UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

PHYSICO-CHEMICAL AND PHARMACOLOGICAL RESEARCH ON SUGAR BORON COMPUNDS

-Summary-

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1. Introduction

Boron and its compounds, though quite studied over time, have not revealed all of their unknowns, and they still continue to surprise through the development of their research.

The first boron compounds ever studied, were those with oxygen, namely boric acid, borax and other borates.

The boric acid is considered a prebiotic agent, causing the spontaneous segregation between calcium and phosphorus from borophosphate evaporite minerals (borate, Mg^{2+}, sulfate, Ca^{2+}, phosphate), providing the necessary phosphate for the phosphorylation of the nucleoside components of the RNA molecules which may have been responsible for the emergence of life on Earth.

Until now, a crucial role has been found for boron only in plants. Boron has been shown to be extremely important for plant growth and development, with a number of functions such as sugar transport, cell wall synthesis and RNA metabolism.

The significance of boron for the prevention of chronic diseases in humans has been recognized for some time. Symptoms of boron deficiency are: decreased bone density and increased urinary excretion of calcium and magnesium, increased incidence of prostate cancer, decreased immune response and the increase of inflammation.

Throughout their research, scientists have discovered a form of boron found in plants, namely calcium fructoborate (CFB), a boric acid ester of fructose. In terms of action, CFB is superior to boric acid/borates and less toxic.

Calcium fructoborate is a food supplement of interest, with many medicinal and therapeutic applications: useful against cancer, arthritis, osteoporosis and even antibiotic-resistant microbial infections.

In view of the above, it was necessary to determine boron as boric acid and/or borate ester from food products and supplements. So, we have developed, optimized and validated, simple, fast, specific and accurate identification and quantitative determination methods such as HPTLC coupled with photodensitometry and UV-VIS spectrophotometry.

Regarding the pharmacological importance of calcium fructoborate and other carbohydrate boron compounds on the prevention of acute or chronic diseases with high recurrence, we have initiated a study on the effects of calcium fructoborate on proinflammatory markers, based on current scientific evidence that inflammatory processes influence cardiovascular health. Both studies have highlighted the conclusive effects of carbohydrate boron compounds in preventing these conditions.

Finally, the study focuses on developing a monograph for calcium fructoborate in order to formalize it in the Romanian Pharmacopoeia – the quality standard for products and substances.

2. The role of natural boron compounds in nutrition and therapeutics

The boron compounds are found in the form of boric acid, borax and other borates.

In humans boron plays an important role in the calcium metabolism.

In nature, boron has a wide distribution at concentrations of 10 mg/kg in the Earth's crust (from 5 mg/kg in basalt to 100 mg/kg in shale) and about 4.5 mg/L in the ocean water. In underground and surface water, the natural boron content is lower.

Boron was involved in prebiotic chemistry and continues to influence the living world on Earth.

Boron is of particular importance for plant growth and development, being involved in a number of functions such as sugar transport, cell wall synthesis and RNA metabolism. In plants, the symptoms of deficiency in boron are common to other nutrient deficiencies.

Contemporary, only two boron compounds (sodium borate and boric acid) are approved in EU as substances for a particular nutritional supplementation or substances that may be added to food.

3. Natural boron compounds with sugars: calcium fructoborate

A new form of boron has been discovered during research, by scientists, in plants, namely calcium fructoborate (CFB). CFB is a complex between fructose, boron and calcium. It identifies naturally in vegetables, fruits, and other food products and can also be obtained by chemical synthesis. Compared with other commercially available forms of boron, it is not only safe but also has a good bioavailability. CFB is a boron-based nutritional supplement, it is a good complementary treatment to osteoporosis, also providing benefits to the cardiovascular system.

4. Borate – a multifaceted problem solver in prebiotic chemistry

Almost all prebiotic chemistry is performed in the laboratory, starting from Stanley Miller's classic effort to the most promising recent results [Becker *et al.*, 2016; Powner *et al.*, 2009]. However, as the first Darwinian biopolymers (RNA, most likely considered today) [Neveu *et al.*, 2013] almost certainly they did not form in the laboratory, it is important to look for real geological and mineralogical contexts favorable to the management of the well-known problems of prebiotic chemistry, especially those found in the "first RNA model" in order to explain the origin of life [Crick, 1968]. These include problems arising from the instability of the RNA molecule.

