Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, No. 7, pp. 547–553, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00

Review

A review of techniques for the analysis of boron in the development of neutron capture therapy agents

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Abstract: The resurgence of interest in neutron capture therapy, which uses boron-10 labelled compounds and neutron irradiation as a technique for the treatment of cancer, has been accompanied by the application of a new generation of techniques for the analysis and imaging of boron in biological matrices. In this review, a description is given of the requirements for boron neutron capture agents and the achievements to date of the analytical techniques.

Keywords: *Boron assay; boron imaging; atomic spectroscopy of boron; electron energy loss spectrometry; boron NMR.*

Introduction

Neutron capture therapy (NCT) for the treatment of cancer is a procedure in which stable and non-toxic boron-10 compounds are targeted to malignant lesions and activated by an incident beam of thermal neutrons [1]. The short range (10 μm) cytotoxic α-radiation released in the neutron capture reaction can cause cell death. This two-step mechanism permits the regression of malignant cells in the midst of normal cells. In this sense NCT is analogous to photoradiation therapy which currently uses porphyrins to localize in the tumour (usually glioblastoma is the target), followed by irradiation with red laser light which penetrates the skin to a limited extent. NCT has the potential advantage of being able to treat tumours which are more deeply seated, with the use of a more penetrating (epithermal) neutron beam.

The involvement of boron compounds in NCT relies on the very high neutron cross-section or capture probability of the ^{10}B atom (3838 barn) compared with the biologically ubiquitous C, H, N and O atoms (0.003, 0.33, 1.8 and 0.0002 barn, respectively).

The nuclear reaction which occurs following neutron capture is:

$$^{10}B + {}^{1}n \rightarrow {}^{7}Li^{*} + \alpha\ (2.31\ MeV)$$
$$\downarrow$$
$$^{7}Li + \gamma\ (478\ keV).$$

The 2.31 MeV of kinetic energy given to the Li and α-particle is dissipated rapidly within a 10-μm radius, while the γ-ray is highly penetrating and is readily detected by an external scintillation counter (the intensity of this γ-ray is reduced to half by about 70 mm of water or biological tissue).

The concept of NCT was recognized in the 1930s, soon after the discovery of the neutron, and this led to clinical trials in the 1950s, conducted at Brookhaven National Laboratory and Massachusetts General Hospital. However, the trials were regarded as producing no significant therapeutic advantage and were discontinued in 1961. The main reason for failure was that the boron compounds were non-specific in terms of their localization, and neutron beams of the necessary quality were not available. Subsequent to the initial US trial, a revised protocol was developed in Japan, and over 100 brain tumour patients were treated by intraoperative thermal NCT. The 5-year survival is 40–50% for NCT, compared with <5% for conventional therapy [2].

World interest in NCT has revived in recent years with the development of new compounds and improved neutron sources [3]. The principal target tumours are the deeply seated, and therefore inoperable, glioblastoma multiforme and melanoma brain metastases [3]. Active development of epithermal neutron beams and the associated research programs is occurring at the European Community Joint Research Centre at Petten (Netherlands), Idaho National Engineering Laboratory and Brookhaven National Laboratory (USA), and the Australian Nuclear Science and Technology Organization. Clinical trials are scheduled to begin in the USA and Europe in 1991.

Boron Compounds for NCT

The clinical trials on glioblastoma by a combination of surgery and NCT with thermal neutrons have been conducted by Hatanaka in Japan over the past 10 years using the sodium salt of mercapto-undecahydrododecaborate ($Na_2B_{12}H_{11}SH$ — referred to as sodium borocaptate or BSH) [2]. Recently, three patients with advanced metastatic melanoma have been treated by Mishima using *p*-boronophenylalanine (BPA) and thermal neutrons, with encouraging results [4]. Fairchild's group [5] reported successful NCT of a rat glioma using the oxidized form of BSH, $Na_4B_{12}H_{11}S$—$SB_{12}H_{11}$ (known as BSSB).

However, the compounds being used in these clinical trials cannot be regarded as ideal, in terms of tumour specificity and/or boron carrying capacity. Consequently, a number of groups are involved in the attempt to synthesize a new generation of boronated molecules [6].

