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UMI
PHARMACOKINETICS, METABOLISM, AND DOSE OPTIMIZATION SIMULATION STUDIES OF SODIUM BOROCAPTATE FOR BORON NEUTRON CAPTURE THERAPY OF MALIGNANT GLIOMAS

DISSERTATION

Presented in Partial Fulfillment of the Requirement for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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**********

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ABSTRACT

Sodium mercaptoundecahydro-c/oso-dodecaborate (Na₂B₁₂H₁₁SH) referred to as sodium borocaptate, or BSH, is a drug that has been used clinically for boron neutron capture therapy (BNCT) of malignant brain tumors in Japan since 1967 and in Europe since 1998. BNCT is based on a nuclear capture reaction that occurs when boron-10, a stable isotope, is irradiated with low energy neutrons to produce high-energy radiation. BSH was administered as a one-hour intravenous infusion to brain tumor patients who were candidates for surgical excision of their tumors at three dose levels 26.5, 44.1, or 88.2 mg BSH/kg, and samples were collected for 120 hours. The disposition of boron, following administration of BSH, was consistent to a three-compartment open model with first-order elimination from the central compartment and was linear over the dose range used for the study. Total body plasma boron clearance was 14.37±3.51 mL/min and the harmonic mean terminal boron half-life was 77.79 hours. Based on compartmental pharmacokinetics, computer simulations were performed and suggested that a multiple BSH dose paradigm would not improve tumor boron uptake parameters relative to a single dose. Urinary metabolites of BSH were detected using electrospray ionization mass spectrometry (ESI-MS). Chemical structures of the BSH metabolites were proposed that were supported by the observed boronated ions detected during ESI-
MS scans of the patient urine and included: BSH sulfinic acid (BSOH), BSH sulfinic acid (BSO₂H), BSH disulfide (BSSB), BSH thiosulfinate (BSOSB), and a BSH-S-cysteine conjugate. A liquid chromatography-mass spectrometry (LC-MS) assay was developed for quantitation of BSH in biological fluids. Using this assay, preliminary BSH pharmacokinetic data was determined from a subset of the patients. BSH pharmacokinetics was consistent with a two-compartment open model with first-order elimination from the central compartment. The total body plasma BSH clearance was 95.65 ± 30.82 mL/min and the terminal half-life was 3.61 hours. Between 73 and 89% of the BSH dose was excreted unchanged in the urine within 24 hours. The boronated BSH metabolites residing in the body for the last three sampling days has yet to be identified.
This work is dedicated to my mother and father.
ACKNOWLEDGMENTS

I wish to thank my adviser, Dr. Alfred Staubus, for his financial and educational support during my graduate studies, and for his patience with me during the times in which we were preparing both manuscripts, and my dissertation.

I thank Dr. Robert Williams for serving on my dissertation committee, for showing an active interest in my scientific progress, and for providing me with the opportunity to be accepted to graduate school.

I thank Dr. Rolf Barth for his advice, patience, encouragement, and support during my graduate studies.

I thank Dr. Weilian Yang, Dianne Adams, and Joan Rotaru for assisting me with various experiments, and for performing the boron analysis and protein concentration determinations used in this dissertation.

I thank Dr. Werner Tjarks, Dr. Albert Soloway, and their laboratory staff for taking an interest in my research and for providing help and boron chemistry expertise.

I thank Dr. Kari Green-Church, Nan Kleinholz, Ben Jones, Dr. Johnnie Brown, and Robin Gates at the Campus Chemical Instrumentation Center Mass Spectrometry Facility. Without their patience, expertise, and willingness to try new methods, none of the mass spectrometry reported in the dissertation would have been possible, and I am forever grateful.
I thank Dr. Peter Swaan and Dr. Larry Robertson for serving on my dissertation committee.

I would like to thank Dr. Ferketich and Dr. Moeschberger for the statistical consultation and assistance that was provided for this study.

I would also like to thank Dr. Bernard Spielvogel from Boron Biologicals, Inc., for generously providing me with cesium salts of BSSB and BSOSB for use during my studies.

This work was partially supported by grant DE-FG02-95EF62059 from the United States Department of Energy.
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CHAPTER 1

INTRODUCTION

Significant advances have been made during the past 20 years toward the treatment of various cancers. Unfortunately, the treatments for cancers of the central nervous system (CNS) have not shared the same success. Dose limiting toxicities, the blood-brain barrier (BBB), and complex tumor biology, are some reasons for the lack of success of brain tumor chemotherapy. Because of the aggressive nature of these cancers, brain tumor research remains extremely important. Efforts are being focused on developing different treatment strategies to overcome the complications that have limited the success of regimes presently used to treat malignant brain tumors.

GLIOBLASTOMA MULTIFORME AND ANAPLASTIC ASTROCYTOMA

Primary CNS tumors are the third leading cause of cancer death in men under the age of 55, and the fourth leading cause of cancer death in women under the age of 35 (1). Malignant gliomas are characterized as being highly infiltrative, having a low propensity to metastasize, and possessing rapid growth capacity with tumor mass doubling times of only a few weeks (2). The prognosis for patients treated with surgical resection and current chemo-radiotherapy protocols for glioblastoma multiforme (GBM), a high-grade
glioma, rarely exceeds 12 months from the time of initial diagnosis (3). High-grade gliomas, such as GBM and anaplastic astrocytoma (AA), exhibit similar histopathologic features. Both are characterized by cytoplasmic and nuclear pleomorphism (dense, hyperchromatic nuclei), varying degrees of anaplasia, and multi-nucleated giant cells (2). GBM is histopathologically differentiated from AA by the presence of necrosis, exuberant endothelial/pericytic infiltration, high cellularity and vascular proliferation (2). Clinical symptoms of GBM include change in personality, emotional lability, epilepsy, hallucinations, and headaches (2).

CURRENT TREATMENT FOR MALIGNANT GLIOMAS

Currently, the chemotherapeutic agent used most frequently for treating malignant brain tumors is Carmustine (BCNU). BCNU is a nitrosourea compound that produces a cytotoxic response by forming both DNA cross-links, and carbamoylation of cellular amino groups (1). A variety of other chemotherapeutic agents (Table 1.1.) have been studied for treatment of malignant brain tumors although none of the compounds listed in Table 1.1. have proven more efficacious than BCNU (2). The standard brain tumor treatment protocol using surgery, BCNU, and radiotherapy is as follows (2): BCNU (80 mg/m²) is administered as a daily 20 minute infusion for 1-3 days, starting about 14 days after surgical tumor resection. Radiotherapy is commenced simultaneously with BCNU administration. Additional treatment cycles may be given in six to eight week intervals depending on individual dose tolerance.
Dose limiting toxicities of BCNU are hematologic suppression, interstitial pneumonia with extensive fibrosis, seizures and thrombosis (2), all of which may result in lethality.

The above surgery-BCNU-radiation treatment regime results in a median survival of 52 weeks, 5-20% patient survival at 18 months and 4% at five years after initiation of treatment (2). Unfortunately, one-third of the patients treated with the BCNU-radiation protocol have no statistically significant clinical improvement of their brain tumors (2).

**BINARY TREATMENT MODALITIES**

The quest for the ability to selectively destroy malignant cells while simultaneously sparing normal contiguous cells has led to the development of binary systems. Binary treatment systems involve the use of two components such that each component is relatively innocuous yet when combined, yield a potent cytoidal effect. The rationale is that each component of the binary system can be manipulated individually to maximize selectivity to the tumor. There are several examples of binary treatments being investigated for clinical efficacy. Radiation sensitizers are compounds that are delivered to tumors that will cause the tumor to be more susceptible to damage from ionizing radiation (4,5). Photodynamic therapy involves the activation of cytotoxic compounds by light (6). Transfecting genes to tumor cells to increase their sensitivity to chemotherapeutic agents is also considered to be a binary treatment system (7,8). The binary system that will be the focus of this study is boron neutron capture therapy (BNCT), and is explained in detail below (9,10).
**RATIONALE FOR USING BINARY TREATMENT (BNCT) FOR MALIGNANT GLIOMAS**

Because AA and GBM have a low propensity to metastasize to other organ sites, the possibility of an increase in tumor-free survival becomes realistic if local tumor eradication can be achieved. Surgical removal of the solid tumor mass may leave behind areas of the tumor which are inaccessible by surgery and/or undetected areas of microscopic tumor infiltration, which could become the foci for disease re-occurrence. Therefore, a post-surgical treatment of the operative site with a binary system could be a means of destroying residual infiltrating and inoperable tumor areas and should be an efficacious treatment for such non-metastasizing cancers.

**BORON NEUTRON CAPTURE THERAPY**

BNCT is a binary treatment modality that involves delivery of boron-10, a stable isotope, to the tumor and subsequent irradiation with a beam of low energy (thermal) neutrons that cause the boron-10 nuclei to undergo a nuclear reaction. This nuclear reaction yields high linear energy (LET) \(^4\)He (\(\alpha\)-particles) and recoiling lithium-7 nuclei (Fig 1.1.). Neither the delivery of boron-10 compounds clinically available, nor irradiation with thermal neutrons are by themselves sufficient to cause tissue damage. The ability of BNCT to destroy malignant cells arises from the \(\alpha\)-particles and lithium-7 nuclei that are formed from the capture reaction. High LET radiation, such as \(\alpha\)-particles, are characterized by high energy transfer per unit path length and therefore,
have a range of <10 μm in human tissue effectively limiting the cytocidal effect to the cell containing the boron-10 which produced the capture reaction (11). This characteristic of high LET radiation provides BNCT the selectivity to destroy boron-10 containing cells while simultaneously sparing cells that do not contain boron-10. High LET radiation is equally toxic to hypoxic, oxygenated or quiescent cells.

\[ ^{10}\text{B} + ^{1}\text{n} \rightarrow ^{4}\text{He} + ^{7}\text{Li} \ (6\%) \]
\[ \downarrow \]
\[ ^{4}\text{He} + ^{7}\text{Li} + \gamma \ (94\%) \]

Figure 1.1. Diagram of boron neutron capture reaction.

EARLY CLINICAL TRIALS OF BNCT

During the late 50's and early 60's, several boron-containing compounds were evaluated in animal models for their biological suitability for BNCT. Two compounds were found, among the many that were screened, to possess favorable tumor localizing properties. These were p-carboxy-benzeneboronic acid and sodium decahydrodecaborate (Na₂B₁₀H₁₀). These two compounds were found to be nontoxic, and gave tumor:brain ratios of 5-8 which persisted for several hours (12,13). Boron-10 enriched analogs of these two compounds were synthesized and evaluated in clinical trials for BNCT of
malignant brain tumors at Massachusetts General Hospital and the Massachusetts Institute of Technology. Although the patient's tumors were destroyed, the clinical trials were failures due to the severe and often lethal radiation damage to cerebral vascular endothelium (14-16). The vascular damage was attributed to the presence of high blood boron concentrations during the initiation of BNCT (10).

**CRITERIA OF BORON DELIVERY AGENTS FOR BNCT**

Soloway et al. (10) states that the success of a boron delivery agent for BNCT is dependant on four clearly defined parameters:

1. Having the tumor:normal tissue boron concentration ratio greater than 1.
2. The boron delivery agent must have sufficiently low toxicity such that doses can be given to achieve clinically useful boron levels.
3. The tumor:blood boron ratio must be sufficiently high during BNCT.
4. Achieving tumor boron-10 concentrations in the range of 20-35 μg/g.

The importance of these parameters can be explained by examining the radiological and biological processes that occur during BNCT. Having the tumor:normal tissue and tumor:blood boron concentration ratios greater than one during BNCT are essential to limit toxicity. If blood and normal tissue adjacent to the treatment site contain significant levels of boron during BNCT, they will be irradiated with thermal
neutrons and thus, subjected to lethal high LET radiation produced from the capture reaction. The result will be that normal contiguous tissue and associated blood vessels will be irreparably damaged. Such toxicity occurred as a result of poor tumor selectivity during early clinical trials of BNCT for brain tumors in the late 1950s and early 1960's as is stated above (14).

A minimum number of boron atoms (20-35 μg of boron-10 per gram of tumor) must be delivered to the tumor (17,18), and a sufficient dose of thermal neutrons must be absorbed by the boron-10 nuclei in order to sustain the lethal capture reaction necessary to eradicate the tumor (9,10,19). Theoretically, only a few α-particles are necessary to kill a cell although probability and statistical considerations require that many are needed to exert a reliable cytotoxic effect; thus, the requirement for large numbers of boron-10 atoms per cell. Consequently, a boron delivery agent must have sufficiently low toxicity such that doses can be given that will result in therapeutically useful boron levels in the tumor at the time of BNCT.

**SODIUM BOROCAPTATE**

Sodium borocaptate (Na₂B₁₂H₁₁SH) or BSH (Fig. 1.2.) is a boron compound that posses rather peculiar physico-chemical properties. The boron cage, or polyhedral borane, portion of the BSH molecule (B₁₂H₁₁)²⁻ has extreme thermal stability and was recovered unchanged at temperatures of up to 810°C (20). From the molecular formula, it can be deduced that BSH is a di-sodium salt. The free acid hydrated form of the polyhedral borane portion of the molecule (H₂B₁₂H₁₁), is an extremely strong acid,
having pKa values comparable to sulfuric acid (20,21). BSH is freely soluble in water and despite it being a di-sodium salt, is also freely soluble in organic solvents such as acetonitrile. The boron cage acts as a strong electrolyte, but simultaneously possess hydrophobic properties comparable to organic aromatic molecules (21). The combination of these properties makes isolation of boron cage containing molecules, such as BSH, impossible by conventional separation methods (21).

BSH was first shown to have tumor localizing properties in mice bearing subcutaneously planted ependymoblastomas by Soloway et al. (22) in 1967. That same year, Dr. Hatanaka started a clinical trial in Japan for BNCT of malignant brain tumors using BSH as a boron-10 delivery agent.

The results from Dr. Hatanaka’s work on BNCT have generated considerable critical regard, but nonetheless had yielded some promising results. In the time period from 1968 to 1997, 146 patients with malignant brain tumors had been treated with BNCT using BSH. In general, the surgical and BNCT protocol used by Dr. Hatanaka was as follows (23): Patients underwent a craniotomy and surgical tumor excision to remove as much of the tumor as was feasible. Then, at various times thereafter, BSH was administered using a 1-2 hour intravenous or intracarotid infusion. The surgical site from which the tumor had been resected was irradiated using a neutron beam at varying times after BSH administration.

The five-year survival rate for the patients treated by Dr. Hatanaka was 25.7% (23). In a subset of 38 patients with high-grade gliomas (GBM and AA), the reported mean survival time was 44 months (24). Among this group of glioma patients was a pair of long-term (>10 years) survivors whom had been diagnosed with glioblastoma
multiforme (24). Both of these patients had no reduction in the quality of life and were able to resume their careers post-treatment (24). Considering the above stated prognosis for high-grade gliomas, these results are quite remarkable. Also, there was also no report of radiation injury to normal brain with the exception of one patient who received an exceptional dose of neutrons during BNCT (24).

COMMENTS ON EARLY CLINICAL TRIALS OF BSH

The results from Dr. Hatanaka’s BNCT clinical trial using BSH have been criticized for a number of reasons:

1. A number of patients treated by BNCT had received prior radiotherapy for their tumors (9).
2. The time interval between surgery, administration of BSH and BNCT were varied (9).
3. There was a lack of patient randomization (9).
4. There was uncertainty in the true histopathology of the resected tumors (9).
5. BSH was administered using multiple routes.
6. The neutron beam used for patient irradiation had poor tissue penetration ability (9).
7. There was no knowledge concerning the metabolic fate of BSH.
The first five points above can be explained as a lack of experimental standardization in the studies reported by Dr. Hatanaka (23,24). A number of patients had received treatment for their tumors prior to being treated by BNCT (24). This makes forming conclusions about the efficacy of BNCT difficult since the observed clinical outcome may not be the result of the experimental variable, but may be a result of previous treatment, or the combination of treatments. The time interval between tumor resection, drug administration, and BNCT were varied (9) in a manner such that it was difficult to form any conclusion about the effect that each parameter may have had on the clinical outcome. Lack of patient randomization was also noted (9) which would lead to statistical complications when attempting to support a hypothesis. The tumors, which were resected from treated patients, were of multiple histopathological tumor types and the consistency of their diagnosis has been questioned (9). BSH was administered to patients using either intravenous or intraarterial infusion (23,24), which further complicates interpretation of the clinical results by adding extra experimental variables.

The most important criticism of Dr. Hatanaka’s work concerns the neutron beam that was used. An overwhelming majority of Dr. Hatanka’s patients were irradiated at the Musashi Institute of Technology reactor. This reactor has a very low neutron flux and required 3-5 hours of irradiation time for the patient to receive an adequate dose of neutrons (9). Furthermore, the depth of penetration for this neutron beam is no more than 6 cm (9). Thus, effective BNCT was limited to superficial tumors as opposed to deeply penetrated tumors. Both of these factors certainly contribute to reducing the potential efficacy of BNCT.
Although BSH has been used in humans for the past thirty years, little is known concerning its biological fate. It has been well established that BSH can readily oxidize to two dimeric forms in aqueous, oxygenated media (10) (Fig 1.2.). To date, all of the human pharmacokinetic and biodistribution reports for BSH have been from data where the boron concentration of various biological matrices was determined after administration of BSH (19,23,25-29). Analytical methods used to determine boron concentration are unable to distinguish parent drug (BSH) from any boronated metabolites and/or oxidation products that may have formed in vivo.

**REASON FOR STUDY**

Because of the above stated deficiencies in the BNCT clinical studies using BSH, it was necessary to perform a phase I clinical trial with a pharmacokinetic component. The specific goals of the pharmacokinetic study that will be reported here are as follows:

- Characterize boron pharmacokinetics in brain tumor patients under controlled conditions.
- Perform a detailed analysis of boron renal plasma clearance.
- Assure accurate diagnosis of resected tumors so that detailed analysis of boron biodistribution, relative to tissue type, can be established.
- Provide a scientific rationale for choosing a BSH dosing paradigm to maximize efficacy of BNCT.
• Develop a sensitive analytical method specific for BSH and identify possible biotransformation products.

Characterizing the boron pharmacokinetics, including detailed analysis of renal plasma boron clearance, following administration of BSH to brain tumor patients is necessary to assess the potential efficacy of BNCT, as well as for optimizing boron delivery to the tumor using BSH. Accurate and consistent tumor diagnosis is essential to evaluate the effect of tumor histopathology on the uptake of boron following BSH administration. Accurate histopathologic diagnosis is also critical in determining a BSH dosing paradigm to optimize BNCT for specific tumor types. A reliable and sensitive assay for quantifying BSH in biological matrices is essential to determine the pharmacokinetic behavior of BSH. Characterization of the biological fate of BSH may lead to identification of the tumor selective boronated species as well as providing information for potential molecular improvements to maximize tumor boron delivery.
<table>
<thead>
<tr>
<th>Carmustine (BCNU)</th>
<th>Teniposide</th>
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<tr>
<td>Procarbazine</td>
<td>Topotecan</td>
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<td>Cisplatin</td>
<td>Camptothecin</td>
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<td>Carboplatin</td>
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<td>Nitrogen Mustard</td>
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<td>Thiotepa</td>
<td>Thioguanine</td>
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<td>Bleomycin</td>
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<td>Taxol</td>
<td>Suramin</td>
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<td>Vincristine</td>
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<td>Mitoguazone</td>
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<tr>
<td>Etoposide</td>
<td>Streptozocin</td>
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Table 1.1. Summary of agents used for chemotherapy of malignant brain tumors (14).
Figure 1.2. Reaction scheme showing oxidation products formed from aqueous BSH solutions.
BIBLIOGRAPHY


CHAPTER 2

BORON PHARMACOKINETICS FOLLOWING INTRAVENOUS ADMINISTRATION OF BSH TO BRAIN TUMOR PATIENTS

INTRODUCTION

Boron disposition following intravenous infusion of BSH was characterized as part of a phase I clinical trial. The following chapter describes the methods and results of the boron pharmacokinetic study.

METHODS

PATIENT SELECTION

Twenty-five patients (10 males and 15 non-pregnant females) with pre-operative diagnoses of either GBM or AA, who had no previous radiation or chemotherapy treatment for their tumors, were admitted to the study. Pre-operative diagnoses were based on clinical histories, neurological examinations, axial computed tomographic scans and magnetic resonance imaging scans. Three of the patients were treated at The Ohio...
State University Medical Center and twenty-two patients at the Beijing Neurosurgical Institute (BNI). Patients who tested positive for hepatitis B antibodies, human immunodeficiency virus, or who had Karnofsky Performance Scale, a measure of a patients overall ability to perform certain physical activities, scores less than 80 were excluded in order to optimize the uniformity of the study population.

**CLINICAL PATIENT MONITORING**

Although there have been no reports of toxicity resulting from BSH at the doses used for this study, clinical data of renal, hepatic, and hematopoietic functions were monitored before infusion of BSH and 12, 24, 36, 48, and 72 hours and 7 days after administration. Clinical data that was collected during the study included the following:

- Electrocardiographic monitoring
- Blood urea nitrogen
- Creatinine
- Bilirubin
- Alkaline phosphatase
- Aspartate aminotransferase
- Alanine aminotransferase
- Hemoglobin
- Hematocrit
- White blood cell count
- Platelet count
SODIUM BOROCAPTATE

BSH was purchased as a drug substance from Centronic, Ltd. (Croydon, UK). The drug substance was converted to a drug product at the Pharmaceutical Service Division, University of Iowa (Iowa City, IA), by dissolving the BSH in triple-distilled water, after which it was sterile filtered, then lyophilized and dispensed in sterile vials. The vials were flushed with nitrogen to minimize the oxidation of BSH to its dimeric forms, BSSB (B\textsubscript{12}H\textsubscript{11}SSB\textsubscript{12}H\textsubscript{11}) and BSOSB (B\textsubscript{12}H\textsubscript{11}SOSB\textsubscript{12}H\textsubscript{11}) (Fig. 1.2.).

Chemical analysis on the drug substance for the presence of oxidized dimers was performed at the Idaho National Engineering and Environmental Laboratory (INEEL) using an isocratic high-performance liquid chromatography (HPLC) assay (1). Injections of 10 \( \mu \)L to 50 \( \mu \)L were made onto a 250 x 8 x 3 mm column, packed with 5 \( \mu \)m particles of Nucleosil C18 resin (Machery-Nagel, River Vale, NJ). The mobile phase was a 1:1 solution of methanol: aqueous 5 mM tetrabutylammonium sulfate adjusted to pH of 8.1 with a flow rate of 0.5 mL/min. Detection was performed using a photodiode array detector at 205, 215 and 225 nm. The oxidation products, BSSB and BSOSB, represented 0.24% of the total drug composition as determined by the HPLC assay (1). Although this HPLC assay does provide chromatographic resolution of BSH, BSOSB and BSSB, it is not sensitive enough to detect these molecules at physiologically relevant concentrations.

Headspace gas chromatography was used to measure the residual oxygen content inside the sealed drug vials at INEEL. Manual injections were made onto a Chrompack
PLOT, fused silica, 25 m x 0.53 mm column, coated with MolSieve (5 Å) (Chrompack, Inc., Raritan, NJ), with a film thickness of 50 μm, which was equilibrated at 40°C. Helium was used as the mobile phase and the chromatograms were monitored by thermal conductivity detection. The oxygen content remained stable in the range of 4 to 5% through the duration of the study (1).

**BSH ADMINISTRATION**

The amount of drug administered to each patient (15, 25 or 50 mg boron/kg corresponding to 26.5, 44.1 or 88.2 mg BSH/kg) was determined according to their body weight. Three patients were in the 15 mg boron/kg dose group, three in the 25 mg boron/kg dose group, and 19 in the 50 mg boron/kg dose group. The appropriate number of vials (each containing 457.7 mg of lyophilized BSH) was reconstituted with 19.55 mL of sterile water and the required amount of BSH was added to a Viaflex plastic bag and diluted to a final volume of 500 mL with sterile normal saline. The 500 mL solution was then infused intravenously during a one-hour period.

**SURGICAL TUMOR RESECTION**

A craniotomy was performed within 2 to 14 hours following termination of the BSH infusion for the purpose of tumor resection and collection of tissue samples. During the resection, multiple samples of solid tumor, infiltrating tumor, and adjacent normal brain were collected. The collected tissue samples were then sectioned and processed as
pairs, one section for histopathological examination and the other for boron concentration
determination. Tissue samples were examined histologically and the percent solid tumor,
infiltrating tumor, normal brain, hemorrhagic and necrotic areas were estimated. The
portion of this study relating to tumor boron pharmacokinetics will be discussed in detail
in Chapter 6.

