Study regarding the identification from natural sources, synthesis and physico-chemical characterization of new bioactive boron containing compounds

–SUMMARY–

Scientific Leader
Prof. univ. dr. Johny NEAMŢU

PhD Student
Carmen Nicoleta OANCEA

CRAIOVA
2018
# Table of contents

Keywords .................................................................................................................................................. 3

1. Introduction ........................................................................................................................................ 4

2. Natural boron compounds .................................................................................................................. 5

3. Fructoboric acid identification from plant sources by the LC-MS method .................................... 6

4. Synthesis, isolation, physico-chemical characterization and quantitative determination of fructoboric acid and zinc fructoborate ..................................................................................... 7

5. *In vitro* and *in vivo* toxicity of the two synthesized compounds: fructoboric acid and zinc fructoborate ........................................................................................................................................ 9

6. Conclusions ......................................................................................................................................... 11
Keywords

- Boron
- Mineralogy
- Plants
- Bacteria
- Toxicity
- Chromatography
- Speciation
- Enzymes
- Pharmacology
- Fructoboric acid
- Zinc fructoborate
- FTIR
- UV-VIS
- LC-MS
- *In vitro*
- *In vivo*
- Histology
1. Introduction

Boron is a dynamic element of vital importance in animal and human biology. This has become more and more clear since recent research has shown that it has surprising and significant effects for animal and human health. Boron, the fifth element of the periodic table, found in group 13, is the only nonmetal in this group. Being a small atom with three electrons on the last orbital, the boron uses covalent bonds to fill its orbitals. Boron atom does not donate protons, but accepts hydroxyl ions. Boron binds to various elements, forming tetrahedral or trigonal arrangements. It has a strong attraction for oxygen, resulting in borates. When boron binds to four oxygen atoms forming a tetrahedral form, the resulting compound is called borate anion ($\text{B(OH}_4\text{)}^-$). When boron binds to three oxygen atoms, the resulting compound has a triple-planar arrangement, denominated orthoboric acid ($\text{B(OH}_3\text{)}$).

Boron has a strong tendency to form complexes with organic molecules having adjacent hydroxyl groups. Boron can interact with important biological substances, including polysaccharides, pyridoxines, riboflavines, dehydroascorbic acid and pyrimidine nucleotides. It forms a strong bond with furanose cis-diols such as erythritol, ribose and apiosis, the latter being present in the cell walls of the plants. Adenine nicotinamide dinucleotide ($\text{NAD}^+$ – nicotinamide adenine dinucleotides) and adipine nicotinamide dinucleotide phosphate ($\text{NAD}^+$ – nicotinamide adenine dinucleotide phosphate) contain ribose components. These molecules are active in energy metabolism and therefore their binding will have repercussions on the processes of the various metabolic pathways. Unique boron chemistry allows it to react with many other metabolites and enzymes, thus being able to modify mineral and energy metabolism in plants, animals and humans. Boron has two commonly encountered isotopes $^{10}\text{B}$ and $^{11}\text{B}$. Thanks to a large neutron capture area, $^{10}\text{B}$ is an excellent absorbent. In nature, boron does not exist in elemental form, but in the form of compounds whose chemical structure is complex. The most common forms of compounds are borax, boric acid, boron oxide, etc.

The significance of boron for the prevention of chronic diseases in humans has been known for some time in the global community. Symptoms of boron deficiency are unspecific, including arthritis, bone loss, immune deficiency, and osteoporosis. It is suggested that boron affects bone minerals by modulating serum levels of steroid hormones (estrogen and testosterone) and their metabolism. Moreover, the boron level in the bones and blood increases with age and health and decreases with disease states.

Considering the above, the use of calcium fructoborate in therapy becomes more and more interesting. Another important compound has been identified in nature as it is fructoboric acid. Obtained by synthesis in the laboratory the fructoboric acid, identical to natural compounds, can lead to its use as a nutritional supplement.
2. Natural boron compounds

Many of the world's countries have low boron content in the soil, while high levels exist in countries such as the US, Turkey, Brazil, Russia or China.

Boron is present especially in soil and water. The terrestrial bark contains the largest amount, the boron having a concentration of 10-20 ppm in the soil.

Boron is present everywhere in nature, even in the atmosphere, where it has a concentration of 0.5 ng/m³ of air. In water it has different concentrations of less than 0.3 mg/l at the surface of the water and an average of 0.03 mg/l in drinking water. In plants, the amount of boron varies depending on the amount of boron in the soil, which can be improved by adding boron fertilizers. They act especially at the level of alkaline soils.

