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PRE-CLINICAL BNCT GLIOBLASTOMA RESEARCH. ICP-AES BORON DETERMINATION METHOD. STUDIES ON ^{10}B BORON BIODISTRIBUTION IN MICE'S ORGANS

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Abstract. *To accompany boron neutron capture therapy for cancerous tumours, there has been an optimised ICP-AES method for boron determination in animals' organs tissues. This technique is characterised by its universalism. The approach comprises preliminary acid decomposition at high temperatures and pressure (if necessary), ICP-AES boron determination in the gained solutions analysing comparative samples basing on a single element solution. Its validity is proved by the spike experiment and mass sample variation test. The ICP-AES method is used during the evaluation of BPA and BSH accumulation in organs and tissues when intravenously injecting U87 glioblastoma medication to SCID mice of SPF-status.*

Keywords: *BNCT, BPA, BSH, atomic emission spectroscopy, organs tissues, sample preparation, boron determination*

1. INTRODUCTION

Boron Neutron Capture Therapy (BNCT) is a binary form of radiotherapy based on selective cell destruction of cancerous tumours e.g. anaplastic astrocytoma and glioblastoma. The main principle of this therapy is the selective destruction of only those cells which contain enough of ^{10}B isotope. The product of thermal neutron capture reaction is an alpha particle with high linear energy and lithium nucleus transfer. During this reaction, the alpha particle track length does not exceed the cell size (less than 10 μm).

When doing previous clinical trials, the only neutron sources were nuclear reactors. More than 1500 patients were treated with the BNCT method worldwide. This method proved its effectiveness not only in dealing with cancerous brain tumours, but also with soft tissues, parenchymatous organs and skin tumours [1, 2]. Using reactors for BNCT revealed a number of problems e.g. device setting inability at health-care facilities, expensive maintenance, etc. To introduce this method to clinical practice we started creating a particle accelerator. At Budker Institute of Nuclear Physics SB RAS, an accelerator-based epithermal neutron source was constructed. It was used for BNCT pre-clinical experiments on cell cultures and laboratory animals [3, 4, 5].

For successful boron neutron capture reaction realization, which would lead to tumour cells death, it is necessary to insert enough boron nuclei into the infected tissues. Nowadays, boronophenylalanine (BPA) and borocaptate (BSH) are widely used for the BNCT method. The BPA molecule contains 1 B atom while BSH has 12 B atoms.

Initially, BPA was elaborated as a delivery agent to treat melanoma. In the studies, we detected selective accumulation in malignant tumours, particularly in 9L rat gliosarcoma [6, 7]. Tumour cells' active accumulation mechanism can be explained by an increased first-type amino-acid transport. Its accumulation selectivity depends on the blood-brain barrier penetrability disorder [8].

BNCT effectiveness will be reached at ^{10}B concentration ranging between 20-35 μg/kg in tumour tissues [9]. So, conducting pre-clinical studies on the biodistribution of containing ^{10}B samples in the living organisms is an important scientific challenge. Nowadays, boron determination is done using inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma atomic emission spectroscopy (ICP-AES). Also a combination of the listed methods is used along with visualization methods (Positron Emission Tomography (PET) [10], Secondary-Ion Mass Spectrometry (SIMS) [11], etc.).

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The use of multi-element methods to detect the only element (B) can be explained by the high-speed responses with a wide dynamic range (4-6 orders of magnitude). The listed spectral methods have a low error in the determination under repeatability conditions (<1-2%) and allow us to use certified standards to build the calibration dependency. The technique of boron determination in animals' tissues requires the following features such as universalism, provision with analyte low limits of detection (LODs) and limits of quantification (LOQs).

In modern scientific literature there have been described ^{10}B accumulation trends in cells [12-16], in mice, hamsters, and rats' organs and tissues [12, 13, 15, 17-21], in patients' blood, serum, and urine [22-25], and in the overview [26] they discuss other techniques of boron determination (including spectral ones – ICP-AES), visualisation method and capabilities of the methods were described.

Boron determination has been studied in plants, cow milk and liver, kidneys and blood serum [27]. Thus, the authors discussed separately matrix interferences of the decomposing solution (acids matrix effect) and spectral interferences of the iron. The validity check of boron determination in the liver has shown boron concentrations found are significantly lower than in published resources. In this work, there are also such metrological characteristics as LODs, LOQs, accuracy, and precision. A similar work [28] is devoted to boron detection in animals' tissues.