**BORON ANALYSIS BY DIRECT CURRENT PLASMA – ATOMIC EMISSION SPECTROSCOPY**

Boron content of collected tissue, urine and plasma samples was determined using
a Spectraspan VB direct current plasma-atomic emission spectrometer (DCP-AES)
(Applied Research Laboratories, Brea, CA) at The Ohio State University Department of
Pathology and has been described in detail (2). This method required digestion of tissue
samples in a concentrated sulfuric acid – 70% hydrogen peroxide cocktail, which was
able to digest samples without the need for high pressures or temperatures (2). The DCP-
AES method was validated in a number of biological matrices including, blood, plasma,
tumor tissue, liver, skin and cell suspensions (2). The plasma source was argon gas
heated to a temperature of 6000-7000K. The monitored wavelength was 249.773 nm and
the argon nebulizer gas flow was 7 L/min (2). This method was shown to be applicable
to a variety of boron containing compounds, including BSH, with a precision of <±5%
deviation from the mean (2). The lower limit of detection for this method was 0.5 μg
boron per gram of tissue (2).
BLOOD AND URINE SAMPLING SCHEDULES

Blood samples (12 mL aliquots) were collected into heparinized tubes before the infusion, and at 15 and 30 minutes, and 1, 2, 4, 7, 13, 24, 48, 72, 96, and 120 hours after the start of the infusion of BSH. The blood samples were subsequently centrifuged and the plasma fraction was recovered for boron analysis using the DCP-AES method, as described above. Pooled urine samples were collected before the infusion and at 0-3, 3-6, 6-9, 9-12, 12-24, 24-48, 48-72, 72-96, and 96-120 hours after start of the BSH infusion. The urine samples were also analyzed for boron content using the DCP-AES method. The plasma boron concentration-time data and urinary boron concentration-time data were used for all subsequent boron pharmacokinetic studies.

PHARMACOKINETIC ANALYSIS

Previous studies, as well as this one, have characterized BSH pharmacokinetics by monitoring boron concentrations in tissues, blood/plasma, and urine. Therefore, the reported boron pharmacokinetics correspond to the hybrid of BSH pharmacokinetics, as well those of the boronated metabolites and/or oxidation products that are discussed in Chapter 4.

For the purpose of model selection, the geometric mean plasma boron concentration-time profile for the 50 mg boron/kg dose group (n=17) (corresponding to 88.2 mg BSH/kg) was used, along with the corresponding scheduled sampling times. (Table 2.1.) A three-compartment open model system with zero-order input and first-
order elimination from the central compartment (Fig. 2.1.), and the analogous two-compartment open model system were evaluated as potential models for boron disposition. The proposed models were fit to the geometric mean plasma boron concentration-time profile using WinNonlin (Pharsight Corporation, Mountain View, CA). 60-minute infusions of 3500 mg boron (50 mg boron/kg x 70 kg) were used as input parameters for both the two, and three-compartment model fits of the geometric mean data. Distribution of residuals, F-test and Akaike Information Criterion (AIC) were used as statistical indicators to justify model selection (3,4).

Following model selection, each patient’s (n=23) plasma boron concentration-time profile was individually used to fit a three-compartment pharmacokinetic model [Eq. (2.1.)] using WinNonlin computer software. Patients #8 and #22, both in the 50 mg boron/kg dose group, could not be analyzed due to incomplete blood sampling, and therefore were removed from the pharmacokinetic study. The exact infusion time (τ), and blood sampling times, as opposed to scheduled infusion and sampling times, were calculated for each patient to properly evaluate the boron pharmacokinetics following administration of BSH. Patient boron concentration-time data was analyzed using a weighting factor of $1/y^2$. 
Equation 2.1.

\[ C_p = A(e^{-\alpha t'} - e^{-\alpha t}) + B(e^{-\beta t'} - e^{-\beta t}) + C(e^{-\gamma t'} - e^{-\gamma t}) \]

Where: 
- \( A, B \) and \( C \) = exponential coefficients for fast, intermediate and terminal disposition phases, respectively.
- \( \alpha \) = macroscopic first-order fast disposition rate constant.
- \( \beta \) = macroscopic first-order intermediate disposition rate constant.
- \( \gamma \) = macroscopic first-order terminal disposition rate constant.
- \( t \) = time from start of the infusion
- \( t' \) = time interval from end of the infusion (\( t' = t - \tau \))

In order to determine if there were saturable kinetics over the dose range that was used for the study, median values for total body plasma clearance (\( C_{lt} \)) and the terminal disposition half-life (\( \gamma \)), two dose-sensitive pharmacokinetic parameters, were examined by the Kruskal-Wallis test, a nonparametric equivalent to a one-way analysis of variance (5). The sample size in the 15 (\( n=3 \)) and 25 mg boron/kg groups (\( n=3 \)), relative to the 50 mg boron/kg group (\( n=17 \)), was too small to assess the normality assumption, which would have been required for a one-way ANOVA statistical model.
RESULTS

PHARMACOKINETIC MODEL SELECTION

To justify model selection, comparative analyses were made between the three-compartment open model and the analogous two-compartment fits to the geometric mean plasma boron concentration-time profile for the dose of BSH corresponding to 50 mg boron/kg. Figures 2.2. and 2.3. show semi-logarithmic plots of the fits of the geometric mean boron concentration-time data to the two and three-compartment models, respectively. Examination of the plot of the weighted residuals for Y (plasma concentration) versus X (minutes) for the two-compartment fit (Fig. 2.4.), reveals a non-random, systematic distribution of weighted residuals, relative to the same plot for the three-compartment model fit (Fig. 2.5.). The equation for calculating the weighted residual, assuming that a weighting scheme of $1/y^2$ was used to fit the data, is shown in Eq. (2.2.).

\[
\text{(weighted residual)} = \left[ \frac{(\text{observed value})}{(\text{theoretical value})} \right] \times \left[ \frac{1}{(\text{observed value})^2} \right]^{1/2}
\]

As can be seen [Eq. (2.2.)] the weighted residual is a measure of the deviation of the observed plasma concentrations from the theoretical predicted plasma concentration and
can be positive or negative, depending on the value of the predicted point relative to the observed point. A concentration-time profile, which has been properly fitted, will have a random scatter of observed data points around the predicted curve. Systematic deviations of the data from the theoretical curve, such as the ones in Fig. 2.4., suggest an inappropriate model selection (3).

The AIC was also examined to provide data for model selection. As shown in Eq. (2.3.), the AIC is a term that evaluates the number of sampling points, the number of estimated parameters, and the sum of squared residuals from competing models to aid in appropriate model selection (4,6). The fit producing the lowest AIC value is, according to this test, the proper model (4,6). The AIC value derived from the two-compartment model fit was 47.48072, and the AIC value from the three-compartment model fit was 28.42711.

Equation 2.3.

\[ \text{AIC} = N \ln(\text{SS}) + 2p \]

Where: 
- \( N \) = number of sampling points
- \( \text{SS} \) = sum of squared residuals
- \( p \) = number of estimated parameters

An F-test was performed on the weighted sum of squared residuals (WSSR) for the two proposed models [Eq. (2.4.)] (3,6). The calculated value of \( F (F_{\text{calc}}) \) for the two proposed model fits was 17.83. The critical value (\( F_{\text{crit}} \)), at \( p=0.05 \), for a two-tailed F test was 8.43 (5). The null hypothesis for the F test is that the simpler model, the model with the fewest number of parameters, is the correct model (3,6).
Since $F_{\text{calc}}>F_{\text{crit}}$, the null hypothesis was rejected on statistical grounds ($p=0.05$). Hence, based on the F test, the two-compartment model was inadequate to characterize the boron disposition.

Equation 2.4.

$$F = \left( \frac{SS_j - SS_k}{SS_k} \right) \left( \frac{df_k}{df_j - df_k} \right)$$

Where: $df_n$ = degrees of freedom for models k and j; and $df_j > df_k$

$SS_n$ = sum of squared residuals for model k and j

The results from all three of these tests, the distribution of residuals, AIC, and the F test, unanimously support the selection of a three-compartment model to describe boron disposition. There are certain scenarios where the results from these tests may conflict, and the strengths and weaknesses of each test should be considered when selecting a pharmacokinetic model (6). Since all three tests arrive at the same conclusion, these arguments do not need to be considered. Due to the results of these tests, the three-compartment model was validated as being the minimum number of compartments necessary to adequately characterize boron disposition following administration of BSH.

**BORON PHARMACOKINETICS FOLLOWING INTRAVENOUS INFUSION OF BSH**

Each patient's boron plasma concentration-time profile was used to fit the classical three-compartment open model system with zero-order input and first-order elimination from the central compartment (Fig. 2.1.). Plasma concentration-time data
from 23 patients were used to fit the pharmacokinetic model, data from two patients were not included due to incomplete sampling. Three patients were in the 15 mg boron/kg group, three in the 25 mg boron/kg group and 17 in the 50 mg boron/kg dose group. Semi-logarithmic plots of the fitted patient boron plasma concentration-time profiles are shown individually in Fig. 2.6. through Fig. 2.28. The resultant pharmacokinetic parameters are statistically summarized in Table 2.2.

Median values for total body plasma boron clearance (ClT) and the terminal boron disposition half-life (γ), two dose sensitive pharmacokinetic parameters, were examined by the Kruskal-Wallis test, a nonparametric equivalent to a one-way analysis of variance (5), in order to determine if there were saturable kinetics over the dose range that was studied. There was no statistical evidence to claim that there was a difference in the values of total body plasma boron clearance and terminal boron disposition half-lives over the three doses administered during the study. It was concluded, based on this statistical data, that boron disposition after BSH administration was linear over the boron dose range 15 to 50 mg/kg and, therefore, pharmacokinetic parameters obtained for each dose group were appropriately averaged together in Table 2.2.

The mean total body plasma boron clearance was 14.37±3.51 mL/min. The disposition half-lives reported in Table 2.2. are harmonic mean values (7). The harmonic mean half-lives for all three doses were 0.58, 6.45 and 77.79 hours for the α, β and γ phases, respectively.
The plasma: blood boron concentration ratio was measured in 16 randomly selected, paired plasma and blood samples and was calculated to be $1.29 \pm 0.22$ (mean±SD) (Table 2.3.). This indicated that boronated species in the blood apparently had preferential binding to plasma proteins rather than erythrocytes. Using this ratio, the corresponding blood clearance and volumes of distribution were calculated. The total body blood boron clearance was $18.14 \pm 4.53$ mL/min and the corresponding blood values for $V_1$ and $V_{dss}$ were $10.00 \pm 3.04$ L and $83.44 \pm 27.09$ L, respectively.

**DISCUSSION**

The pharmacokinetic parameters reported here, as well as those previously reported in the literature (1,8-12), characterize boron disposition following administration of BSH, *not* the disposition of BSH. The assays that have been used to determine boron concentrations cannot distinguish between the parent drug (BSH) and any boronated metabolites and/or oxidation products that may have been formed *in vivo*. Although this is the case, it should be noted that the success of BNCT is not dependent upon the chemical form of the boron, but rather the absolute amount of boron in the tumor and the corresponding tumor: normal brain and tumor: blood concentration ratios.
It is also critical to realize that boron pharmacokinetic analysis, such as the one described in this Chapter, will remain essential for evaluating the potential success of any boron delivery system used for the purpose of BNCT.

Boron disposition following i.v. infusion of BSH was fit to a classical three-compartment open model system with zero-order input and first-order elimination from the central compartment. There was no statistically detectable Michaelis-Menten (saturable) behavior over the boron dose range of 15 to 50 mg/kg, indicating linear disposition. The harmonic mean terminal boron disposition half-life ($\gamma$) was 77.79 hours, and the mean total body plasma boron clearance was 14.37±3.51 mL/min. Using the empirically determined plasma:blood boron concentration ratio of 1.29, total body blood boron clearance was calculated as 18.54±4.53 mL/min.

Several reports have appeared in the literature, using various methods and study designs, for determining the disposition of boron following administration of BSH (8-12). The pharmacokinetic parameters from this study are in reasonable agreement with those reported by Stragliotto et al. (9) in which blood was sampled for seven days and boron blood concentration-time data were fitted to a three-compartment model. They (9) reported a mean terminal disposition half-life ($\gamma$) of 66.2 hours and total body blood clearance of 14.7±7.0 mL/min. A pharmacokinetic report by Horn et al. (8) fitted both two and three compartment models to patient boron concentration-time data. Some patient's data were used to fit a two-compartment model and others were used to fit a three-compartment model (8). A report by Gabel et al. (12) fitted a two-compartment model to patient boron concentration-time profiles and reported a mean total body blood clearance of 32.8±5.2 mL/min, a value approximately twice that of both the calculated
total body blood clearance from this study, and the reported value for total body blood
clearance reported by Stragliotto et al. (9). This discrepancy, in part, may have been due
to the fact that blood sampling did not continue for 120 hours (12). Therefore, according
to our pharmacokinetic analysis, the AUC calculated by Gabel et al. (12) would have
been underestimated and consequently, their apparent total body clearance would have
been overestimated ($\text{Cl}_T = \text{Dose}/\text{AUC}$). Underestimation of the terminal half-life caused
by insufficient blood sampling was also apparent in the data of Haselsberger et al. (11),
who collected samples for 96 hours and fitted a two-compartment model to patient boron
concentration-time data and calculated a mean terminal half-life of 16.77±7.31 hours, a
value approximately 4.5 times less than the value determined from this study. Kageji et
al. (10) also reported pharmacokinetic parameters from studies in which blood was
sampled for three to four days, which according to this pharmacokinetic study, was
insufficient to properly characterize boron disposition following administration of BSH.
Kageji et al. (10) fitted a bolus two-compartment model to patient concentration-time
data even though BSH was administered by a one-hour i.v. infusion, which would have
resulted in an inaccurate estimate of the AUC and would have further complicated
calculation of AUC dependent pharmacokinetic parameters. Based on our
pharmacokinetic study, we conclude that a three-compartment model is necessary to
describe boron disposition following administration of BSH and that accurate blood
sampling should be carried out for at least 120 hours to adequately characterize the
terminal disposition phase and AUC.

Mehta and Lu (13) attempted to generate an allometric pharmacokinetic model for
extrapolating total body plasma boron clearance and Vdss from various animal models to
humans. The allometric equation [Eq. (2.4.)] has been used to extrapolate pharmacokinetic parameters, as a function of body weight, across various experimental animal models for prediction in humans (14,15). The allometric model was based on data collected from mouse, rat, rabbit and human studies (13).

Equation 2.4.

\[ Y = aW^b \]

Where:  
Y = scalable parameter  
W = body weight  
a = allometric coefficient  
b = allometric exponent

The parameters \( a \) and \( b \), in the allometric model, are determined by regression analysis \((\ln Y = \ln a + b\ln W)\). Mehta and Lu reported 0.127 and 1.577 as the values for \( a \), and 0.68 and 0.87 as the values for \( b \), for scaling Cl\( T \) and Vdss, respectively. Using their allometric model (13) and the average weight of patient in this study (63.4 kg), the predicted mean total body plasma boron clearance was calculated to be 35.57 mL/min and the calculated predicted value for Vdss was 57.56 L. The extrapolated total body plasma clearance was approximately 2.5 fold higher than the value reported from this study, but the extrapolated value for Vdss was reasonably close to the value of 64.68±21.00 L reported from this study.
This discrepancy in total body clearance may have been due to: 1. quantitative and qualitative differences in the metabolic profiles for BSH among the various test species and 2. the consequence of possible underestimation of the AUC values, due to inappropriate sampling or model selection, on the clearance values from the underlying animal and human studies that were used to generate the allometric model.

It has been suggested, by a number of investigators (9,12,16,17,18), that in vivo, BSH binds to serum albumin through a disulfide bond and that this may explain the long terminal boron half-life observed after BSH administration. Reported terminal half-lives of serum albumin in humans, determined from administering $^{125}\text{I}$ radiolabeled albumin, range from 14-20 days with 8.4-10.6% of the intravascular albumin pool catabolized per day (19,20). The reported terminal half-life values for albumin are much greater than the mean terminal boron half-life of 77.79 hours determined from this pharmacokinetic study. If the boronated species in the blood were covalently bound to albumin through a disulfide linkage, then the boron terminal half-life should have approached the terminal half-life for albumin. Since this was not the case, it is unlikely that there was significant covalent interaction between boronated species in the blood and serum albumin. This is supported by reports suggesting an ionic interaction, as opposed to covalent interaction, between BSH and serum albumin (21,22). Based on the boron pharmacokinetic data presented in this Chapter, an ionic interaction appears to be more plausible.
CONCLUSIONS

The present chapter described in detail, boron disposition following intravenous infusion of BSH. The pharmacokinetic parameters determined from this study agree with published reports describing boron disposition following BSH administration, when the reports were based on similar study designs (blood sampling for at least 120 hours following administration). Discrepancies between the results presented here and those from reports with inadequate study design have been explained. Pharmacokinetic evidence was also presented to suggest an ionic, as opposed to covalent, interaction between boron species in the blood and serum albumin.
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Table 2.1. Geometric mean plasma boron concentrations (μg/mL) for patients in the 50 mg boron/kg dose group.
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<th>Parameter</th>
<th>15 mg/kg (n=3)</th>
<th>25 mg/kg (n=3)</th>
<th>50 mg/kg (n=17)</th>
<th>All Doses (n=23)</th>
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<td>V1 (L)</td>
<td>7.59±2.73</td>
<td>7.26±2.37</td>
<td>7.86±2.44</td>
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<td>V2 (L)</td>
<td>6.51±3.50</td>
<td>7.06±4.06</td>
<td>8.50±3.67</td>
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<td>V3 (L)</td>
<td>53.35±27.36</td>
<td>41.42±8.52</td>
<td>47.18±20.06</td>
<td>47.23±19.41</td>
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<td>Vdss (L)</td>
<td>67.46±25.58</td>
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<td>Clr (mL/min)</td>
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<td>α (min⁻¹)</td>
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<td>β (min⁻¹)</td>
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<td>γ (min⁻¹)</td>
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Table 2.2. Mean (±SD) pharmacokinetic parameters, based on plasma boron concentrations, after i.v. administration of varying doses of BSH.

n = number of patients in each category
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<th>Blood Boron Concentration (µg/mL)</th>
<th>Plasma:Blood Boron Concentration Ratio</th>
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Table 2.3. Sixteen randomly chosen paired plasma and corresponding blood boron concentrations for determination of the plasma:blood boron concentration ratio.
Figure 2.1. Classical three-compartment open model system with zero-order input and first-order elimination from the central compartment where 1 = central compartment, 2 = shallow tissue compartment, 3 = deep tissue compartment, Ro = zero-order input (constant-rate infusion), k10 = first-order elimination rate constant, k12, k21, k31, k13 = first-order distribution rate constants.
Figure 2.2. Geometric mean plasma boron concentration-time profile from patients in the 50 mg boron/kg dose group (n=17), used to fit a two-compartment open model system with zero-order input and first-order elimination from the central compartment.
Figure 2.3. Geometric mean plasma boron concentration-time profile from patients in the 50 mg boron/kg dose group (n=17), used to fit a three-compartment open model system with zero-order input and first-order elimination from the central compartment.
Figure 2.4. Plot of weighted residuals versus X (minutes) from the fit of a two-compartment open model with zero-order input and first-order elimination from the central compartment to the geometric mean boron concentration-time data from patients in the 50 mg boron/kg dose group.
Figure 2.5. Plot of weighted residuals versus X (minutes) from the fit of a three-compartment open model with zero-order input and first-order elimination from the central compartment to the geometric mean boron concentration-time data from patients in the 50 mg boron/kg dose group.
Figure 2.6.  Semi-logarithmic plot of the plasma boron concentration-time profile from patient #1, used to fit a three-compartment open model.
Figure 2.7. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #2, used to fit a three-compartment open model.
Figure 2.8. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #3, used to fit a three-compartment open model.
Figure 2.9. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #4, used to fit a three-compartment open model.
Figure 2.10. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #5, used to fit a three-compartment open model.
Figure 2.11. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #6, used to fit a three-compartment open model.
Figure 2.12. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #7, used to fit a three-compartment open model.
Figure 2.13. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #9, used to fit a three-compartment open model.
Figure 2.14. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #10, used to fit a three-compartment open model.
Figure 2.15. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #11, used to fit a three-compartment open model.
Figure 2.16. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #12, used to fit a three-compartment open model.
Figure 2.17. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #13, used to fit a three-compartment open model.
Figure 2.18.  Semi-logarithmic plot of the plasma boron concentration-time profile from patient #14, used to fit a three-compartment open model.
Figure 2.19. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #15, used to fit a three-compartment open model.
Figure 2.20. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #16, used to fit a three-compartment open model.
Figure 2.21. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #17, used to fit a three-compartment open model.
Figure 2.22. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #18, used to fit a three-compartment open model.
Figure 2.23. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #19, used to fit a three-compartment open model.
Figure 2.24. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #20, used to fit a three-compartment open model.
patient 21 - 2900 mg boron/60 minutes

Figure 2.25. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #21, used to fit a three-compartment open model.
patient 23 - 2600 mg boron/60 minutes

Figure 2.26. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #23, used to fit a three-compartment open model.
Figure 2.27. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #24, used to fit a three-compartment open model.
Figure 2.28. Semi-logarithmic plot of the plasma boron concentration-time profile from patient #25, used to fit a three-compartment open model.
BIBLIOGRAPHY


CHAPTER 3

DETERMINATION OF RENAL PLASMA BORON CLEARANCE FOLLOWING ADMINISTRATION OF SODIUM BOROCAPTATE TO GLIOMA PATIENTS

INTRODUCTION

Gabel et al. (1) reported that 91.3±10.1% (mean±SD) of the corresponding boron dose was excreted in the urine within 96 hours of BSH administration. Haselsberger et al. (2) reported that 81.9±8.6 of the administered boron dose was recovered in the urine within 24 hours of dosing, and a report by Stragliotto et al. (3) stated that 50-80% of the administered boron dose was excreted within 96 hours of BSH administration. All of these published results suggest that renal excretion of boron from the body following BSH administration is the most important route of elimination. As such, the following Chapter describes a thorough analysis of renal plasma boron clearance using the patient urinary boron concentration-time data.
METHODS

URINARY BORON CONCENTRATION DETERMINATION

As part of the protocol for the clinical study, as described in Chapter 2, urine samples were collected from each patient before BSH administration and during the following time intervals after administration: 0-3, 3-6, 6-9, 9-12, 12-24, 24-48, 48-72, 72-96, and 96-120 hours. The urine samples were assayed for boron concentration in the Department of Pathology at The Ohio State University. The urinary boron concentration-time data were used for the determination of boron renal plasma clearance that is described in this Chapter.

CALCULATION OF BORON RENAL PLASMA CLEARANCE

Boron renal plasma clearance was calculated individually for each urine collection time interval, using Eq. (3.1.), where $\Delta A_{ex}$ was the measured amount of boron excreted in the urine on the interval $t_n$ to $t_{n+1}$ ($\Delta A_{ex} = \text{urine boron concentration} \times \text{urine void volume}$), and $\Delta A_{UC}$ was the area under the plasma boron concentration-time curve from the same time interval.

Equation 3.1.

$$Cl_r = \frac{\Delta A_{ex}}{\Delta A_{UC}}$$
CALCULATION OF ΔAUC AS A FUNCTION OF TIME FOR A THREE-COMPARTMENT OPEN MODEL

Equations were derived, using the three-compartment pharmacokinetic model (Fig. 2.1.), to calculate accurate ΔAUC values for each patient's urine collection time interval, using each individual patient's pharmacokinetic parameters. The AUC, for any time interval $t_n$ to $t_{n+1}$, can be expressed as the following equation [Eq. (3.2.)].

\[
\text{AUC}_{t_n \rightarrow t_{n+1}} = \int_{t_n}^{t_{n+1}} Cpd\,dt
\]

Since the drug administration was a zero-order process (constant-rate infusion), two AUC equations were derived, one for the AUC during the infusion (up-slope), and the other for the AUC after termination of the infusion (down-slope). All differential equations were integrated using the method of Laplace transformation (4-6). The Laplace transform for the differential equation describing the change in the amount of boron in the central compartment during the up-slope, as a function of time, is shown in Eq. (3.3.).
\[
\bar{A} = \frac{Ro(s+k21)(s+k31)}{s(s+\alpha)(s+\beta)(s+\gamma)}
\]

Where:
- \( \bar{A} \) = Laplace transform for the amount of boron in the central compartment
- \( Ro \) = zero-order infusion rate = Dose/infusion time
- \( k21 \) and \( k31 \) = microscopic first-order distribution rate constants
- \( \alpha \) = rapid first-order macroscopic rate constant
- \( \beta \) = intermediate first-order macroscopic rate constant
- \( \gamma \) = terminal first-order macroscopic rate constant
- \( s \) = Laplace domain variable

The corresponding Laplace transform for the up-slope AUC equation, derived for the same time interval 0 to \( \tau \) (infusion time), is shown in Eq. (3.4.).

\[
\overline{\text{AUC}_{0 \rightarrow \tau}} = \frac{Ro(s+k21)(s+k31)}{s^2(s+\alpha)(s+\beta)(s+\gamma)}
\]

The Laplace transform for the differential equation describing the change in the amount of boron, as a function of time, within the central compartment after termination of the i.v. infusion (time interval \( \tau \) to \( \infty \)) is shown in Eq. (3.5.).
\[
\overline{A} = \frac{A_{\text{max}}[(s + k_{21})(s + k_{31})] + B_{\text{max}} k_{21}(s + k_{31}) + C_{\text{max}} k_{31}(s + k_{21})}{(s + \alpha)(s + \beta)(s + \gamma)}
\]

Where: 
- \(A_{\text{max}}\) = maximum amount of boron in the central compartment at the end of the infusion (at time = \(t\))
- \(B_{\text{max}}\) = maximum amount of boron in the shallow tissue compartment at the end of the infusion (at time = \(t\))
- \(C_{\text{max}}\) = maximum amount of boron in the deep tissue compartment at the end of the infusion (at time = \(t\))

The corresponding Laplace transform for the down-slope AUC equation, derived for the time interval \(\tau\) to \(\infty\), is shown in Eq. (3.6.).

\[
\overline{\text{AUC}}_\tau \rightarrow \infty = \frac{A_{\text{max}}[(s + k_{21})(s + k_{31})] + B_{\text{max}} k_{21}(s + k_{31}) + C_{\text{max}} k_{31}(s + k_{21})}{s(s + \alpha)(s + \beta)(s + \gamma)}
\]

Equations (3.3.) through (3.6.) were integrated and the final integrated equations, for both the up-slope and down-slope AUC's, are shown in Appendix A.
To estimate the amount of the boron dose excreted in the urine from $t_0$ to infinity, the amount of the boron dose excreted from 120 hours after the start of BSH infusion to infinity was calculated using Eq. (3.7).

\[
A_{\text{ex}}_{120 \to \infty} = \frac{(\Delta A_{\text{ex}}/\Delta t)^{120}}{\lambda}
\]

Where: $A_{\text{ex}}_{120 \to \infty} = \text{amount of boron excreted on the time interval } t_{120} \text{ to } t_0$

$(\Delta A_{\text{ex}}/\Delta t)^{120} = \text{renal excretion rate at 120 hours}$

$\lambda = \text{first-order rate constant obtained from log-linear regression of terminal } \Delta A_{\text{ex}}/\Delta t \text{ data}$

Boron renal excretion rates $(\Delta A_{\text{ex}}/\Delta t)$ were calculated for the last three urine collections; 72, 96, and 120 hours $(\Delta A_{\text{ex}}/\Delta t = \text{amount excreted during time interval/length of time interval})$ in all patients. Semi-logarithmic plots of the renal excretion rate $(\Delta A_{\text{ex}}/\Delta t)$ versus the mid-point time $(t_{\text{mid}} = (t_n + t_{n+1})/2)$ for the corresponding urine collection periods, revealed a log-linear decay in the renal excretion rate for the last three days of sampling (24-hour urine collections) in all but 6 of the 23 patient profiles. Since the calculated boron renal excretion rates were average values over the entire 24-hour
collection period, they are appropriately used in conjunction with the corresponding mid-point time for the urine collection period. To avoid overestimating the fraction of the dose excreted from 120 hours to infinity, the renal excretion rate for each patient was extrapolated, by using the first-order rate constant obtained from log-linear regression analysis of individual patient urinary excretion rate-time data, so that the value for the renal excretion rate at the end point of the last collected urine void (120 hours), as opposed to the mid-point time, would be used in Eq. (3.7.). The extrapolated renal excretion rate then was divided by the value of first-order rate constant obtained from the terminal slope of the urinary excretion rate-time data [Eq. (3.7.)]. The resultant value was the amount of boron excreted from the last sampling time to infinity.

