A Simple and Rapid Method for Measurement of \(^{10}\text{B-para}\)-Boronophenylalanine in the Blood for Boron Neutron Capture Therapy Using Fluorescence Spectrophotometry

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Boron/Neutron/Capture/Therapy.

Background and Purpose; \(^{10}\text{B}\) deriving from \(^{10}\text{B-para}\)-boronophenylalanine (BPA) and \(^{10}\text{B}\)-borocaptate sodium (BSH) have been detected in blood samples of patients undergoing boron neutron capture therapy (BNCT) using prompt gamma ray spectrometer or Inductively Coupled Plasma (ICP) method, respectively. However, the concentration of each compound cannot be ascertained because boron atoms in both molecules are the target in these assays. Here, we propose a simple and rapid method to measure only BPA by detecting fluorescence based on the characteristics of phenylalanine. Material and Methods; \(^{10}\text{B}\) concentrations of blood samples from human or mice were estimated by the fluorescence intensities at 275 nm of a BPA excited by light of wavelength 257 nm using a fluorescence spectrophotometer. Results; The relationship between fluorescence to increased BPA concentration showed a positive linear correlation. Moreover, we established an adequate condition for BPA measurement in blood samples containing BPA, and the estimated \(^{10}\text{B}\) concentrations of blood samples derived from BPA treated mice were similar between the values obtained by our method and those by ICP method. Conclusion; This new assay will be useful to estimate BPA concentration in blood samples obtained from patients undergoing BNCT especially in a combination use of BSH and BPA.

INTRODUCTION

\(^{10}\text{B-para}\)-boronophenylalanine (BPA) and \(^{10}\text{B}\)-borocaptate sodium (BSH) have been used for boron neutron capture therapy (BNCT).\(^1\)–\(^5\) \(^{10}\text{B}\) nucleus has markedly large cross section to capture slow neutrons in comparison with body composition elements. The \(^{10}\text{B}\) (n, \(\alpha\)) \(^{7}\text{Li}\) reaction releases a high linear energy transfer (LET) \(\alpha\) particle and a recoiling \(^{7}\text{Li}\) ion with an average total kinetic energy of 2.34 MeV.

Their tracks do not exceed one cell diameter. Therefore if these compounds accumulate in tumour cells or tumour tissue and they receive thermal neutrons or epi-thermal neutrons, the tumours can be destroyed efficiently and selectively. As the effectiveness of BNCT is related primarily to the selective accumulation of the boron compounds in the tumor relative to the surrounding normal tissues, concentration of boron compounds should be monitored carefully for efficient killing of the tumour cells. In the clinical setting of BNCT, the \(^{10}\text{B}\) concentration in blood should be monitored to estimate radiation dose delivered to tumour and normal tissues. As previously reported, BPA and BSH are combined to ensure such efficient killing.\(^6\)–\(^10\) The relative BNCT effect varies depending on the combination of \(^{10}\text{B}\) compound and tissue or organ even at a same \(^{10}\text{B}\) concentration and neutron fluence. This effective RBE has been determined by experiments on each normal tissue assuming that \(^{10}\text{B}\) compound is distributing in the tissue at equal concentration in blood. \(^{10}\text{B}\) concentration in blood has been measured by prompt gamma ray spectrometer, or ICP method.\(^11\)–\(^13\) However,

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doi:10.1269/jrj.09015
these methods can not distinguish $^{10}$B concentration of BPA and BSH in blood samples. Therefore, a simple and rapid method to measure $^{10}$B concentration of each compound separately is needed to accurately estimate X-ray equivalent dose. We proposed a simple and rapid method to measure only BPA by detecting fluorescence at 275 nm of a BPA compound excited by light of wavelength 257 nm using a fluorescence spectrophotometer based on the characteristics of phenylalanine.

MATERIALS AND METHODS

Measurement of BPA concentration in ethanol

A stock solution of BPA (1,300 or 1,600 ppm $^{10}$B) was used for all experiments. BPA solutions were diluted with ethanol, and 1 ml solution was poured into the cuvette and placed in the cell of the fluorescence spectrophotometer (F-2000; Hitachi, Tokyo, Japan). The fluorescence spectrophotometer was set at 700 V for detection amplification. Excitation and emission wavelengths were 257 nm and 275 nm, respectively. These wavelengths were determined from the fact that phenylalanine can be measured in these wavelength with little influence by the other amino acids such as tyrosine and tryptophan.14) This was evidenced in Fig 2a.