In terms of tumour localization capabilities, the most successful boron compounds so far investigated contain one B atom per molecule. For example, the use of BPA against melanoma is based on it being a precursor for melanin synthesis, and it achieves tumour-to-blood ratios >6 when used for melanoma xenografts in animal studies [7]. Similarly, borono-thiouracil (BTU), based on thiouracil as a false precursor for melanin synthesis, accumulates well in melanoma xenografts [8]. These compounds are applicable to melanoma treatment, with the only drawback being the relatively low boron content of the molecule. Efforts are being made to improve the delivery of boron with these compounds, by (i) resolution of the more active L-BPA from the racemic mixture, (ii) sugar derivatives of BPA for better patient acceptance, and (iii) the development of derivatives carrying the decaborane ($B_{10}H_{14}$) or dodecaborane ($B_{12}H_{16}$) cluster compounds.

For NCT of glioma, BSH is the compound of choice, although it does not have a demonstrated specificity for the tumour. However, BSH is non-toxic, and passes through lesions in the blood–brain barrier to develop therapeutic concentrations in the tumour. The clearance from normal brain tissue appears to be somewhat faster than from the tumour.

The porphyrin and the phthalocyanine groups of compounds have been the subject of much study for photodynamic therapy, because of their ability to localize in glioma. Boronated porphyrin derivatives are being developed by Kahl [9] and boronated phthalocyanines by Alam [10]. Preliminary work has shown good tumour uptake.

The other categories of agents under development are boronated purines, pyrimidines and their nucleosides, which may function as fraudulent bases and so be incorporated into the tumour cell. It is also possible to administer boron-containing macromolecules, such as monoclonal antibodies responsive to specific tumour receptors, and some efforts are being directed at the use of encapsulating liposomes or lipoproteins to carry hydrophobic boron compounds into the tumour.

Analytical Methodology

One of the developments required for adequate biological testing of new boron compounds is an analytical methodology for the determination of boron pharmacokinetics. Since the evaluation of potentially useful compounds for toxicity and tumour incorporation involves administering various doses to a number of laboratory animals, the need for an accurate and rapid boron assay is evident.

A point for consideration is that natural boron contains only 20% ^{10}B so that enrichment is required for the boron to be used in neutron capture. This additional expense can be avoided in some of the development phases if neutron irradiation is not needed, so that natural boron can be used.

There are two other considerations involved in setting up the assay procedure. Firstly, whether the biological tissue need be digested to release the boron-containing compound, and secondly, whether the complex boron compound need be converted to a simpler chemical species such as boric acid. Table 1 provides a summary of methods which are applicable to quantitative boron determination for biodistribution studies of BNCT compounds, and these are discussed in more detail below.

A second analytical development stems from a need to know more specifically the localization of the boron within the tumour cell. There are two sites, namely the cell membrane and the cell nucleus, which are presumed to be the key targets for the cytotoxic event. For instance, a monoclonal antibody responsive to

Table 1
Methods for the determination of boron concentration applicable to the evaluation of BNCT compounds in biological tissue

Sample pretreatment	Analytical technique	Limit of detection	Reference
Neutron irradiation	Prompt gamma spectrometry	1 ppm	[11, 12]
$H_2SO_4/H_2O_2/Sn^{++}$	Colorimetry/dianthrimide	1 ppm	[39]
RF combustion–O_2	Colorimetry/curcumine	0.5 ppm	[40]
$NaOH/Na_2CO_3$ fusion	Colorimetry/Methylene Blue	0.1 ppm	[14]
$HClO_4/H_2O_2$	ICP–atomic emission	0.05 ppm	[18]
Na_2CO_3 fusion	ICP–mass spectrometry	0.01 ppm	[20]
CaO ignition	Fluorimetry/quinizarin	0.1 ppm	[15]
Na_2CO_3 fusion	Chemical ionization MS	0.1 ppm	[22]
Neutron irradiation	^{4}He mass spectrometry	0.01 ppm	[23]

a cell surface associated antigen may carry boron to the cell membrane, but the boron may be metabolized or internalized. The particles produced when ^{10}B captures a neutron travel for only 10 μm at most. If this event occurs too far away from the nucleus, it may not kill the cell. Thus, techniques for cellular imaging are being used to provide a better understanding of the mechanism of incorporation of boron and its metabolic fate. The imaging methods are listed in Table 2 and described below.