RESULTS

FRAGMENT OF THE BORON DOSE EXCRETED FROM 120 HOURS TO INFINITY

The cumulative fraction of the boron dose excreted in the urine was calculated at each collection time period for the 23 patients included in the pharmacokinetic study. These values were averaged and are shown graphically in Figure 3.1. An average of 84.9% ± 13.5% (mean ± SD) of the boron dose was excreted at 120 hours after start of the BSH infusion, indicating that renal excretion was the major route of elimination of boronated species.

Semi-logarithmic plot of the boron renal excretion rate (ΔAex/Δt) versus the mid-point time (t_{mid} = (t_n + t_{n+1})/2), for the corresponding urine collection periods, revealed a
log-linear decay in the renal excretion rate for the last three days of sampling (24-hour urine collections) in all but 6 of the 23 patient profiles (Fig 3.2.-3.12.). Log-linear regression analysis was performed on the $\Delta A_{ex}/\Delta t$-time data and the value for the slope ($\lambda$) of the regression line through the data points was determined for each patient. Using Eq. (3.7.), the amount of boron excreted from the last sampling point infinity was calculated for those 17 patients and the results are statistically summarized in Table 3.1. The calculated percent of the boron dose excreted from the last measured urine collection period (120 hours) to infinity was $0.47\% \pm 0.24\%$ therefore; the mean amount of boron excreted from $t_0$ to infinity was approximately 85.37%.

**RENAL PLASMA BORON CLEARANCE**

Using Eq. (3.1.) and the AUC equations in Appendix A, renal plasma boron clearance was calculated for each urine collection time period using each individuals pharmacokinetic parameters. The individual renal plasma boron clearance values for each patient were plotted versus the mid-point times of the corresponding urine collection period as shown in Figure 3.19. The supporting data for Fig. 3.19. are shown in Appendix B.
DISCUSSION

As can be seen in Fig. 3.1., 84.9% of the administered boron dose was excreted in the urine at 120 hours after start of the infusion, indicating that renal excretion was the major route of elimination of the boronated species. This result is consistent with other reports that concluded that renal excretion of boron was the major route of elimination following administration of BSH (1-3). Accordingly, patients with decreased renal function would require an adjustment of the BSH dose to avoid clinically unacceptable boron blood levels at the time of BNCT. The calculated value for the percent of the boron dose excreted in the urine from \( t_0 \) to infinity was 85.37%, indicating that the remaining 14.63% of the boron dose was eliminated by some other route. The most likely route of elimination for the remaining boron dose would have been biliary excretion in the feces; although there were no bile or fecal samples collected to test this hypothesis.

Because the majority of the administered dose was excreted in the urine and the apparent plasma boron elimination behavior was first-order, \( a \) priori it was expected that the renal plasma boron clearance values would have been constant and independent of the urine sampling time. However, as shown in Fig. 3.19., the renal plasma boron clearance values for each patient were not independent of the urine sampling time. Rather, when all of the renal plasma boron clearance values were plotted together \( versus \) the mid-point times for the corresponding urine collection intervals, a well-defined trend appeared that was inconsistent with what would have been predicted of clearance values from a single component, first-order system. Renal plasma boron clearance demonstrated initial values
that were much higher than those calculated for the last three days of sampling. Two
days after administration of BSH, renal plasma boron clearance reached an asymptotic
value of approximately 3-4 mL/min, which was three to four times greater than the
average rate of urine production (1 mL/min) and 30-40 times smaller than the normal
glomerular filtration rate (GFR) (120 mL/min). This result suggests that boronated
species present during the last three days of the study were moderately bound to blood
constituents and/or were undergoing renal tubular reabsorption.

Horn et al. (7) reported a diuretic effect after single-dose administration of BSH to
brain tumor patients, which was characterized as an increase in urine production and
minor changes in GFR. In one patient, however, the GFR was reduced by almost 50%
(7). This reported diuretic effect does not explain the change in renal plasma boron
clearance that was observed during this study. Assuming no significant change in GFR,
the diuretic effect of BSH would have merely diluted the boron concentration in the urine
without significantly altering the renal excretion rate. For this study, renal plasma boron
clearance was determined by first calculating the amount of boron in each urine
collection interval [Eq. (3.1.)], normalizing any increase in urine production.
Furthermore, the reduction in renal plasma clearance was observed in all patients and
lasted for the entire sampling interval.
CONCLUSIONS

85.37% of the boron dose, administered as BSH, was excreted in the urine from $t_0$ to infinity. The remaining 14.63% was excreted by some other route, presumably biliary. There was a significant, well-defined change in renal plasma boron clearance after administration of BSH. The observed time-dependent renal plasma boron clearance values suggest that BSH was metabolized or oxidized \textit{in vivo} to form multiple boronated species. Thus, the apparent renal plasma boron clearance was a composite value, consisting of individual renal plasma boron clearance values for the different boronated species present in the plasma, as well as their time dependent plasma concentration-time profiles. This hypothesis is supported by recently published data (8) stating that a portion of the BSH within patient glioblastoma tissue has been chemically altered which may be due to \textit{in vivo} metabolism, and by data using thin-layer chromatography and electrospray ionization mass spectrometry to determine BSH biotransformation products which will be explained in detail in Chapter 4.
### Table 3.1

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Table 3.1. Statistical summary of data for determination of the fraction ($f$) of the boron dose excreted from the last urine sampling time (120 hours) to infinity. $\lambda$ is the first-order rate constant obtained from log-linear regression analysis of the terminal urinary excretion rate-time data (n=17). Aex is the amount of boron excreted, $\Delta\text{Aex}/\Delta t$ is the boron renal excretion rate, and SD is the standard deviation.
Figure 3.1. Plot of the cumulative fraction of the boron dose excreted in urine (±SD), following intravenous administration of BSH, *versus* corresponding urine collection interval. Each point represents $n=23$. 
Figure 3.2. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #1.

Figure 3.3. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #4.
Figure 3.4. Semi-logarithmic plot of renal boron excretion rate ($dAex/dt$) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #5.

Figure 3.5. Semi-logarithmic plot of renal boron excretion rate ($dAex/dt$) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #6.
Figure 3.6. Semi-logarithmic plot of renal boron excretion rate \( \frac{dA_{ex}}{dt} \) \textit{versus} the mid-point time \( t_{mid} = \frac{(t_n + t_{n+1})}{2} \) for the corresponding urine collection periods for patient #7.

Figure 3.7. Semi-logarithmic plot of renal boron excretion rate \( \frac{dA_{ex}}{dt} \) \textit{versus} the mid-point time \( t_{mid} = \frac{(t_n + t_{n+1})}{2} \) for the corresponding urine collection periods for patient #9.
Figure 3.8. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #10.

Figure 3.9. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #12.
Figure 3.10. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #13.

Figure 3.11. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #16.
Figure 3.12. Semi-logarithmic plot of renal boron excretion rate \( \frac{dA_{ex}}{dt} \) versus the mid-point time \( t_{mid} = (t_n + t_{n+1})/2 \) for the corresponding urine collection periods for patient #17.

Figure 3.13. Semi-logarithmic plot of renal boron excretion rate \( \frac{dA_{ex}}{dt} \) versus the mid-point time \( t_{mid} = (t_n + t_{n+1})/2 \) for the corresponding urine collection periods for patient #19.
Figure 3.14. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #20.

Figure 3.15. Semi-logarithmic plot of renal boron excretion rate (dAex/dt) versus the mid-point time ($t_{mid} = (t_n + t_{n+1})/2$) for the corresponding urine collection periods for patient #21.
Figure 3.16. Semi-logarithmic plot of renal boron excretion rate \( \frac{dA_{ex}}{dt} \) versus the mid-point time \( t_{mid} = \frac{t_n + t_{n+1}}{2} \) for the corresponding urine collection periods for patient #23.

Figure 3.17. Semi-logarithmic plot of renal boron excretion rate \( \frac{dA_{ex}}{dt} \) versus the mid-point time \( t_{mid} = \frac{t_n + t_{n+1}}{2} \) for the corresponding urine collection periods for patient #24.
Figure 3.18. Semi-logarithmic plot of renal boron excretion rate \((dAex/dt)\) versus the mid-point time \((t_{mid} = (t_n + t_{n+1})/2)\) for the corresponding urine collection periods for patient #25.
Figure 3.19. Plot of renal plasma boron clearance versus mid-point time for the corresponding urine collection period for all patients (n=23).


CHAPTER 4

INVESTIGATION OF URINARY METABOLITES AND OXIDATION PRODUCTS OF SODIUM BOROCAPTATE USING THIN LAYER CHROMATOGRAPHY AND ELECTROSPRAY IONIZATION MASS SPECTROMETRY

INTRODUCTION

Determining the metabolic fate of a drug is a major focus of drug development efforts, since the identification of the metabolites of a drug can help predict or explain possible toxic side effects, pharmacokinetics, and biologically active agents (1). Elucidation of the metabolic scheme of a drug may also lead to identification of new potential synthetic drug candidates, which may posses improved efficacy relative to the reference compound (1).

Although BSH has been used clinically for BNCT for over thirty years, very little is known about its metabolic fate. This, in part, may be due to the stability of the polyhedral borane and its resistance to analytical methods usually employed to determine drug metabolism. Be this as it may, BSH contains a sulphydryl group in its chemical structure (Fig. 1.2.) and biotransformation of xenobiotics, via the sulphydryl group, has many well-characterized pathways (1-8):
• Glutathione conjugation
• S-glucuronidation
• S-methylation
• S-oxidation
• Disulfide formation (dimerization)
• Mercapturic acid formation

Due to the chemical and thermal stability of the \((\text{B}_{12}\text{H}_{12})^{2-}\) polyhedral borane anion (9), it is unlikely that any significant biotransformation of BSH will occur on the boron cage portion of the molecule. A similar polyhedral borane anion \((\text{B}_{10}\text{H}_{10})^{2-}\) was administered to brain tumor patients in the early 60’s and was reported to be, based on chemical and NMR data, excreted unchanged in the urine (32). As such, efforts for determining the biotransformation of BSH have been focused on the possibility of metabolism by modification of the sulfhydryl function using the above specified biotransformation pathways.

The following chapter describes the identification of BSH metabolism in patient’s urine samples using thin-layer chromatography and electrospray ionization time-of-flight mass spectrometry (ESI-MS), and the methods that were used to correlate chemical structures of putative BSH biotransformation products to empirically observed ESI-MS boron ion clusters.
METHODS

SCREENING PATIENT URINE SAMPLES FOR BORONATED COMPOUNDS USING NORMAL PHASE THIN-LAYER CHROMATOGRAPHY

Urine samples from a subset of the brain tumor patients were examined for the presence of boronated compounds using a normal phase thin-layer chromatography (TLC) procedure. As part of the protocol for the phase I clinical study (Chapter 2), urine samples were collected from patients before dosing and during the following time intervals after the start of the 60 minute intravenous infusion of BSH: 0-3, 3-6, 6-9, 9-12, 12-24, 24-48, 48-72, 72-96, and 96-120 hours. BSH standards, used for TLC spot comparison, were prepared by dissolving BSH in blank human urine. 20-50 µL aliquots of patient urine and standards were spotted onto silica gel TLC plates (Merck, Darmstadt, Germany). The mobile phase was (4:1) ethyl acetate:methanol (Fisher Scientific, Pittsburgh, PA). The TLC plates were developed in the mobile phase to a distance of 10 to 11 cm, which took approximately 30 minutes. Boron spot visualization was achieved by spraying a PdCl solution (0.5g PdCl₂ dissolved in 27mL concentrated HCl and subsequently diluted to 1 liter with deionized water) onto each plate, after it had been developed in the mobile phase, and heating with a laboratory heat gun for approximately 20 seconds (10). Because of the limited sensitivity of this TLC method for detecting boronated compounds, analysis of patient urine samples was limited to samples that were collected from the first 24 hours after termination of the infusion of BSH. Rf values for the detected boron spots were calculated for spot identification (11).
SCREENING PATIENT URINE SAMPLES FOR PROTEIN BOUND BORON COMPOUNDS USING NORMAL PHASE THIN-LAYER CHROMATOGRAPHY

Urine samples from patients in the 88.2 mg BSH/kg dose group were examined using TLC for the presence of protein bound boron compounds. Sample application and chromatographic conditions were the same as described above. For comparison, each sample was tested in duplicate on two separate TLC plates, one for boron spot visualization and the other for protein spot visualization. Boron spot visualization was performed as described above. Protein spot visualization was performed using an ethanolic ninhydrin solution technique (11-12). A 0.3% ninhydrin (Sigma Chemical Company, St. Louis, MO) solution was made in HPLC grade ethanol (Fisher Scientific, Pittsburgh, PA) and was applied to the TLC plate after it was developed in the mobile phase. Once the ninhydrin solution was applied, the plate was gently heated with a laboratory heat gun, which will cause the ninhydrin to form colored complexes with proteins (11-12). The PdCl treated TLC plate was then visually compared to the analogous plate treated with the ninhydrin solution.

TREATING PATIENT URINE SAMPLES WITH DITHIOTHREITOL TO IDENTIFY THE PRESENCE OF DISULFIDE BONDS IN BSH METABOLITES

Patient urine samples were treated with dithiothreitol (DTT) to test for the presence of disulfide bonds in the chemical structure of the boronated compounds which,
on the selected TLC system, had significantly different Rf values from BSH. The experimental conditions used for the disulfide reduction, have been shown to reduce dimerized BSH (BSSB) and oxidized dimer (BSOSB) back to the parent BSH (13). Approximately 20 mg of DTT (Sigma-Aldrich Chemical Company, St. Louis, MO) were added to 1 mL of the patient urine and vortex mixed. The pH of the DTT urine solution was adjusted to approximately 13 by addition of 10 M aqueous sodium hydroxide (Fisher Scientific, Pittsburgh, PA). The solution was then purged with nitrogen, sealed, and incubated at room temperature in the dark for 12 days. After the incubation period, the DTT urine solution was adjusted to a pH of approximately 3 with 5 M hydrochloric acid (Fisher Scientific, Pittsburgh, PA) and the urine solution was extracted twice with equal volumes of ethyl acetate (Fisher Scientific, Pittsburgh, PA) to remove the DTT. The aqueous fraction was then immediately tested using the above stated TLC protocol for boron compounds. For comparison, the treated urine sample (aqueous fraction) was tested along with the untreated sample. The ethyl acetate extracted fraction was also tested for the presence of boronated compounds.

**URINE SAMPLE PREPARATION FOR ELECTROSpraY IONIZATION MASS SPECTROMETRIC ANALYSIS**

Urine samples from two patients (patient #20 and #14), who had both received an i.v. dose of 88.2 mg /kg of BSH, were tested for the presence of boronated ions by ESI-MS. A series of scans (two positive ion and one negative ion) were performed in order to correlate empirically observed boronated ions to putative chemical structures of possible
BSH metabolites. Cesium salts of BSSB and $^{10}\text{BSOSB}^{10}$ were obtained as generous gifts from Boron Biologicals, Inc. (Fancy Gap, VA), and were used to identify those respective compounds in the patient urine samples. For the positive ion scans, patient urine samples were diluted in a solution of (1:1) methanol:5mM aqueous tetrabutylammonium (TBA) acetate (Sigma Chemical Company, St. Louis, MO) and, for a separate positive ion scan, (1:1) methanol:5mM aqueous tetrapentylammonium (TPA) bromide (Fluka Chemical Company, St. Louis, MO). For negative ion experiments the patient urine samples were diluted with methanol (Fisher Scientific, Pittsburgh, PA). In all cases the urine samples were centrifuged after dilution at 10,000 rpm for 5 minutes and the supernate was infused into the electrospray source at a rate of $5 - 10 \mu L \text{ min}^{-1}$.

**ELECTROSPRAY IONIZATION WITH TIME-OF-FLIGHT MASS DETECTION INSTRUMENTATION AND CONDITIONS**

All experiments were performed on a Micromass Q-TOF™ II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray), operated in both positive and negative ion modes. Polyalanine and alanine (Sigma Chemical Company, St Louis, MO) was used for positive ion mass calibration of 100 – 2000 m/z. Optimal electrospray ionization (ESI) conditions were: capillary voltage 3050 V, source temperature 110°C, and a cone voltage of 60 V. The ESI nebulization and drying gas was nitrogen. The collision gas flow was set to zero in order to increase instrument sensitivity. The linear quadrupole (Q1) was set to optimally pass ions from 50 – 2000 m/z into the pusher region of the time-of-flight (TOF) mass analyzer were they
were scanned from 500-2000 m/z for positive ion determinations and from 50-1200 m/z for negative ion determinations, both using a one second integration time. Data was acquired in continuum mode until acceptable averaged data was obtained (10-20 minutes). The averaged spectra were evaluated for the presence of mass clusters that had an isotope pattern identifiable to molecules containing multiple boron atoms. All boronated ions detected in patient samples using this ESI method were assumed to have originated from the administration of BSH. Although DCP-AES boron measurements were able to detect boronated compounds in all collected urine samples (Chapter 3), due to low sensitivity, ESI-MS analysis was limited to the patient urine samples that were collected for the first 24 hours following termination of the infusion of BSH for both positive ion scans, and for the first 48 hours for the negative ion scan.

ESI MS-MS experiments were performed on reference standard BSSB to provide additional information for making a correlation between observed boron ion clusters and putative metabolite/oxidation structures. For MS-MS product ion analysis (low energy collision induced dissociation (CID)), the quadropole was set to pass precursor ions of selected masses to the hexapole collision cell. Argon was used as the collision gas and product ion spectra were acquired with the TOF analyzer. Electrospray source parameters for the MS-MS CID experiments were essentially the same as above, and the collision energy was optimized for each compound.

Accurate mass measurements were performed by adding sodium iodide, as an internal standard (lock mass), to the sample after it had been infused into the electrospray source. A cluster ion of the sodium iodide which was closest in mass to the analyte was chosen as the lock mass.
The concentrations of the analyte and chosen lock mass were adjusted so that the relative ion peak heights were similar. Using a lock mass, the mass accuracy of the analyte is typically within 10 - 20 ppm (parts-per-million).

\textit{Correlation of observed boronated ions to possible BSH metabolite chemical structures}

The polyhedral borane portion of BSH has a di-negative charge at all practical pH values (9,23). As such, BSH and its metabolites were detected as multi-cation, pseudo-molecular ions during positive ion scans. BSH and its metabolites, formed detectable positive ions by complexing with both sodium ions and the quaternary ammonium compound, TBA. Reference spectra for BSH, $^{10}$BSOSB$^{10}$, and BSSB were used to identify these respective compounds in the patient urine samples. Based on known thiol biotransformation reactions, chemical structures for putative BSH metabolites were proposed. After the metabolite chemical structures were proposed, pseudo-molecular weights were calculated for each metabolite using all possible perturbations of positive ion formation; taking into account the chemical structures of the proposed BSH metabolites, the proposed metabolite charge state, and the molecular weights of sodium and TBA. After comparing the boronated ions that were detected in the urine samples to all the calculated pseudo-molecular ion values, chemical structures of several metabolites were found to be consistent to the boron ion clusters that were observed in the urine samples. The positive ion scans were repeated using a different quaternary ammonium compound (TPA). The rationale being that since BSH and its metabolites were forming
positively charged complexes with TBA, the observed ions should shift m/z, corresponding to the difference in mass of the cations when TPA is substituted for TBA. A third independent scan was performed with the urine samples using negative ion mode. Samples from patient #20 were tested using all three experimental conditions (TBA, TPA, and negative ions) while samples from patient #14 were not tested using TPA.

RESULTS

NORMAL PHASE THIN-LAYER CHROMATOGRAPHY METABOLISM STUDIES

Initial testing of patient urine samples by TLC suggested the presence of multiple boronated compounds in the urine samples that were collected within 12 hours following administration of BSH. Figure 4.1. shows the TLC plates corresponding to the parallel ninhydrin and PdCl experiment. As can be seen in Fig. 4.1., the PdCl treated TLC plate clearly shows the presence of multiple boronated compounds in the 6-9 hour urine sample from patient #20. The behavior of BSH in this TLC system was reproducible, with one well-defined spot with an approximate Rf value of 0.46 (spot A in Fig. 4.1.). The Rf values for the boronated spots in the patient urine sample were 0.50 for spot B and 0.35 for spot C. There also was some spotting at the origin suggesting the presence of another boron spot with an Rf value close to zero (Fig. 4.1.). Since the Rf values for spot B (0.50) and BSH (0.46) differ by only 8%, this result is inconclusive as to if spot B consists of a compound or compounds chemically different than BSH. There was a 24%
difference in the Rf values for spot C relative to BSH (spot A). The time zero sample for patient #20 showed no spotting and thus, no detectable boron or interfering compounds.

As can be seen from Figure 4.1., BSH had no reactivity to the ninhydrin test due to the lack of any colored complexes in lane 1 (BSH standard). There were also no colored complexes formed at the Rf values corresponding to spots B and C in the patient urine sample. However, there was an intense color reaction at the origin in both the 6-9 hour urine sample and the time zero urine sample, indicating the presence of proteins or amino acids at those Rf values (Fig. 4.1.).

Figure 4.2. shows the results from the DTT treatment experiment. Before DTT treatment, there were multiple boronated spots in the 6-9 hour urine sample from patient #2. The pattern of boron spots is similar to the pattern seen in the 6-9 hour urine sample from patient #20 and also was similar to all others tested. The Rf values for spots B and C in Figure 4.2. were 0.51 and 0.34, respectively. After treatment of the patient urine by DTT, there was only one discernable boron spot, with an Rf value indistinguishable from the measured value for BSH. The ethyl acetate extract was tested by TLC for the presence of boronated compounds. There were no detectable boron compounds in the ethyl acetate extract.
Mass spectra shown in Figures 4.3.- 4.6. correspond to the parent compound BSH. The negative ion data (Fig. 4.3.) shows a doubly charged negative boron ion cluster at 87.0 m/z that correlated with BSH \((B_{12}H_{11}SH)^{-2}\), which had a calculated m/z of 86.9 and was identical to spectra from BSH reference material. Each isotope peak for the 87.0 m/z cluster was 0.5 AMU apart, indicating that the ion possessed a double negative charge, which is consistent with the predicted ion. The ion cluster at 87.0 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 24 hours after start of the BSH infusion. There also was a singly negative charged boron ion cluster at 197.1 m/z that corresponded with BSH \([(B_{12}H_{11}SH)^{-} + Na^{+}]^{+}\), which had a calculated m/z of 196.9 and was identical to spectra from BSH reference material. The ion cluster at 197.1 m/z was detectable in all urine samples from patient #14 and patient #20 which were collected over the first 24 hours.

