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Electrospray Ionization Mass Spectrometry Coupled To Reversed-Phase Ion-Pair High-Performance Liquid Chromatography for Quantitation of Sodium Borocaptate and Application To Pharmacokinetic Analysis

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We have developed a quantitative assay using electrospray ionization mass spectrometry coupled to reversed-phase ion-pair liquid chromatography (LC/MS) for quantitation of sodium borocaptate (BSH) in human plasma. The assay was developed using a Micromass Q-TOF II mass spectrometer equipped with an orthogonal electrospray source. The mobile phase was a 1:1 solution of methanol and 5 mM aqueous tetrabutylammonium acetate flowing at 0.2 mL/min, and the chromatography was performed using a Machery-Nagel Nucleosil C18 column. Plasma samples from patients who had received an intravenous infusion of sodium borocaptate ($Na_2B_{12}H_{11}SH$), frequently referred to as BSH, were prepared for analysis by precipitation with acetonitrile. Following this, the supernatants were collected, and 40 μL was injected onto the column for analysis. The LC/MS assay was linear over a BSH plasma concentration range of 20−0.5 μg/mL with acceptable variability for both intra- and interassay precision. The LC/MS assay was used to generate pilot pharmacokinetic data for the plasma disposition of BSH in humans. The disposition of BSH was found to be consistent with a two-compartment model with first-order elimination from the central compartment. The mean total body plasma clearance was 95.7 ± 30.8 mL/min and the harmonic mean terminal half-life was 3.6 h.

Boron neutron capture therapy (BNCT) is based on a nuclear capture reaction that occurs when ^{10}B, a nonradioactive constituent of natural elemental boron, is irradiated with low energy neutrons to produce high-energy α particles and recoiling ^{7}Li nuclei. Since BNCT is a binary system, neutron irradiation can be initiated when the tumor-to-normal-tissue ^{10}B concentration ratio is highest. In theory, this can result in greater selectivity and efficacy for treating locally aggressive cancers, such as malignant brain tumors. For BNCT to be successful, a sufficient amount of the ^{10}B-containing drug must be delivered to the tumor and enough neutrons must be absorbed by the ^{10}B atoms to produce a lethal $^{10}B(n,\alpha)^{7}Li$ capture reaction. Detailed information on various aspects of BNCT are provided in recent reviews by Barth et al.,[1] Soloway et al.,[2] and Coderre and Morris.[3]

Sodium undecahydromercapto-*closo*-dodecaborate, commonly referred to as sodium borocaptate ($Na_2B_{12}H_{11}SH$), or BSH, is a polyhedral borane that has been used clinically for BNCT of brain tumors in Japan[4,5] and Europe.[6] A high-performance liquid chromatographic (HPLC) assay has been developed[7] for BSH that uses monobromobimane to form an ultraviolet (UV)-absorbing adduct with the drug. This has been used to detect BSH in a pharmacokinetic study in rats.[8] We previously developed an HPLC assay for characterization of the pharmaceutical formulation of BSH,[9] but that assay was unable to detect the BSH at physiologically relevant levels. Using our HPLC assay[9] as a starting point, we now report a sensitive, specific, and reliable assay for BSH using electrospray ionization (ESI) that could be used to determine the pharmacokinetics of the drug in plasma. The LC/MS assay described in the present report has two advantages over existing assays for BSH: First, the selectivity afforded by mass spectrom-

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etry is a significant improvement over those assays using UV spectroscopic detection.[7−9] Second, the BSH does not need to be derivatized for detection, thereby reducing sample preparation time as compared to that previously reported.[7,8] As described below, using this assay, we have obtained pilot pharmacokinetic data for the plasma disposition of BSH in brain tumor patients who had received this drug as part of a phase I biodistribution study.

