ascorbic acid (Vitamin C) assay

The quantitative determination of vitamin C was performed using a European Pharmacopoeia compendiale titrimetric method: a quantity of tablets powder containing 80 mg calcium ascorbate was dissolved in a mixture of 10 mL of diluted sulphuric acid and 80 mL of carbon dioxide-free water. One mL of starch solution was added, and the obtained solution was titrated with 0.05 M iodine until a persistent violet-blue colour was obtained [24]. This titration procedure is widely accepted and is appropriate for testing the amount of vitamin C in the tablets, liquids, and fruits and vegetables [25]. A titrimetric method with more comprehensive limits (± 10%) is also described in USP ascorbic acid tablets monograph for the assay of Vitamin C.

The reagents used were: iodine potassium iodide (0.05 M) and distilled water.

1 mL of 0.05 M iodine is equivalent to 10.66 mg of $C_{12}H_{14}CaO_{12}$, $2H_2O$.

$$Calcium\ ascorbate\ \frac{mg}{tb} = \frac{V \times 10.66 \times M}{G_S} \quad (1),$$

where, V = volume of 0.05M iodine solution used in titration (mL); M = average tablet mass (mg); G_s = mass of tablet powder (mg). Acceptability criteria: 605.0 mg/tb ± 5% (574.75 ÷ 635.25 mg/tb).

Hesperidin assay

The quantitative determination of hesperidin was performed by a reversed-phase high-performance liquid chromatography (HPLC) method with UV detection at λ = 280 nm. The mobile phase consists of 0.1% phosphoric acid:methanol in a ratio of 60:40 (v:v), with a flow rate of 1 mL/min and a column temperature of 25°C. Injection volume was 10 µL, and a C18 chromatographic column (150 mm length x 4.6 mm i.d.) used 5 µm particle size was used [26]. The system suitability was tested. The standard external method was used for the quantification of hesperidin.

There were used a JASCO HPLC System, FALC ultrasound bath, OHAUS analytical balance, Hesperidin CRS (EDQM), 85% Phosphoric acid (Merck), Methanol (Merck), water for HPLC.

$$Hesperidin\ \frac{mg}{tb} = \frac{A_S \times G_R \times M}{A_R \times G_S} \quad (2),$$

where, A_S - peak area corresponding to hesperidin in the sample solution; G_R - the mass of hesperidin CRS (mg); M = average tablet mass (mg); A_R - peak area corresponding to hesperidin in the reference solution; G_S = mass of tablet powder (mg). Acceptability criteria: 8 mg/tb ± 10% (7.20 ÷ 8.80 mg/tb).

In vitro dissolution study

The release of ascorbic acid in prolonged-release tablets was assessed by an *in vitro* dissolution test: 900 mL distilled water as dissolution medium, baskets dissolution apparatus, 50 rpm, 480 minutes dissolution time, 37 ± 0.5°C temperature. In addition, the amount of dissolved calcium ascorbate was assessed by adapting volumetric titration used for vitamin C assay; sampling times were one and 8 hours [27].

Acceptability criteria: The dissolved calcium ascorbate content must be 20 ÷ 40% dissolved after one hour and at least 70% after 8 hours.

An *in vitro* dissolution apparatus SR 8 - PLUS - Hanson Research was used.

$$Q_1 = \frac{V \times E_1 \times 900}{605} \quad (3),$$

$$Q_2 = \frac{V \times E_2 \times 900}{605} \times \frac{100}{900} \times Q_1 \quad (4),$$

where, Q_1 = calcium ascorbate dissolved after 1 hour (%); Q_2 = calcium ascorbate dissolved after 8 hour (%); E_1 = equivalent of 0.01N iodine solution used for titration after 1 hour = 1.066; E_8 = equivalent of 0.025N iodine solution used in the titration after 8 hours = 2.665.

Results and Discussion

Non-acidic character testing (Neutrality testing)

Three prolonged-release Vitamin C formulas were tested compared to the innovative dietary supplement. The pH value of the existing formulas was around 3.52 for the first product and 4 for the other two products based on vitamin C. The assessed products contain vitamin C in the form of ascorbic acid (therefore, they have an acidic pH). For the proposed innovative formula, the average pH value obtained was 6.39. The test (experimental) formula has a neutral pH (6.44) compared to the acidic pH of the existing tested products (3.52, 4.22 and 3.92, respectively) (Figure 2).

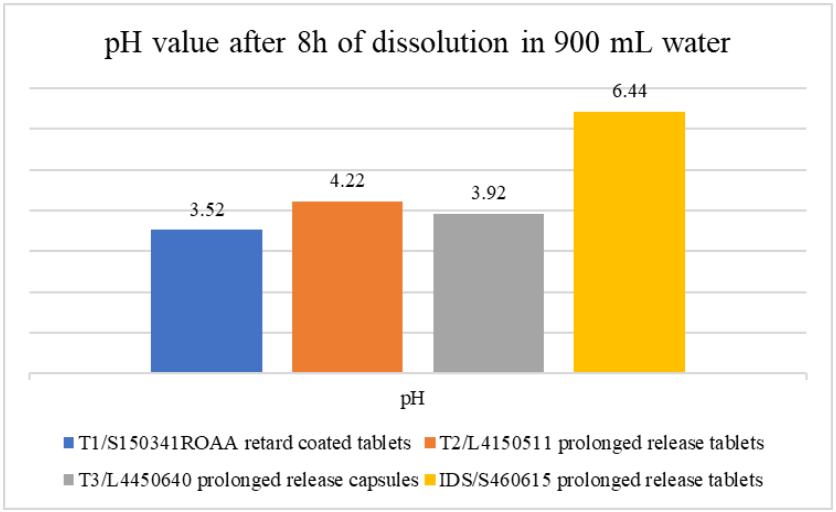

Figure 2.
The pH determination of the innovative dietary supplement (IDS) compared to three slow-release conventional ascorbic acid existing formulas (Product T1-T3)

Vitamin C assay

To quantify the ascorbic acid content in the developed formula, we analysed six samples of the innovative dietary supplement.