Fig. 4.4. summarizes data using TPA to form positive ions and shows a singly charged positive boron ion cluster at 1069.3 m/z that corresponded to BSH \([(B_{12}H_{11}SH)^{-} + 3TPA^{+}]^{+}\), which had a calculated m/z of 1069.5 and was identical to spectra produced from BSH reference material. The ion cluster at 1069.3 m/z was detectable in all urine samples from patient #20 that were collected over the first 24 hours. Also shown in Fig.
4.4. is a singly charged positive boron ion cluster at 793.9 m/z that corresponded to BSH 
\[ (\text{B}_{12}\text{H}_{11}\text{SH})^{2+} + 2\text{TBA}^{+1} + \text{Na}^{+1} \]^{+1}, which had a calculated m/z of 793.9 and was identical to spectra produced from BSH reference material. The ion cluster at 793.9 m/z was detectable in all urine samples from patient #20 that were collected over the first 12 hours.

Fig. 4.5. summarizes data using TBA to form positive ions shows a singly charged positive boron ion cluster at 901.0 m/z that corresponded to BSH 
\[ (\text{B}_{12}\text{H}_{11}\text{SH})^{2+} + 3\text{TBA}^{+1} \]^{+1}, which had a calculated m/z of 901.3 and was identical to spectra from BSH reference material. The ion cluster at 901.0 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 24 hours. Also shown in Fig. 4.5. is a singly charged positive boron ion cluster at 681.8 m/z that corresponded to BSH 
\[ (\text{B}_{12}\text{H}_{11}\text{SH})^{2+} + 2\text{TBA}^{+1} + \text{Na}^{+1} \]^{+1}, which had a calculated m/z of 681.8 and was identical to spectra from BSH reference material. The ion cluster at 681.8 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 12 hours. Figure 4.6. shows the computer predicted isotope pattern for the boron ion cluster at 901.0 m/z 
\[ (\text{B}_{12}\text{H}_{11}\text{SH})^{2+} + 3\text{TBA}^{+1} \]^{+1} along with the matching empirically observed ion from the patient urine that was attributed to the same chemical structure.

BSH SULFENIC ACID (BSOH)

The mass spectra shown in Figures 4.7.- 4.9. were determined to attributable to the presence of BSH sulfenic acid (BSOH) in the patient urine samples. Fig. 4.7. summarizes negative ion data and shows a doubly charged negative boron ion cluster at
94.0 m/z which approximately correlated with BSOH \((B_{12}H_{11}SOH)^2\), which has a calculated m/z of 94.8. Although this mass spectrum contains interfering peaks, a doubly charged boron isotope pattern is discernable. The ion cluster at 94.0 m/z was detectable in all urine samples from patient #14 and patient #20 which were collected over the first 24 hours. Also shown in Fig. 4.7. is a singly charged negative boron ion cluster at 213.1 m/z that corresponded to BSOH \([(B_{12}H_{11}SOH)^2 + Na^+]^-\), which had a calculated m/z of 212.9. The ion cluster at 213.1 m/z was detectable in all urine samples from patient #20 that were collected over the first 24 hours and in all urine samples from patient #14 that were collected over the first 12 hours.

Fig. 4.8. summarizes data using TPA to form positive ions and shows a singly charged positive boron ion cluster at 809.9 m/z that approximately correlated to BSOH \([(B_{12}H_{11}SOH)^2 + 2TPA^+ + Na^+]^+\), which had a calculated m/z of 809.1. The ion cluster at 809.9 m/z was detectable in all urine samples from patient #20 that were collected over the first 12 hours. Also shown in Fig. 4.8. is a singly positive charged boron ion cluster at 1084.4 m/z that approximately correlated to BSOH \([(B_{12}H_{11}SOH)^2 + 3TPA^+]^+\), which had a calculated m/z of 1085.5. The ion cluster at 1084.4 m/z was detectable in all urine samples from patient #20 that were collected over the first 24 hours.

Fig. 4.9. summarizes data using TBA to form positive ions and shows a singly charged positive boron ion cluster at 916.1 that approximately correlated to BSOH \([(B_{12}H_{11}SOH)^2 + 3TBA^+]^+\), which had a calculated m/z of 917.3. The ion cluster at 916.1 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 24 hours. Accurate mass determination on the 917.1 m/z peak in
the observed ion cluster from the patient urine sample deviated by 19 parts-per-million (ppm) from the value which was calculated using the predicted ion chemical formula $[(\text{B}_{12}\text{H}_{11}\text{SOH})^2 + 3\text{TBA}^+]^+\].

BSH SULFINIC ACID (BSO$_2$H)

The mass spectra in Figures 4.10.- 4.12. were determined to be attributable to the presence of BSH sulfinic acid (BSO$_2$H) in the patient urine samples. Fig. 4.10. summarizes negative ion data and shows a doubly charged negative boron ion cluster at 103.0 m/z that corresponded to BSO$_2$H (B$_{12}$H$_{11}$SO$_2$H)$^2$, which had a calculated m/z of 102.9. The ion cluster at 103.0 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 48 hours.

Fig. 4.11. summarizes data using TPA to form positive ions and shows a singly charged positive boron ion cluster at 1100.3 m/z that approximately correlated to BSO$_2$H $[(\text{B}_{12}\text{H}_{11}\text{SO}_2\text{H})^2 + 3\text{TPA}^+]^+$, which had a calculated m/z of 1101.4. The ion cluster at 1100.3 m/z was detectable in all urine samples from patient #20 that were collected over the first 24 hours.

Fig. 4.12. summarizes data using TBA to form positive ions and shows a singly charged positive boron ion cluster at 715.9 m/z that approximately correlated to BSO$_2$H $[(\text{B}_{12}\text{H}_{11}\text{SO}_2\text{H})^2 + 2\text{TBA}^+ + \text{Na}^+]^+$, which had a calculated m/z of 713.7. The ion cluster at 715.9 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 12 hours. Also shown in Fig. 4.12. is a singly charged positive boron ion cluster at 931.1 m/z that approximately correlated to BSO$_2$H.
[(B_{12}H_{11}SO_{2}H)^{-2} + 3\text{TBA}^{+}]^{+1}$, which had a calculated m/z of 932.0. The ion cluster at 931.1 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 24 hours.

**BSH-S-CYSTEINE CONJUGATE (BSH-CYS)**

The mass spectra shown in Figures 4.13.- 4.15. were attributable to the presence of a BSH-S-cysteine conjugate (BSH-CYS) in the patient urine samples. Fig. 4.13. summarizes negative ion data and shows a singly charged negative boron ion cluster at 338.2 m/z that corresponded to BSH-CYS $[(B_{12}H_{11}SSC_{3}H_{5}NO_{2})^{-3} + 2\text{Na}^{+}]^{-1}$, which had a calculated m/z of 338.1. The ion cluster at 338.2 m/z was detectable in the 0-3 hour urine sample from patient #14 and in urine samples from patient #20 that were collected from 3 hours to 9 hours after start of the BSH infusion.

Fig. 4.14. summarizes data using TPA to from positive ions and shows a singly charged positive boron ion cluster at 890.0 m/z that approximately correlated to BSH-CYS $[(B_{12}H_{11}SSC_{3}H_{5}NO_{2})^{-3} + 2\text{TPA}^{+1} + 2\text{H}^{+1}]^{+1}$, which had a calculated m/z of 890.9. The ion cluster at 890.0 m/z was detectable in all urine samples from patient #20 that were collected over the first 9 hours. Fig. 4.14. also shows a singly charged positive boron ion cluster at 914.0 m/z that approximately correlated with BSH-CYS $[(B_{12}H_{11}SSC_{3}H_{5}NO_{2})^{-3} + 2\text{TPA}^{+1} + \text{H}^{+1} + \text{Na}^{+}]^{+1}$, which had a calculated m/z of 912.9. The ion cluster at 914.0 m/z was detectable in the urine samples from patient #20 that were collected from 3 hours to 9 hours after start of the BSH infusion. Also included in Fig. 4.14. is a singly charged positive boron ion cluster at 1189.3 m/z that corresponded
to BSH-CYS \([(B_{12}H_{11}SSC_3H_5NO_2)^3 + 3TPA^{+1} + H^{+1}]^{+1}\), which had a calculated m/z of 1189.2. The ion cluster at 1189.3 m/z was detectable in all urine samples from patient #20 that were collected over the first 24 hours.

Fig. 4.15. summarizes data using TBA to form positive ions and shows a singly charged positive boron ion cluster at 1262.4 m/z that corresponded to BSH-CYS \([(B_{12}H_{11}SSC_3H_5NO_2)^3 + 4TBA^{+1}]^{+1}\), which had a calculated m/z of 1262.3. The ion cluster at 1262.4 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 12 hours. Also shown in Fig. 4.15. is a singly charged positive boron ion cluster at 1020.9 m/z that approximately correlated with BSH-CYS \([(B_{12}H_{11}SSC_3H_5N_0_{2})^{+ + 3TBA^{+1} + H^{+1}]^{+1}\), which had a calculated m/z of 1020.1. The ion cluster at 1020.9 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 24 hours.

**BSH DIMER DISULFIDE (BSSB)**

The mass spectra shown in Figures 4.16.- 4.20. were attributable to the presence of dimerized BSH (BSSB) in the patient urine samples. Fig. 4.16. summarizes negative ion data and shows a singly charged negative boron ion cluster at 416.4 m/z that approximately corresponded to BSSB \([(B_{12}H_{11}SSB_{12}H_{11})^{+1} + 3Na^{+1}]^{-1}\), which had a calculated m/z of 414.7. The ion cluster at 416.4 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected over the first 6 hours.

Fig. 4.17. summarizes data using TPA to form positive ions and shows a singly charged positive boron ion cluster at 1841.5 m/z that approximately correlated to BSSB
[(B_{12}H_{11}SSB_{12}H_{11})^4 + 5TBA^+]^+$, which had a calculated m/z of 1839.1. The ion cluster at 1841.5 m/z was detectable in all urine samples from patient #20 that were collected over the first 12 hours.

Fig 4.18. summarizes data using TBA to form positive ions and shows a singly charged positive boron ion cluster at 1315.6 m/z that correlated to BSSB 

[(B_{12}H_{11}SSB_{12}H_{11})^4 + 4TBA^+ + H^+]^+$, which had a calculated m/z of 1316.6. Also shown in Figure 4.18., for comparison, is the same boron ion cluster obtained from BSSB reference material. The ion cluster at 1315.6 m/z was detectable in all urine samples from patient #14 and patient #20, which were collected between 3 to 12 hours after start of the BSH infusion. Figure. 4.19. shows a singly charged positive boron ion cluster at 1559.9 m/z that approximately correlated to BSSB 

[(B_{12}H_{11}SSB_{12}H_{11})^4 + 5TBA^+]^+$, which had a calculated m/z of 1558.1. Also shown in Figure 4.19., for comparison, is the same boron ion cluster obtained from BSSB reference material. The ion cluster at 1559.9 m/z was detectable in all urine samples from patient #14 and patient #20 that were collected for the first 12 hours. Accurate mass determination of the 1557.8 m/z peak in the observed ion cluster from the patient urine sample deviated by 4.5 ppm from the value which was calculated using the predicted ion chemical formula 

[(B_{12}H_{11}SSB_{12}H_{11})^4 + 5TBA^+]^+$.

ESI MS-MS scans were performed to provide further data for compound identification. The mass spectra shown in Figure 4.20. correspond to ESI MS-MS spectra performed on the parent ion peak at 1557.8 m/z from both the patient urine sample and BSSB reference standard. One major boronated fragmentation product, having a mass cluster at 1372.7 m/z, was observed for CID of the parent ion (1557.8 m/z) in both the
patient urine samples and BSSB reference material. This result indicated that the boronated parent ion at 1557.8 m/z in the BSSB reference standard had the same chemical structure as the boronated parent ion at 1557.8 m/z in the patient urine sample, thus confirming the presence of BSSB in the patient urine sample.

BSH DIMER THIOSULFINATE (BSOSB)

The mass spectra shown in Figures 4.21. and 4.22. were attributed to the presence of BSH thiosulfinate (BSOSB). Fig. 4.21. summarizes data using TPA to form positive ions and shows a singly charged positive boron ion cluster at 1580.7 m/z that approximately corresponded to BSOSB [(B_{12}H_{11}SOSB_{12}H_{11})^{4-} + 4TPA^{+} + Na^{+}]^{+}, which had a calculated m/z of 1578.7. The ion cluster at 1580.7 m/z was detectable in the urine samples from patient #20 that were collected between 3 to 9 hours.

Fig. 4.22. summarizes data using TBA to form positive ions and shows a singly charged positive boron ion cluster at 1573.9 m/z that corresponded to BSOSB [(B_{12}H_{11}SOSB_{12}H_{11})^{4-} + 5TBA^{+}]^{+}, which had a calculated m/z of 1574.1. The ion cluster at 1573.9 m/z was detectable in urine samples from patient #20 that were collected between 6 hours to 12 hours, and in urine samples from patient #14 that were collected between 3 to 12 hours after start of the BSH infusion. Accurate mass determination on the 1574.8 m/z peak in the observed ion cluster from the patient urine sample deviated by 10.9 ppm from the value which was calculated using the predicted ion chemical formula [(B_{12}H_{11}SOSB_{12}H_{11})^{4-} + 5TBA^{+}]^{+}. Fig. 4.22. also shows a singly charged positive boron ion cluster at 1332.6 m/z that approximately correlated to BSOSB.
[(B$_{12}$H$_{11}$SOSB$_{12}$H$_{11}$)$^+$ + 4TBA$^{+1}$ + H$^{+1}$]$^{+1}$, which had a calculated m/z of 1331.5. The ion cluster at 1332.6 m/z was detectable in the urine sample, from patient #14 and patient #20 that was collected between 9 to 12.

The BSOSB metabolite was not detected during negative ion scans. This most likely was due to a lack of instrument sensitivity for this molecule in negative ion mode. Although this was the case, BSOSB was detected during both positive ion experiments.

**DISCUSSION**

The normal phase TLC method described in this Chapter provided a fast and relatively inexpensive method to screen patient urine samples for the presence of boronated compounds. Boronated compounds where found in patient urine samples, which had considerably different Rf values from a BSH standard (Fig. 4.1.). Since TLC is a procedure which can separate compounds, based on their differential partitioning between stationary and mobile phases, it was concluded that the patient samples contained at least one boronated compound which was chemically different than the parent drug BSH. Since BSH was the only compound administered to the patients, the boronated spot, which was chromatographically resolved from the BSH spot, was assumed to be a result of *in vivo* biotransformation or oxidation of BSH. Efforts were undertaken to use preparative TLC methods to isolate the boronated metabolites but failed to yield encouraging data.

The qualitative nature of TLC analysis was exploited to generate chemical and structural information for the BSH metabolites. As was stated in Chapter 2, it has been
hypothesized that BSH may bind \textit{in vivo} to serum albumin (14-18). Even though it was concluded, using the boron pharmacokinetic data, that there was no apparent covalent interaction between boronated compounds in the plasma and serum albumin (Chapter 2), a simple ninhydrin procedure was performed to examine the possibility of protein bound BSH metabolites in the patient urine samples. There was no color reaction at the Rf values corresponding to the BSH metabolite spot although there was an intense color reaction at the origin of the TLC plate (Fig. 4.1.). Since there was also detection of boronated species at the origin, it was concluded that there may be protein bound BSH metabolites in the patient urine samples and indeed, cysteine conjugated BSH (BSH-CYS) was detected by electrospray mass spectrometry as is described below. It was also concluded, based on these TLC data, that there were non-protein bound boronated metabolites in the urine of patients who received BSH.

As was stated in Chapter 1, BSH has a well-characterized oxidation pathway yielding both dimerized (BSSB) and thiosulfinate (BSOSB) products in oxygenated aqueous solution (10,19). As such, the BSSB and BSOSB were suspected to be metabolic or \textit{in vivo} oxidation products resulting from BSH administration. Since BSSB and BSOSB contain disulfide linkages in their chemical structures, a DTT treatment was performed on patient urine samples to determine the effect of disulfide reducing conditions on the Rf values for the BSH metabolites. Comparison of TLC’s from DTT treated, and untreated patient urine clearly shows the disappearance of all boronated spots with Rf values significantly different than BSH after DTT treatment (Fig. 4.2.). Therefore, on the basis of this TLC data, it was concluded that disulfide linkages were a part of the chemical structure of the BSH metabolites.
Urine samples from patients #20 and #14 were investigated for the presence of boronated metabolites using a direct infusion electrospray ionization mass spectrometry (ESI-MS) method (20, 21). Since boron has two stable isotopes of atomic masses 10 and 11, which have approximate natural abundances of 19% and 81%, respectively (24), molecules containing multiple boron atoms in their chemical structures will have readily identifiable isotope pattern resulting from statistical permutations of the isotope abundances (Figure 4.16.). As such, molecules containing multiple boron atoms could be identified, based on their isotope pattern, from electrospray mass spectra and correlated to possible chemical structures using multiple independent mass spectral scans.

The application of various counter-ions to produce stable detectable pseudo-molecular ions for ESI-MS analysis has been described for various organic compounds (25-28), and specifically for boron ESI-MS analysis (29). Using this type of approach, the chemical structures for several putative metabolites of BSH were correlated to observed boron ion clusters from patient urine samples.

It is apparent that some of the observed apparent base mass peaks for the reported boron ion clusters do not exactly match the calculated molecular weights of the pseudo-molecular or molecular ions for the proposed BSH metabolites. Having stated this, it needs to be recognized that in every case, the calculated molecular weight of the proposed BSH metabolite was found within 2 m/z (usually 1 m/z) of the observed apparent base molecular ion and that the calculated pseudo-molecular or molecular ion was contained in the observed boron ion cluster. For both BSOH and BSO₂H, the negative ion data yielded boronated ion clusters that had apparent base mass peaks that were indistinguishable from the calculated metabolite molecular weights. The same is
true of BSH-CYS; there was both positive and negative ion mass spectral data that was indistinguishable from the predicted pseudo-molecular ions of BSH-CYS. Reference material of BSSB resulted in one boron mass cluster, at 1315.5 m/z, that was practically indistinguishable from the mass cluster observed in the urine samples (Figure 4.18.) and one mass cluster, at 1557.8, that had an apparent base peak mass difference of 2 m/z when compared to the patient’s urine data (Figure 4.19.). Ions were chosen for ESI MS-MS determinations, from the 1557.8 boron ion mass cluster, and yielded identical CID daughter ion data from both the patient’s urine samples, and BSSB reference material (Fig. 4.20.). This result suggested that although there was an apparent base peak mass discrepancy for this particular ion cluster (1557.8), they were the same molecule. When accurate mass measurements could be made, there was sufficient agreement between the measured mass and the calculated mass to suggest that the ions that were being measured were originated from the proposed structures. Accurate mass determinations were made for ions resulting from BSOH, BSSB and BSOSB. Scans of $^{10}$BSOSB$^{10}$ reference material did not produce ions that would have been consistent with $^{10}$BSOH, indicating that the presence of ions correlated to BSOH in the patient urine samples did not originate from fragmentation of BSOSB in the ESI source.

The reason for the apparent mass discrepancy in some of the ESI spectra is not entirely clear but is probably due to the various factors such as the boron isotope distribution, possible free-radical formation inside the ESI source, and low metabolite ion abundance. These reasons are not offered as explanations, but rather as insight into the problems that have been reported when performing mass spectral analysis on boron compounds. It has been recognized that mass spectral analysis of heavily chlorinated
compounds can lead to apparent molecular ions, which differ from the predicted molecular weight value, and that the difference can be as great as 2 m/z (28). Since boron, like chlorine, has isotopes that are in considerable proportions, it is reasonable to assume that there may be a similar phenomenon occurring with mass spectral analysis of heavily boronated compounds. There also have been reports of significant deviations in the boron natural abundance isotope ratio found in various plants and geologic materials (30) and that these deviations may be used to identify distinct sources of boron in geologic samples (24). In one instance, the natural abundance variability of boron prevented normal operation of computer software that was used for boron quantitation and isotope dilution studies (30). The synthesis of BSSB has been characterized (31), and it was documented that an exceptionally stable free-radical \( \text{BS}^+ \) can be formed from BSSB that may also complicate the mass spectral analysis of such compounds.

Because reliable reference standard material for many of the suspected metabolites does not presently exist, several scans were performed, using various chemical and instrument conditions, to correlate the observed boronated ion mass spectra to putative metabolite chemical structures. Two positive ion (TBA and TPA), and one negative ion scans were performed on the urine samples from patient #20 while one positive (TBA), and one negative ion scans were performed on the urine samples from patient #14. For the positive ion experiments, two different ion-pair reagents (TBA and TPA) were used which formed detectable positively charged pseudo-molecular ions with the boron cage compounds. Since the ion-pair reagents have differing molecular weights, the clusters they formed had different molecular weights and served as an independent determination of the boronated ion molecular weight.
A third independent scan was performed in negative ion mode, in which no ion-pair reagent was added, and the ions were detected in their native negative charge states.

Both BSH sulfenic acid ($B_{12}H_{11}SOH$) (BSOH), and BSH sulfinic acid ($B_{12}H_{11}SO_{2}H$) (BSO$_2$H), were correlated to observed boronated ions in patient urine samples by ESI-MS. Oxidation of thiol groups to sulfenic acids and sulfinic acids can be catalyzed in vivo by a number of enzyme systems (1, 2, 4, 5, 7, 8). Flavin-containing monooxygenase (FMO) is an NADPH and oxygen-dependant microsomal enzyme system that serves as a sulfur, nitrogen, and phosphorus oxygenase in mammalian systems (1). Cytochrome P450, specifically CYP3A4, is an NADPH and oxygen-dependant microsomal enzyme that is capable of oxidizing thiol groups (22). Although these two enzyme systems can both have simultaneous affinity for the same substrate, there exists a limited generalization in that the more nucleophilic a sulfur atom, the more likely that it will be a substrate for FMO (1, 5, 8). Considering the nucleophilicity of BSH, it would likely have a higher affinity for FMO relative to P450.

Both the BSH dimer disulfide (BSSB) and the corresponding thiosulfinate (BSOSB) were identified in the patient urine samples. As stated in Chapter 1, both of these compounds can be formed from spontaneous oxidation of BSH in aqueous media (Fig. 1.2.). Whereas direct dimerized disulfide formation is not a common enzymatic reaction, it has been reported to occur through a series of FMO catalyzed reactions (8). The sulfur atom of a sulfenic acid (BSOH) can become the site of nucleophilic substitution by a thiol group (BSH), yielding the dimerized disulfide (BSSB) and one mole of water (8). It is not clear from this ESI-MS data if the formation of the BSSB and BSOSB was a spontaneous chemical reaction or if it was enzymatically mediated.
Perhaps the most interesting identified biotransformation product was a BSH-S-cysteine conjugate. Although there are well-characterized examples of in vivo amino acid conjugation, phase II reactions have not been shown to involve cysteine as a conjugation agent (6). Rather, the formation of cysteine-S-conjugates have been characterized for many drugs as a multi-step, multi-enzyme process (1, 3, 5-8). The first step in this chain of chemical reactions is the formation of the glutathione-S-conjugate (BSH-GSH), which is catalyzed by glutathione-S-transferase (3, 5, 6). This conjugation reaction occurs by formation of a disulfide bond between the ligand (BSH) and the cysteinyi residue of the tripeptide γ-glutamyl-cysteinyl-glycine or glutathione (3, 5, 6).

The next step is hydrolysis of the glutamic acid residue at the γ-glutamyl bond of glutathione by the enzyme γ-glutamyl transferase (γ-GT) (3). γ-GT has been found in the luminal membrane of the biliary epithelium, in the bile canalicular membrane of hepatocytes, in the epithelium of the small intestine, in the renal tubule brush-border membrane, and in the basolateral membrane of renal tubule cells (3). Since the active site of γ-GT is extracellular, the glutathione-S-conjugate must be transported out of the cells (renal or hepatic) in order to act as a substrate (3). It is usually assumed that the carrier that secretes glutathione will also secrete glutathione-S-conjugates and that this is the most likely mechanism of transport to the extracellular active site of γ-GT (3). Following hydrolysis by γ-GT, the glycine residue is hydrolyzed by the enzymes aminopeptidase M or cysteinylglycine dipeptidase (3). Both of these enzymes seem to be co-localized in the cell membrane with γ-GT (3). The net result of these two enzyme catalyzed hydrolytic reactions is that the only residue remaining bound to the drug from the original glutathione tripeptide is cysteine, thus forming the cysteine-S conjugate. All
of the enzymes involved in the formation of the cysteine-S conjugate from the glutathione-S-conjugate are extremely efficient and have been characterized to completely hydrolyze substrates at millimolar concentrations (3). The glutamic acid and glycine liberated from these hydrolysis reactions is recycled and used for other biochemical needs (6).

Neither the glutathione-S-conjugate of BSH or the γ-glutamyl hydrolysis intermediate were detected in the patient urine samples. The absence of detectable amounts these chemical species in the urine can be explained by the biochemical reactions that occurred. Glutathione-S-conjugates are readily excreted by carrier-mediated transport into the bile (3,6). The glutathione-S-conjugates may then be subject to the above stated hydrolysis reactions or, without any appreciable enterohepatic recycling, excreted into the feces completely bypassing the systemic circulation. This explanation appears to be plausible considering that according the principle of mass-balance, 14.63% of the boron dose was unaccounted for in the urine. The absence of detectable γ-glutamyl hydrolysis intermediate may be explained considering the high activity and co-localization of the hydrolyzing enzymes. Once the glutamic acid residue was hydrolyzed, the glycine residue will then be hydrolyzed before the intermediate can re-enter the systemic circulation.

