Fourier Transform Infrared (FTIR) Spectrometry for the Assay of Polyhedral Boron Compounds in Plasma and Pharmaceutical Formulations

Yohanes Setiawan,[1] Tor Rise,[1] and Douglas E. Moore[1,2]

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The determination of polyhedral boron compounds such as sodium borocaptate directly in blood plasma is described using FTIR with computed nonlinear background subtraction of the water absorption band. The procedure can be performed in less than 15 min to a sensitivity level of 5 ppm boron (at a signal/noise ratio of 2.5), which is satisfactory in the clinical application of such compounds in neutron capture therapy of cancer. For boron compounds with suitable organic solubility, extraction from plasma into carbon tetrachloride is described as an alternative approach not requiring computed subtraction and capable of achieving a sensitivity level of 1 ppm. Both the boron and the lipid content of liposome formulations containing the polyhedral boron compounds can be measured simultaneously by FTIR. After extraction into $CHCl_3$:CH_3OH (1:1) or dispersion in ethanol, the extracts are evaporated to dryness and redissolved in carbon tetrachloride for FTIR assay.

KEY WORDS: boron neutron capture therapy; polyhedral boron compounds; Fourier transform infrared spectrometry; quantitative analysis in plasma; quantitative analysis of liposome formulations.

INTRODUCTION

Neutron capture therapy (NCT)[3] for the treatment of cancer is a binary procedure wherein stable and nontoxic boron-10 compounds are targetted to malignant lesions, there to be activated by an incident beam of thermal neutrons (1). The short-range (10-μm) cytotoxic α-radiation released in the neutron capture reaction can initiate reproductive cell death. The binary selectivity of this mechanism permits the regression of malignant cells in the midst of normal cells. NCT has the potential advantage of being able to treat tumors which are deeply seated, with the use of a penetrating (epithermal) neutron beam. While two boron-containing compounds (*p*-boronophenylalanine and sodium undecahydrododecaborate, $Na_2B_{12}H_{11}SH$, also called sodium borocaptate or BSH) have been approved for use in clinical trials of NCT, new compounds are being synthesized and tested in the continuing search for an agent having ideal physical and biological behavior. The analytical aspects of these developments have been reviewed (2).

In the clinical application of NCT the boron concentration in the patient needs to be monitored frequently, to determine the appropriate time to begin the neutron irradiation of the tumor. This assay must be both rapid and robust. The blood is the most readily sampled tissue for boron analysis and, from accumulated pharmacokinetic data, that can be correlated with the tumor boron concentration. Inductively coupled plasma atomic emission spectrometry (ICP-AES) following acid digestion provides precise boron analysis capability down to less than 0.02 ppm (3). However, because of the digestion step, even the most streamlined operation of this procedure will take 45 min to provide the boron concentration in blood plasma. The neutron activation or prompt-gamma technique is capable of rapid measurement but requires a 1-g sample to be neutron irradiated and has a sensitivity limited to about 2.5 ppm boron-10 (4). Magnetic resonance imaging of boron-11 is under development as a noninvasive technique for *in vivo* boron concentration estimation (5).

As an alternative, use can be made of the infrared absorption band which occurs at about 2500 cm^{-1} due to the boron–hydrogen stretching vibration for compounds containing the icosahedral boron cage structure of 10 to 12 boron atoms. This high-energy region of the infrared is relatively free from overlap arising from absorption by most solvents and other molecules in the system. Additionally, the B–H stretch band is not significantly influenced by interaction from the surrounding atoms. Fourier Transform infrared (FTIR) instrumentation has enhanced the capabilities of this spectroscopic technique, especially in the area of sensitivity and speed of assay. Lu and Munro (6) have presented FTIR as a method for the measurement of BSH in aqueous solution and urine. They showed that the strong interfering absorption due to water can be successfully removed by the use of a short-pathlength cell and background subtraction techniques and achieved a detection limit of 10 μg/mL for BSH (5 ppm boron).

We have extended this study to demonstrate the use of FTIR for the determination of BSH directly in blood plasma. This is achieved with the use of nonlinear baseline correction and is capable of providing a result in less than 15 min to a sensitivity level more than adequate for the clinical situation. For those boron compounds with suitable solubility in organic solvents, extraction from plasma into a nonabsorbing solvent provides an alternative approach.

Another application of FTIR to NCT research is to the assay of formulations containing the polyhedral boron compounds. For example, there is much interest in the use of liposomes and lipoproteins as vehicles for the delivery of boron compounds to tumor cells (7,8). A convenient assay procedure is needed to determine the boron content of the formulation to be administered to the animal for biodistribution studies. Extraction into an organic solvent followed by FTIR assay of the solution provides a convenient and rapid method to accomplish the determination of not only the bo-

[1] Department of Pharmacy, The University of Sydney, Sydney 2006, Australia.