Lüneburgite (borosulfate magnesium evaporite) could be a phosphorylating agent itself under non-aqueous conditions, under which phosphate esters may form thermodynamically. Synthetic lüneburgite was obtained by mixing and evaporation of calcium oxide, boric acid, phosphoric acid, sulfuric acid, and magnesium oxide in suitable ratios.

Borate containing vaporized mineral assemblies provide phosphate and phosphorylates the ribonucleosides regiospecific; thus borate becomes a multilateral solution to solve problems in prebiotic chemistry (the phosphate problem of the "RNA first model" for the origin of life).

The experiments suggest that a relatively simple geological evaporation containing borophosphate minerals could address the instability of the ribose (by complexation of borate with sugars) [Kim *et al.*, 2011; Sleep *et al.*, 2011], the difficult formation of unstable phosphate esters in water (attenuated by evaporation of water) [Furukawa *et al.*, 2015] and the problem of calcium phosphate in the presence of calcium [Kim *et al.*, 2016; Saladino *et al.*, 2012].

5. Obtaining boron compounds with sugars

Calcium fructoborate (CFB) is a food supplement of interest, with multiple medicinal and therapeutic applications; for example, it proved to be an effective antioxidant [Scorei *et al.*, 2005], useful against cancer [Scorei & Popa, 2010] effective in reducing inflammation associated with arthritis [Scorei *et al.*, 2011]. CFB was also documented with potential use in the treatment of skin diseases [Miljkovic & Pietrzkowski, 2000] and in an attempt to reduce the growth rate of hair follicles [Miljkovic, 1999b].

In view of the above, with regard to the therapeutic usefulness of calcium fructoborate it is even more interesting to obtain other boron compounds with carbohydrates. In this sense, for the synthesis of these compounds, I have chosen Hunter's patent documentation and support, from 2016 (US Patent No. US9421216 B2 – „Compositions and methods for borocarbohydrate complexes").

The synthesis reactions for the new carbohydrate boron compounds were obtained using following the following equations:

$$C_6H_{12}O_6 + H_3BO_3 \rightarrow C_6H_{10}O_6B(OH)_2^- + H_3O^+$$

$$C_6H_{10}O_6B(OH)_2^- + C_6H_{12}O_6 \rightarrow (C_6H_{10}O_6)_2B^- + 2H_2O$$

$$CaCO_3 + 2H_3O^+ \rightarrow Ca^{2+} + CO_2 + 3H_2O$$

$$MgCO_3 + 2H_3O^+ \rightarrow Mg^{2+} + CO_2 + 3H_2O$$

$$Ca^{2+} + 2(C_6H_{10}O_6)_2B^- \rightarrow Ca[(C_6H_{10}O_6)_2B^-]_2$$

$$Mg^{2+} + 2(C_6H_{10}O_6)_2B^- \rightarrow Mg[(C_6H_{10}O_6)_2B^-]_2$$

All the synthesis reactions were carried out to scale and optimized by setting the appropriate molar ratios between the reagents (carbohydrates, boric acid, calcium carbonate, magnesium carbonate).

Although the synthetic reactions seem simple, in theory at least, a closer investigation reveals a high complexity due to specific isomers of carbohydrates: the presence of different types with open chains, forms having a furanose ring or a pyranose ring, each with its own stereoisomeric configuration (alpha or beta) to the anomeric carbon atom.

To further complicate things, the boric acid molecule forms diester complex links with two -OH groups of a carbohydrate molecule, and because fructose has five -OH groups in the vicinal position, many esters can be formed with each stereoisomer.

6. Identification and quantitative determination of boron compounds with sugars by the UV-VIS spectrophotometric method

A series of studies to optimize boron assay conditions were performed using azomethine H as a chelating agent (coloring agent). Azomethine H (8-hydroxy-1-(salicylideneamino)-3,6-naphthalenedisulfonic acid) is a Schiff base of salicylaldehyde and H-acid (1-amino-8-naphthol-3,6-disulfonic acid) [Matsuo *et al.*, 2004; Bulearca *et al.*, 2014].