For people, the main source of boron is food. The richest sources of boron are fruits and vegetables. Dairy products, meat as well as most cereals are less important sources of boron. Essentially, the optimal amount of boron for the body can be obtained through a diet rich in vegetal. Although a tolerable daily dose has not been demonstrated, various research on human subjects and animals suggested a required dose of 1–13 mg/day (WHO/FAO/IAEA, 1996).

However, a considerable number of people consume less than 1 mg boron per day. People who have adequate amounts of vegetables and fruits in the diet can accumulate up to about 6 mg/day of boron. Since ordinary diets do not provide optimal health for the population, it is necessary to find adjunctive methods for boron intake such as dietary supplements.

Boron compounds are also found in a wide range of consumer products, such as boron and silicate glassware, soaps, detergents, preservatives, adhesives, porcelain, cosmetics, enamels, leather, carpets and even precious stones.
3. **Fructoboric acid identification from plant sources by the LC-MS method**

Fructoboric acid is a sugar-borate ester complex with a boron molecule linked to one or two fructose molecules to form fructoborate mono or diester. This complex is obtained industrially by the reaction of boric acid with fructose. Natural identical esters of fructoborate are found in some fruits and vegetables. Numerous studies have shown that borates are essential in plant growth processes such as normal wall cell development, membrane function and lignin biosynthesis; it is also important for human health. Fructoboric acid has antioxidant activity. Recent studies have suggested that borates can help regulate hormones and vitamin D in a short-term intake. Scorei *et al.* have found a negative correlation between borate intake and the incidence of human health problems.

Since it has been shown that boron is essential for plant growth, various methods have been developed to characterize the plant-boron-borate complexes. Matoh *et al.* purified the boron-polyglucid complex from the radish roots, hydrolyzing it further and characterizing boron in the complex by NMR. Hu *et al.* using assisted laser desorption matrix coupled with Fourier transformation mass spectrometry to analyze soluble boron complexes with high performance liquid chromatography and gas chromatography coupled with mass spectrometry. To further understand the primary functions of the plant sugar-borate complexes and human health, a more effective screening method is needed to identify this complex compound in different samples, especially at low concentrations.

In this study, we set up an analytical method to identify fructoborate using liquid chromatography coupled with mass spectrometry. The method was optimized by using fructoboric acid as a standard and then applied to identify the fructoborate complex naturally present in different fruit powders. Basic chromatographic conditions were designed and selected after testing the various conditions affecting HPLC analysis. The XBridge BEH Amide column was used due to good retention for fructoborate. The mobile phase with acetonitrile and 0.1 M ammonium acetate (9:1) was used to obtain a good peak and resolution. Fructoboric acid screening in apricot and raisin samples demonstrates the potential of this analytical method for identifying and quantitating fructoboric acid at low concentrations in fruits, vegetables, nuts and seeds. In the future, the method could be further optimized to quantify fructoboric acid in different plant samples as well as in biological samples.
4. Synthesis, isolation, physico-chemical characterization and quantitative determination of fructoboric acid and zinc fructoborate

At present, in the food supplements industry, the only boron-based products are the calcium fructoborate (CaFB) complex, boron citrate, boron aspartate, boron glycinate chelate, boron ascorbate, boric acid and sodium tetraborate.

The most scientifically researched and the only nutritional boron supplement found in nature is fructoboric acid, a naturally occurring sugar-borate ester that offers potential for human health. Fructoboric acid contains three forms of borate (diester, monoester and boric acid), all of which are biologically active at both intracellular (free boronic acid) and extracellular (such as fructose-borate diester and monoester). At the cellular and molecular level, fructoboric acid is superior to boric acid, showing a complex "protective" effect against the inflammatory response. Oral fructoboric acid is effective in relieving the symptoms of physiological response to stress, including inflammation of mucous membranes, discomfort associated with osteoarthritis and bone loss, and supporting cardiovascular health. Clinical studies have shown the ability of fructoboric acid to effectively modulate molecular markers associated with inflammatory mechanisms, mainly at elevated serum levels of C-reactive protein (CRP).

Recent research has also focused on obtaining zinc complexes, an extremely important element for human health. Decreased Zn concentration in the blood of elderly people correlates with inflammation in almost all organs. It is well known that inflammation is the first step in the development of neurological diseases, diabetes, atherosclerosis and cancer. The intestinal absorption of Zn is enhanced by the preparation of Zn macropolymer complexes, such as Zn orotate. However, it has been identified that this compound stimulates the development of liver cancer in animal models. Therefore, other Zn chelating polymers are needed for additional.

Consequently, all of these data indicate that supplementing the diet with fructoboric acid complexes with zinc may prevent the development of cancer and various inflammatory diseases.