Measurement of BPA concentration in human blood

Blood samples obtained from a healthy man volunteer (35 years old) was analysed within 24 h. An aliquot of 200 μl of blood was added to each 1.5 ml tube, and 1–10 μl of BPA (6.5–65 ppm $^{10}$B) and 1.2 μl of BSH (30 ppm $^{10}$B) stock solution were added to the blood samples. After vortex-mixing the sample for a few seconds, blood was centrifuged at 15,000 rpm for 2 minutes, and 50 μl of the supernatant containing plasma fraction was poured in another 1.5 ml tube. A 12-fold volume of ethanol (600 μl) was added to the plasma and the tube was vortex-mixed. Samples were centrifuged at 15,000 rpm for 2 minutes. After centrifugation, supernatants were poured into the collection tube, which was part of a syringeless filter device (Mini-Uniprep, Whatman, NJ, USA). The solution was pushed through polytetrafluoroethylene filter media (pore size, 0.45 μm) to remove proteins present in the plasma solution. Filtrated solutions were diluted with ethanol to concentrations (20-fold), and 1 ml solutions were poured into the cuvette and placed in the cell of the fluorescence spectrophotometer.

Measurement of BPA concentration in mice blood

BPA solution (0.3 or 0.6 ml of 1,600 ppm $^{10}$B) was administered intraperitoneally to C3H mice. Twenty minutes after injection of BPA, 0.3 ml of heparin solution (1,000 unit/ml of heparin sodium solution, Ajinomoto, Tokyo, Japan) was injected. Five minutes after injection of heparin, 500–1,000 μl of blood was collected from the eye’s artery. An aliquot of 50 μl of blood was added to each 1.5 ml tube, and 0.5–1.5 μl of BPA (16–48 ppm $^{10}$B) were added to the blood samples. After vortex-mixing the sample for a few seconds, blood was centrifuged at 15,000 rpm for 2 minutes, and 20 μl of the supernatant containing plasma fraction was poured in another 1.5 ml tube. A 12-fold volume of ethanol (240 μl) was added to the plasma and the tube was vortex-mixed. Samples were centrifuged at 15,000 rpm for 2 minutes. After centrifugation, supernatants were poured into the collection tube, which was part of a syringeless filter device (Mini-Uniprep, Whatman, NJ, USA). The solution was pushed through polytetrafluoroethylene filter media (pore size, 0.45 μm) to remove proteins present in the plasma solution. Filtrated solutions were diluted with ethanol to concentrations (20-fold), and 1 ml solutions were poured into the cuvette and placed in the cell of the fluorescence spectrophotometer.

ICP method

Plasma samples were obtained after centrifugation of human and mice blood as described previously. Twenty micro-litter of plasma were diluted with 5 ml water and stored in the fridge. Inductively Coupled Plasma (ICP) method was performed as following the recommended instruction. The concentration of boron in each plasma sample was determined by ICPS-1000TR (Shimazu, Kyoto, Japan).

RESULTS AND DISCUSSION

The relationship between BPA concentration in ethanol and fluorescence showed a positive linear correlation until high intensities of fluorescence were achieved (Fig. 1). Our measurement condition was therefore suitable for detecting Fig. 1. Relationship between BPA concentration and fluorescence intensity at 275 nm of blood. The fluorescence value of only ethanol was subtracted at all points. The equation of this line was $y = 332x + 9.26$ ($R^2 = 0.9996$).
BPA concentration without fluorescence saturation. A similar slope for the scattered plot was obtained if water or phosphate-buffered solution was used instead of ethanol as the solvent (data not shown), suggesting that soluble BPA was detected without the influence of solvents. Ethanol, even in the absence of BPA, emits fluorescence at 275 nm after excitation by light at 257 nm. The value for ethanol without BPA was 130–180 and the obtained fluorescence value of only ethanol was subtracted from the total fluorescence value of BPA and ethanol solution.