[2] To whom correspondence should be addressed.

[3] *Abbreviations used:* NCT, neutron capture therapy; ICP-AES, inductively coupled plasma atomic emission spectrometry; FTIR, Fourier transform infrared; BSH, sodium undecahydrododecaborate (also called sodium borocaptate); DBM, diethyl *p*-[1,2-dicarba-*closo*-dodecaboran-1-yl]benzyl(formamido)malonate; PDB, *n*-pentyl-1,2-dicarba-closo-dodecaborane; DBA, *p*(1,2-dicarba-*closo*-dodecaborane)benzyl alochol.

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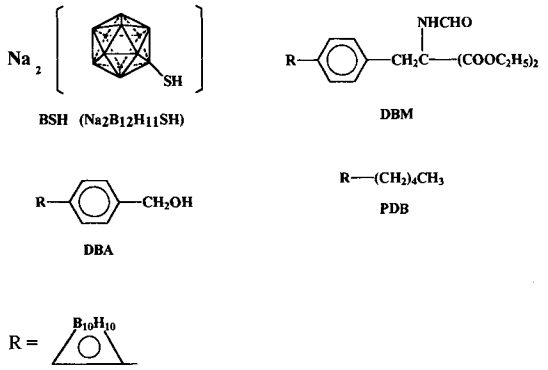

Fig. 1. Structures of the polyhedral boron compounds used in this study.

ron content but also the lipid content of the liposome preparation.

MATERIALS AND METHODS

Chemicals

Disodium mercaptoundecahydro-*closo*-dodecaborate (BSH) was from the Callery Chemical Co., (Callery, PA), while diethyl *p*-[1,2-dicarba-*closo*-dodecaboran-1-yl]benzyl(formamido)malonate (DBM), *n*-pentyl-1,2-dicarba-*closo*-dodecaborane (PDB), and *p*-(1,2-dicarba-*closo*-dodecaborane)benzyl alcohol (DBA) were synthesized in our laboratory (9). Human serum albumin was obtained from Sigma Chemical Co., (St Louis, MO). Carbon tetrachloride, chloroform, and methanol were of analytical grade from Ajax Chemicals (Sydney, Australia). All water used was doubly distilled in an all-glass system. Human plasma was obtained from the Sydney Blood Bank.

FTIR Spectroscopy

Infrared spectra were recorded with a Bio-Rad FTIR spectrometer Model FTS 20/80, equipped with a Bio-Rad 3200 Data Station and a wide-range mercury cadmium telluride detector. A resolution of 8 cm^{-1} with a 6-mm aperture was used, and 256 scans were collected and apodized with a triangular function for each sample. A Specac variable-pathlength liquid cell with calcium fluoride windows was used to record spectra of all liquid samples. Generally, a pathlength of 1 mm was used for CCl_4 solutions and of 50 μm for aqueous solutions. For quantitative measurements with small volume samples, a Specac microcell with calcium fluoride windows and a fixed pathlength of 1 mm was used.

Calibration Solutions

Standard solutions of DBA were prepared containing 5 mg/mL (5000 ppm) of boron in CCl_4. (Caution: In principle, any polyhedral boron compound can be used for calibration, but the use of decaborane and chlorinated solvents is to be avoided, as this combination is shock sensitive and can detonate violently and unpredicably.) The solutions for calibration were derived from a series of dilutions ranging in concentration from 0 to 500 μg boron/mL. BSH standard solution was prepared containing 10 mg/mL of boron in water, and dilutions were made to provide solutions containing 0 to 500 μg boron/mL.

Analysis of Boron in Aqueous Samples

Direct Measurement. Aliquots of the BSH stock solution were added to either aqueous solution or human plasma samples, to give boron concentrations in the range between 0 and 200 μg/mL (ppm). Each sample was examined in the variable-pathlength liquid cell with calcium fluoride windows.