Azomethine H was used for the quantitative determination of boron in aqueous solution.

The principle of the method is based on the quantitative complexation of azomethine H with the borate anion from food supplements containing boric acid or borates (sodium tetraborate or borax). The transfer of borate between the boric acid/borate and azomethine H is performed instantaneously and quantitatively, due to the large differences between the association constants [Bulearca *et al*., 2014].

The boron concentration from food supplements (VitaCab, Organic Boron, Osteoremed) and calcium ascorbate borate complex (raw material) was determined spectrophotometrically by coupling with azomethine H.

The spectrophotometric method boron is simple and low cost, requiring no performance equipment or digestion of samples to be analyzed.

In the presence of boron, azomethine H solution shows a maximum absorption at 415 nm, after 30 minutes of maintaining stable in the dark and at a temperature of 23 ± 0.5^{0}C.

Following the high resolution continuous flame atomic absorption spectroscopy (HR-CS AAS), the spectrophotometric assay method of boron by coupling with azomethine H can be successfully used for standardization of manufacturing batches and continuous flow determinations, necessary in the supplements industry.

7. Simultaneous determination of boric acid and calcium fructoborate in nutritional supplements, via HPTLC coupled with photodensitometry

In plant products, boron is found as monoester and diester borate, along with boric acid (BA) [Dincă & Scorei, 2013; Matsunaga & Nagata, 1995; Matsunaga *et al.*, 1996]. It was therefore expected that obtaining borate esters would represent the key to the physiological activity of boron [Goldbach *et al.*, 2007; Hunt, 2012; Nielsen, 2008]. Bis-fructose ester of boric acid has been found in plant products (*radix*, *herba*), fruits, seeds, honey, and in some foodstuff. Thus, the ester is a natural fructoborate important in human nutrition [Biţă *et al.*, 2017; Mogoşanu *et al.*, 2016]. Calcium fructoborate (CFB), being a natural sugar borate ester, is the most studied boron-based compound [Duval et al. 2012].

The HPTLC method has been used for the first time to identify, quantify and differentiate CFB from BA, from different food supplements (bulk, tablets and capsules)

[Biţă *et al.*, 2017].

The validation process for the optimized HPTLC method was realized taking into account the following criteria: specificity, linearity, precision, accuracy, detection limit (LOD) and quantification limit (LOQ), and robustness.

From an economic point of view, the chromatographic method is cheap, simple and effective and can be conveniently used for the continuous quality control for batches of the nutritional supplements industry.

Research has shown that the presence of other active compounds (glucosamine sulphate, chondroitin sulphate, vitamin D, methylsulfonylmethane, polyphenolic compounds) and excipients do not interfere with the analysis of calcium fructoborate in nutraceutical products.

The HPTLC method has also been successfully used for the determination of calcium fructoborate in various herbal medicinal products or food products.

8. The NMR assay of boron compounds with sugars

Boric acid forms esters and complexes with a wide variety of mono-, di- and polyhydroxylated compounds [Woods, 1996]. Some of the most stable boric acid esters are complexes in which boric acid is a bridge between the two carbohydrate molecules, for example fructose-boron-fructose. The examination of the complexation of boron in plant extracts through nuclear magnetic resonance (NMR) ^{11}B has shown that most of the boron is associated with diester diols and hydroxycarboxylic acids complexes, in radish and apple [Matsunaga & Nagata, 1995]. Fructose is the most important molecule for complexation with boron. Later, this hypothesis was verified [Brown & Shelp, 1997; Hu *et al.*, 1997] after the isolation and characterization from higher plants of soluble boron complexes. Calcium fructoborate (CFB) is most commonly found in fruits and vegetables.

Various forms of fructoborate were obtained, with a certain content of fructoborate magnesium salts or calcium salts (MgFruB and CaFruB), alongside with complexes like magnesium or calcium obtained with glucose (MgGluB and CaGluB), mannose (MgManB and CaManB), galactose (MgGalB and CaGalB) and sucrose (MgSucB and CaSucB).