Based on the ESI-MS description of the BSH biotransformation products, a biotransformation pathway for BSH has been hypothesized (Fig. 4.23.). Although the data presented in this Chapter does not rigorously support the idea that BSO₂H and BSOSB are secondary metabolites, the order of the reactions depicted in Figure 4.23. are reasonable. Attempts to establish the correct sequence of biotransformation by
performing precursor-order determinations (33) on the urine ESI-MS metabolite data failed to yield promising results. This is most likely due to the fact that precursor-order determination is sensitive to the early phase of metabolite formation. Since all the identified metabolites appeared in the first patient urine sample (0-3 hours), it was difficult to draw any conclusion from the precursor-order data.

CONCLUSIONS

Multiple boronated compounds were detected by TLC techniques in the urine of patients who had been administered BSH. Using TLC experiments, it was determined that disulfide bonds were part of the chemical structure of the boronated metabolites. It was also determined, using ninhydrin TLC studies, that there were metabolites of BSH that were not protein bound, although there may have been the presence of a protein (amino acid) bound metabolite. ESI-MS scans were used to correlated observed boron ion clusters to chemical structures of putative BSH metabolites, including those that contained disulfide bonds and a cysteine-S-conjugate. A biotransformation pathway was proposed (Fig. 4.23.), based on general biochemical knowledge, although further work is needed for definitive characterization. Although the patient urine samples were stored at -20°C until analyzed, there was a significant time delay (approximately two years) between collecting the patient samples and having the ability to use ESI-MS to scan for boronated compounds. Because of this time delay, it is possible that some of the compounds that were identified as apparent metabolites are actually products of chemical degradation of either the parent drug, or other metabolites. The metabolite chemical
structures that have been correlated to the ESI-MS, ESI-MS-MS, and accurate mass data, are reasonable biotransformations for sulphhydryl containing compounds. Further work, possibly using various \textit{in vitro} metabolic screening techniques, will be needed to conclusively establish the presence and biological importance of these proposed BSH metabolites and their impact on using BSH for BNCT. Although DCP-AES boron measurements were able to detect boronated compounds in all collected urine samples (Chapter 3), due to low sensitivity, ESI-MS analysis was limited to the patient urine samples that were collected for the first 24 hours following termination of the infusion of BSH for both positive ion scans, and for the first 48 hours for the negative ion scan. The presence of a boronated ion cluster, that is consistent with the chemical structure for the proposed BSH biotransformation product BSO$_2$H, was the only biotransformation product that was detected in the patient's urine after 24 hours, and was undetectable after 48 hours. The boronated BSH biotransformation products residing in the body for the last 3 days of sampling (48 hours – 120 hours post dose) have yet to be identified.
Figure 4.1. Left: TLC treated with ninhydrin solution for the visualization of proteins. Lane 1 is a 1 mg/mL BSH urine standard. Lane 2 is the 6-9 hour urine sample from patient #20. Lane 3 is the time zero urine sample from patient #20. Right: TLC treated with PdCl solution to visualize boronated compounds. The lane assignment is identical to the left side TLC. Rf values for the boronated spots was as follows: A=0.46, B=0.50, and C=0.35.
Figure 4.2. TLC showing the results of treating the 6-9 hour urine sample from patient #2 (15 mg boron/kg dose group) with dithiothreitol (DTT) for 12 days. Lane 1 corresponds to the untreated 6-9 hour urine sample and lane 2 corresponds to the DTT treated urine sample. The Rf values for the detected boron spots are as follows: A=0.49, B=0.51, and C=0.34.
Figure 4.3. Negative ion ESI mass spectra of BSH obtained from the 0-3 hour urine sample from patient #20. The cluster at 87.0 m/z corresponded to the doubly charged negative ion \((B_{12}H_{11}SH)^{-2}\). The cluster at 197.1 corresponded to the singly charged negative ion \([(B_{12}H_{11}SH)^{-2} + Na^{+1}]^{-1}\).
Figure 4.4. Positive ion ESI mass spectra of BSH obtained from the 0-3 hour urine sample from patient #20, using TPA to form positive ions. The cluster at 1069.3 m/z corresponded to the singly charged positive ion [(B_{12}H_{11}SH)^2 + 3TPA^+]^+. The cluster at 739.9 m/z corresponded to the singly charged positive ion [(B_{12}H_{11}SH)^2 + 2TPA^+ + Na^+]^+. 
Figure 4.5. Positive ion ESI mass spectra of BSH obtained from the 0-3 hour urine sample from patient #20 using TBA to form positive ions. The cluster at 681.8 corresponded to the singly charged positive ion \([(B_{12}H_{11}SH)^2 + 2\text{TBA}^+ + \text{Na}^+]^+\). The cluster at 901.0 m/z corresponded to the singly charged positive ion \([(B_{12}H_{11}SH)^2 + 3\text{TBA}^+]^+\).
Figure 4.6. Above: Computer predicted isotope pattern for the singly charged positive ion corresponding to $[(B_{12}H_{11}SH)^{-2} + 3TBA^{+}]^{+}$. Below: Positive ion ESI mass spectrum of BSH obtained from the 0-3 hour urine sample from patient #20 using TBA to form positive ions. The cluster at 901.0 m/z corresponded to the singly charged positive ion $[(B_{12}H_{11}SH)^{-2} + 3TBA^{+}]^{+}$.
Figure 4.7. Negative ion ESI mass spectra, obtained from the 0-3 hour urine sample from patient #20, which were attributed to BSOH. The cluster at 94.0 m/z corresponded to the doubly charged negative ion \((B_{12}H_{11}SOH)^{-2}\). The cluster at 231.1 m/z corresponded to the singly charged negative ion \([(B_{12}H_{11}SOH)^{-2} + Na^{+}]^{-1}\).
Figure 4.8. Positive ion ESI mass spectra using TPA to form positive ions, obtained from the 0-3 hour urine sample from patient #20, which were attributed to BSOH. The cluster at 809.9 m/z corresponded to the singly charged positive ion \([\text{BtaH}n\text{SGH}]^- + \text{ITPA}^- + \text{Na}^+\]^+. The cluster at 1084.4 m/z corresponded to the singly charged positive ion \([\text{BaHuSOH}]^- + \text{STPA}^-]^+\). The cluster at 1084.4 m/z corresponded to the singly charged positive ion \([\text{B}_{12}\text{H}_{11}\text{SOH}]^- + 2\text{TPA}^+ + \text{Na}^+]^+. The cluster at 1084.4 m/z corresponded to the singly charged positive ion \([\text{B}_{12}\text{H}_{11}\text{SOH}]^- + 2\text{TPA}^+ + \text{Na}^+]^+\).
Figure 4.9. Positive ion ESI mass spectrum using TBA to form positive ions, obtained from the 0-3 hour urine sample from patient #20, which was attributed to BSOH. The cluster at 916.1 m/z corresponded to the singly charged positive ion [(B_{12}H_{11}SOH)^{-2} + 3TBA^{+}]^{+1}.
Figure 4.10. Negative ion ESI mass spectrum, obtained from the 0-3 hour urine sample from patient #20, that was attributed to BSO₂H. The cluster at 103.0 m/z corresponded to the doubly charged negative ion (B₁₂H₁₁SO₂H)²⁻.
Figure 4.11. Positive ion ESI mass spectrum using TPA to form positive ions, obtained from the 0-3 hour urine sample from patient #20, which was attributed to BSO₂H. The cluster at 1100.3 m/z corresponded to the singly charged positive ion \([\text{B}_{12}\text{H}_{11}\text{SO}_2\text{H}]^+ + 3\text{TPA}^+]^+\).
Figure 4.12. Positive ion ESI mass spectra using TBA to form positive ions, obtained from the 0-3 hour urine sample from patient #20, which were attributed to BSO$_3$H. The cluster at 715.9 m/z correlated to the singly charged positive ion $[(\text{B}_{12}\text{H}_{11}\text{SO}_2\text{H})^- + 2\text{TBA}^+ + \text{Na}^+]^+$. The cluster at 931.1 m/z corresponded to the singly charged positive ion $[(\text{B}_{12}\text{H}_{11}\text{SO}_2\text{H})^- + 3\text{TBA}^+]^+$.  

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Figure 4.13. Negative ion ESI mass spectrum, obtained from the 6-9 hour urine sample from patient #20, that was attributed to BSH-CYS. The cluster at 338.2 m/z corresponded to the doubly charged negative ion $[(B_{12}H_{11}SSC_3H_2NO_2)^3 + 2Na^+]^{-1}$. 
Figure 4.14. Positive ion ESI mass spectra using TPA to form positive ions, obtained from the urine samples from patient #20, that were attributed to BSH-CYS. The cluster at 890.0 m/z correlated to the singly charged positive ion \([(B_{12}H_{11}SSC_3H_9NO_2)^{-3} + 2TPA^{+1} + 2H^{+1}]^{+1}\), which was present in the 0-3 urine hour sample. The cluster at 914.0 m/z correlated to the singly charged positive ion \([(B_{12}H_{11}SSC_3H_9NO_2)^{-3} + 2TPA^{+1} + H^{+1} + Na^{+1}]^{+1}\) and the cluster at 1189.3 m/z corresponded to the singly charged positive ion \([(B_{12}H_{11}SSC_3H_9NO_2)^{-3} + 3TPA^{+1} + H^{+1}]^{+1}\), both were present in the 3-6 hour sample.
Figure 4.15. Positive ion ESI mass spectra using TBA to form positive ions, obtained from the urine samples from patient #20, which were attributed to BSH-CYS. The cluster at 1262.4 m/z correlated to the singly charged positive ion $\left[\left(B_{12}H_{11}SSC_3H_3NO_2\right)^{-3} + 4\text{TBA}^{+1}\right]^{+1}$, which was present in the 3-6 hour sample. The cluster at 1020.9 m/z corresponded to the singly charged positive ion $\left[\left(B_{12}H_{11}SSC_3H_3NO_2\right)^{-3} + 3\text{TBA}^{+1} + \text{H}^{+1}\right]^{+1}$, which was present in the 0-3 hour sample.
Figure 4.16. Negative ion ESI mass spectrum, obtained from the 0-3 hour urine sample from patient #20, which was attributed to BSSB. The cluster at 416.4 m/z corresponded to the singly charged negative ion \([(\text{B}_{12}\text{H}_{11}\text{SSB}_{12}\text{H}_{11})^{-4} + 3\text{Na}^{+1}]^{-1}\).
Figure 4.17. Positive ion ESI mass spectrum using TPA to form positive ions, obtained from the 3-6 hour urine sample from patient #20, which was attributed to BSSB. The cluster at 1841.5 m/z corresponded to the singly charged positive ion $[(B_{12}H_{11}SSB_{12}H_{11})^{\text{-}} + 5\text{TPA}^{+}]^{+}$.
Figure 4.18. Above: Positive ESI mass spectrum obtained from BSSB reference material. The cluster at 1315.6 m/z corresponded to the singly charged positive ion \([\text{B}_{12}\text{H}_{11}\text{SSB}_{12}\text{H}_{11}]^+ + 4\text{TBA}^+ + \text{H}^+\)^+. Below: Positive ESI mass spectrum, which was correlated to BSSB, obtained from 3-6 hour urine sample from patient #20.
Figure 4.19. Above: Positive ESI mass spectrum obtained from BSSB reference material. The cluster at 1558.8 m/z corresponded to the singly charged positive ion \[ (\text{Bi}_2\text{H}_n\text{SSBi}_2\text{H}_n)^{+} + \text{5TBA}^{+} \] \[^{+} \]. Below: Positive ESI mass spectrum, which was correlated to BSSB, obtained from the 6-9 hour urine sample from patient #14.
Figure 4.20. Above: Positive ESI MS-MS daughter ion spectra from the 1557.8 m/z ion in BSSB reference standard using TBA to form positive ions. Below: Positive ESI MS-MS daughter ion spectra from the 1557.8 m/z ion in the 0-3 hour urine sample from patient #14, using TBA to form positive ions.
Figure 4.21. Positive ion ESI mass spectrum using TPA to form positive ions, obtained from the 6-9 hour urine sample from patient #20, which was attributed to BSOSB. The cluster at 1580.7 m/z corresponded to the singly charged positive ion $[\text{Bi}_{2}\text{H}_{11}\text{SOSB}_{11}\text{H}_{11}]^{4} + 4\text{TPA}^{+} + \text{Na}^{+}]^{+}$. 
Figure 4.22. Positive ion ESI mass spectra using TBA to form positive ions, obtained from the 9-12 hour urine sample from patient #20, which were attributed to BSOSB. The cluster at 1573.9 m/z correlated to the singly charged positive ion $[(B_{12}H_{11}SOSB_{12}H_{11})^4 + 5TBA^+]^+$. The cluster at 1332.6 m/z correlated to the singly charged positive ion $[(B_{12}H_{11}SOSB_{12}H_{11})^4 + 4TBA^+ + H^+]^+$. 
Figure 4.23. Proposed BSH biotransformation pathway based on ESI-MS urinary metabolite data of BSH biotransformation products in the urine of patients who had received BSH.
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CHAPTER 5
REVERSE PHASE ION-PAIR HPLC ELECTROSPRAY MASS SPECTROMETRIC ASSAY FOR SODIUM BOROCAPTATE AND THE APPLICATION TO PHARMACOKINETICS

INTRODUCTION

As stated in Chapter 1, one of the goals of this study was to develop a sensitive and specific analytical method for determining the concentration of BSH in biological fluids. The present Chapter describes a reverse phase ion-pair electrospray mass spectrometry liquid chromatographic assay (LC-MS) for BSH, and the application of this assay for determining the pharmacokinetics of BSH.

METHODS

REAGENTS

BSH was purchased from Centronic (Croydon, UK) and its characterization has been described in detail in Chapter 2. Tetrabutylammonium acetate (1.0 M solution), polyalanine, and alanine were purchased from Sigma Chemical Company (St. Louis,
MO). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Blank human plasma was purchased from the American Red Cross for use in preparing analytical standards. Dicesium undecahydrododecaborate (Cs₂B₁₂H₁₂) (B12) was obtained as a gift from Callery Chemical Company (Pittsburgh, PA) and was used as an internal standard. Ultrapure (18 MΩ) laboratory deionized water was obtained from a Barnstead (Dubuque, IA) Nanopure Diamond ultrafiltration unit.

**INSTRUMENTATION**

The BSH LC-MS assay was developed using a Micromass Q-TOF™ II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray), operated in positive ion mode. Polyalanine and alanine was used for positive ion mass calibration range of 100 – 2000 m/z. Electrospray conditions were as follows: capillary voltage 3050 V, source temperature 150°C, capillary voltage 70 V, and source block temperature 90°C. The ESI nebulization and drying gases were nitrogen. In order to increase the assay sensitivity, the flow of collision gas was set to zero. The linear quadrupole (Q1) was set to pass ions from 850 – 950 m/z into the pusher region of the time-of-flight (TOF) mass analyzer were they were scanned for BSH and B12 pseudo-molecular ions. Masslynx (Micromass, Wythenshawe, UK) computer software was used to control all settings and operation of the QTOF II™ and the liquid chromatography system described below.
**HPLC CONDITIONS**

The liquid chromatographic/autosampler system consisted of a Waters Alliance 2690 Separations Module (Waters, Milford, MA). Chromatography was performed using a 250 x 2.1 mm narrow-bore Nucleosil C18 column (Machery-Nagel, River Vale, NJ) with a 5 μm particle size which was preceded by a 7.5 x 3.2 mm guard column that was filled with the same packing material. The mobile phase was (1:1) methanol:5mM aqueous TBA. The mobile phase flow rate was maintained at 0.2 mL/min and was split post-column using a micro-splitter valve (Upchurch Scientific, Oak Harbor, WA) to approximately 45 μL/min for introduction to the ESI source. The total assay time was ten minutes per injection.

**PREPARATION OF STOCK SOLUTIONS AND PLASMA CALIBRATION STANDARDS**

Stock solutions were prepared by dissolving lyophilized BSH in deionized water for a target concentration of 1 mg/mL. After dissolving, the aqueous BSH solution was immediately aliquoted into screw-top borosilicate tubes, purged with nitrogen, capped, and frozen at -20°C until used. A stock solution of 1 mg/mL Cs₂B_{12}H_{12} (B12) internal standard was similarly prepared and stored.
Plasma calibration standards were prepared by spiking blank human plasma (1 mL) with an appropriate volume of BSH stock solution for a resultant concentration range of 20 μg/mL to 0.5 μg/mL. 10 μL of B12 internal standard stock solution was added to each 1 mL plasma standard, for an internal standard concentration of 10 μg/mL. Plasma calibration standards were prepared fresh for each assay.

ASSAY PROCEDURE

Since the boron concentration had already been determined for the patient plasma samples (Chapter 2), it was possible to estimate a dilution factor so that the BSH concentration would be within the linear range for the LC-MS assay. Using the appropriate dilution factor, patient plasma samples were diluted using blank human plasma to a final volume of 1 mL. A 1 mL aliquot was removed from the patient plasma samples not requiring dilution. To each 1 mL patient plasma sample, 10 μL of B12 internal standard solution was added. After the addition of internal standard, a 200 μL aliquot was removed from each 1 mL patient sample and calibration standard, and placed into a disposable polypropylene microcentrifuge tube. 400 μL of acetonitrile was then added to each tube for the purpose of plasma protein precipitation. The precipitated samples were then centrifuged at 10,000 rpm for 10 minutes and the supernate was removed and placed into autosampler vials containing 250 μL glass inserts. 40 μL of each sample was injected onto the HPLC column for analysis.

Each standard was injected once and each patient sample was injected in duplicate. The arithmetic mean BSH concentration was calculated for the duplicate
patient injections and was used for subsequent BSH pharmacokinetic analysis. In order to assure instrument reliability, a control sample of known concentration (20 µg/mL) was injected after every ten injections and at the end of each assay. The control sample had to be within 5% of the target concentration for the instrument to be considered stable.

**EXTRACTED ION CHROMATOGRAMS**

Since the instrument was collecting mass spectral data from a range of masses (850 – 950 m/z), it was necessary to extract ions from the total ion chromatogram (TIC) that were specific for the pseudo-molecular ions of BSH [(B₁₂H₁₁SH)²⁺ + 3TBA⁺]⁺ and B₁₂ [(B₁₂H₁₂)²⁺ + 3TBA⁺]⁺. Ions were extracted from the TIC over a mass range of 866.5 - 870.5 m/z for B₁₂ and 898.5 - 902.5 for BSH. Each extracted ion chromatogram was then smoothed and integrated, using the Masslynx computer software, to determine the area under the curve for each chromatographic peak.

**CALIBRATION CURVES**

For each standard, a ratio (BSH:B₁₂) was calculated using the corresponding areas for BSH and B₁₂ peaks obtained from the respective extracted ion chromatograms. The peak area ratios were then plotted *versus* the corresponding BSH concentration and each curve was fitted by linear regression analysis. The instrument was calibrated prior to each assay.
ASSAY PRECISION/ACCURACY DATA

To determine the within-run precision, a set of 10 μg/mL BSH standards (n=9) was prepared and assayed in series. The arithmetic mean assayed BSH concentration, standard deviation, coefficient of variation, and percent error from the target BSH concentration were all calculated from the resulting data.

Three sets of BSH standards; 20, 5, and 0.5 μg/mL representing high, medium and low concentrations; were assayed in triplicate on three separate days to determine the between-run precision. The high, medium, and low BSH concentrations were then pooled over each of the three separate assays and the arithmetic mean concentration, standard deviation, coefficient of variation (CV), and percent error from the target BSH concentration was calculated for each concentration level.

BSH PHARMACOKINETIC ANALYSIS

The plasma BSH concentration-time profiles of four patients in the 50 mg boron/kg dose group (Patient #10, #13, #14, and #17), each having a histopathological diagnosis of AA or GBM, were used to obtain information for the pharmacokinetics of BSH. The exact infusion time (τ), and blood sampling times, as opposed to scheduled infusion and sampling times, were calculated for each patient to properly evaluate the BSH pharmacokinetics. Semi-logarithmic plots of the BSH plasma concentration-time
data revealed an apparent bi-exponential decay that was characteristic of a two-compartment open model with zero-order input and first-order elimination from the central compartment. Each patient's plasma concentration-time profile was then used to fit the two-compartment model, using WinNonlin computer software, and the results have been statistically summarized. Patient BSH concentration-time data was analyzed using a weighting factor of $1/y^2$.

To gain insight for the renal pharmacokinetics of BSH, two patient urinary BSH concentration-time profiles (patient #13 and #17) were determined by assaying the urine samples, which were collected for the first 24 hours, for BSH concentration using the LC-MS assay described above. Equations describing the AUC for the selected two-compartment pharmacokinetic model were derived (Appendix C) and integrated using the method of Laplace transformation, as outlined in Chapter 3. The renal plasma BSH clearance was then calculated using Eq. (5.1.) where $\Delta\text{Aex}$ was the measured amount of BSH excreted in the urine on the interval $t_n$ to $t_{n+1}$ ($\Delta\text{Aex} = \text{urine BSH concentration} \times \text{urine void volume}$), and $\Delta\text{AUC}$ was the area under the plasma BSH concentration-time curve from the same time interval.

Equation 5.1.

$$\text{Cl}_r = \frac{\Delta\text{Aex}}{\Delta\text{AUC}}$$
Since the biotransformation of BSH has been described (Chapter 4), and both the boron and BSH plasma and renal pharmacokinetics have been evaluated for patients #13 and #17, pooled BSH metabolite pharmacokinetic parameters for those two patients were calculated. The apparent pooled BSH metabolite $AUC_{met}^{0\rightarrow\infty}$ can be calculated as the difference between the $AUC_{boron}^{0\rightarrow\infty}$ determined from the boron pharmacokinetic analysis and the $AUC_{BSH/boron}^{0\rightarrow\infty}$ determined from the BSH pharmacokinetic analysis [Eq. (5.2.)]. Since the AUC parameter is dependant on amount, and 88.2 mg BSH/kg is equivalent to 50 mg boron/kg, the $AUC_{BSH/boron}^{0\rightarrow\infty}$ parameter for BSH must be divided by 1.764 to covert it to equivalent boron units.

Equation 5.2.

$$AUC_{met}^{0\rightarrow\infty} = AUC_{boron}^{0\rightarrow\infty} - AUC_{BSH/boron}^{0\rightarrow\infty}$$

Where:

- $AUC_{met}^{0\rightarrow\infty}$ = apparent pooled boronated metabolite AUC from time zero to infinity.
- $AUC_{boron}^{0\rightarrow\infty}$ = boron disposition AUC, following administration of BSH, from time zero to infinity.
- $AUC_{BSH/boron}^{0\rightarrow\infty}$ = BSH AUC from time zero to infinity, converted to boron equivalents.

The apparent pooled BSH metabolite $AUC_{met}^{120\text{hrs}\rightarrow\infty}$ can be calculated by dividing the plasma boron concentration at 120 hours by the terminal gamma boron disposition rate
constant [Eq. (5.3.)]. This calculation assumes that the measured plasma boron concentration at 120 hours was composed of BSH metabolites, and not of the parent drug. Given the short value for the terminal half-life of BSH, relative to the terminal half-life of boron, this appears to be a reasonable assumption.

\[
\text{AUC}_{\text{met}}^{120 \text{hrs} \to \infty} = \frac{\text{Cp}(120 \text{hrs})}{\gamma}.
\]

Where: \(\text{Cp}(120 \text{hrs}) = \) plasma boron concentration at 120 hours.
\(\gamma = \) first-order terminal boron disposition rate constant.

The apparent pooled metabolite \(\text{AUC}_{\text{met}}^{0 \to 120 \text{hrs}}\) can then be calculated as the difference between the apparent pooled metabolite \(\text{AUC}_{\text{met}}^{0 \to \infty}\) and the apparent pooled metabolite \(\text{AUC}_{\text{met}}^{120 \text{hrs} \to \infty}\) [Eq. (5.4.)].

\[
\text{AUC}_{\text{met}}^{0 \to 120 \text{hrs}} = \text{AUC}_{\text{met}}^{0 \to \infty} - \text{AUC}_{\text{met}}^{120 \text{hrs} \to \infty}
\]

The amount of pooled metabolites excreted during the 120-hour urine collection period \((\text{Aex}_{\text{met}}^{0 \to 120})\) can be calculated as the difference in the amount of boron excreted during the 120 hours, and the amount of BSH excreted during the same collection period [Eq. (5.5.)]. Again, the amount of BSH excreted needs to be converted to the corresponding boron equivalence by dividing by 1.764.
Equation 5.5.

\[ A_{\text{ex}}^{\text{met}}_{0\rightarrow120\text{hrs}} = A_{\text{ex}}^{\text{boron}}_{0\rightarrow120\text{hrs}} - A_{\text{ex}}^{\text{BSH/boron}}_{0\rightarrow120\text{hrs}} \]

Where:
- \( A_{\text{ex}}^{\text{met}}_{0\rightarrow120\text{hrs}} \) = amount of pooled metabolites excreted during the 120 hour urine collection period.
- \( A_{\text{ex}}^{\text{boron}}_{0\rightarrow120\text{hrs}} \) = amount of boron excreted, after administration of BSH, during the 120 hour urine collection period.
- \( A_{\text{ex}}^{\text{BSH/boron}}_{0\rightarrow120\text{hrs}} \) = amount of BSH excreted during the 120 hour urine collection period, converted to boron units.