EXPERIMENTAL SECTION

Instrumentation. The BSH LC/MS assay was developed using a Micromass Q-TOF II (Micromass, Wythenshawe, U.K.) mass spectrometer equipped with an orthogonal electrospray source (Z-spray), operated in the positive ion mode. Sodium iodide was used for the positive ion mass calibration range of 100−2000 m/z. Electrospray conditions were as follows: capillary voltage, 3050 V; source temperature, 150 °C; cone voltage, 70 V; and source block temperature, 90 °C. The ESI nebulization and drying gases were nitrogen. To increase the assay sensitivity, the flow of collision gas was set to 0. The linear quadrupole (Q1) was set to pass ions from 100 to 2000 m/z into the pusher region of the time-of-flight (TOF) mass analyzer where they were scanned from 850 to 950 m/z for BSH and B12 pseudo-molecular ions. Masslynx v.3.4. (Micromass, Wythenshawe, U.K.) computer software was used to control all of the settings and operation of the QTOF II mass spectrometer 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 × 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 × 3.2-mm guard column that was filled with the same stationary phase. The mobile phase was a 1:1 solution of methanol and 5 mM aqueous tetrabutylammonium acetate (TBA). The pH of the mobile phase was not modified after preparation and was ∼8.5. The mobile phase flow rate was maintained at 0.2 mL/min and was split postcolumn using a microsplitter valve (Upchurch Scientific, Oak Harbor, WA) to ∼45 μL/min for introduction to the ESI source. The total assay time was 10 min/injection.

Reagents. Natural abundance BSH (19.6% in the isotopic form ^{10}B and 80.4% as ^{11}B) was purchased as a drug substance from Centronic, Ltd. (Croydon, U.K.). This was converted to a lyophilized drug product by the Pharmaceutical Service Division, University of Iowa (Iowa City, IA), and was stored in sterile vials, which had been flushed with nitrogen to reduce the oxygen content to <5%, thereby minimizing oxidation. Oxidation products of BSH were <0.25% of the total drug product and included BSSB ($B_{12}H_{11}SSB_{12}H_{11}$) and BSOSB ($B_{12}H_{11}SOSB_{12}H_{11}$), both of which were measured using an HPLC assay.[9] Tetrabutylammonium acetate (1.0 M solution), and sodium iodide were purchased from Sigma-Aldrich Chemical Co. (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 dodecahydrododecaborate ($Cs_2B_{12}H_{12}$) (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.

Clinical Study. A Phase I clinical study of the biodistribution and pharmacokinetics of BSH in patients with a diagnosis of malignant glioma was performed at The Ohio State University Medical Center and the Beijing Neurosurgical Institute and has been reported in detail elsewhere.[9] Patients who were admitted to the study had a preclinical diagnosis of either glioblastoma multiforme (GBM) or anaplastic astrocytoma (AA), both of which are high-grade, primary brain tumors. Each patient received a 1-h i.v. infusion of BSH at doses of 26.5 mg/kg (n = 3), 44.1 mg/kg (n = 3), or 88.2 mg/kg (n = 19), which corresponded to 15, 25, or 50 mg/kg of boron, respectively. Blood samples were collected at time intervals ranging 15 min to 120 h following termination of the infusion and were stored frozen (−20 °C) until analyzed for total boron concentration using direct-current plasma atomic emission spectroscopy (DCP-AES).[10] Only a subset of samples from patients in the 88.2 mg BSH/kg dose group were used for this study.

Preparation of Stock Solutions and Calibration Standards. Stock solutions were prepared by dissolving lyophilized BSH in deionized water and diluting this solution to a final concentration of 1 mg/mL. Immediately following this, the aqueous BSH solution was aliquoted into screw-top borosilicate tubes, purged with nitrogen, sealed, and frozen at −20 °C until they were used. A stock solution of 1 mg/mL $Cs_2B_{12}H_{12}$, designated B12, was used as an internal standard and was prepared in a similar way and stored at −20 °C. Plasma calibration standards were prepared by spiking blank human plasma (1 mL) with an appropriate volume of BSH stock solution to produce a concentration range of 20−0.5 μg/mL. A 10-μL portion of the 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 at the time each assay was performed.

Assay Procedure. Since the boron concentration had already been determined for the patient plasma samples,[9] 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 patients' plasma samples not requiring dilution. To each 1 mL plasma sample, 10 μL of the B12 internal standard solution was added. After the addition of the internal standard, a 200-μL aliquot was removed from each patients' sample and calibration standard and placed into a polypropylene microcentrifuge tube. A 400-μL portion of acetonitrile then was added to each tube to precipitate the plasma protein, following which the samples were vortex mixed and centrifuged at 10 000 rpm for 10 min. The supernatants then were removed and placed into autosampler vials containing 250-μL glass inserts. A 40-μL portion of each sample was injected onto the HPLC column for analysis. The recovery of BSH from the plasma protein precipitation was evaluated using our previously reported DCP-AES method[10] and was ∼97% (unpublished observations). Each standard was injected once, and each patient sample was injected in duplicate. The arithmetic mean BSH concentration was calcu-

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lated and was used for subsequent BSH pharmacokinetic analysis. To ensure instrument reliability, a control sample of known BSH concentration (20 μg/mL) was assayed after every 10 injections and at the end of each assay.