The anomeric region of the ^{13}C spectrum may be used to identify signals of the free sugar molecules thoroughly mixed with the sugar-borate complex. The amount of free carbohydrate compared to the total full anomeric region allows to calculate the percentage of free sugar. This value is used to determine which sugar-metal combination ensures the most efficient formation of the complex.

From the results, it was established that in the current manufacturing conditions, fructose leads to the high concentration of sugar-borate complex and there is a small difference observed between the complex magnesium fructoborate and calcium fructoborate.

9. Comparative microbiological research on boric acid and calcium ascorbatoborate

The bacteriostatic or antiseptic effect of boric acid has long been known. Boron compounds are micronutrients essential for many organisms and also plays an important role in plant life. However, in large amounts, boron is toxic to living cells. For all living organisms, there is a small gap between the boron deficit and the toxic levels of this trace element. Boron is involved in quorum sensitivity of microorganisms, an important mechanism in determining the antimicrobial activity [Houlsby *et al.*, 1986; Watanabe *et al.*, 1988; Reichman *et al.*, 2009].

Ascorbatoborate complexes were obtained in the form of salts with Ca^{2+} ions by rapid precipitation from aqueous solution. The complexes were prepared as mono-chelate (1:1) and bis-chelate (1:2) borate esters, readily soluble in water, but subject to slow hydrolytic cleavage of boric acid and ascorbate in aqueous solution as indicated by NMR studies [Kose & Zümreoglu-Karan, 2012].

The biological material of the study consisted of a wild type of *S. aureus* isolated from the pharynx. The strain of *S. aureus* methicillin resistent was evaluated using the agar diffusion technique [Watts, 2002; Cavalieri *et al.*, 2005]. Disks with oxacillin (30 µg) (Oxoid Ltd., Basingstoke, Hampshire, England) were used. The antimicrobial action of calcium ascorbatoborate esters (mono- and diester) and boric acid against *S. aureus* were also determined by the agar diffusion method [Perez *et al.*, 1990].

It has been found that calcium ascorbatoborate diester has an increased bactericidal action than the boric acid. The results for boric acid were similar to those in other scientific papers. For calcium ascorbatoborate monoester, the best antimicrobial activity was recorded.

10. The effects of calcium fructoborate on some biochemical parameters: double-blind, placebo-controlled clinical trial

Calcium fructoborate is a natural occurring molecule present in fruits, a commercial product manufactured by a patented process, as described for the first time by Miljkovic *et al*. (1999a) (US Patent No. US5962049) [Miljkovic *et al*., 2009]. It was shown, previously, that CFB supports antiinflammatory response [Scorei *et al.*, 2010, 2011; Scorei & Scorei, 2013].

A recently published clinical research has shown that CFB can reduce discomfort in the knee joint and improve flexibility as measured by subjective assessments such as the WOMAC – The Western Ontario and McMaster Universities Osteoarthritis Index and the McGill index [Reyes-Izquierdo *et al.*, 2014] and can modulate the inflammation-associated markers [Scorei, 2011; Scorei *et al.*, 2011; Scorei & Rotaru, 2011; Reyes-Izquierdo *et al.*, 2012].

CFB reduced the level of the C-reactive protein (CRP) [Scorei *et al.*, 2011; Scorei & Scorei, 2013] a protein of immune response recognition, a sensitive marker of inflammation [Danesh *et al.*, 2004; Li & Fang, 2004; Scorei & Rotaru, 2011; Militaru *et al.*, 2013].

In general, current scientific evidence supports the hypothesis that cardiovascular health is directly related and influenced by the inflammatory processes of the body [Ross, 1999].

If this is true, cardiovascular status can be monitored by measuring certain inflammatory biomarkers [Armstrong *et al.*, 2006; Packard & Libby, 2008].

Evaluating concomitant lipid levels, homocysteine and CRP was proposed to assess the risk of cardiovascular disease [Yoldas *et al.*, 2007] and coronary diseases [Bhupathiraju *et al.*, 2007; Khandanpour *et al.*, 2009; Wierzbicki, 2007].