The authors [29] used ICP-AES as one of the boron determination methods. Boron was inserted as a form of borane. They determined it in the liver, kidneys, muscles, and tumours after microwave digestion in the nitric-phosphoric acid mixture (to reduce boron loss as a highly volatile compound). They discuss matrix effect from the acids used and Fe, K, Na, P, S present components.

In the works [27, 30] they fully discussed instrument memory effects analysing mixtures with a large amount of boron and their correction using mannitol and ammonia solutions.

The works [31, 32] are worth-mentioning. To determine a large number of microelements including B the authors used an AES method with plasma spectra excitation source, and as an alternative, the two-jet arc plasmatron [33]. The main advantage of this source in comparison with ICP is the high plasma power, 15 kW. It allows introducing a solid sample into plasma without acid digestion. However, preliminary lyophilisation is necessary. This procedure is lengthy and depends on the initial sample.

The works described above showed that the quantification of boron used the ICP-AES techniques developed before the 2000s [27-29]. Thus, there is no specific information on what kind of spectral and nonspectral interferences appear while analysing one or another tissue, organ. In the analysis of the observed work, instrumental conditions have not been discussed. This aspect is very important since the registration analytic signal at optimal conditions may lead to a decrease in LODs, LOQs and increase analysis precision. All of this shows the necessity of further studies using modern devices.

2. Materials and Methods

Animal testing has been approved by the NSU ethical committee. The research has been done on 8-10-week-old severe combined immunodeficiency (SCID) male mouse outbreed with SPF-status. 18-21 days before the experiment U87 human glioblastoma cells were prepared with the concentration of 100,000 cells per 1 μl and intracranially injected to get subcutaneous volume formation. Commercially available substances (previously enriched with ^{10}B), such as L-p-boron phenylalanine (BPA) and borocaptate (BSH), were used as a boron monoisotopic ^{10}B targeting delivery substance.

BPA and BSH substances were injected (into the retro-orbital sinus) at the concentrations of 350 μg/g and 100 μg/kg respectively. To estimate BPA and BSH biodistribution at regular intervals (1, 2, 3 and 4 after the injection) the animals were euthanized, their organs of interest were taken out and later frozen and kept at T= -20°C.

2.1 Animal Tissue and Organ Preparation

To dissolve mice's organs and tissues (blood, brain, tumour, kidneys, liver, spleen) oxidizing acids were used: nitric acid HNO3 (extra pure grade 27-5), hydrogen peroxide H2O2 (medical, 30%).

To dissolve blood, kidneys, and spleen HotBlock Pro series was used. These materials were decomposed in open vessels. To the sample with a mass of 0.20-1.0 g, nitric acid was added and heated to 90 °C, then held for 30-60 min to get a clear solution. To dissolve samples with a large amount of fat, such as the brain, tumour, and liver, we used microwave accelerated reaction system MARS-5. To the sample with a mass of 0.10-1.0 g, a mixture of nitric acid and hydrogen peroxide (1:0.2-0.5) was added. The sample dissolution program consisted of two stages. During the first stage, autoclaves were being heated to 90°C for 15 min and then held for 5-10 min to stabilize the pressure in them. The second stage lasted for 20-30 min at a temperature of 180°C.

2.2 ICP-AES

The boron level was determined by the ICP-AES method using high-resolution spectrometer iCAP-6500 (Thermo). Samples were injected using a peristaltic pump. The introduction system comprised Sea Spray nebulizer and Tracey Spray Chamber. The analytical signal was registered with an axial view of plasma. Two analytic lines B 249.678 nm and B 249.773 nm were used. Instrumental conditions are in Tab.1. We used a signal-element solution of boron ions (Russian State Standard 7345-96) to build calibrated dependences. The final results were received by averaging results for two analytic lines. Boron definition in blood, liver, and spleen was done at B 249.678 nm.

Table 1. Instrumental conditions*

Parameters	Value
Power, watt	1150
Peristaltic pump velocity, rpm	60
Argon flow rate, l/min	0.70

*other instrumental conditions were used according to manufacturer's recommendations.

3. Results and Discussion

3.1 Choosing conditions of Sample Preparation for Animal Organs and Tissues

Typically, the sample is introduced into the plasma as an aerosol of a slightly acidic solution. It is important for the solution received after sample preparation to be homogeneous, clear (without inclusions or sample residues) and totally containing the required component.