Solvent Extraction. Aliquots of the DBA stock solution were added to (a) aqueous solutions, (b) aqueous solutions containing human serum albumin, and (c) human plasma samples, to give boron concentrations between 0 and 200 μg/mL (ppm). Each sample of 5 mL was extracted three times with 25 mL of CCl_4. The CCl_4 extracts were combined and evaporated to dryness at 35°C under vacuum. The res-

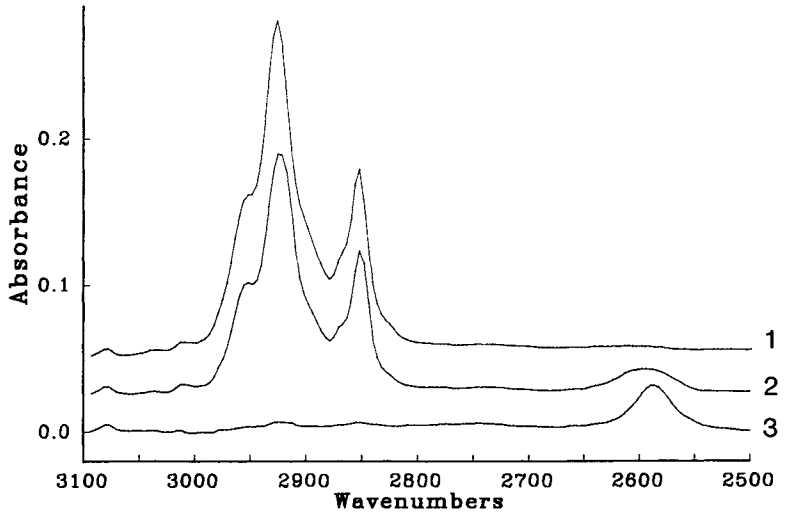

Fig. 2. Infrared spectrum of (1) phosphatidylcholine–dicetyl phosphate mixture (10 mg/mL) in CCl_4, (2) PDB-containing liposome extract in CCl_4, and (3) boron compound PDB (30 ppm) in CCl_4.

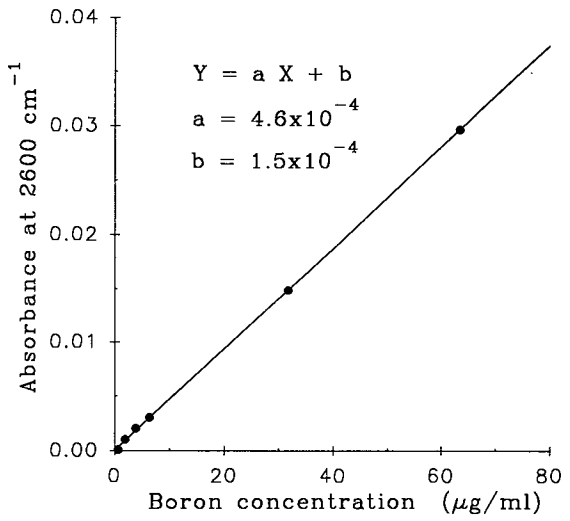

Fig. 3. Calibration curve for FTIR determination of DBA in CCl_4 (1-mm pathlength).

idue was reconstituted with 1 mL of fresh CCl_4 and examined in the microcell by FTIR.

Analysis of Boron in Liposome Formulations

Formulations containing the boron compounds DBM and PDB were prepared according to the following recipe:

Phosphatidylcholine (50 mg), dicetyl phosphate (10 mg), cholesterol (20 mg), and DBM (10 mg) or PDB (5 mg) were dissolved in 6 mL of methanol/chloroform (2:1). The organic solvent was removed using a rotary evaporator to form a lipid film on the 100-mL round-bottomed flask wall, and phosphate-buffered saline (5 mL) was added to initiate the spontaneous formation of bilayer lipid vesicles. The liposome suspension was extruded three times through a capillary-type membrane filter (Anapore, 0.1 μm).

For analysis, two extraction methods were used. (i) One-tenth milliliter of the liposome suspension was diluted with 0.9 mL of phosphate-buffered saline, then extracted with 2 mL of chloroform/methanol (1:1), the lower (organic) layer collected, and the upper layer subjected to two further extractions with 1 mL of chloroform. (ii) One-tenth milliliter of liposome suspension was dispersed directly in 2 mL of ethanol. In both cases, the organic phase was evaporated to dryness at 35°C under vacuum, and the residue dissolved in 1.0 mL CCl_4 and examined in the microcell by FTIR. Three aliquots of each liposome preparation were analyzed.

RESULTS AND DISCUSSION

The structures of the boron compounds used in this study, BSH, DBA, DBM, and PDB, are given in Fig. 1. The IR spectrum of DBA in CCl_4 (spectrum 3 in Fig. 2) displays the B–H absorption band at 2600 cm^{-1} which is characteristic of the polyhedral boron compounds and can be used for quantification. This is identical for all the carborane compounds, which contain 2 carbon atoms with 10 boron atoms in a neutral dodecahedron structure. For BSH, a dianion with 12 boron atoms, the absorption in aqueous solution occurs at 2500 cm^{-1}. For both types of compounds this band has a similar molar absorptivity.