Extracted Ion Chromatograms. Since the instrument was collecting 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 pseudomolecular ions of BSH $[(B_{12}H_{11}SH)^{2-} + 3TBA^{1+}]^{1+}$ and B12 $[(B_{12}H_{12})^{2-} + 3TBA^{1+}]^{1+}$. Ions were extracted from the TIC over a mass range of 866.5−870.5 m/z for B12 and 898.5−902.5 m/z for BSH. To achieve maximum sensitivity, the extracted ion chromatograms accounted for all major isotope peaks for both BSH and B12. Although the BSH and B12 coeluted from the column, each molecule could be distinguished on the basis of the difference in weight of the respective pseudomolecular ion, from the extracted ion chromatograms, and integrated independent of each other. Each extracted ion chromatogram then was smoothed and integrated using Masslynx v.3.4. 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, which were obtained from the respective extracted ion chromatograms. The peak area ratios then were plotted versus the corresponding BSH concentration, and each curve was fitted by linear regression analysis. The instrument was recalibrated prior to each assay.

Assay Precision and Accuracy Determination. To determine the intra-run precision, a set of 10 μg/mL BSH standards ($n = 9$) was prepared and assayed sequentially. The arithmetic mean BSH concentration, standard deviation, coefficient of variation, and percent error from the theoretical BSH concentration were calculated from the resulting data. Three sets of BSH standards representing high, medium, and low concentrations (20, 5, and 0.5 μg/mL) were assayed in triplicate on three separate days to determine the inter-run precision. The concentration data then were pooled over each of the three separate assays, and the arithmetic mean concentration, standard deviation, coefficient of variation (CV), and percent error from the theoretical BSH concentration were 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 were used to obtain information for the pharmacokinetics of BSH. The exact infusion time and blood sampling times, rather than the scheduled infusion and sampling times, were calculated for each patient to properly evaluate the pharmacokinetics of BSH. Semilogarithmic plots of the BSH plasma concentration−time data revealed an apparent biexponential decay, which 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 then was used to fit the two-compartment model, using WinNonlin (Pharsight Corporation, Mountain View, CA) computer software, and the results were statistically summarized.

RESULTS AND DISCUSSION

Previously reported pharmacokinetic analyses of BSH have been based upon data derived from analytical methods that determined the total boron concentration of samples, irrespective of its chemical form following the administration of the drug.[4−6,9,12−16] Although these studies have provided data necessary for the development of BSH as a therapeutic agent, the true pharmacokinetics of the drug in humans has remained undetermined. We have developed an LC/MS assay for the quantitation of BSH in plasma and have used this to generate pilot data for the pharmacokinetics of BSH in brain tumor patients. The experimental conditions for the developed assay were designed to overcome the difficulties of coupling an ion-pair HPLC method, specifically when using quaternary ammonium compounds as ion-pairing agents,[17,18] to an electrospray mass spectrometer. Several other mass spectrometers were evaluated for the development of this assay, but only the Micromass Q-TOF II with the orthogonal ESI source (Z-spray) was found to be adequate. The Micromass Z-spray ESI source was resistant to precipitation of ion-pairing reagents that usually would cause a quenching of analyte ionization (fouling). The assay performed reliably over a wide concentration range of BSH and we were able to routinely run the mobile phase for more than 5 h without any appreciable fouling of the ESI source. Splitting the chromatographic effluent before introduction to the ESI source also significantly improved the performance of the assay, presumably by increasing the ESI efficiency.