When determining low content analyte in objects of animal origin, the main difficulty is a residual quantity of undecomposed organic compounds which leads to nonspectral influence occurrence.

The analysed samples were divided into two groups according to their ability to decompose in acid and the amounts of residue after dissolution. The first group comprised organs that dissolved relatively fast and the residue was absent or minimal. They were blood, kidney, and spleen. They are dissolved in open vessels using HotBlock at a temperature of 90-100 ° C. If there was some undecomposed residue, we used filtration to avoid blocking transporting capillary. We determined boron concentration before and after this procedure (Fig.1). As seen in Fig.1, filtration does not influence the results achieved, in particular, it does not have an understating effect.

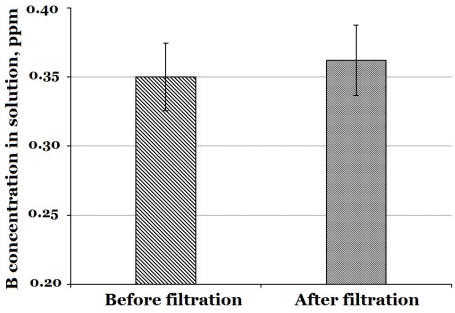

Figure 1. Boron concentration in the solution after kidney decomposing (n=4, P=0.95)

The second group comprised the brain, tumour, and liver. After dissolving them in an open vessel there is a significant amount of undecomposed organic material because of large proportions of lipids. For this reason, harsher conditions such as elevated temperatures and pressure were used to decompose the organs listed above. These conditions were created in the microwave accelerated reaction system MARS-5.

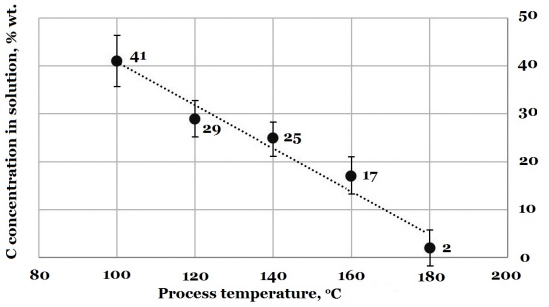

Figure 2. The dependence of carbon concentration in the solution on the decomposition temperature

To reduce the nonspectral effects for the second group, samples were studied in relation to the dependence of carbon quantity in the solution on the acid decomposition temperature (Fig.2).

We defined residual carbon in the gained solution by the spectrometer iCap-6500 using 247.856 nm and 426.726 nm analytic lines. To build a calibrated dependence, we used a solution based on gallic acid ($C_7H_6O_5$). One can see that the least amount of carbon in the solutions (~ 2 %) is at 180°C. This temperature provides a clear homogenous solution. This temperature was used in further work.

3.2 ICP-AES Instrumental Conditions for Boron Determination

Optimal instrumental conditions allow obtaining a high analytical signal and low background fluctuations. AES optimum power provides the best conditions for 249.678 and 249.773 nm B analytic lines excitation. Using the appropriate pump and argon flow rate leads to the effective introduction of sample solution into AES and creates necessary aerosol density in the spray chamber.

While analysing the solutions, the preferable argon flow rate is known to be ranging between 0.50-0.60 l/min [34]. To reach the lowest LODs and LOQs at the constant argon flow rate 0.60 l/min we varied rf power from 1000 to 1300 watt and peristaltic pump velocity from 25 to 60 RPM. We determined that the minimum LODs of the indicated analytic lines is reached at 1150 watt. Figure 3 demonstrates data for the 249.678 nm analytic line. Further peristaltic pump velocity increase leads to the analytic signal decrease and, as a consequence, LODs and LOQs enhancement which agrees with the previously obtained data [34].