Figure 3 shows the calibration curve for DBA in CCl_4 solution using a 1-mm pathlength. From the absorbance values of the major B–H stretching band, a straight line was found with a correlation coefficient (R^2) equal to 0.9987. The detection limit of boron for DBA (or other polyhedral boron compounds) in CCl_4 was estimated to be 1 ppm (at a signal/noise ratio of 2.5).

On the other hand, the use of water as a solvent results in a substantial masking of the B–H band of BSH because water is a strong absorber in the midinfrared region, and only a small amount of energy can pass through the sample. Quantification of boron compounds in aqueous solutions using FTIR requires the subtraction of the large background and the use of the short-pathlength cell. However, the high surface tension of water is an impediment to filling a short-pathlength cell. In this experiment, 50 μm was the optimal pathlength chosen, and each spectrum was baseline-corrected using the nonlinear baseline correction (Bio-Rad FTIR software) before quantitative measurement. The IR spectrum of BSH in water and plasma, after the baseline correction, displays the B–H absorption band at 2500 cm^{-1}. The relationship between the absorbance at 2500 cm^{-1} (50-μm pathlength) and the concentration of BSH in human plasma was linear, with the regression equation

$$y = 2.7*10^{-3} + 9.7*10^{-5}x$$

where x is the concentration of boron in ppm. This calibration was essentially the same as that obtained for BSH in simple aqueous solution. Note that the baseline correction procedure leads to a nonzero intercept even though a straight-line plot was obtained (R^2 = 0.9932). Integration of

Table I. Recovery of Boron Added as DBA to 5 mL of Water

Boron in water added (ppm)	Boron in CCl_4 expected (ppm)	Boron in CCl_4 found ± SD[a] (ppm)	Recovery of added boron ± CV (%)
94.0	470	465 ± 5.1	99.0 ± 1.1
18.8	94.0	93.9 ± 2.3	98.8 ± 2.4
9.40	47.0	46.5 ± 2.2	99.0 ± 4.6
1.88	9.40	8.7 ± 0.7	92.6 ± 7.3
0.94	4.70	4.6 ± 0.5	97.3 ± 10.8
0.38	1.88	1.4 ± 1.1	74.2 ± 58.7

[a] Standard deviation of three replicates.

Table II. Recovery of Boron Added as DBA to 5 mL of Phosphate-Buffered Saline (PBS), pH 7.4, Containing Human Serum Albumin (HSA)

Boron in PBS–BSA added (ppm)	Boron in CCl_4 expected (ppm)	Boron in CCl_4 found ± SD[a] (ppm)	Recovery of added boron ± CV (%)
82.9	415	400 ± 9.6	96.4 ± 2.3
16.6	82.9	81.5 ± 4.6	98.3 ± 5.6
8.29	41.5	40.4 ± 0.8	97.4 ± 2.0
1.66	8.29	7.8 ± 0.7	93.9 ± 8.0
0.83	4.15	3.9 ± 0.7	94.4 ± 18.2
0.33	1.66	1.3 ± 0.9	80.5 ± 56.0

[a] Standard deviation of three replicates

the area under the peak after baseline correction gave a similar result. The detection limit of boron for BSH in aqueous solution or plasma was estimated to be 5 ppm B (at a signal/noise ratio of 2.5), which was similar to that found in urine by Lu and Munro using baseline subtraction (6). Thus, in an NCT protocol involving BSH as the boron carrier, where the blood boron concentration would be of the order of 20 ppm, FTIR could be used to monitor that concentration in the blood of patients after administration of BSH prior to neutron irradiation. However, the precision of the assay for concentrations below 10 ppm would be improved if a lower boron detection limit could be achieved.

The limiting factor in the above assay stems from the interfering water absorption. For polyhedral boron compounds with adequate nonaqueous solubility, an alternative approach is to extract the boron compound into CCl_4 for FTIR measurement directly without baseline correction. DBA was used to test the efficiency of the extraction approach. Various amounts of DBA were added to (i) aqueous solutions, (ii) phosphate-buffered saline, pH 7.4, containing human serum albumin, and (iii) human plasma, and the aqueous phase was extracted three times with CCl_4. The extraction recovery was performed in triplicate for each concentration. The results for DBA in aqueous solution, in phosphate-buffered saline containing human serum albumin, and in human plasma using the microcell are shown in Tables I, II, and III, respectively.