The wide variety of analytical techniques being employed for boron determination and imaging is a reflection of the multi-disciplinary nature of NCT research. Much of the instrumentation described here is highly sophisticated and expensive. Consequently, many of these techniques are available only at the major research centres.

1. *Quantitative determination of boron*

Prompt gamma spectrometry. Among the investigators of BNCT, many have used prompt γ-ray spectrometry for the determination of trace amounts of ^{10}B in animal tissue [11, 12]. This method is essentially neutron activation analysis, relying on the detection of the γ-ray of 478 keV emitted from the disintegrating ^{10}B nucleus after absorption of a thermal neutron.

Although prompt gamma spectrometry has the advantage of not involving the pretreatment or digestion of samples, and can be used with live subjects, the requirement of ^{10}B as well as the neutron source restricts its application, particularly in the development stages involving pharmacokinetic studies with new boron-containing agents in animal-tumour model systems. The detection limit of prompt gamma spectrometry is about 1 ppm, i.e. 1 μg ^{10}B per g of tissue [13]. Since the requirement of NCT is a concentration of ^{10}B in excess of 20 ppm, this sensitivity is adequate for confirming the presence of suitable amounts of ^{10}B in the tumour site of a patient after administration of the boron-containing compound.

Colorimetric assay. The colorimetric assay methods represent the application of chemical techniques to avoid the need for neutron irradiation. All rely on the breakdown or digestion of the biological tissue to release the boron compound, followed by chemical conversion to a species such as boric acid or tetrafluoroborate which is then conjugated to a colour reagent. The method with greatest sensitivity for boron (detection limit 0.1 ppm) involves alkali fusion, treatment with hydrofluoric acid, and complexation of the tetrafluoroborate so formed with Methylene Blue [14].

The principal drawback to these techniques has been encountered in terms of interferences from oxidizing agents and competing ions in

Table 2
Methods used for visualizing and/or imaging boron in biological samples

Sample type	Method	Sample pretreatment	Reference
Tissue section	α-Track etch	n-Irradiation	[24, 25, 26]
Tissue section	Secondary ion mass spectrometry (SIMS)	Freeze etch/dry	[27, 28]
Cell incubate	Electron energy loss spectra (EELS)	Cell fixation microtome section	[33, 34]
Whole animal	Nuclear magnetic resonance spectra (^{11}B-NMR)	None	[35, 36]
Whole animal	Positron emission tomography (^{19}F or ^{11}C-PET)	None	[37, 38]

the complexation step. The consequences of these interferences is that some strong oxidizing agents are not available for the tissue digestion step. The use of alkali fusion requires a relatively long time and is prone to loss of sample by evaporation.

The major deficiencies of the other colorimetric assay methods are either a lack of sensitivity, the risk of loss of boron because of the long tissue digestion step, or the need for neutron irradiation during the development process. The spectrofluorimetric method using quinizarin [15] is highly sensitive for boron but requires ignition with calcium oxide at 550°C before mixing with the reagent in concentrated sulphuric acid.

Atomic spectroscopy of boron. The emission spectrum of excited atomic boron is characteristic of the element, and one classical method which takes advantage of this has been reported [16]. In this procedure the sample of tissue containing boron is placed without pretreatment on a boron-free graphite electrode, on which it is excited in an electric arc, and the spectrum recorded on a spectrograph using UV-sensitive photographic plates. Germanium in the form of germanium oxide was used as internal standard, and the emission line at 265.118 or 259.254 nm for germanium was correlated with those of boron at 249.773 and 249.678 nm, respectively. By this means, a relative standard deviation of 15% was achieved for the estimation of boron in tissue samples from animals dosed with BSH.

Conventional atomic absorption and emission spectrometric measurement of boron has not been completely satisfactory since boron is one of the more refractory elements. Thus the complex boron cage compounds used in NCT are not completely dissociated in the flame, and this leads to variable results at the ppm level [11, 17].