Our LC/MS assay was linear over a BSH plasma concentration range of 20−0.5 μg/mL. Figure 1 shows representative extracted ion chromatograms for both BSH and the internal standard (B12) from a plasma calibration standard. The extracted ion chromatograms for all of the patients' plasma samples demonstrated acceptable peak shapes and were identical to the extracted ion chromatograms from the plasma calibration standards for both the BSH and the internal standard. Since ∼97% of the BSH was recovered following protein precipitation with acetonitrile, plasma protein binding of BSH was not a significant factor. As seen from the extracted ion chromatograms from blank human plasma samples, there were no interfering peaks for both BSH and B12. All calibration standard curves used for patient sample quantitation had R^2 values greater than 0.99.

A statistical summary of the intra-run precision data is shown in Table 1. The arithmetic mean BSH concentration was 10.59 ± 0.56 μg/mL (10 μg/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 inter-run precision data is shown in Table 2. The calculated arithmetic mean concentrations and corresponding standard deviations for the high (20 μg/mL), medium (5 μg/mL), and low (0.5 μg/mL) BSH concentrations were 19.99 ± 0.61, 4.71 ± 0.15, and 0.63 ± 0.10 μg/mL, respectively, and the corresponding calculated CV values were

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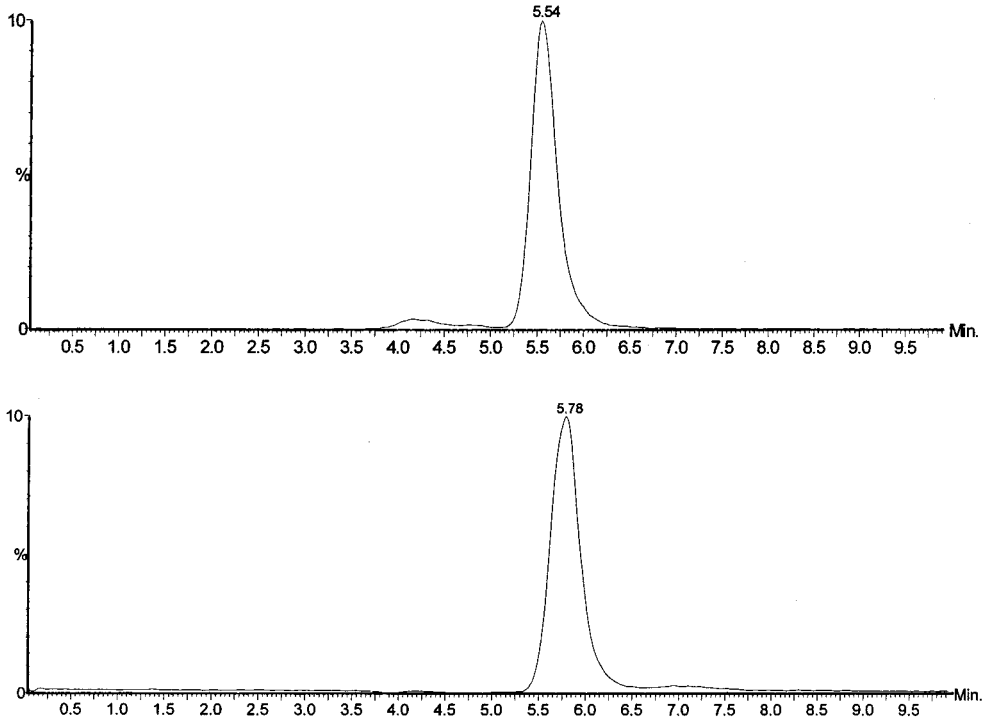

Figure 1. Upper: Extracted ion chromatogram from a plasma calibration standard for the mass range 866.5−870.5 *m*/*z*, which corresponded to the internal standard pseudomolecular ion $[(B_{12}H_{12})^{2-} + 3TBA^{1+}]^{1+}$. Lower: Extracted ion chromatogram from a plasma calibration standard for the mass range 898.5−902.5 *m*/*z*, which corresponded to the BSH pseudo-molecular ion $[(B_{12}H_{11}SH)^{2-} + 3TBA^{1+}]^{1+}$. The units of the *x* axis were in minutes.

Table 1. Statistical Summary of the Intrarun Precision Data for the LC/MS Assay of BSH in Plasma

	BSH concn (μg/mL) ($n = 9$)[a]
	10.34
	10.84
	10.79
	10.73
	9.35
	11.08
	10.19
	10.72
	11.23
mean	10.59
SD	0.56
CV	0.053
error from target (%)	5.57

[a] The target BSH concentration was 10 μg/mL. All concentrations reported as μg/mL.