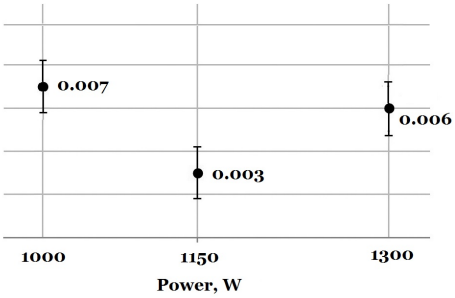

Figure 3. LODs for B 249.678 nm at AES varied power, ppm

3.3 Matrix Effects

When determining boron in organs and tissues, a part of nonspectral influences is connected with spraying. After samples decomposition, the solution contains up to 2% organic compounds and high acid concentration. This significantly changes the solution density and leads to aerosol particle growth, i.e. ICP sample injection effectiveness decrease. An internal standard introduction [15, 16, 24, 27-29] solves these problems. In the beginning, we used a “universal” Internal standard, i.e Y solution at 0.1 ppm (324.228 and 371.029 nm) for all samples. However, noticeable matrix effects were discovered to reveal themselves only in the brain and liver. Their accumulated boron concentration is higher than 1 μg/kg, so a simple consequent sample dilution provides analyte

determination without precision compromisation (Tab.2). From the table we see, when analysing the 0.1 g/ml matrix concentration solution, the result is lower than the one with the internal standard. While analysing the 0.01 and 0.005 g/ml matrix concentration solution, the received results almost coincide with the internal standard using results.

Table 2. B concentration in the liver sample at different dilution indices with and without internal standard (n=3, P=0.95)

Measuring conditions	B, μg/g	RSD, %
Without internal standard, Sample C=0.1 g/ml	2.00±0.09	2.8
Internal standard, Sample C=0.1 g/ml	2.38±0.19	5.1
Consequent dilution, sample C=0.01 g/ml	2.18±0.12	1.9
Consequent dilution, sample C=0.005 g/ml	2.20±0.09	1.7

Figure 4 demonstrates significant spectral interferences caused by Fe in tissues and organs (Fig. 4*a* and *b*). It was described in [27, 28] works. Spectral line Fe I 249.653 nm and non-identified line 249.695 nm strongly affect the B I 249.678 nm. Due to the spectrum profile (Fig. 4*a*) one can suppose that 249.6916 nm line belongs to Fe II (NIST spectral line database proves it) [35]. However, Tables of spectral lines [36] and iTeva software have only Fe I 249.699 nm line. Noticeable Fe spectrum interferences are seen detecting B at 249.773 nm line. According to Fig. 4*b* Fe I 249.782 nm line influences it much. The most apparent spectral matrix influences are seen at 249.773 nm line in blood, spleen and liver samples. These influences make the results overstated. To get sufficient results for the samples it is relevant to use 249.678 nm line.

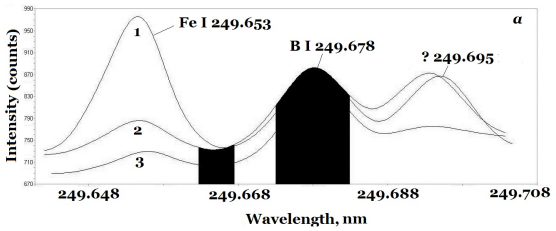

Figure 4a. Spectra profile for 249.678nm for samples: 1 – spleen, 2 – kidney, 3 – brain

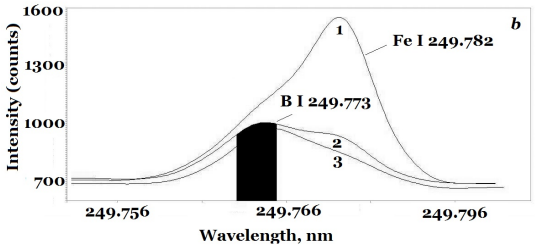

Figure 4b. Spectra profile for 249.773 nm for samples: 1 – spleen, 2 – kidney, 3 – brain

3.4 Data Validation

The final stage of method development to determine boron in organs and tissues is data validation. The method validity was checked by the spike experiment and mass sample variation test. During the spike experiment, we established that a discovered concentration satisfactorily coincides with the inserted one, and inter-laboratory precision does not exceed 20%. The data show (Tab.3) 249.773 nm analytic line result for blood overvalued. Found boron concentrations from different samples mass are comparable (Tab.4). The received results point out even boron distribution in organs (tissues).

Table 3. The spike experiment for different organs (n=3, P=0.95), ppm

Organ	Inserted	Found		Recovery, %
		249.678 nm	249.773 nm	
Blood	0.052	0.051±0.013	0.061±0.015	98-120
Kidney	0.10	0.10±0.03	0.10±0.03	100
Liver	0.25	0.23±0.02	0.23±0.03	92

Table. 4. Boron detection results at liver sample mass variation (n=3, P=0.95), μg/g

Sample code	Sample mass, g	B level, μg/g
1 pl	0.4080	1.7±0.3
	0.7294	1.5±0.4
7 pl	0.4546	10±3
	0.4547	10±2

Tables 3 and 4 show that sample preparation does not influence ICP-AES analysis results. ICP-AES technique of B determination provides express analyte measurement with high precision. LODs of boron are 0.003 ppm. During the sample conversion, LOQs of boron are 0.015 μg/g. Inter-laboratory precision ranges from 2 to 20%.