Synthesis of the two compounds used fructose and boric acid from Merck Millipore (Darmstadt, Germany) and zinc powder from Sigma-Aldrich (St. Louis, United States).

The Zn-fructoborate (mono and diester) type esters were synthesized based on the general method of obtaining the various fructoborates.

To determine the amount of Zn in the newly formed complex, we have chosen to use the UV-VIS spectrophotometric method. The analysis was performed with a T80 UV/VIS spectrometer from PG Instruments (Leicestershire, United Kingdom).

The thermal analysis of the two compounds was performed with a PerkinElmer DIAMOND TG/DTA thermo-balancing, which simultaneously acquires the TG, DTG, DTA and DSC curves. A linear heating regime at a rate of 10 K/min was applied in an oxidizing atmosphere (air) to 1000°C. The gas flow was in both cases of 150 cm$^3$/min$^{-1}$, which produces uniformity of the temperature field in the
sample and ensures the removal of decomposition products. Or used alumina crucibles, both as a reference and for sample. Each time the basic lines for the desired curves were drawn under the same conditions as for the sample measurements. By subtracting the baseline from the curves plotted experimentally for the sample, the instrumental effect on the measurement is eliminated, leaving only the one due to the studied samples.

An FTIR-FRONTIER TWO spectrophotometer, controlled by a specialized software, Spectrum v.10.5.1 (Perkin Elmer Inc.), was used for the assay. The software allows both qualitative analysis by identifying characteristic bands compared to similar substances found in the SPECTRUM SEARCH database and quantitative analysis, SPECTRUM QUANT, which uses either Lambert-Beer law at a given wavelength or methods chemistry, PCR, PLS or PLS+, in which the entire spectral range obtained can be used.

The samples were analyzed using ZnSe crystal ATR analysis technique, which allows the recording of an IR spectrum in the range of 4000–650 cm\(^{-1}\) or KRS 5, which allows the recording of an IR spectrum in the range of 4000–400 cm\(^{-1}\). There were 16 scans, with a resolution of 4 cm\(^{-1}\), in the above-mentioned areas.

The optimization of the laboratory synthesis of zinc fructoborate by the reaction between fructoboric acid and activated metal zinc was carried out by carrying out several tests in which the best yields were obtained using the Büchi reactor.

Following the optimization of the laboratory synthesis, two food-grade purity substances, zinc fructoborate and fructoboric acid, respectively, were obtained as finished products, which were characterized according to the 10\(^{th}\) Romanian Pharmacopoeia edition (1993), in the form of technical data sheets.
5. *In vitro* and *in vivo* toxicity of the two synthesized compounds: fructoboric acid and zinc fructoborate

Zinc (Zn) is an essential element that is extremely necessary in cellular functions. It is involved in the glutaminergic transmission (signaling molecule) in the brain, in the antioxidant response and is a cofactor of many enzymes and transcription factors.

Abnormal Zn homeostasis causes a variety of health problems including growth retardation, immunodeficiency, hypogonadism and neuronal and sensory dysfunctions. Experiments on animals have shown that during aging a Zn deficiency may occur which is associated with a decrease in brain function. Zinc homeostasis is achieved via Zn and permeable channels, highlighting the physiological relevance of Zn in life. A bioinformatic study of the human genome has shown that about 10% of all proteins can bind to Zn.

Zn physiological supplementation in the elderly restores thymus endocrine activity and innate immune response (NK cell cytotoxicity) and increases the survival rate in elderly mice. Therefore, Zn supplementation is useful to increase longevity, since Zn-binding proteins can regain their original protective burden against oxidative damage with a beneficial impact on immune response.

The interaction between Zn and boric acid was characterized by the low acute toxicity of zinc borate (ZB) with an LD\(_{50}\) value of greater than 10 g/kg body weight in rats compared to sodium tetraborate pentahydrate with an LD\(_{50}\) (mean lethal dose) of 3.3 g/kg body weight. (ZB and sodium tetraborate pentahydrate have equivalent boron concentration).

There was no evidence of toxic effects when administering 1000 mg ZB/kg body weight/day in a 28-day repeated dose gavage study corresponding to an equivalent dose of 50 mg boron/kg body weight.

The lowest level of adverse effects observed (LOAEL) for testicular outcome is 26 mg B/kg body weight. The recommended zinc level in humans can interact with boron to reduce the risk of toxic effects. The level of zinc in soft tissue in humans is twice as high as in the same type of tissue in laboratory animals. Zn has also been shown to protect against testicular toxicity of cobalt and cadmium.