A more effective atomic spectroscopy method for the assay of BNCT compounds in biological samples is based on inductively coupled plasma atomic emission spectrometry (ICP–AES) [18]. One requirement for ICP–AES is that the sample must be converted to a homogeneous solution before it is aspirated into the plasma. A simple and effective method for biological tissue digestion was developed using perchloric acid and hydrogen peroxide at 70°C for 1 h [18]. An alternative approach used nitric acid in a Teflon-lined bomb at 150°C for 3 h [19].

The acceptance of the ICP–AES technique by the majority of NCT research teams has been assured, not only because ^{10}B and neutron irradiation are not required, but it also avoids the necessity to convert the complex BNCT compound to a simpler species. The method has proven capable of providing the precision for boron (±2%), and the sensitivity (0.05 ppm) needed for biodistribution studies. The ICP source with a plasma temperature of the order of 8000 K reduces all species to free atoms, with a significant population in the excited state from which emission then occurs. The optimum wavelength for the detection of boron is 249.678 nm, because of the absence of interfering emission from other atoms.

The ICP principle has also been extended to provide a method for introducing the sample in mass spectrometry. The technique of ICP–MS is about an order of magnitude more sensitive than ICP–AES, and is capable of discrimination between the two isotopes [20]. ICP–MS has not been as readily available as ICP–AES, but is now being used by NCT researchers, particularly in the study of uptake of boron compounds by cells in culture. In such experiments, a single culture dish may contain 2×10^5 cells, amounting to about 0.1 mg of cellular mass, so that the capacity to detect 10 ng of boron is clearly an advantage [21].

Other methods. Chemical ionization mass spectrometry has been used to determine the isotope ratio $^{10}B/^{11}B$ following conversion of the compound to boric acid then methyl borate [22]. Helium mass spectrometry can also be used following neutron irradiation [23]. However, the complexity of the work-up in the former case, or the specialized equipment for the latter, render both as lesser choices.

2. *Imaging of boron in biological tissues*

α-Track etch. The α-track etching technique in tissue sections has been proposed for the quantitation of the ^{10}B isotope in tissues following neutron irradiation, and has the important ability to determine the microscopic distribution of ^{10}B in histological sections of the tumour. The tissue section is placed in contact with a detector film, such as carboxymethylcellulose or Lexan, then neutron irradiated, stained, reversed and etched with potassium hydroxide [24]. For quantitation,

the total areas of the tracks (under microscope) are related to a standard curve based on the corresponding analysis of a series of homogenates containing known amounts of boric acid.

Recently, a similar neutron capture radiography was described by which quantitative assessment of the boron distribution in whole body animal sections can be made [25]. A high resolution α-track autoradiogram development using a computer controlled microscope stage claims a resolution of the order of 2–4 μm [26].

Secondary ion mass spectrometry. Although the ICP–AES or MS techniques are capable of providing sub-ppm detection limits for boron, such limits are altered when the amount of available sample is <1 ml. Electron probe microanalysis (or ion microscopy) extends the range of sample size down to the 100 pl (picolitre) level while maintaining sub-ppm detection. Secondary ion mass spectrometry (SIMS) has been applied in the early days of its development to semi-conductor and metallurgical research. Recently, biomedical applications of the technique have been reported, and among them is boron analysis. The technique employs sputtering of the surface atoms from relatively small areas (typically, 250 × 250 μm) of a tissue sample by an energetic primary ion beam. The secondary ions generated from the sample are detected in a mass analyser. The elemental detection sensitivities in the SIMS analysis are a function of the relative ease of ionizing the respective elements sputtered from the surface [27].

While this technique can be used for quantifying boron in very small samples, the particular application of SIMS is to the determination of intracellular boron concentration, due to the likelihood that there is an uneven biodistribution of the boron compound. After calibration with ICP–AES, by using homogenates of cultured cells incubated with a boron compound, the technique can be applied to cells cultured on silicon chips with BSH included in the culture medium, then freeze-fractured and freeze-dried for ion microanalysis. The ion microscope measures dry weight concentrations, and a cellular water content needs to be assumed to determine the mean boron concentration in the site sampled. Thus uptake of BSH was measured in the treated cells to be: nuclear, 25 ± 6 ppm; cytoplasmic, 84 ± 24 ppm [28, 41].