Despite the influences of many fractions in blood, only BPA should be measured in this method. The fluorescence spectrum between 260 and 360 nm excited by the light 257 nm were shown in Fig 2a. The spectrums for the samples derived from human plasma without BPA (dark blue) were clearly different from the spectrum for ethanol only. This suggests that plasma only emit the light (especially in over 280 nm). However, the light 275 nm were not influenced by the fractions of plasma and increased intensities in 275 nm were observed with higher concentration of BPA (Fig 2a). The scattered plots of the samples derived from human blood before subtraction of the ethanol intensities and after subtraction of those were shown on the left (b) and right (c) of Fig. 2, respectively. In the sample without BPA addition (0 ppm), fluorescence was the same as the intensity of ethanol when used alone (zero in Fig. 2c), suggesting that...
all other components which influence measurement at 275 nm were excluded by our treatments. When the samples were prepared for measurement, plasma samples were diluted with ethanol. We diluted the samples at three types of ratio. Slope values decreased according to the dilution ratio. In case of 120-fold dilution, the shape of the line was quadratic at < 65 ppm $^{10}$B of BPA. The reason for as to why the scattered plots reached the ceiling at lower dilution ratio is unclear, but linear increase with 2.1 and 2.6 of slope value was observed in 300-fold and 240-fold dilution samples, respectively. This slope value means that the range of resolution is < 0.5 ppm $^{10}$B in BPA containing blood sample. We therefore applied a 240-fold dilution in the further study.

To mimic the blood sample of a patient undergoing BNCT, BPA containing blood sample at 13 ppm $^{10}$B was previously prepared for creating scattered plots, in addition to the control scattered plots between $^{10}$B of BPA concentra-

![Fig. 3.](image)

**Fig. 3.** Relationship between BPA concentration and fluorescence intensity at 275 nm in blood samples with (white square) and without (black diamond) previous addition of 13 ppm BPA. The dilution ratio of all blood samples was 240-fold. The equations of lines for the scattered plots from blood samples with previous addition of BPA (white square) and control (black diamond) were $y = 2.3654x + 32.85$ ($R^2 = 0.988$) and $y = 2.3479 + 0.7905$ ($R^2 = 0.9966$), respectively.

![Fig. 4.](image)

**Fig. 4.** Little effects were observed by the presence of BSH on BPA measurement in the blood samples. Thirty ppm $^{10}$B of BSH was added to blood sample containing BPA in each concentration. Result shows mean frequency ± standard error of the mean (SEM) of fluorescence intensities from three independent experiments.

![Fig. 5.](image)

**Fig. 5.** (a) Relationship between BPA concentration and fluorescence intensity at 275 nm in blood samples derived from five nontreated mice. The equations of lines for the scattered plots from each mouse sample were $y = 3.9775x + 27.49$ ($R^2 = 0.9607$), $y = 3.3981x + 14.92$ ($R^2 = 0.9877$), $y = 3.3906x + 22.55$ ($R^2 = 0.9965$), $y = 2.72x + 9.97$ ($R^2 = 0.9945$), $y = 2.5338x + 7.84$ ($R^2 = 0.9959$), respectively. (b) Relationship between added BPA concentration and fluorescence intensity at 275 nm in blood samples derived from mice treated with 0.6 ml (left) and 0.9 ml (right) BPA solutions. The equations of lines for the scattered plots from the samples of 0.6 ml and 0.9 ml treated mouse were $y = 2.4069x + 54.11$ ($R^2 = 0.9442$) (black plots of left panel), $y = 1.8869x + 39.69$ ($R^2 = 0.9923$) (white plots of left panel), and $y = 2.93x + 112.33$ ($R^2 = 0.9982$) (black plots of right panel), respectively.
tions and fluorescence intensities. Two nearly parallel linear scattered plots were obtained between the control and the BPA-added sample (Fig. 3). The equations of lines for the scattered plots from blood samples with previous addition of BPA (white square) and control (black diamond) were \( y = 2.3654x + 32.85 \) (\( R^2 = 0.988 \)) and \( y = 2.3479x + 0.7905 \) (\( R^2 = 0.9966 \)), respectively. Therefore, the slope value was indicated to be almost equal (2.3654 vs 2.3479). It was indicated that the increased fluorescence intensity in the sample with previous addition of BPA was 32.06 at the y intercept after subtraction of background y value. The estimated BPA concentration in the sample was 13.55 ppm (32.06 was divided by 2.3654, slope for pink plots). This estimated value is very similar with 13 ppm as an actual concentration.