The quantification limit for the assay varies according to the size of the aliquot of plasma taken and the volume of CCl_4 used to reconstitute the boron compound for the FTIR measurement. In the present experiment, 5 mL of plasma was used, and the final volume of CCl_4 necessary for the microcell was 1 mL. Thus, in the three aqueous matrices the quantification limit was estimated as 1 ppm, using the coefficient of variation of 10% as the upper limit of acceptable error. In the extraction of plasma with CCl_4, no protein precipitant (such as phosphotungstic acid) was used, thereby avoiding the possibility that DBA could be precipitated and trapped in the protein pellet. In all cases, good recovery of boron was recorded in the CCl_4 phase at levels above the detection limit.

The FTIR technique as described above can be extended to the assay of other polyhedral boron compounds with suitable solubility in organic solvents such as CCl_4. Where the compound is a ionizable weak acid or base, the nonionized fraction may be extracted quantitatively with CCl_4 after the appropriate adjustment of the pH of the aqueous phase.

Boron Compounds in Liposome Formulations

The negatively charged liposomes were formulated using phosphatidylcholine and dicetyl phosphate with the added boron compounds, DBM and PDB. The boron concentration was determined, after extraction, in CCl_4 by FTIR. The absorbance at 2600 cm^{-1} is the same for all carborane compounds, and the standard curve in Fig. 3 was verified as applicable to all. As pointed out by Pidgeon *et al.* (10), liposomal lipid content can also be determined by FTIR using the C–H absorptions in the region 2992 to 2764 cm^{-1}. They used a film of lipid deposited from chloroform solution onto a calcium fluoride plate, but this procedure necessitated the addition of a deuterated compound as internal standard because of the variable thickness of the film. We chose to

Table III. Recovery of Boron Added as DBA to 5 mL of Human Plasma

Boron in plasma added (ppm)	Boron in CCl_4 expected (ppm)	Boron in CCl_4 found ± SD[a] (ppm)	Recovery of added boron ± CV (%)
99.9	500	493 ± 13	98.6 ± 2.6
19.98	99.9	97.0 ± 7.9	97.0 ± 7.9
9.99	49.96	48.7 ± 1.5	97.6 ± 3.0
2.00	9.99	9.8 ± 0.9	98.6 ± 9.1
1.00	5.00	5.0 ± 0.7	99.7 ± 13.4
0.40	2.00	2.4 ± 0.8	120.5 ± 39.5

[a] Standard deviation of three replicates.

Table IV. Boron and Lipid Assay of Liposome Formulations

Liposome formulation No.	Boron compound	Lipid found in liposome extract ± SD[a] (mg/mL)	Boron found in liposome extract ± SD[a] (ppm)	% boron incorporated in liposomes
1	PDB	1.01 ± 0.36	48.3 ± 1.6	4.6
2	PDB	0.95 ± 0.18	50.9 ± 0.2	5.3
3	PDB	0.79 ± 0.40	28.5 ± 1.2	3.7
4	DBM	0.90 ± 0.06	43.5 ± 0.9	4.8

[a] Standard deviation of three replicates.

assay the lipids by direct measurement of the CCl_4 solution in the 1-mm-pathlength microcell, so that an internal standard was not required. Because the liposomes are formulated with defined lipid mixtures, a calibration curve can be constructed using varying amounts of the ingredients, phosphatidylcholine and dicetyl phosphate, mixed at the same ratio as in the liposome formulation. Figure 2 displays the IR spectrum of lipid (spectrum 1) and boron compound (spectrum 3) standards and the boron–liposome extract (spectrum 2) in CCl_4 showing bands due to B–H at 2600 cm^{-1} and C–H between 2850 and 2970 cm^{-1}. The calibration curve for this lipid mixture measured with a 1-mm pathlength using the peak absorbance at 2930 cm^{-1} *versus* the phosphatidylcholine content was linear, with the regression equation

$$y = 2.4*10^{-3} + 0.39x$$

where x is the lipid concentration as milligrams per milliliter ($R^2 = 0.9943$).

The measured values and the calculated boron concentrations per unit volume of sample suspension are presented in Table IV, as well as boron concentrations expressed as a percentage of the lipid content. Triplicate determinations were made for each liposome preparation, with variations generally within 6% when extraction into chloroform/methanol was used (liposome preparations 1 and 3). Potential sources of error in this method are variations in the extraction efficiency and incomplete water removal from the organic extract. The agreement was significantly better, at less than 2%, when the liposome sample was directly dispersed in ethanol before evaporation to dryness (liposome preparations 2 and 4). The FTIR method provides a ready means of determining the exact composition of formulations containing lipid components. This is a significant advantage since a variable proportion of all components is lost during the preparation of the liposomes.

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