The average pooled BSH metabolite renal plasma clearance over the 120-hour urine collection period was then calculated by dividing the amount of pooled BSH metabolites excreted during the 120 hour urine collection interval by the corresponding pooled BSH metabolite AUC from the same time interval [Eq. (5.6.).]

Equation 5.6.

\[ \text{Clr}_{\text{met}} = \frac{A_{\text{ex}}^{\text{met}}_{0\rightarrow120\text{hrs}}}{\text{AUC}_{\text{met}}^{0\rightarrow120\text{hrs}}} \]

Where:
- \( \text{Clr}_{\text{met}} \) = average pooled metabolite renal plasma clearance during the 120 hour urine collection period.
DETERMINATION OF BSH PLASMA PROTEIN BINDING PARAMETERS

An ultrafiltration technique was used to characterize the BSH plasma protein binding (5-7). Samples were prepared by spiking BSH into 2 mL of blank human plasma or pH 7.2 phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$) for a resulting boron concentration range of 275 – 91 µg/mL (482 – 161 µg/mL of BSH). The samples prepared in PBS were used to determine the non-specific binding of BSH to the ultrafiltration membrane. After preparation, the samples were incubated in a 37°C water bath for one hour. After incubation, a one mL aliquot of each sample was tested for boron concentration by the DCP-AES method described in Chapter 2, and the other one mL aliquot was placed into a Amicon ultrafiltration tube (Amicon, Beverly, MA) equipped with an Amicon Diaflo™ 14mm membrane filter which had a 30,000 molecular weight pore size. The tubes were then centrifuged at 1200 rpm until approximately 100 µL (10% of the starting volume) of ultrafiltrate was collected (3 minutes for PBS samples and 10 minutes for plasma samples). The ultrafiltrate was then diluted with laboratory water for boron analysis.

All measured boron values were converted to the corresponding BSH concentrations by multiplying the boron concentrations by 1.764. BSH concentration data were used for all protein binding calculations. Total plasma protein concentration was assayed in triplicate, at the Ohio State University Department of Pathology, using a Pierce Coomassie Protein Assay Reagent Kit and corresponding bovine serum albumin standard (Pierce, Rockford, IL).
Mass balance [Eq. (3.8.)] was used to determine the unbound fraction ($f_u$) of BSH.

$$D_{\text{Total}} = DP + D + D_m$$

Where:
- $D_{\text{Total}}$ = Amount of BSH drug in the system
- $DP$ = Amount of BSH bound to plasma proteins
- $D$ = Amount of free BSH
- $D_m$ = Amount of BSH bound to membrane filter

Using Eq. (3.8.), $f_u$ can be calculated as the ratio of the amount of free BSH to the total amount of BSH ($f_u = D / D_{\text{Total}}$). The amount of BSH bound to the filter is evaluated from the PBS samples, where $DP$ is equal to zero, and therefore, $D_m = D_{\text{Total}} - D$. The fraction of BSH bound to the membrane ($f_m$), for each PBS sample, was then calculated as the ratio of the amount of membrane bound BSH to the total amount of BSH ($f_m = D_m / D_{\text{Total}}$).

The amount of BSH bound to the membrane filter ($D_m$) for each plasma sample was then calculated by multiplying the corresponding $f_m$ from the PBS data, to the total BSH plasma concentration ($D_{\text{Total}}$). The amount of BSH bound to plasma proteins ($DP$) was then calculated using Eq. (3.8.). All calculated BSH amounts ($D$, $DP$, and $D_{\text{Total}}$) were converted to the corresponding molar concentrations for Scatchard plot analysis (7). The Scatchard plot was generated by plotting $[(DP)/(P) + (D)]$ on the y-axis and $(DP)/(P)$ on the x-axis and yields a slope of $-K_a$ (equilibrium association constant) and a y-intercept equal to $N \times K_a$, where $N$ is the number of BSH binding sites per mole of protein (7).
RESULTS

HPLC CHROMATOGRAPHY AND INSTRUMENT CALIBRATION

Figure 5.1 shows representative extracted ion chromatograms for both BSH and internal standard (B12) from a plasma calibration standard. The extracted ion chromatograms for all patient plasma samples were identical to the extracted ion chromatograms from plasma calibration standards for both the BSH and internal standard. There were no interfering peaks for both BSH and B12, as was apparent from the extracted ion chromatograms from blank human plasma samples.

A typical standard curve for the developed LC-MS assay for BSH in plasma is shown in Figure 5.2. All calibration standard curves used for patient sample quantitation had \( R^2 \) values greater than 0.99.

PRECISION DATA

A statistical summary of the within-run precision data is shown in Table 5.1. The arithmetic mean BSH concentration was 10.59 ± 0.56 \( \mu \text{g/mL} \) (10 \( \mu \text{g/mL} \) target value) and the CV was 0.053. The calculated percent error from the target BSH concentration was 5.57%.

A statistical summary of the between-run precision data is shown in Table 5.2. The calculated arithmetic mean concentrations and corresponding standard deviations for the high (20 \( \mu \text{g/mL} \)), medium (5 \( \mu \text{g/mL} \)), and low (0.5 \( \mu \text{g/mL} \)) BSH concentrations were
19.99 ± 0.61, 4.71 ± 0.15, and 0.63 ± 0.10 μg/mL, respectively. The calculated CV values for the high, medium, and low BSH concentrations were 0.03, 0.03, and 0.16, respectively. The percent error from the target value was calculated for the arithmetic mean of the high, medium, and low BSH concentration and were 0.06%, 5.87%, and 20.21%, respectively. Although the CV and percent error at the 0.5 μg/mL BSH concentration level were large, they were considered acceptable since the 0.5 μg/mL is the lowest BSH concentration used for quantitation.

**BSH PHARMACOKINETICS**

The disposition of BSH was modeled as a classical two-compartment open model with zero-order input and first-order elimination from the central compartment. The fitted patient BSH plasma concentration-time profiles are shown in Figures 5.3.-5.6. For comparison, the plasma boron and corresponding BSH (corrected for boron equivalence) concentration-time profiles are shown together in Figures 5.7.-5.10. The resulting BSH pharmacokinetic parameters have been statistically summarized in Table 5.3. The fit from the two-compartment model yielded acceptable variability in the fitted parameters and demonstrated a random distribution of residuals (4). The mean total body plasma BSH clearance was 95.64 ± 30.82 mL/min and the harmonic mean terminal BSH half-life was 3.61 hours. The fraction of the BSH dose excreted unchanged in the urine from time zero to 24 hours was 0.728 and 0.888 for patients #13 and #17, respectively. A plot of the cumulative fraction of the BSH dose excreted in the urine of patient #17 versus the midpoint time for the corresponding urine collection interval is shown in Figure 5.11.
Renal plasma BSH clearance was calculated for patients #13 and #14, at each urine collection interval, and the results are statistically summarized in Table 5.4. The mean renal plasma BSH clearance for patients #13 and #17, was 154.7±60.0 mL/min and 63.5±32.5 mL/min, respectively. Figures 5.12. and 5.13. show semi-logarithmic plots of the renal BSH excretion rate (ΔAex/Δt or dAex/dt) versus midpoint time for each patient and both were linear, characteristic of first-order renal plasma clearance.

**POOLED BSH METABOLITE RENAL PLASMA CLEARANCE**

The average renal plasma clearance of the pooled BSH metabolites for the time interval 0 to 120 hours after administration of BSH was 6.0 mL/min and 3.1 mL/min for patients #13 and #17, respectively. The average renal plasma clearance of the pooled boronated metabolites, for both patients, was considerably lower than the corresponding renal plasma clearance of the parent drug (BSH).

**BSH PLASMA PROTEIN BINDING**

A plot of the free fraction (fu), or fraction unbound to plasma proteins, of BSH versus the corresponding total plasma BSH concentration is shown in Figure 5.14, and was indicative of saturable BSH plasma protein binding. The fraction unbound of BSH ranged from 0.31 at 482 µg/mL of BSH to 0.17 at 189 µg/mL of BSH and had a mean value of 0.22.
The mean±SD plasma protein concentration was 43.1±5.1 mg/mL which was consistent to published values of total human plasma protein concentration (7). Since albumin comprises 60% of all plasma proteins, the concentration of albumin was estimated as 25.9 mg/mL and the corresponding molar concentration of $3.9 \times 10^{-4}$ M was used for Scatchard plot analysis. A Scatchard plot of BSH protein binding is shown in Figure 5.15. The plot was linear, which was indicative of a single class of BSH protein binding sites (11). The equation of the resulting linear regression was $Y = -3073.8X + 16945$ and had an $R^2$ value of 0.91. Accordingly, the equilibrium association constant ($K_a$) was $3073.8 \text{ M}^{-1}$ and the number of binding sites per mole of albumin was 5.5.

**DISCUSSION**

In order to obtain definitive information for the disposition of a drug, a specific and sensitive analytical assay needs to exist for quantitation of the drug in biological fluids. There has been a reported BSH HPLC assay that used monobromobimane to form an (ultraviolet) UV absorbing adduct with BSH that could then be detected (1). There also was a reported rat BSH pharmacokinetic study using the UV BSH HPLC assay (2). Efforts were undertaken to reproduce the reported BSH assay for this study. Despite these efforts, the assay could not be reproduced and as such, a new specific analytical assay, which was capable of detecting BSH at physiologically relevant concentrations, needed to be developed.

The HPLC assay that was used to characterize the BSH pharmaceutical formulation was able to detect BSH at high concentrations (3). But at physiologically
relevant concentrations, this assay was not sensitive enough to detect BSH. Although
this was the case, the chromatographic conditions for the BSH pharmaceutical
formulation HPLC assay (3) were used as initial conditions to develop a specific,
sensitive, and reliable BSH LC-MS assay.

The LC-MS assay reported in this Chapter was linear over a BSH concentration
range of 20 µg/mL to 0.5 µg/mL. Acceptable between-run and within-run precision was
demonstrated for the developed LC-MS assay (Tables 5.1.-5.2.). There was acceptable
chromatographic peak shape for both the BSH and the internal standard (B12) and the
total assay time for each sample was 10 minutes.

The utility of the newly developed BSH LC-MS assay was demonstrated by
determining the BSH concentration-time profile in a subset (n=4) of patients in the 88.2
mg BSH/kg dose group. Because there was a significant time delay in collecting the
patient samples and the development of the LC-MS assay (approximately 2 years), and
the lack of reliable stability information for BSH, accurate characterization of BSH
pharmacokinetics could not be scientifically justified. Although the patient samples had
been frozen at -20°C until assayed, there may have been some degradation of the parent
compound, which could have led to the formation of the oxidation products BSSB and
BSOSB described in Chapter 4. Although this may have been the case, useful
information could still be gained from determining the BSH pharmacokinetics in a subset
of patients. Assuming that any degradation or oxidation reactions would occur as a first-
order chemical process, each BSH concentration would be a given percent lower than its
initial value.
As such, any BSH clearance, AUC, and volumes of distribution obtained from these patients should be interpreted accordingly, but the estimates of the first-order rate constants and their corresponding half-lives should not be affected by the storage time.

BSH disposition was modeled as a two compartment open-model with zero-order input and first-order elimination from the central compartment. This is in contrast to the boron disposition following BSH administration (Chapter 2), which was modeled as a three-compartment open model with zero-order input and first-order elimination from the central compartment. Consequentially, the persistent gamma phase for the boron disposition, which had a harmonic mean half-life of 77.79 hours (Chapter 2), is likely to be composed of one or more BSH metabolites and/or in vivo oxidation products that have apparent first-order plasma elimination. The electrospray mass spectrometric analysis of the patient urine samples described in Chapter 4 was able to detect the presence of a boronated ion cluster, that is consistent with the chemical structure for the proposed BSH biotransformation product BSO$_2$H, and was the only biotransformation product that was detected in the patient’s urine after 24 hours, and was undetectable after 48 hours. The boronated BSH biotransformation products residing in the body for the last 3 days of sampling (48 hours – 120 hours post dose) have yet to be identified.

For comparison purposes, semi-logarithmic plots of the plasma concentration-time profiles for both BSH, corrected for boron equivalence, and total boron are shown for each patient in Figures 5.7. – 5.10. The fitted BSH plasma concentration-time profiles are shown for each patient in Figures 5.3. - 5.6. The $\alpha$ and $\beta$ harmonic mean disposition half-lives of BSH were 0.40 and 3.61 hours, respectively.
Based upon the value of the terminal disposition half-life ($\beta$) for BSH, it can be predicted that the vast majority of the administered BSH dose would be eliminated from the body in the initial 24-hour period after dosing.

The fraction of the BSH dose excreted unchanged in the urine, over the first 24 hours, was estimated for patients #13 and #17 and was 0.728 and 0.888, respectively. This result reinforces the statement in Chapter 3 that patients with decreased renal function will require a BSH dosage adjustment to avoid clinically unacceptable blood boron concentrations during BNCT. Mean renal plasma BSH clearance was estimated for patients #13 and #17, and was $154.7\pm60.0$ mL/min and $63.5\pm32.5$ mL/min, respectively. Pooled boronated metabolite renal plasma clearance was calculated for patients #13 and #17, and was 6.0 mL/min and 3.1 mL/min, respectively. The pooled boronated metabolite renal plasma clearance is not describing the renal pharmacokinetics of any one particular BSH metabolite, but rather can be used to compare the relative renal excretion of parent drug to its boronated metabolites. The pooled boronated metabolite renal plasma clearance for both patients was approximately 95% lower than the parent drug (BSH) renal plasma clearance. This result, along with the terminal half-life of BSH, can be used to explain the change in renal plasma boron clearance that was described in Chapter 3 (Fig 3.19.). Since the BSH is eliminated rapidly and has a higher renal plasma clearance, relative to the pooled boronated metabolites, the composite renal plasma boron clearance reported in Chapter 3 will have higher values for the first 24 hours, due to the presence of BSH. After 24 hours, the vast majority of BSH will have been eliminated, and the remaining boronated metabolites have a significantly lower renal plasma clearance.
Semi-logarithmic plots of the BSH renal excretion rate versus midpoint time for both patients (#13 and #17) revealed a log-linear relationship that is characteristic of first-order renal excretion. Since the vast majority of the BSH dose was excreted unchanged, and the apparent BSH plasma elimination behavior was first-order, first-order renal excretion was expected.

Saturable plasma protein binding can cause changes in the renal clearance of some drugs. Because only free drug, which is not bound to plasma proteins, is filtered by the glomerulus, changes in the free fraction ($f_u$) may have consequential changes in renal clearance by altering the contribution of glomerular filtration. As a result of the higher fraction unbound at high total drug plasma concentrations, the renal clearance may have higher values. The affect of saturable plasma protein binding on clearance and disposition has been modeled for some drugs and has been shown to cause non-linear plasma concentration-time curves (8).

The plasma protein binding behavior of BSH has been characterized. The fraction of BSH unbound ranged from 0.31 at 482 µg/mL of BSH (273 µg/mL boron), to 0.17 at 189 µg/mL of BSH (107 µg/mL boron) and had a mean value of 0.22. Scatchard plot analysis suggests that there is one class of BSH protein binding sites and that there are approximately 5.5 BSH binding sites per mole of albumin with a binding constant ($K_a$) equal to 3073.8 M$^{-1}$ (3.0738 mM$^{-1}$). The molar concentration of albumin was estimated based on the total assayed protein plasma concentration and reported values of the percentage of total plasma protein that is attributable to albumin. One of the functions of albumin is to bind anionic molecules (7) and as such, it has been suspected to interact with BSH, which has been discussed in Chapter 2. The BSH plasma protein
binding parameters reported here are in reasonable agreement with those reported by Zhu Tang et al. (9), who used $^{11}$B NMR to quantitate BSH in aqueous serum albumin solutions. They found a single class of 3-5 binding sites per albumin protein, with a $K_a$ of 1.5-3.0 mM$^{-1}$ (9).

Since the contribution to renal clearance by glomerular filtration is equal to the product of the fraction unbound and the GFR, it can be estimated that the contribution of glomerular filtration on the renal plasma BSH clearance would be approximately 24 mL/min. Since the actual value for the renal plasma BSH clearance is much greater than the filtration clearance, the additional clearance mechanism may be active tubular secretion. Since there was saturable BSH plasma protein binding, and no apparent effect on the renal plasma BSH clearance (Table 5.4.), it was concluded that the renal plasma BSH clearance was independent of plasma protein binding.

**CONCLUSION**

An ESI LC-MS assay for BSH has been developed. The LC-MS assay was linear over a BSH concentration range of 20 µg/mL to 0.5 µg/mL, and had acceptable precision, variability, and reliability. The utility of the LC-MS assay was demonstrated by estimating the BSH pharmacokinetics in a subset of the patients who enrolled in this study. Semi-logarithmic plot of the BSH concentration-time profile showed apparent bi-exponential decay, characteristic of a two-compartment open model. The mean terminal disposition half-life of BSH was 3.61 hours and the vast majority of the BSH dose was excreted unchanged in the urine in 24 hours. Renal plasma BSH clearance was estimated
in two patients and was approximately 154.7 and 63.5 mL/min. Renal plasma clearance of the pooled boronated metabolites of BSH was significantly lower the renal plasma clearance of the parent drug and this may explain the change in the renal plasma boron clearance that was described in Chapter 3. Active renal tubular secretion may be an important excretion pathway of BSH elimination and as such, co-administration of BSH with a renal tubular secretion inhibitor, such as probenicid, may be a means to decrease the renal clearance of BSH.
<table>
<thead>
<tr>
<th>Assayed BSH Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.34</td>
</tr>
<tr>
<td>10.84</td>
</tr>
<tr>
<td>10.79</td>
</tr>
<tr>
<td>10.73</td>
</tr>
<tr>
<td>9.35</td>
</tr>
<tr>
<td>11.08</td>
</tr>
<tr>
<td>10.19</td>
</tr>
<tr>
<td>10.72</td>
</tr>
<tr>
<td>11.23</td>
</tr>
</tbody>
</table>

| Mean       | 10.59 |
| Standard Deviation | 0.56 |
| CV         | 0.053 |
| Error from target (%) | 5.57 |

Table 5.1. A statistical summary of the within-run precision (n=9) data for the developed LC-MS assay for BSH in plasma. The target BSH concentration was 10 μg/mL. All concentrations reported as μg/mL.
Table 5.2. A statistical summary of the between-run precision data for the developed LC-MS assay for BSH in plasma. Target BSH concentrations represent high (20 pg/mL), medium (5 pg/mL), and low (0.5 pg/mL) concentrations. All concentrations reported as µg/mL.
### Table 5.3. Summary of BSH pharmacokinetic parameters for patients (n=4) diagnosed with AA or GBM, who received a BSH dose of 88.2 mg/kg.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_1$ (L)</td>
<td>$11.94 \pm 3.78$</td>
</tr>
<tr>
<td>$k_{10}$ (min$^{-1}$)</td>
<td>$7.99 \times 10^{-3} \pm 5.04 \times 10^{-4}$</td>
</tr>
<tr>
<td>$k_{12}$ (min$^{-1}$)</td>
<td>$0.0120 \pm 0.0109$</td>
</tr>
<tr>
<td>$k_{21}$ (min$^{-1}$)</td>
<td>$0.0123 \pm 8.86 \times 10^{-3}$</td>
</tr>
<tr>
<td>$\alpha$ (min$^{-1}$)</td>
<td>$0.0291 \pm 0.0195$</td>
</tr>
<tr>
<td>$\beta$ (min$^{-1}$)</td>
<td>$3.20 \times 10^{-3} \pm 6.34 \times 10^{-4}$</td>
</tr>
<tr>
<td>$Cl_{t}$ (mL/min)</td>
<td>$95.65 \pm 30.82$</td>
</tr>
<tr>
<td>$\alpha ; t_{1/2}$ (hr)</td>
<td>$0.40$</td>
</tr>
<tr>
<td>$\beta ; t_{1/2}$ (hr)</td>
<td>$3.61$</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameters are expressed as mean ± standard deviation. The reported half-lives are harmonic mean values.
Table 5.4. Statistical summary of the calculated renal plasma BSH clearance values for patients #13 and #17.

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>Renal plasma BSH clearance for patient 13 (mL/min)</th>
<th>Renal plasma BSH clearance for patient 17 (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3 hours</td>
<td>68.1</td>
<td>56.7</td>
</tr>
<tr>
<td>3-6 hours</td>
<td>190.2</td>
<td>68.7</td>
</tr>
<tr>
<td>6-9 hours</td>
<td>161.8</td>
<td>116.2</td>
</tr>
<tr>
<td>9-12 hours</td>
<td>199.0</td>
<td>32.8</td>
</tr>
<tr>
<td>12-24 hours</td>
<td>BSH not detected*</td>
<td>43.1</td>
</tr>
</tbody>
</table>

Mean 154.7 63.5

Standard Deviation 59.9 32.5

* There was no detectable BSH in the 12-24 hour urine sample for patient #13. Due to instrument complications, this sample was not able to be re-assayed.
<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Boron (µg/mL)</th>
<th>BSH (µg/mL)</th>
<th>BSH/boron (µg/mL)</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>279</td>
<td>328.5</td>
<td>186.2</td>
<td>0.67</td>
</tr>
<tr>
<td>120</td>
<td>213</td>
<td>172.8</td>
<td>98.0</td>
<td>0.46</td>
</tr>
<tr>
<td>180</td>
<td>145</td>
<td>115.7</td>
<td>65.6</td>
<td>0.45</td>
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<tr>
<td>240</td>
<td>117</td>
<td>80.33</td>
<td>45.5</td>
<td>0.39</td>
</tr>
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<td>420</td>
<td>103.5</td>
<td>62.74</td>
<td>35.6</td>
<td>0.34</td>
</tr>
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<td>780</td>
<td>51</td>
<td>13.43</td>
<td>7.6</td>
<td>0.15</td>
</tr>
<tr>
<td>1515</td>
<td>18</td>
<td>0.58</td>
<td>0.3</td>
<td>0.02</td>
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Table 5.5. Comparison of boron, BSH, and BSH/boron (BSH concentration converted to boron equivalence) plasma concentration-time data (n=4). The term (f) is the fraction of the boron concentration that is accounted for as BSH.
<table>
<thead>
<tr>
<th>Urine Collection Interval (hr.)</th>
<th>A ex boron (mg)</th>
<th>A ex BSH/boron (mg)</th>
<th>f</th>
</tr>
</thead>
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<tr>
<td><strong>Patient 13</strong></td>
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<tr>
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<td>1060.2</td>
<td>0.98</td>
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<td>853.7</td>
<td>1.14</td>
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<td>9-24</td>
<td>308.1</td>
<td>79.4</td>
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Table 5.6. Comparison of the amount of boron excreted and the amount of BSH/boron (amount of BSH converted to boron equivalence) excreted for each urine collection interval. The term (f) is the fraction of the amount of boron excreted that is accounted for by BSH.
Figure 5.1. Above: Extracted ion chromatogram from a plasma calibration standard for the mass range 866.5 - 870.5 m/z, which corresponded to the internal standard pseudo-molecular ion [(B_{12}H_{12})^{2} + 3TBA^{+}]^+. Below: Extracted ion chromatogram from a plasma calibration standard for the mass range 898.5 - 902.5 m/z, which corresponded to the BSH pseudo-molecular ion [(B_{12}H_{11}SH)^{2} + 3TBA^{+}]^+. The units of the x-axis were minutes.
Figure 5.2. A typical calibration curve for the LC-MS assay of BSH in plasma. The equation for the linear regression of this data set was: \( Y = 0.1385X - 0.0796 \), and the \( R^2 \) value was 0.997.
Figure 5.3. Semi-logarithmic plot of the BSH plasma concentration-time profile from patient #10, used to fit a two-compartment open model with zero-order input and first-order elimination from the central compartment.
Figure 5.4. Semi-logarithmic plot of the BSH plasma concentration-time profile from patient #13, used to fit a two-compartment open model with zero-order input and first-order elimination from the central compartment.
Figure 5.5. Semi-logarithmic plot of the BSH plasma concentration-time profile from patient #14, used to fit a two-compartment open model with zero-order input and first-order elimination from the central compartment.
Figure 5.6. Semi-logarithmic plot of the BSH plasma concentration-time profile from patient #17, used to fit a two-compartment open model with zero-order input and first-order elimination from the central compartment.
Figure 5.7. Semi-logarithmic plot of the concentration-time profiles from patient #10 for both BSH (corrected for boron equivalence), and total boron following BSH administration.
Figure 5.8. Semi-logarithmic plot of the concentration-time profiles from patient #14 for both BSH (corrected for boron equivalence), and total boron following BSH administration.
Figure 5.9. Semi-logarithmic plot of the concentration-time profiles from patient #17 for both BSH (corrected for boron equivalence), and total boron following BSH administration.
Figure 5.10. Semi-logarithmic plot of the concentration-time profiles from patient #13 for both BSH (corrected for boron equivalence), and total boron following BSH administration.
Figure 5.11. Plot of the cumulative fraction of the BSH dose excreted in the urine of patient #17 versus the corresponding midpoint time of the urine collection interval.
Figure 5.12. Semi-logarithmic plot of renal BSH excretion rate versus midpoint time for patient #13.
Figure 5.13. Semi-logarithmic plot of renal BSH excretion rate \textit{versus} midpoint time for patient #17.
Figure 5.14. Plot of the free fraction of BSH ($f_u$) in plasma, versus the corresponding total BSH plasma concentration.
Figure 5.15. Scatchard plot of BSH plasma protein binding. The equation for the linear regression was: $Y = -3073.8X + 16945$ and the $R^2$ value was 0.91.
BIBLIOGRAPHY


CHAPTER 6

SIMULATION STUDIES TO DETERMINE THE OPTIMUM DOSING PARADIGM OF SODIUM BOROCAPATE FOR BORON NEUTRON CAPTURE THERAPY OF MALIGNANT GLIOMAS

INTRODUCTION

One of the goals of this Phase I clinical study was to use pharmacokinetic methods to provide scientific data to suggest an optimum dosing paradigm of BSH to maximize the potential efficacy of BNCT. Despite clinical use of BSH for over thirty years, there is uncertainty concerning the optimum BSH dosing schedule (single vs. multiple doses), the drug administration route (i.v. versus i.c.), and the interval between drug administration and initiation of BNCT (1-3).