Each tablet of the proposed formulation contains 605.77 ± 0.56 mg of calcium ascorbate, within the acceptability range (574.75 ÷ 635.25 mg/tablet). The relative standard deviation is 0.09%, within the acceptance limit, showing that the dosing method is accurate, respecting the repeatability condition.

Hesperidin assay

The system suitability was tested using 0.40 mg/mL hesperidin standard solutions.

The peak parameters (retention time, height, area, peak width at half size), as well as system performance parameters (asymmetry factor, number of theoretical plates), are reproducible, showing a relative standard deviation below 2% (0.55 for time retention, 0.29 for peak area), which demonstrates that the system is stable and efficient. Hence, it corresponds to the proposed purpose.

The HPLC assay of hesperidin in the innovative formula was performed on six product samples from three batches (mean 8.51 + 0.05)

The results of the HPLC assay of hesperidin for six samples of the tested formula are within the standard limits of 8 mg/tab ± 10% (Retention time (min) 7.20 ÷ 8.80 mg/tb).

In vitro dissolution study

In vitro dissolution tests is a vital tool in the development and quality control of drugs, making it possible to evaluate the performance or efficiency of the dosage form in releasing the active substance through the amount dissolved in the dissolution medium when the product is subjected to specific equipment. Calcium ascorbate dissolution test results at one hour and eight hours are presented in Figure 3.

The samples analysed by the dissolution test at one hour have the lowest value obtained of 24.1% and the highest of 25.2%, which are within the acceptability range of 20 ÷ 40%.

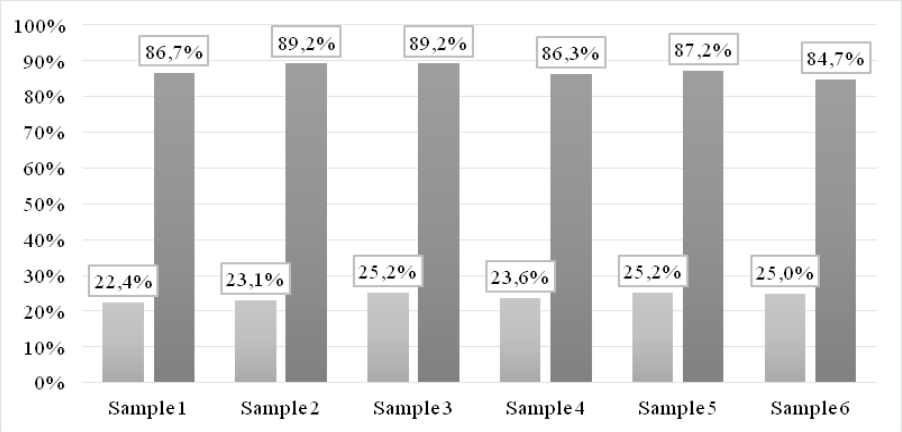

Figure 3.

Dissolved calcium ascorbate (%) after one hour (grey) and after eight hours (dark grey) (IDS Samples 1-6)

The average value of the dissolved calcium ascorbate at 8 hours was 87.2% ± 1.75 of the total amount. Therefore, the samples analysed for the dissolution parameter had the lowest value of 84.7% compared to the minimum imposed limit of 70%, following the acceptability criteria.

From the *in vitro* dissolution study results, it appears that the samples correspond to the requirements regarding the dissolution parameter of calcium ascorbate in the prolonged-release dosage form, the values of % dissolution at one hour being in the range of 20 ÷ 40% (24.1%), and at 8 hours over 70% (87.2%).

Vitamin C, a compound used for decades in medicine, is essential for the human body. The human body cannot synthesise ascorbic acid as it lacks l-gluconolactone oxidase, depending on the oral intake. Lack of vitamin C generates severe or mild deficiencies in humans due to a lack of dietary intake. The food intake of vitamin C is sufficient in a healthy diet rich in fruits and vegetables; as the modern diet lacks these constituents, an increase of need for pharmaceutically formulated ascorbic acid was observed during the last decades. Even if it is a small and hydrophilic molecule, its pharmacological approach is hindered by particularities of pharmacokinetics [28].

Immediate-release *versus* modified-release vitamin C is an essential aspect of the pharmaceutical products, as the hydrophilic nature of ascorbic acid, the absence of passive diffusion across biological membranes displaying a constant and dose-independent half-life [29]; this profile generates a rapid absorption and renal elimination being quantitatively filtered through glomerulus using the gradient of hydrostatic pressure [30]. Using slow-release forms will improve its bio-availability stability, ensuring a controlled release profile. During the COVID pandemic, one of the defence shields was assuring high vitamin C levels in the body [31]. For long-term ascorbic acid use,

high demand for good pharmacokinetic profiles and improved gastric tolerance was welcomed [32, 33]. However, *in vivo*, bioavailability studies and digestibility studies may be developed to perform more accurate pharmacokinetic profiles [27].

The present research has limitations, as it used only *in vitro* studies to assess the release profile of the active compound, ascorbic acid.

Conclusions

Our studies reveal the neutrality of the innovative ascorbic acid dietary supplement, the suitable active substance content of the product, and the delayed pharmaceutical formulation, which extends the duration of beneficial effects on the human body with reduced side effects.

Conflict of interest

The authors declare no conflict of interest.

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