Consequently, all of these data indicate that active borage-based nutritional supplements may delay aging. We therefore tested the in vitro cytotoxicity of the new zinc-boron complex, zinc fructoborate.

The IC\(_{50}\) determined for ZnFB was 0.195 ± 0.005 mM. ZnFB affects the viability and morphology of cultured cells only at a concentration equal to or greater than 0.5 mM. Compared with the other studies, ZnFB exhibited a relatively low toxicity comparable to that of zinc histidine (0.3 mM).

From the point of view of acute toxicity, taking into account the DL50 (> 5000 mg/kg body weight) at po administration, BALB/c, ZnFB and FBA class mice fall into the class of substances with very low toxicity (Category V, practically nontoxic).

From the histopathological point of view, for the three toxicodynamic (acute, sub-chronic and chronic) experiments, normal aspects were revealed for all the internal organs analyzed.
Zinc is necessary for many metabolic processes as a structural, regulatory or catalytic ion. Our study shows that ZnFB, a new naturally active zinc-boron complex, has a low cytotoxic effect on Vero's renal cells. The level of toxicity is lower than that of most used supplements, such as zinc zinc. In conclusion, ZnFB could be successfully used as a new source of Zn\(^{2+}\) supplementation.
6. Conclusions

It has been possible to identify, separate and produce fructoboric acid for the first time in the world. This organic acid is a non-carboxyl natural acid that is found in plants.

The XBridge BEH Amide column was used due to good retention for fructoborate. 90% acetonitrile/10% 0.1 M ammonium acetate was used as a mobile phase to obtain a good peak and resolution.

Screening of fructoboric acid in apricot and raisin samples demonstrates the potential of this analytical method for the identification and quantification of fructoboric acid at low concentrations in fruits, vegetables, nuts and seeds.

In the future, the method could be further optimized to quantify fructoboric acid in different plant samples as well as in biological samples.

Optimization of the laboratory synthesis of zinc fructoborate by reaction between fructoboric acid and activated metal zinc was carried out by carrying out several tests.

The method of analysis used allows easy analysis of boron, being simple and costly.

The HPTLC coupled with MS, to identify plant fructoboric acid, is accurate, fast, selective, and sensitive.

Determination of the molecular composition and evaluation of the stability of fructoboric acid and zinc fructoborate was carried out by UV – VIS, FTIR and TGA analyzes.

In terms of quality standards, so far fructoboric acid does not have a monograph that includes the criteria for identification (purity) and quantitative analysis. Contributing to the accumulation of new data on natural fructoboric acid, research in the PhD thesis opens the way for the preparation of an individual monograph, according to the pharmacopoeia rules.

Following the optimization of the laboratory synthesis, two food-grade purity substances, zinc fructoborate and fructoboric acid, respectively, were obtained as finished products, which were characterized according to the 10th Romanian Pharmacopoeia edition (1993), in the form of technical data sheets.

The $[\text{H}_3\text{O}]^+[(\text{C}_6\text{H}_{10}\text{O}_6)_2\text{B}]^{-4\text{H}_2\text{O}}$ molecular formula, considered for fructoboric acid, proves to be consistent with the results of thermal analysis and FTIR and MS spectroscopy.

Studies conducted with elemental analysis and FTIR spectroscopy led to the chemical formula for zinc fructoborate – $\text{Zn(H}_2\text{O})_6[(\text{C}_6\text{H}_{10}\text{O}_6)_2\text{B}]_2\cdot4\text{H}_2\text{O}$.

Studies on the Vero cell line on in vitro effects of FBA and ZnFB have highlighted the following: (i) cells are more resistant to FBA than ZnFB; (ii) for FBA, cell viability is affected above the minimal toxic concentration of 3 mM; (iii) for ZnFB, cell culture is affected both morphologically and viability (even to a greater extent than positive cytotoxicity control) over the minimal toxic concentration of 0.5 mM.
From acute toxicity point of view, considering the DL$_{50}$ (> 5000 mg/kg body weight), BALB/c mice, ZnFB and FBA are classified as very low toxicity substances (Category V, non-toxic – OECD, 2012). From the histopathological point of view, in the case of the three toxicodynamic experiments (acute, sub-chronic and chronic), all the internal organs analyzed were shown normal.

Zinc is necessary for many metabolic processes as a structural, regulatory or catalytic ion. Our study shows that ZnFB, a new naturally active zinc-boron complex, has a low cytotoxic effect on Vero's renal cells. The level of toxicity is lower than that of most used supplements, such as zinc zinc. In conclusion, ZnFB could be successfully used as a new source of Zn$^{2+}$ supplementation.