Once the pharmacokinetic profile of a drug has been characterized, computer simulation can be a powerful tool to predict the outcome of various administration paradigms. When considering the specific case of BNCT, three pharmacokinetic parameters need to be investigated: the tumor boron concentration-time profile, the tumor:plasma, and the normal brain:plasma concentration ratios. All of these must be
optimized to improve the chances for success for using BSH as a capture agent. This Chapter describes computer simulation studies using the three-compartment open model, with emphasis on the tissue-compartmental pharmacokinetics, with various one-hour BSH i.v. infusion schedules to identify the optimum BSH dosing paradigm. These simulations were performed in an attempt to improve tumor boron uptake parameters by modifying the BSH dosing paradigm and to provide information to direct future studies of using BSH for the purpose of BNCT.

METHODS

TISSUE SAMPLE COLLECTION AND CLASSIFICATION

As described in detail in Chapter 2, a craniotomy was performed within 2 to 14 hours following termination of the BSH infusion for the purpose of tumor resection and tissue sample collection. During the resection, multiple samples of solid tumor, infiltrating tumor, and adjacent normal brain were collected. Each tissue sample was processed into multiple sections and analyzed as pairs, one for section histopathologic examination and the other for determination of the tissue boron concentration using the DCP-AES method described in Chapter 2. Because of the focus of this study, only patients who had a final histopathologic diagnosis of GBM or AA were used to evaluate the tissue boron kinetics (n=18).
Since there was no evidence that histopathologic diagnosis had any affect on the fitted pharmacokinetic parameters, the remaining five patients were not excluded from the boron pharmacokinetic or renal plasma boron clearance studies that were presented in Chapters 2 & 3.

Tissue samples were categorized as normal brain, infiltrating tumor, or solid tumor based on histopathologic examination that showed 80% or greater of the tissue area to be composed of the respective category. Tissue samples with extensive hemorrhagic or necrotic areas were excluded from the tissue boron kinetic analysis.

**DETERMINATION OF AVERAGE TISSUE BORON CONCENTRATIONS**

Due to the high variability and heterogeneity of boron concentrations in the collected tissue samples (normal brain, solid tumor, or infiltrating tumor), it was necessary to examine arithmetically averaged values so that the tumor pharmacokinetics could be evaluated. Geometric averaging of the tissue boron values was not appropriate due to the absence of detectable boron in tissue samples that met the defined histopathologic criteria. This is not to state that some patients had undetectable boron in their sampled tissues, only that multiple sections were taken from each patient’s sampled tissues and some of the sections had undetectable boron.

Mean tissue boron concentrations, for patients in the 50 mg boron/kg dose group having a final diagnosis of AA or GBM (n=14), were calculated by arithmetically averaging values of tissue samples of the same histopathologic type, at approximately the same time post-infusion, while attempting to keep a statistically acceptable number of
determinations for each group. Patients from the other two dose groups, 15 and 25 mg boron/kg, were not used in the tissue boron concentration profile analysis because the tissue boron concentrations from these two dose groups are proportionally smaller than concentrations from the 50 mg boron/kg group at analogous times. Separate analysis of the average tissue concentration-time profiles for the 15 and 25 mg boron/kg groups were not performed because the large variability in the data and relatively few time points in each group would not have resulted in statistically acceptable data. For the purposes of model fitting that is described below, the mean tissue boron values were used in conjunction with the mean time calculated for the corresponding time interval from which the data were averaged.

**DETERMINATION OF AVERAGE TISSUE:Cl BORON CONCENTRATION RATIOS**

The tissue:Cl ratios were calculated using each patient’s individual fitted pharmacokinetic parameters and the integrated equation for the central compartment boron concentration-time profile [Eq. (2.1.)] such that the concentration of boron in the central compartment (plasma) at the exact time of tissue sampling was calculated. The individual calculated tissue:Cl ratios were then averaged using the same method which was used to average the tissue boron concentration-time data. Because of linear pharmacokinetic behavior of boron disposition over the doses studied, tissue samples (normal brain, infiltrating tumor, or solid tumor) from all of the patients diagnosed with AA or GBM (n=18) were used in the concentration-ratio analysis.
Consequently, the analysis of tissue:C1 boron concentration ratio-time data included values from patients in all three dose groups in order to add to the number of samples for statistical considerations.

**COMPUTER SIMULATION OF COMpartMENTAL PHARMACOKINETICS**

Differential equations describing the boron concentration-time profile for tissue compartments 2 (C2) and 3 (C3) of the validated three-compartment model with zero-order input and first-order elimination from the central compartment were derived and integrated using the method of Laplace transformation (Appendix D & E). Using these integrated equations and the integrated equation for the central compartment [Eq. (2.1.)], programs were written for the WinNonlin computer software to simulate the concentration-time profile in C1, C2, and C3 (Appendices E-G) to correlate C2 or C3 to the mean tumor and normal brain tissue boron concentration-time data. To expand the applicability of the programs shown in Appendices F-H, multiple dose summation algorithms were added to allow for simulation of multiple dose scenarios.

Values for the plasma volume of distribution of the shallow tissue compartment (V2), and the deep tissue compartment (V3), from the three-compartment open model (Fig. 2.1.) were calculated using Eq. (6.1.) and (6.2.), which were derived from a three-compartment open model, assuming steady state conditions, where distribution clearance between the central compartment and each tissue compartment were equal. Arithmetic mean values of V2 and V3 were used for the purpose of compartmental simulations.
Simulations of the boron concentration-time profiles for the central compartment (plasma-C1), compartment two (shallow tissues-C2), and compartment three (deep tissues-C3) of the selected three-compartment model were performed using WinNonlin computer software. Boron concentration-time profiles for compartments C1, C2, and C3 were simulated using a one-hour infusion of 50 mg boron/kg, and an average subject weight of 70 kg. The calculated average weight of patients in the study was 63.4 kg, but an average weight of 70 kg was used for extrapolation to an American or European population. This weight discrepancy did not affect the results since the shape, but not the absolute magnitude, of the simulated curves were evaluated relative to the actual measured values. Once each compartment boron concentration-time profile was simulated, the corresponding C2:C1 and C3:C1 profiles were calculated and compared graphically to the mean tissue:C1 boron concentration ratios.
FITTING MODIFIED TISSUE COMPARTMENT EQUATIONS TO MEAN TISSUE BORON CONCENTRATION-TIME DATA

To provide further evidence of a correlation between the tissue boron values and one of the pharmacokinetic tissue compartments, the mean tissue boron concentration-time profiles from the 50 mg boron/kg dose group, from each histologic category (normal brain, solid tumor and infiltrating tumor), were fitted to modified integrated equations for boron disposition in compartments C2 and C3 of the selected three-compartment model. A term \( f \) was added to each exponential coefficient in the integrated equations describing the C2 and C3 concentration-time profiles. The \( f \) term normalized the tissue boron concentration-time data for the fraction of boron in the corresponding peripheral compartment that was accounted for in the tissue samples as shown in Eq. (6.3.).

Equation 6.3.

\[
C_n(t) = f \lambda_1\left(e^{-\alpha t} - e^{-\alpha t'}\right) + f \lambda_2\left(e^{-\beta t} - e^{-\beta t'}\right) + f \lambda_3\left(e^{-\gamma t} - e^{-\gamma t'}\right)
\]

Where:

- \( n = \) The number of the tissue compartment (2 or 3)
- \( \lambda_n = \) Coefficient of the exponential term
- \( f = \) Fraction of boron within compartment (n) contained in the tissue sample
- \( t = \) Time from start of infusion
- \( t' = \) Elapsed time from termination of infusion
The volumes and rate constants that comprised the exponential coefficients were set to constant values corresponding to the average parameters determined from the boron pharmacokinetic study (Chapter 2) such that the only parameter that was fitted was the $f$ term. The proposed models [Eq. (6.3.1)] were fit to the mean tissue boron concentration-time data using a one-hour infusion of 3500 mg boron (50 mg/kg x 70 kg) with equal weights. To determine if there were significant differences in $f$ estimates between the three tissue types, 95% confidence intervals were constructed.

**USING COMPUTER SIMULATIONS TO EVALUATE VARIOUS BSH DOSING PARADIGMS**

Different dosing schedules were examined using computer simulation of the C1, C2, and C3 concentration-time profiles, as well as the simulated C2:C1 and C3:C1 boron concentration ratio-profiles. All the doses used in the simulations were either equal fractions of 3500 mg boron (88.2 mg BSH/kg x 70 kg), or repeated 3500 mg boron doses, each given as one-hour i.v. infusions, with differing time intervals between each dose. A single 3500 mg i.v. infusion, corresponding to the experimental conditions used for this study, was used for comparison purposes. Once the boron concentrations were simulated, the corresponding ratios were calculated and these have been summarized for 6, 12, 24, and 48 hours following termination of the last dose. These times were selected for comparison since they represent potential times for BNCT following administration of BSH.
RESULTS

APPARENT BORON VOLUME OF DISTRIBUTION FOR THE SHALLOW TISSUE COMPARTMENT (V2) AND THE DEEP TISSUE COMPARTMENT (V3)

Values for the apparent boron volume of distribution for the shallow tissue compartment (V2) and the deep tissue compartment (V3) were necessary to perform computer simulations and fitting experiments with the tissue compartment concentration-time data. The values for V2 and V3 were calculated (n=23) to be 8.05±3.61 L and 47.23±19.41 L, respectively.

MEAN TISSUE BORON CONCENTRATIONS AND TISSUE:CI BORON CONCENTRATION RATIOS

A summary of the mean boron tissue concentration values for patients diagnosed with AA or GBM in the 50 mg boron/kg dose group, along with their corresponding time intervals and number of samples in each group are shown in Table 6.1. For computer fitting purposes, the mean tissue boron concentration values were used in conjunction with the mean time calculated for the corresponding time interval.

A summary of the mean tissue:plasma (C1) boron concentration ratios for samples taken from all patients diagnosed with AA or GBM, along with the corresponding time interval, and number of samples in each group are shown in Table 6.2. The calculated mean tissue (normal brain, solid tumor, and infiltrating tumor) boron
concentration ratios were compared graphically to the computer simulated compartmental ratio values, as shown in Fig. 6.1. It was observed that the mean tissue:plasma (C1) boron concentration ratio showed minimal change from the 3-7 hour clustered points to the 13-14 hour clustered time points, characteristic of the C2:C1 simulated concentration ratio profile. The C3:C1 concentration ratio increased approximately four-fold over the same time interval, which was inconsistent with the observed tissue:plasma boron concentration ratio data.

FITTING MODIFIED TISSUE COMPARTMENT EQUATIONS TO MEAN TISSUE BORON CONCENTRATION-TIME DATA

Semi-logarithmic plots of the fitted mean tissue boron concentration-time profiles are shown in Fig. 6.2.-6.4. The fits of the modified C2 equation to mean tissue boron concentration-time data demonstrated a superior randomness in the distribution of residuals and lower AIC values relative to the analogous fits of the modified C3 equation, suggesting mean tissue boron concentration-time data, for all three tissue types, shows greater correlation to C2 relative to C3.

The fitted estimates for $f$ using the C2 derived equation were 0.065, 0.043, and 0.027 for solid tumor, infiltrating tumor and normal brain, respectively. To determine if there were significant differences in $f$ estimates between the three tissue types, 95% confidence intervals were constructed. Three confidence intervals were constructed: solid tumor versus normal brain, infiltrating tumor versus normal brain, and solid tumor versus infiltrating tumor. If the 95% confidence interval for the difference in $f$ did not
contain a zero, then there was a significant difference between the two tissue types. The results suggest that the \( f \) for solid tumor (0.065) was significantly larger than \( f \) for normal brain (0.027) (95% confidence interval 0.0250-0.0524) and infiltrating tumor (0.043) (95% confidence interval 0.0096-0.0378). The \( f \) for infiltrating tumor was significantly larger than the \( f \) for normal brain (95% confidence interval 0.0037-0.0272).

**COMPUTER COMPARTMENTAL SIMULATIONS OF VARIOUS DOSING SCHEDULES OF BSH**

Different fractionated BSH dose schedules were examined using the above stated compartmental simulation approach. Figure 6.5. was the simulated plasma and tissue compartment boron concentration-time profiles representing a single 60 minute infusion of 3500 mg boron (88.2 mg BSH/kg) and corresponded to the experimental dosing conditions used in this study. Figure 6.6. was the simulated plasma and tissue compartment boron concentration-time profile representing four 60 minute infusions of 875 mg boron (3500 mg boron total dose) administered at six hour time intervals. Figure 6.7. was the simulated plasma and tissue compartment boron concentration-time profile representing four 60 minute infusions of 875 mg boron (3500 mg boron total dose) administered at 24 hour time intervals. Figure 6.8. was the simulated plasma and tissue compartment boron concentration-time profile representing two 60 minute infusions of 1750 mg boron (3500 mg boron total dose) administered at three hour time intervals.
Figure 6.9. was the simulated plasma and tissue compartment boron concentration-time profile representing four repeated 60 minute infusions of 3500 mg boron administered at 24 hour time intervals.

Once the boron concentrations were simulated, the corresponding concentration ratios were calculated and have been summarized in Tables 6.3.-6.5. for 6, 12, 24 and 48 hours following termination of the last dose. These times were selected for comparison since they represent potential times for BNCT following administration of BSH. The set of dose simulations representing four-repeated 60-minute i.v. infusions of 3500 mg boron each, given at 24-hour intervals are summarized in Table 6.6. for 6, 12, and 18 hours and correspond to the proposed multiple dose/multiple irradiation BNCT paradigm suggested by Gabel et al. (3). The single dose regimen appears to give the highest C2 concentration at each comparable time point, as well as C2:C1 ratio values equal to or higher than all fractionated multiple dose schedules. Repeated 50 mg boron/kg multiple dosing resulted in higher absolute C2 concentrations, but did not improve the C2:C1 ratio over what is achievable using a single dose.

DISCUSSION

Evaluation of any dosing paradigm for BNCT requires that three parameters need to be investigated: the tumor boron concentration-time profile, the tumor:plasma, and the normal brain:plasma boron concentration ratios. All of these must be optimized to improve the chances for success using BSH, or any other boron delivery drug, as a neutron capture agent.
Most importantly, a sufficient quantity of boron-10 must be localized in the tumor and the concentrations in contiguous normal brain and in the vascular compartment should be as low as possible to minimize damage of normal tissues.

Because of the high variability in tumor boron uptake and the relatively few time intervals at which the tumors were sampled, we could not fit the tumor boron concentration-time profile to its own discreet equation or incorporate a separate tumor compartment into the existing pharmacokinetic model. But because a pharmacokinetic compartment can be composed of tissues of many different histologic types, which share similar pharmacokinetic profiles but differ in their magnitude of drug binding, a correlation is possible between the tissue kinetics and the pharmacokinetic behavior exhibited by one of the tissue compartments in the three-compartment model.

A pharmacokinetic compartment is defined as an amount of drug that acts kinetically like a distinct, homogenous, well mixed amount of material (4,5). Although this may seem like an ambiguous definition, at times a pharmacokinetic compartment can be shown to have distinct, real world physiological relevance. This point has been addressed by various authors (6-8), and in some cases physiologic significance has been attributed to the characteristics of a peripheral tissue pharmacokinetic compartment.

It was observed that the mean tissue:C1 boron concentration ratio (Fig. 6.1.) showed minimal detectable change from the 3-7 hour sampling times to the 13-14 sampling times, characteristic of the C2:C1 simulated concentration ratio profile. The C3:C1 concentration ratio increased approximately four-fold over the same time interval, which was inconsistent with the observed tissue:C1 ratio data (Fig. 6.1.). These data are supported by those of Gabel et al. (3), who presented a plot of tumor:blood boron
concentration ratios for up to 72 hours after BSH infusion. Visual inspection of their plot suggests that their tumor:blood boron concentration ratios were essentially constant between 12 to 72 hours, which is characteristic of the simulated C2:C1 profile. Thus, according to the tissue:plasma boron concentration ratio analysis, normal brain, solid, and infiltrating tumor exhibited kinetic behavior identifiable with the shallow tissue compartment C2.

To further substantiate this observation, the programs used to simulate the C2 and C3 boron concentration-time profile were modified to provide models that could be fitted to the tissue boron concentration-time data. These models were designed to normalize the compartmental boron concentration-time profile for the fraction of the compartmental boron concentration contained in the tissue samples. In essence, these fits were attempts to superimpose the simulated curves for the C2 and C3 profiles on to the mean tissue boron concentration-time data (Fig. 6.2.-6.4.). The tissue boron concentration-time data were consistent with C2, whereas the fits for C3 showed marked deviation, especially at the 13-14 hour time points. Mean tissue boron concentration-time data fitted to C2, demonstrated a superior randomness in the distribution of residuals and lower AIC values relative to the mean tissue boron concentration-time profiles fitted to C3. This set of experiments complements and reinforces the observation from the boron concentration ratio data that the normal brain, solid and infiltrating tumor exhibited pharmacokinetic behavior identifiable to the C2 shallow tissue compartment.
However, it is not being suggesting that the normal brain and tumor samples were a pharmacokinetic compartment by themselves, but merely that they appeared to be part of the C2 compartment and differed in the degree of their binding of the boronated species and therefore showed kinetic profiles superimposable by some fraction to C2.

There was a statistical difference in the fitted fraction parameter demonstrating a higher fractional amount of boron in solid and infiltrating tumor, relative to normal brain (9). This result would be expected for any successful boron delivery agent used for BNCT. In other words, a greater amount of boron needs to partition into the tumor, both solid and infiltrating, relative to the surrounding normal brain tissue.

Correlating normal brain, solid, and infiltrating tumor to C2 allowed for investigation of several dosing schedules, using computer simulations, in the attempt to improve the biodistribution of boron following administration of BSH. The dosing conditions used for the simulations were based upon clinical applicability with respect to the infusion time intervals, and the equal fractionation of the highest dose (88.2 mg BSH/kg) used in our study. Based on these criteria, several sets of simulations were performed. As can be seen from the semi-logarithmic plots of the simulated boron concentration-time compartmental profiles for each dosing paradigm (Fig. 6.5.-6.8.), the central compartment C1 and the shallow tissue compartment C2 reach a rapid equilibrium within a few hours after administration and decline in parallel after equilibrium is attained. Since the boron disposition of normal brain, solid, and infiltrating tumor after BSH administration have been correlated to C2, this has the consequence of the inability to use a multiple dosing paradigm of BSH to achieve a larger tumor:plasma boron concentration ratio which is essential minimize potential toxicity.
A summary of the results from the simulations using equal fractionated one-hour i.v. infusion doses of BSH (Figs. 6.5.-6.8.) are shown in Tables 6.3.-6.5., and clearly show that the C2:C1 ratio cannot be improved over what is attainable for a single dose.

As is also shown in Tables 6.3.-6.5., multiple dosing of BSH only would be beneficial had the tumor boron disposition followed the C3 compartment profile. Had this been the case, significant improvements in the tumor:blood boron concentration ratio and modest improvements in absolute boron concentrations would have been predicted for up to 24 hours following termination of the last infusion.

It has been suggested (3) that four sequential i.v. infusions of BSH at 24-hour intervals along with tumor neutron irradiations 12 to 18 hours after each BSH infusion would be the best dosing paradigm. This has been tested using our computer simulation approach with four one-hour i.v. infusions of 3500 mg boron (88.2 mg BSH/kg x 70 kg) per dose, and the results are summarized in Table 6.6. While large multiple doses of BSH will yield higher absolute C2 concentrations, the C2:C1 concentration ratios would not be improved over what is attainable using a single dose. From our simulated data, it appears that this dosing paradigm would produce higher concentrations of boron in the C2 compartment, but also would result in proportionally higher C1 compartment concentrations that may result in significant toxicity.
CONCLUSION

Due to the apparent kinetic correlation between tumor, normal brain and compartment C2, multiple i.v. doses of BSH will not improve the tumor:plasma boron ratio over what is achievable for a single infusion dose, although absolute tumor boron concentrations might be increased with corresponding increases in blood boron concentrations. This is supplemental to the conclusion from a previous tissue biodistribution study that a single dose of BSH, administered i.v., will not result in therapeutically useful boron concentrations in GBM or AA (9).
Table 6.1. Arithmetic mean boron concentrations in solid tumor, infiltrating tumor, and normal brain for patients in the 50 mg boron/kg dose group with post-operative diagnosis of glioblastoma multiforme or anaplastic astrocytoma. Tissue concentrations are expressed mean±SD with units of µg/mL.

n = number of samples in histologic category in each averaged time interval.
Table 6.2. Mean tissue:plasma (C1) boron concentration ratios for all patients diagnosed with anaplastic astrocytoma or glioblastoma multiforme. Boron concentration ratios are expressed as mean±SD when n>1.

n = number of samples in each histopathologic group.
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<th>Fractionated dose every 6 hours x 4</th>
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<td>48 hours</td>
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Table 6.3. Simulated compartmental boron concentrations (µg/mL) and their concentration ratios at various times following single and fractionated one-hour BSH i.v. infusion dose schedules. All doses represent one-hour infusions of BSH (total dose equal to 3500 mg boron corresponding to 88.2 mg BSH/kg x 70 kg) administered in equal fractions when more than one dose is given. Corresponds to simulated multiple dose curves in Figure 6.6.

^ Corresponds to time interval from termination of last BSH infusion.
Table 6.4. Simulated compartmental boron concentrations (μg/mL) and their concentration ratios at various times following single and fractionated one-hour BSH i.v. infusion dose schedules. All doses represent one-hour infusions of BSH (total dose equal to 3500 mg boron corresponding to 88.2 mg BSH/kg x 70 kg) administered in equal fractions when more than one dose is given. Corresponds to simulated multiple dose curves in Figure 6.7.

^ Corresponds to time interval from termination of last BSH infusion.
Table 6.5. Simulated compartmental boron concentrations (µg/mL) and their concentration ratios at various times following single and fractionated one-hour BSH i.v. infusion dose schedules. All doses represent one-hour infusions of BSH (total dose equal to 3500 mg boron corresponding to 88.2 mg BSH/kg x 70 kg) administered in equal fractions when more that one dose is given. Corresponds to simulated multiple dose curves in Figure 6.8.

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Corresponds to time interval from termination of last BSH infusion.
Table 6.6. Simulated central compartment and tissue compartment C2 boron concentrations (pg/mL) and their concentration ratios at various times following repeated one-hour BSH i.v. infusion dose schedules. BSH administered as a one-hour infusion of 3500 mg boron (88.2 mg/kg BSH x 70 kg) every 24 hours for four days representing multiple dose and multiple irradiation BNCT schedule, as shown in Figure 6.8.
Figure 6.1. Plot of tissue compartment:C1 boron concentration ratios from computer simulations and mean patient tissue:plasma (C1) boron concentration ratios. Tissue boron concentration ratios are represented as mean±SD when n>1.
Figure 6.2. Above: Semi-logarithmic plot of modified C2 equation fitted to mean normal brain tissue boron concentration-time data from patients in the 50 mg boron/kg dose group diagnosed with AA or GBM.

Below: Semi-logarithmic plot of modified C3 equation fitted to mean normal brain tissue boron concentration-time data from patients in the 50 mg boron/kg dose group diagnosed with AA or GBM.
Figure 6.3. Above: Semi-logarithmic plot of modified C2 equation fitted to mean solid tumor tissue boron concentration-time data from patients in the 50 mg boron/kg dose group diagnosed with AA or GBM.

Below: Semi-logarithmic plot of modified C3 equation fitted to mean solid tumor tissue boron concentration-time data from patients in the 50 mg boron/kg dose group diagnosed with AA or GBM.
Figure 6.4. Above: Semi-logarithmic plot of modified C2 equation fitted to mean infiltrating tumor tissue boron concentration-time data from patients in the 50 mg boron/kg dose group diagnosed with AA or GBM.