Table 2. Statistical Summary of the Interrun Precision Data for the LC/MS Assay of BSH in Plasma

	target concn, μg/mL[a]		
	20	5	0.5
	19.88	4.57	0.62
	19.41	4.47	0.60
	20.33	4.54	0.62
	19.98	4.78	0.74
	19.53	4.91	0.76
	21.44	4.77	0.75
	19.65	4.86	0.49
	19.67	4.81	0.51
	20.00	4.65	0.55
mean	19.99	4.71	0.63
SD	0.61	0.15	0.10
CV	0.03	0.03	0.16
error from target (%)	0.06	5.87	20.21

[a] Target BSH concentrations represent high (20 μg/mL), medium (5 μg/mL), and low (0.5 μg/mL) concentrations.

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 larger than the others tested, these were acceptable, since 0.5 μg/mL was set as the limit-of-quantitation for the LC/MS assay.

The utility of our LC/MS assay for BSH was convincingly demonstrated by determining the plasma concentration−time profile in a subset ($n = 4$) of patients in the 88.2 mg BSH/kg dose group for the pilot pharmacokinetic analysis. Since there was a 2-year interval between collection of the patients' samples and the development of the LC/MS assay, we did not attempt to comprehensively characterize the pharmacokinetics of BSH using this assay because of questions relating to compound stability. However, unpublished data (Bauer, W. F., personal communication) indicate that the parent compound is stable for several years at −21 °C. Be that as it may, we have obtained useful preliminary information on the pharmacokinetics of BSH in a subset of patients. If any degradation or oxidation reaction occurred as a first-order chemical process, it would not be unreasonable to assume that the initial concentration of BSH for each sample would have been an equal percent higher than its assayed value. Therefore, any BSH clearance, AUC, and volumes of distribution obtained from these patients should be interpreted with some

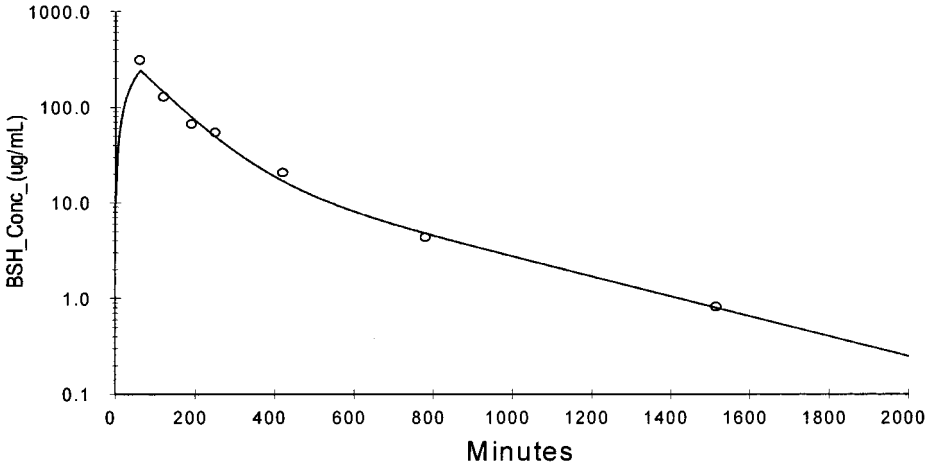

Figure 2. Semilogarithmic plot of the BSH plasma concentration−time profile from patient no. 13, used to fit a two-compartment open model with zero-order input and first-order elimination from the central compartment.

Table 3. Summary of BSH Pharmacokinetic Parameters for Patients Diagnosed with AA or GBM Who Received a BSH Dose of 88.2 mg/kg[a]

parameter	
$V1$ (L)	11.9 ± 3.8
$k10$ (min^{-1})	$7.99 \times 10^{-3} \pm 5.04 \times 10^{-4}$
$k12$ (min^{-1})	0.0120 ± 0.0109
$k21$ (min^{-1})	$0.0123 \pm 8.86 \times 10^{-3}$
α (min^{-1})	0.0291 ± 0.0195
β (min^{-1})	$3.20 \times 10^{-3} \pm 6.34 \times 10^{-4}$
Cl_T (mL/min)	95.7 ± 30.8
α $t_{1/2}$ (hr)	0.40
β $t_{1/2}$ (hr)	3.6

[a] Pharmacokinetic parameters are expressed as mean ± standard deviation. The reported half-lives are harmonic mean values. ($n = 4$).