Electron energy loss spectroscopy. One of the new generation systems for delivery of boron for NCT involves the use of specific monoclonal antibodies (MAb). Methods have been developed for the conjugation of dodecaborane groups to monoclonal antibodies to the level of 1500 boron atoms per MAb. This has been achieved without significantly affecting the specificity of the antibodies against the appropriate cell lines [29, 30]. Calculations show that this level of boron incorporation is close to that required for effective killing of the cells based on a uniform distribution of the boron [31]. What is not known is whether such a uniform distribution will prevail, or whether specific localization occurs to certain cell components.

It is perhaps fortuitous that boron conjugation with MAb provides a means whereby the time course and spatial distribution of B-MAb can be studied in detail by the application of electron energy loss spectroscopy (EELS) in the transmission electron microscope. Submicron spatial resolution is achievable and the method is highly sensitive to elements with low atomic number, such as boron, carbon, nitrogen, etc. Given that the biologically ubiquitous elements will not provide information on endocytosis of biomolecules, the use of boron as marker is almost ideal for EELS detection. The net effect is that B-MAb distributions in cells can be studied as a function of incubation time before fixing.

The principle of EELS is as follows [32]. When an electron beam passes through an electron microscope specimen, it undergoes a number of interactions. In one such interaction, it ejects an electron from an atom, and in so doing it loses energy. The amount of energy lost (ΔE) depends upon, and is a characteristic of, the atom, so that by measuring ΔE the atom can be characterized. By focusing the electron beam onto a selected area of the specimen, the atoms in that area can be characterized from ΔE. By measuring the number [$I(\Delta E)$] of incident electrons which have lost the characteristic energy, the concentration of the element can be determined. Thus an elemental map can be constructed to provide an image of the boron localization in the cell.

For example, EELS imaging was used with boronated protein-A to obtain indirect detection of specific antigenic sites to wheat germ agglutin in acinar cells at a spatial resolution of

0.5 nm [33]. In another study [34], colorectal cancer cells were exposed to a specific antibody carrying 500 boron atoms per molecule for varying times. Initially, boron was seen on the microvilli at the cell surface. Within 5 min, boron had penetrated the cell membrane and had reached the nuclear membrane. By 30 min boron was seen in proximity to chromosomes undergoing mitosis. These preliminary findings suggest that cellular boron penetration occurs, and have important implications for microdosimetry.

Boron nuclear magnetic resonance. One advantage, apart from the cost, of using ^{11}B in the development stages of NCT lies in the possibility of using nuclear magnetic resonance for both spectroscopy and imaging. Although ^{10}B also has a nuclear magnetic moment, the sensitivity of its detection is much less than for ^{11}BN. Preliminary work has shown that ^{11}B imaging can be used to detect and quantitate boron uptake non-invasively in animal tumour models [35, 36]. The major problem to be overcome for whole animal imaging arises from the short spin–spin relaxation time (T_2) of the ^{11}B signal. NMR imaging technology has been designed for tissue protons with a T_2 >100 ms. ^{11}B in solution has a T_2 of only 6 ms, and in tissue this falls to <1 ms, making the signal broad and indistinct, unless altered probe electronics are available so that it can be measured in the shorter time scale.

Other methods. It is particularly important to be able to determine the optimum time after administration of the boron compound to the patient for neutron irradiation to be commenced. The only alternative procedures involve ionizing radiation — either prompt gamma spectroscopy, or positron-emitting isotopes in positron emission tomography [37]. The former lacks the ability to provide a resolution less than that determined by the size of the γ-ray collimator. The latter would require the rapid chemical incorporation of carbon-11 (half-life 20.5 min), or fluorine-18 (half-life 1.9 h), and the knowledge that the boron and positron-emitter remain attached to the molecule following its administration to the patient. This appears to have been achieved with the synthesis and testing of ^{18}F–BPA, obtained readily by reaction of BPA with acetyl hypofluoride [38]. The use of ^{11}C presents greater difficulty in incorporation into the appropriate boronated molecule.

Acknowledgements — This project has been funded by the New South Wales State Cancer Council, and the Australian Institute for Nuclear Science and Engineering.

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[Received for review 6 February 1990; revised manuscript received 23 April 1990]