Below: Semi-logarithmic plot of modified C3 equation fitted to mean infiltrating tumor tissue boron concentration-time data from patients in the 50 mg boron/kg dose group diagnosed with AA or GBM.
Figure 6.5. Semi-logarithmic plot of the simulated plasma and tissue compartment boron concentration-time profiles representing a single 60-minute i.v. infusion of 3500 mg boron (88.2 mg BSH/kg).
Figure 6.6. Semi-logarithmic plot of the simulated plasma and tissue compartment boron concentration-time profile representing four 60-minute infusions of 875 mg boron (3500 mg boron total dose) administered at six-hour time intervals.
Figure 6.7. Semi-logarithmic plot of the simulated plasma and tissue compartment boron concentration-time profile representing four 60-minute infusions of 875 mg boron (3500 mg boron total dose) administered at 24-hour time intervals.
Figure 6.8. Semi-logarithmic plot of the simulated plasma and tissue compartment boron concentration-time profile representing two 60-minute infusions of 1750 mg boron (3500 mg boron total dose) administered at three-hour time intervals.
Figure 6.9. Semi-logarithmic plot of the simulated plasma and tissue compartment boron concentration-time profile representing four repeated 60-minute infusions of 3500 mg boron administered at 24-hour time intervals.
BIBLIOGRAPHY


CHAPTER 7

SUMMARY

A pharmacokinetic model was developed to describe the boron plasma disposition after intravenous administration of BSH. The disposition of boron was modeled as a three-compartment open model with first-order elimination from the central compartment. The harmonic mean terminal boron disposition half-life was 77.79 hours, and the mean total body plasma boron clearance was 14.37±3.51 mL/min. Using the empirically determined plasma:blood boron ratio of 1.29, total body blood boron clearance was calculated as 18.54±4.53 mL/min. The boron pharmacokinetic parameters reported in this study are in reasonable agreement with previously published boron pharmacokinetic parameters when the study designs were adequate.

A pharmacokinetic correlation was established between the shallow tissue pharmacokinetic compartment (C2) and the collected solid tumor, infiltrating tumor, and normal brain samples. Computer simulations of various boron disposition parameters, using several fractionated and repeated intravenous BSH dosing schedules, were evaluated using the tissue–C2 correlation.
Due to the apparent kinetic correlation between tumor, normal brain and the pharmacokinetic compartment C2, multiple i.v. doses of BSH will not improve the tumor:plasma boron ratio over what is achievable using a single infusion dose, although absolute tumor boron concentrations might be increased with corresponding increases in blood boron concentrations, which increases the probability of toxicity.

Multiple boronated compounds were detected in the urine of patients who had been administered BSH by TLC techniques. Using TLC experiments, it was determined that disulfide bonds were part of the chemical structure of the boronated metabolites. It was also determined, using ninhydrin TLC studies, that there were metabolites of BSH that were not protein bound, although there may have been the presence of a protein (amino acid) bound metabolite. ESI-MS scans were used to correlate observed boron ion clusters to chemical structures of putative BSH metabolites including: BSH sulfenic acid (BSOH), BSH sulfinic acid (BSO₂H), BSH disulfide (BSSB), BSH thiosulfinate (BSOSB), and a BSH-S-cysteine conjugate (BSH-CYS). The metabolite chemical structures that have been correlated to the ESI-MS, ESI-MS-MS, and accurate mass data, are reasonable biotransformations for sulphydryl containing compounds. The biological importance of these metabolites has yet to be evaluated. The presence of a boronated ion cluster, that is consistent with the chemical structure for the proposed BSH biotransformation product BSO₂H, was the only biotransformation product that was detected in the patient’s urine after 24 hours, and was undetectable after 48 hours. The boronated BSH biotransformation products residing in the body for the last 3 days of sampling (48 hours – 120 hours post dose) have yet to be identified.
It has been established that the ESI-MS scans can be performed on brain tissue homogenates (data not shown) such that, the molecular weights of the boron compounds localized in the tumor tissue may be determined. This could lead to the identification of the tumor localizing boronated species and possible improvements in tumor boron uptake.

An ESI LC-MS assay for BSH has been developed. The utility of the LC-MS assay was demonstrated by evaluating the BSH pharmacokinetics in a subset of the patients who enrolled in this study. Semi-logarithmic plots of the BSH concentration-time profile showed apparent bi-exponential decay, characteristic of a two-compartment open model. The mean terminal disposition half-life of BSH was 3.61 hours and the vast majority of the BSH dose was excreted unchanged in the urine in 24 hours. Renal plasma BSH clearance was estimated in two patients and was approximately 154.7 and 63.5 mL/min. Renal plasma clearance of the pooled boronated metabolites of BSH was significantly lower the renal plasma clearance of the parent drug and this may explain the observed change in the renal plasma boron clearance. Active renal tubular secretion may be an important excretion pathway of BSH elimination and, as such, co-administration of BSH with a renal tubular secretion inhibitor, such as probenicid, may be a means to decrease the renal clearance of BSH, effectively decreasing the total body BSH clearance and increasing the AUC. The net result may be a longer circulation time for BSH and, were BSH identified as a tumor localizing compound, higher tumor boron concentrations.
APPENDIX A

AUC EQUATIONS DERIVED FOR A THREE COMPARTMENT OPEN MODEL WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
\[ \text{AUC}_0 \rightarrow \tau = \frac{R_0(k_31 + k_21)}{Vd \alpha \beta \gamma} + \left[ \frac{R_0(k_31 + k_21 - \alpha)}{Vd(-\alpha)(\beta - \alpha)(\gamma - \alpha)} \right] e^{-\alpha t} + \left[ \frac{R_0(k_31 + k_21 - \beta)}{Vd(-\beta)(\alpha - \beta)(\gamma - \beta)} \right] e^{-\beta t} + \left[ \frac{R_0(k_31 + k_21 - \gamma)}{Vd(-\gamma)(\alpha - \gamma)(\beta - \gamma)} \right] e^{-\gamma t} \]

\[ + \frac{R_0 k_21 k_31}{Vd} \left[ \frac{\alpha \beta (\gamma t - 1) - \alpha \gamma - \beta \gamma}{(\alpha \beta \gamma)^2} + \frac{1}{\alpha^2(\beta - \alpha)(\gamma - \alpha)} e^{-\alpha t} + \frac{1}{\beta^2(\alpha - \beta)(\gamma - \beta)} e^{-\beta t} + \frac{1}{\gamma^2(\alpha - \gamma)(\beta - \gamma)} e^{-\gamma t} \right] \]

\[ \text{AUC} \quad \tau \rightarrow \infty = \frac{A_{\text{max}} + B_{\text{max}} + C_{\text{max}}}{Vd \times k10} + \]

\[ \left[ \frac{A_{\text{max}}}{Vd} \left[ (k_{21} - \alpha)(k_{31} - \alpha) \right] + \frac{B_{\text{max}}}{Vd} k_{21} \left( k_{31} - \alpha \right) + \frac{C_{\text{max}}}{Vd} k_{31} \left( k_{21} - \alpha \right) \right] e^{-\alpha t'} \]

\[ + \left[ \frac{A_{\text{max}}}{Vd} \left[ (k_{21} - \beta)(k_{31} - \beta) \right] + \frac{B_{\text{max}}}{Vd} k_{21} \left( k_{31} - \beta \right) + \frac{C_{\text{max}}}{Vd} k_{31} \left( k_{21} - \beta \right) \right] e^{-\beta t'} \]

\[ + \left[ \frac{A_{\text{max}}}{Vd} \left[ (k_{21} - \gamma)(k_{31} - \gamma) \right] + \frac{B_{\text{max}}}{Vd} k_{21} \left( k_{31} - \gamma \right) + \frac{C_{\text{max}}}{Vd} k_{31} \left( k_{21} - \gamma \right) \right] e^{-\gamma t'} \]
Vd = Volume of Distribution of the Central Compartment

Ro = Infusion rate

\( \alpha \) = Fast disposition first-order rate constant

\( \beta \) = Intermediate disposition first-order rate constant

\( \gamma \) = Slow disposition first-order rate constant

k10 = first-order elimination rate constant

k21 and k31 = first-order distribution rate constants

\( t \) = time

\( \tau \) = infusion time

\( t' = t - \tau \)

A_{max} = Amount in Central Compartment at time = \( \tau \)

B_{max} = Amount in shallow tissue compartment at time = \( \tau \)

C_{max} = Amount in deep tissue compartment at time = \( \tau \)
APPENDIX B

CALCULATED RENAL PLASMA BORON CLEARANCE VALUES FOR EACH PATIENT
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Tn = endpoint time for the urine collection interval
Tn' = Tn - τ (infusion time)
Aex = amount of boron excreted
Clr = Renal plasma boron clearance
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<td>193.8</td>
<td>13.36</td>
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<td></td>
<td>2700</td>
<td>2640</td>
<td>171.78</td>
<td>8.67</td>
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<td>4140</td>
<td>4080</td>
<td>24.64</td>
<td>2.54</td>
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<td>5580</td>
<td>5520</td>
<td>12.075</td>
<td>1.74</td>
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<td>7020</td>
<td>6960</td>
<td>0</td>
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</tbody>
</table>

Tn = endpoint time for the urine collection interval
Tn' = Tn – τ (infusion time)
Aex = amount of boron excreted
Clr = Renal plasma boron clearance
APPENDIX C

AUC EQUATIONS DERIVED FOR A TWO COMPARTMENT OPEN MODEL WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
$$AUC_0 \rightarrow \tau = \frac{Ro}{\alpha \beta V_1} e^{-\alpha t} + \frac{Ro}{(-\alpha)(\beta - \alpha)V_1} e^{-\alpha t} + \frac{Ro}{(-\beta)(\alpha - \beta)V_1} e^{-\beta t}$$

$$+ \frac{Ro}{(\alpha \beta)^2 V_1} \left[ \frac{1}{(\alpha - \beta)} \left( \alpha^2 e^{-\beta t} - \beta^2 e^{-\alpha t} \right) + \alpha \beta t - \alpha - \beta \right]$$

$$AUC_{\tau \rightarrow \infty} = \frac{A_{\max} k_{21}}{\alpha \beta V_1} + \frac{A_{\max} (k_{21} - \alpha)}{(-\alpha)(\beta - \alpha)V_1} e^{-\alpha t'} + \frac{A_{\max} (k_{21} - \beta)}{(-\beta)(\alpha - \beta)V_1} e^{-\beta t'}$$

$$+ B_{\max} k_{21} \left[ \frac{1}{\alpha \beta V_1} + \frac{1}{(-\alpha)(\beta - \alpha)} e^{-\alpha t'} + \frac{1}{(-\beta)(\alpha - \beta)V_1} e^{-\beta t'} \right]$$
V1 = Volume of Distribution of the Central Compartment
Ro = Infusion rate
α = Fast disposition first-order rate constant
β = Intermediate disposition first-order rate constant
k21 = first-order distribution rate constant
t = time
τ = infusion time
t' = t - τ
A_{max} = Amount in Central Compartment at time = τ
B_{max} = Amount in shallow tissue compartment at time = τ
APPENDIX D

EQUATION DESCRIBING THE CONCENTRATION-TIME PROFILE FOR THE SHALLOW TISSUE COMPARTMENT (C2) FOR A THREE-COMPARTMENT OPEN MODEL, WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
C2 = \frac{R_{o}k_{12}(k_{31} - \alpha)}{V2\alpha(\beta - \alpha)(\gamma - \alpha)} \left( e^{-\alpha t'} - e^{-\alpha t} \right) + \frac{R_{o}k_{12}(k_{31} - \beta)}{V2\beta(\alpha - \beta)(\gamma - \beta)} \left( e^{-\beta t'} - e^{-\beta t} \right) + \frac{R_{o}k_{12}(k_{31} - \gamma)}{V2\gamma(\alpha - \gamma)(\beta - \gamma)} \left( e^{-\gamma t'} - e^{-\gamma t} \right)

Where:

\text{V2} = \text{Volume of Distribution of the Shallow Tissue Compartment}

R_{o} = \text{Infusion rate}

\alpha = \text{Fast disposition first-order rate constant}

\beta = \text{Intermediate disposition first-order rate constant}

\gamma = \text{Slow disposition first-order rate constant}

k_{31} = \text{first-order distribution rate constant}

t = \text{time}

\tau = \text{infusion time}

t' = t - \tau

k_{12} = \frac{\alpha\beta + \alpha\gamma + \beta\gamma - k_{21}k_{31} - \left( \frac{\alpha\beta\gamma}{k_{21}k_{31}} \right) + (k_{31} + k_{21})}{k_{31}}

\frac{k_{21}}{k_{31}} \left( \frac{k_{31}(\alpha + \beta + \gamma - k_{31}) - \alpha\beta - \alpha\gamma - \beta\gamma + k_{21} \left( \frac{\alpha\beta\gamma}{k_{21}k_{31}} \right)}{k_{31} - k_{21}} \right)
APPENDIX E

EQUATION DESCRIBING THE CONCENTRATION-TIME PROFILE FOR THE DEEP TISSUE COMPARTMENT (C3) FOR A THREE-COMPARTMENT OPEN MODEL, WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
\[ C_3 = \frac{R \rho \left( k_{21} - \alpha \right)}{V_3 \rho (\beta - \alpha) (\gamma - \alpha)} \left( e^{-\alpha t'} - e^{-\alpha t} \right) + \frac{R \rho \left( k_{21} - \beta \right)}{V_3 \rho (\alpha - \beta) (\gamma - \beta)} \left( e^{-\beta t'} - e^{-\beta t} \right) + \frac{R \rho \left( k_{21} - \gamma \right)}{V_3 \rho (\alpha - \gamma) (\beta - \gamma)} \left( e^{-\gamma t'} - e^{-\gamma t} \right) \]

Where:

- \( V_3 \) = Volume of Distribution of the Shallow Tissue Compartment
- \( R \rho \) = Infusion rate
- \( \alpha \) = Fast disposition first-order rate constant
- \( \beta \) = Intermediate disposition first-order rate constant
- \( \gamma \) = Slow disposition first-order rate constant
- \( k_{21} \) = first-order distribution rate constants
- \( t \) = time
- \( \tau \) = infusion time
- \( t' = t - \tau \)

\[ k_{13} = \frac{k_{31} (\alpha + \beta + \gamma - k_{31}) - \alpha \beta - \alpha \gamma - \beta \gamma + k_{21} \left( \frac{\alpha \beta \gamma}{k_{21} k_{31}} \right)}{k_{31} - k_{21}} \]
APPENDIX F

COMPUTER PROGRAM WRITTEN FOR WINONLIN SOFTWARE FOR SIMULATION OF THE CONCENTRATION-TIME PROFILE OF THE CENTRAL COMPARTMENT FOR A THREE-COMPARTMENT OPEN MODEL WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
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\[ \text{del} = t - ti \]
\[ t_{\text{star}} = \max(0, \text{del}) \]
remark \( k_0 \) is equal to infusion rate
remark \( d \) is equal to dose
remark \( a_1, b_1 \) and \( c_1 \) are exponential coefficients
remark \( t_i \) is equal to \( \tau \) (infusion time)
\[ k_0 = \frac{d}{t_i} \]
\[ a_1 = k_0 \times (k_{21} - \alpha) \times (k_{31} - \alpha) \]
\[ a_1 = a_1 / (v \times \alpha \times (\gamma - \alpha) \times (\beta - \alpha)) \]
\[ b_1 = k_0 \times (k_{21} - \beta) \times (k_{31} - \beta) \]
\[ b_1 = b_1 / (v \times \beta \times (\gamma - \beta) \times (\alpha - \beta)) \]
\[ c_1 = k_0 \times (k_{21} - \gamma) \times (k_{31} - \gamma) \]
\[ c_1 = c_1 / (v \times \gamma \times (\alpha - \gamma) \times (\beta - \gamma)) \]
\[ \text{amt} = a_1 \times \exp(-\alpha \times t_{\text{star}}) - \exp(-\alpha \times t) + \]
\[ b_1 \times \exp(-\beta \times t_{\text{star}}) - \exp(-\beta \times t) + \]
\[ c_1 \times \exp(-\gamma \times t_{\text{star}}) - \exp(-\gamma \times t) \]
\[ \text{sum} = \text{sum} + \text{amt} \]
\[ \text{next} \]
\[ f = \text{sum} \]
\[ \text{end} \]
\[
\text{seco} \]
\[ d = \text{con}(2) \]
\[ t_i = \text{con}(4) - \text{con}(3) \]
\[ k_0 = \frac{d}{t_i} \]
\[ a_1 = k_0 \times (k_{21} - \alpha) \times (k_{31} - \alpha) \]
\[ a_1 = a_1 / (v \times \alpha \times (\gamma - \alpha) \times (\beta - \alpha)) \]
\[ b_1 = k_0 \times (k_{21} - \beta) \times (k_{31} - \beta) \]
\[ b_1 = b_1 / (v \times \beta \times (\gamma - \beta) \times (\alpha - \beta)) \]
\[ c_1 = k_0 \times (k_{21} - \gamma) \times (k_{31} - \gamma) \]
\[ c_1 = c_1 / (v \times \gamma \times (\alpha - \gamma) \times (\beta - \gamma)) \]
\[ \text{cmax} = a_1 \times (1.0 - \exp(-\alpha \times t)) + \]
\[ b_1 \times (1.0 - \exp(-\beta \times t)) + \]
\[ c_1 \times (1.0 - \exp(-\gamma \times t)) \]
\[ k_{10} = \alpha \times \beta \times \gamma / (k_{21} \times k_{31}) \]
\[ e_1 = \alpha \times \beta + \alpha \times \gamma + \beta \times \gamma \]
\[ e_2 = k_{21} \times (\alpha + \beta + \gamma) \]
\[ e_3 = e_1 - e_2 - k_{10} \times k_{31} + k_{21} \times k_{21} \]
\[ k_{12} = e_3 / (k_{31} - k_{21}) \]
\[ k_{13} = (\alpha + \beta + \gamma) - (k_{10} + k_{12} + k_{21} + k_{31}) \]
\[ a = d^* (k_{21} \times \alpha * (\alpha - k_{31}) / (v \times (\gamma - \alpha) \times (\beta - \alpha))) \]
\[ b = d^* (k_{21} \times \beta * (k_{31} - \beta) / (v \times (\gamma - \beta) \times (\alpha - \beta))) \]
\[ c = d^* (k_{21} \times \gamma * (k_{31} - \gamma) / (v \times (\gamma - \alpha) \times (\beta - \gamma))) \]
\[ k_{10 \_hl} = -\text{dlog}(0.5)/k_{10} \]
\[ \alpha_{hl} = -\text{dlog}(0.5)/\alpha \]
beta_hl=-dlog(.5)/beta
gamma_hl=-dlog(.5)/gamma
auc = (a/alpha) + (b/beta) + (c/gamma)
cl=d/auc
aumcb=(a/(alpha*alpha) + b/(beta*beta) + c/(gamma*gamma))
aumc=aumcb + ti*auc/2.d0
mrt=(aumc/auc) - ti/2
vss=d*((aumc/auc) - ti/2.d0)/auc
cld2=k12*v
v2=cld2/k21
cld3=k13*v
v3=cld3/k31
end

EOM
APPENDIX G

COMPUTER PROGRAM WRITTEN FOR WINONLIN SOFTWARE FOR SIMULATION OF THE CONCENTRATION-TIME PROFILE OF THE SHALLOW TISSUE COMPARTMENT (C2) FOR A THREE-COMPARTMENT OPEN MODEL WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
Model I
remark three compartment constant infusion model - Peripheral #2
remark Christopher Gibson
remark The Ohio State University College of Pharmacy
remark Summer 2000
remark
remark Constants - #doses
remark dose 1
remark start time
remark endtime
remark etc. for each dose
command
nparm 6
nsec 0
pnames 'v2', 'k21', 'k31', 'alpha', 'beta', 'gamma'
end
func1
j=0
ndose = con(1)

do i=1 to ndose
j=j+3
if x <= con(j) then goto red
endif
next
red:
ndose = i-1
sum=0
j=0

do i=1 to ndose
j=j+3
t=x - con(j)
d=con(j-1)
ti=con(j+1) - con(j)
del=t-ti
tstar=max(0,del)
remark k0 is equal to infusion rate
remark d is equal to dose
remark a2 b2 and c2 are exponential coefficients
remark ti is equal to tau (infusion time)
remark e3 is equal to k12
k0=d/ti
el = alpha * beta + alpha * gamma + beta * gamma
el = el - k21 * k31
el = el - ((alpha * beta * gamma)/(k21 * k31)) * (k31 + k21)
el = el/k31
e2 = k31 * (alpha + beta + gamma - k31) - alpha * beta
e2 = e2 - alpha * gamma - beta * gamma
e2 = e2 + k21 * ((alpha * beta * gamma)/(k21 * k31))
e2 = e2 / (k31 - k21)
e2 = e2 * (k21/k31)
e3 = el - e2
a2 = k0 * e3 * (k31 - alpha)
a2 = a2 / (v2 * alpha * (gamma - alpha) * (beta - alpha))
b2 = k0 * e3 * (k31 - beta)
b2 = b2 / (v2 * beta * (gamma - beta) * (alpha - beta))
c2 = k0 * e3 * (k31 - gamma)
c2 = c2 / (v2 * gamma * (alpha - gamma) * (beta - gamma))
amt=a2*(exp(-alpha*tstar) - exp(-alpha*t)) + &
   b2*(exp(-beta*tstar) - exp(-beta*t)) + &
   c2*(exp(-gamma*tstar) - exp(-gamma*t))
sum=sum+amt
next
f=sum
end

EOM
APPENDIX H

COMPUTER PROGRAM WRITTEN FOR WINNONLIN SOFTWARE FOR SIMULATION OF THE CONCENTRATION-TIME PROFILE OF THE DEEP TISSUE COMPARTMENT (C3) FOR A THREE-COMPARTMENT OPEN MODEL WITH ZERO-ORDER INPUT AND FIRST-ORDER ELIMINATION FROM THE CENTRAL COMPARTMENT
Model 1
remark three compartment constant infusion model - Peripheral #3
remark Christopher Gibson
remark The Ohio State University College of Pharmacy
remark Summer 2000
remark***********************************************************************
remark Constants - #doses
remark dose 1
remark start time
remark endtime
remark etc. for each dose
command
nparm 6
nsec 0
pnames 'v3', 'k21', 'k31', 'alpha', 'beta', 'gamma'
end
func1
j=0
ndose = con(1)

do i=1 to ndose
j=j+3
if x <= con(j) then goto red
endif
next

red:
ndose = i-1
sum=0
j=0

do i=1 to ndose
j=j+3
t=x - con(j)
d=con(j-1)
ti=con(j+1) - con(j)
del=t-ti
tstar=max(0,del)
remark k0 is equal to infusion rate
remark d is equal to dose
remark a1 b1 and c1 are exponential coefficients
remark ti is equal to tau (infusion time)
k0=d/ti
\[
a_3 = k_{31} \cdot (\alpha + \beta + \gamma - k_{31}) - \alpha \cdot \beta \\
a_3 = a_3 - \alpha \cdot \gamma - \beta \cdot \gamma \\
a_3 = a_3 + k_{21} \cdot \left(\frac{\alpha \cdot \beta \cdot \gamma}{k_{21} \cdot k_{31}}\right) \\
a_3 = \frac{a_3}{(k_{31} - k_{21})} \\
a_3 = k_0 \cdot a_3 \cdot (k_{21} - \alpha) \\
a_3 = \frac{a_3}{(v_3 \cdot \alpha \cdot (\beta - \alpha) \cdot (\gamma - \alpha))} \\
b_3 = k_{31} \cdot (\alpha + \beta + \gamma - k_{31}) - \alpha \cdot \beta \\
b_3 = b_3 - \alpha \cdot \gamma - \beta \cdot \gamma \\
b_3 = b_3 + k_{21} \cdot \left(\frac{\alpha \cdot \beta \cdot \gamma}{k_{21} \cdot k_{31}}\right) \\
b_3 = \frac{b_3}{(k_{31} - k_{21})} \\
b_3 = k_0 \cdot b_3 \cdot (k_{21} - \beta) \\
b_3 = \frac{b_3}{(v_3 \cdot \beta \cdot (\alpha - \beta) \cdot (\gamma - \beta))} \\
c_3 = k_{31} \cdot (\alpha + \beta + \gamma - k_{31}) - \alpha \cdot \beta \\
c_3 = c_3 - \alpha \cdot \gamma - \beta \cdot \gamma \\
c_3 = c_3 + k_{21} \cdot \left(\frac{\alpha \cdot \beta \cdot \gamma}{k_{21} \cdot k_{31}}\right) \\
c_3 = \frac{c_3}{(k_{31} - k_{21})} \\
c_3 = k_0 \cdot c_3 \cdot (k_{21} - \gamma) \\
c_3 = \frac{c_3}{(v_3 \cdot \gamma \cdot (\alpha - \gamma) \cdot (\beta - \gamma))} \\
amt = a_3 \cdot (\exp(-\alpha \cdot t_{\star}) - \exp(-\alpha \cdot t)) + \\
\quad b_3 \cdot (\exp(-\beta \cdot t_{\star}) - \exp(-\beta \cdot t)) + \\
\quad c_3 \cdot (\exp(-\gamma \cdot t_{\star}) - \exp(-\gamma \cdot t)) \\
sum = \sum + amt \\
next \\
f = \sum \\
end \\

EOM
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**CHAPTER 2**


**CHAPTER 3**


**CHAPTER 4**


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CHAPTER 6


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