Also, we examined the influence of BSH on our BPA measurement, and the result showed that no influences by the presence of 30 ppm \(^{10}\text{B} \) of BSH were observed in the fluorescence intensities for \(^{10}\text{B} \) of BPA (Fig. 4). It is clear that we can measure the \(^{10}\text{B} \) concentrations of BPA by this method using blood samples simultaneously containing two compounds. In the present clinical use of BPA with BSH, approximate concentrations for each compound have been estimated by the expected concentrations for \(^{10}\text{B} \) of BSH, which had been obtained from attenuation curve of BSH concentrations. Therefore, the results are clearly showing that an application of our method to clinical setting for BNCT will improve the measurement of \(^{10}\text{B} \) concentrations for BPA and BSH.

To mimic the patient situation, BPA treated mice were analysed. Because of the small blood volume, we could not estimate the background intensity in a BPA treated mouse unlike in human. Therefore, we first estimated an average of background intensity from five mice. BPA was added in the blood samples derived from five mice, and five linear lines followed by scattered plots were drawn with a good correlation (Fig. 5a). The reason why the slopes of each line in control mice were discord should be because of different hematocrit values among mice, as BPA must be distributed to plasma fraction. The average of the y intercepts from five values (between 7.84 and 27.49) was 16.55 (Fig. 5a). The y intercepts in mice samples were higher than those in human blood, because fluorescence may be emitted from heparin. In the preparation of mice samples, heparin is needed to collect the blood without blood-curdling into a tube. In contrast, human blood was collected into tube containing EDTA-2Na and this is not influent in the fluorescent intensities of samples (data not shown). Next, scattered plots were described in BPA treated mouse (Fig. 5b). The left and right graphs are showing that the 0.6 and 0.9 ml of BPA stock (1,600 ppm \(^{10}\text{B} \) of BPA) were administrated intraperitoneally, respectively. The y intercepts in the lines of the left graph (Fig. 5b, black and white plots) were 54.11 and 39.69. The values after the subtraction of background value (16.55) were 23.14 and 37.56, respectively; therefore, the BPA concentrations from the lines were 12.26 ppm and 15.61 ppm (divided by each slope value 1.8869 and 2.4069, respectively). In the right graph of Fig. 5b, the values at the y intercept in black and white plots were 95.78 and 153.9 after the subtraction of background value, respectively. Therefore, concentrations in 0.9 ml treated mice were 32.69 ppm and 59.84 ppm, respectively.

We next compared our new method to ICP method in the measurement of blood concentration of \(^{10}\text{B} \) in BPA treated mice. As shown in the left graph of Fig. 6, good correlation was observed between fluorescence intensities and \(^{10}\text{B} \) concentrations for BPA. In the graph, the y intercept was 56.637, and the value after the subtraction of background

![Fig. 6. The comparison of the new method and ICP method in the samples derived from a BPA treated mouse. Left panel is showing the relationship between fluorescence intensity and added BPA concentration. The equations of lines for the scattered plots were \( y = 3.2477x + 56.637 \) (\( R^2 = 0.9756 \)). Right panel is showing the relationship between the relative values for boron evaluated by ICP and added BPA concentration from the same mouse. The equation of line for the scattered plots was \( y = 0.01x + 0.109 \) (\( R^2 = 0.9988 \)).](http://jrr.jstage.jst.go.jp)
CONCLUSION

We established a new method to measure only BPA in blood sample by detecting fluorescence based on the characteristics of phenylalanine. This method will be especially useful for BPA measurement in patients undergoing BNCT by concomitant use of BPA and BSH.

ACKNOWLEDGEMENT

The authors are grateful to Yuka Yamamoto for taking blood samples. This research was supported by the grant of Initiative for Nuclear Fundamental Strategic Research.

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Received on February 9, 2009
Revision received on April 2, 2009
Accepted on April 9, 2009
J-STAGE Advance Publication Date: June 9, 2009