Table 4. Comparison of Boron, BSH, and BSH/BoronPlasma Concentration−Time Data[a]

		concn, μg/mL			
	time (min.)	boron	BSH	BSH/boron	f[b]
patient A	60	279	328.5	186.2	0.67
	120	213	172.8	98.0	0.46
	180	145	115.7	65.6	0.45
	240	117	80.33	45.5	0.39
	420	103.5	62.74	35.6	0.34
	780	51	13.43	7.6	0.15
	1515	18	0.58	0.3	0.02
patient B	60	306	312.9	177.4	0.58
	120	171	129.8	73.6	0.43
	190	115	67.38	38.2	0.33
	250	99	55.11	31.2	0.32
	420	63	20.98	11.9	0.19
	780	42.5	4.395	2.5	0.06
	1515	21.6	0.827	0.5	0.02
patient C	60	291	287.6	163.04	0.56
	120	205.5	160.6	91.04	0.44
	180	141	114.1	64.68	0.46
	240	135	76.4	43.31	0.32
	420	108	16.1	9.13	0.08
	780	63	2.4	1.36	0.02
patient D	60	301.5	321.9	182.48	0.61
	130	193.5	173.5	98.36	0.51
	185	142.5	130.2	73.81	0.52
	240	112.5	69.3	39.29	0.35
	425	78	51.4	29.14	0.37
	595	51	20.9	11.85	0.23
	1365	21.5	2	1.13	0.05

[a] BSH concentrations have been converted to equivalent amounts of boron. [b] The term (f) is the fraction of the boron concentration that is accounted for as BSH ($n = 4$).

caution. However, the estimates of the first-order rate constants and their corresponding half-lives would not have been affected by the storage time.

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. A representative fitted patient BSH plasma concentration−time profile is shown in Figure 2, and the resulting BSH pharmacokinetic parameters have been summarized statistically in Table 3. The fit from the two-compartment model yielded acceptable variability in the fitted parameters and demonstrated a random distribution of residuals.[11] The mean total body plasma clearance of BSH was 95.7 ± 30.8 mL/min, and the harmonic mean terminal half-life of BSH was 3.6 h. On the basis of the value of the terminal disposition half-life for BSH, it can be predicted that the vast majority of the administered BSH dose would be eliminated from the body during the initial 24-hour period after dosing. This is in contrast to the model that we have developed to describe the boron disposition in plasma following administration of BSH.[9] This was a three-compartment open model with zero-order input and first-order elimination from the central compartment. Table 4 summarizes a comparison of the assayed concentration of BSH to the total boron measurements, as determined from our previous study,[9] for each patient. Since 88.2 mg/kg of BSH is equivalent to 50 mg/kg of boron, the BSH concentrations were converted using the appropriate correction factor to an equivalent amount of boron for comparison. It is noteworthy that a significant fraction of the boron in the patients' plasma at 120 min after administration is in a chemical form other than the parent drug, BSH. It is unknown if this was due to metabolism of BSH or if it was an artifact of the long plasma storage time. The terminal phase for the disposition of boron, which had a harmonic mean half-life of 77.8 hours,[9] probably was composed of one or more metabolites of BSH or in vivo oxidation products or both that have apparent first-order

elimination. Electrospray mass spectrometric analysis of the patients' urine samples 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_2H.[19] This was the only biotransformation product that was detected in the patients' urine after 24 h, and it was undetectable after 48 hours.[19] The boronated BSH biotransformation products residing in the body for the last 3 days of sampling (48 h − 120 h post dose) have yet to be identified.

The importance of the in vivo metabolites and oxidation products of BSH should not be underestimated, especially since they apparently have a much lower clearance from the body relative to the parent drug. Additional studies using fresh samples collected from patients receiving BSH would be needed to completely characterize the pharmacokinetics of BSH as well as to describe the relationship between the disposition of BSH and the disposition of boron following administration of the drug.

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