3.5 BPA and BSH Accumulation

Developed ICP-AES technique of B determination in mice's organs and tissues was used to estimate BPA and BSH accumulation effectiveness and to build medication elimination kinetic curves. Table 5 demonstrates all found concentrations for blood, tumour, brain, kidney, and liver (C±ΔC). The experiment showed BPA and BSH concentration was decreasing monotonously for 4 hours. The most absolute B concentrations were in liver and kidney samples.

For BPA, B concentration ratio of tumour/blood (Fig.5) ranged from 1.1:1 to 2.2:1 and was 1.3; 1.6; 1.1 and 2.2. Taking into account the large variability of the found boron values and small selection (3 animals per period), ΔC is at acceptable level. Received B concentrations in tumour for 1 and 2 hours' period coincide within ΔC. For BSH, B concentration ratio of tumour/blood (Fig.5) ranged from 0.44:1 to 1.1:1 and was 0.44; 0.53; 1.1 and 1.0. The ratio of tumour/blood over time increased which confirm necessary long period medication insertion to reach necessary 10B concentration in tumour.

So, for BPA the optimum BNCT period for SCID mice with orthotopic tumour xenotransplant is exactly

after medication insertion, while BSH requires longer accumulation time.

4. Conclusion

As a result of the study, we developed the ICP-AES method for B determination in animals' organs and tissues. LODs of boron are 0.003 ppm, and LOQs of boron are 0.015 μg/g. Inter-laboratory precision does not exceed 20%.

We determined organs with maximum B accumulation: liver and kidney. The maximum BPA tumour/blood ratio of 2.2 is reached at the 4h time period. However, the absolute B level is low, its maximum is reached at 1 and 2h time period. The maximum BSH tumour/blood ratio is 1.1 reached at 3h time period. We can conclude for BPA the optimum BNCT period for SCID mice with orthotopic tumour xenotransplant is exactly after medication insertion while BSH requires longer accumulation.

Table 5. B concentration results for liver sample variation (a – blood, b – tumour, c – brain, d – kidney, e – liver, f – tumour/blood).

Med.		1 h	2 h	3 h	4 h
BPA	a	9±2	7±2	7±10	1.6±0.2
	b	12±3	11±2	8±4	3.6±1.2
	c	4.9±0.3	5.3±0.5	5±8	1.9±0.3
	d	39±4	24±8	19±26	7±3
	e	10±2	7±3	6±10	1.6±0.1
	f	1.3	1.6	1.1	2.2
BSH	a	13.3±1.9	3±3	2.8±0.9	1.2±0.3
	b	5.9±0.7	1.6±1.4	3±3	1.2±0.3
	c	0.69±0.11	0.20±0.09	0.17±0.02	0.12±0.02
	d	14±4	5±3	3.3±0.4	4±5
	e	14.5±0.3	9.1±1.4	4.3	6.5±0.2
	f	0.44	0.53	1.1	1.0

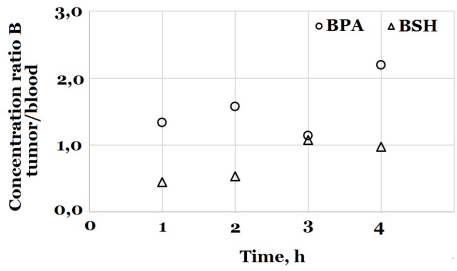

Figure 5. B concentration tumour/blood ratio depending on time for BPA and BSH.

Alternative B delivery method to tumours is using liposomes [13]. This approach increases ^{10}B accumulation effectiveness in the infected tissues. It is worth mentioning that developed ICP-AES determination of boron includes all steps of analysis, so no extra research is needed.

The research is done in the «SPF-vivarium» Federal Research Center Institute of Cytology and Genetics SB RAS. All the experiments on animals were approved by the institutional bioethics committee and correspond with guidelines for accommodation and use of laboratory animals, published by US NIH (№ 85-23, revised in 1985).

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