Other general markers of inflammation, such as interleukins (IL) IL-1β and IL-6 also provide useful information on cardiovascular risk [Su *et al.*, 2013]. Also, IL-1β is increasingly recognized as a factor of inflammation and proatherogenic biomarker [Vicenová *et al.*, 2009].

The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania, on May 3rd, 2012. In addition, the study was conducted in accordance with the criteria outlined in the 1975 Helsinki Declaration, updated in 2008 [WMA, 2013]. An informed consent form was signed by each participant, before commencing the study (ClinicalTrials.gov, ISRCTN90543844, May 24th, 2012).

Participants were randomly distributed in three groups, with the same number of men and women per group. Two participants in Group A (N = 28 initial) and a participant in the group C (initially N = 27) were excluded because they did not fit the criteria. The final number of participants analyzed in each group was 26.

The results suggest that the use of CFB at a daily dose of 112 mg for 30 days can significantly reduce proinflammatory and proaterogenic markers – TC, LDL-cholesterol, triglycerides, CRP and homocysteine while increasing the level of HDL-cholesterol (considered a protective lipid). Moreover, the supplementary use of CFB at a dose of 112 mg a day, appear to have a statistically significant inhibitory effect on the pro-inflammatory cytokines, such as IL-1 β, IL-6 and MCP-1.

In order to verify and confirm the effects of CFB on proinflammatory markers, additional clinical trials with larger groups of subjects are required. Also, a longer-term study could determine the long-term benefit of CFB supplementation.

11. Research on the development of a monograph for calcium fructoborate (*Calcii fructoboras*)

In Romania, the quality control of pharmaceutical raw materials is carried out by the control laboratories of drug manufacturers and the National Agency of Medicine and Medical Devices (ANMDM). Quality standards established or approved by ANMDM, with the approval of the Ministry of Health, include [Popovici & Lupuleasa, 2011]:

- Pharmacopoeia, if officinal products and substances;
- State standard for the quality control of substances and products manufactured in commercial establishments under the jurisdiction of the state;
- STAS for the control of industrial products with various applications;
- Internal quality standard for products made by a specific manufacturer;
- Manufacturing sheet or technological sheet of the manufacturer to import certain products not related to the pharmacopoeia or other quality standards.

The 10th of the Romanian Pharmacopeia (1993) includes general monographs, individual monographs and monographs for general methods of analysis. Individual monographs for substances are presented in alphabetical order, taking into account the Latin and Romanian names, chemical structure, molecular formula, relative molecular mass, description, solubility, identification, quantitative determination, preservation conditions, pharmacological action and uses [FR X, 1993; Popovici & Lupuleasa, 2011].

Identified in fresh fruit and vegetables, calcium fructoborate (CFB) is a soluble boron source, a natural sugar borate ester. CFB contains three forms of borate (diester, monoester and boric acid), all biologically active, intracellular (free boric acid) and extracellular reactive (fructoborate monoester and diester).

There is no monograph in the literature on calcium fructoborate so far, which includes the quality standards for quantitative identification and determination criteria. This is mainly due to the relative novelty of the substance in the nutritional supplements market and the small number of manufacturers for this compound both worldwide and in Romania.

The research within the PhD thesis contributes to the accumulation of new data on calcium fructoborate, thus opening the way for the development of an individual monograph, following all the pharmacopoeial rules.

In accordance with FR X (1993), the proposed monograph entitled the "Calcii fructoboras" contains all the necessary individualization active compound to formalize the future and drug substance, given its pharmacological actions (anti-inflammatory, antiosteoporotic).

▪ definition of the active substance:

- the Latin name;
- the Romanian name;
- chemical structure;
- molecular formula;
- relative molecular mass;
- description.

▪ establishing the specific purity parameters (criteria):

- solubilities;
- identification reactions: with ammonium oxalate, silver nitrate, phenylhydrazine in sulfuric acid, by thin layer chromatography (after derivatization with chlorogenic acid and UV examination at 365 nm);
- the aspect of the solution;
- acidity;
- mineral impurities: arsenic, chlorides, heavy metals, sulphates;
- organic impurities: slightly carbonizable substances (glucose, sucrose), oxalates;

▪ quantitative determination: HPTLC coupled with photodensitometry;

- conservation conditions;
- pharmacological action and uses.