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High-performance liquid chromatographic assay for sodium mercaptoundecahydrododecaborate in rat tissues

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Abstract

Mercaptoundecahydrododecaborate (BSH) is an important agent for the boron neutron-capture therapy (BNCT). A sensitive high-performance liquid chromatographic (HPLC) method was developed for measuring BSH concentrations in rat tissues. Various tissue samples containing the drug were homogenized in a 1:1 (g/ml) mixture with phosphate buffered saline. The samples were then deproteinised with 4 volumes of acetonitrile and centrifuged. An aliquot of the supernatant was dried and reconstituted in 200 μl of Tris-HCl buffer. The samples were subjected to precolumn derivatization using the thiol reactive monobromobimane (mBB). The drug–mBB adduct was resolved by isocratic elution from a C_{18} reversed-phase column. The optimized mobile phase was methanol–0.02 *M* phosphate buffer (43:57, v/v) containing 0.01 *M* tetrabutylammonium dihydrogen phosphate as the ion-pairing agent with the final pH adjusted to 7.0. The flow-rate was set at 2.0 ml/min. The adduct was monitored by UV absorption at 373 nm. The analysis was completed in less than 15 min. The detection limit was 0.5 μg/ml (0.25 μg of boron). The assay method was linear over a concentration range of 0.5 to 50 μg/ml. This assay method could be used to evaluate the BSH concentrations in different tissues in studies on the targeted delivery of BSH.

1. Introduction

The boron-10 enriched compound sodium mercaptoundecahydrododecaborate (BSH), the structure of which is shown in Fig. 1, is one of the most useful agents for the boron neutron-capture therapy (BNCT) in the treatment of cancers [1,2]. BNCT is a binary procedure. First, stable and non-toxic boron-10 compounds are localized to malignant tumors. The tumor is then bombarded with thermal neutrons and the resultant cytotoxic α-radiation (with a short range of 10 μm) can cause cell death. The binary specificity of this mechanism permits the selective destruction of tumor cells in the presence of neighboring normal cells.

Though several methods are being employed for boron determination in biological samples, most of these require expensive instrumentation and/or involve complicated and time-consuming procedures [3,4]. Moreover, these methods cannot be used to specifically measure BSH concentrations. Recently, a method using Fourier

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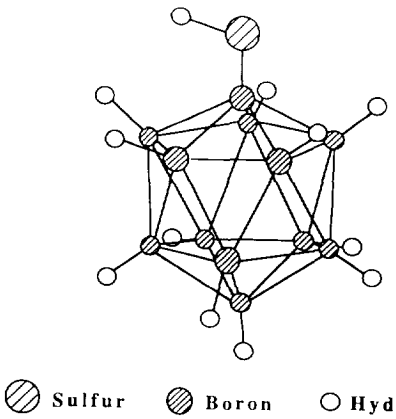

Fig. 1. Structure of mercaptoundecahydrododecaborate (BSH).

transform infrared spectroscopy (FT-IR) has been used for quantitative analysis of BSH in plasma and urine [5–7]. Although the method is simple and rapid, it is based on the measurement of the B–H band and is not specific for BSH measurement.

The ability of monobromobimane (mBB) to react with thiol groups such as cystine and N-acetylcystine provides a good mechanism for BSH derivatization [8–10]. Recently, a high-performance liquid chromatographic (HPLC) method for BSH measurement in rat plasma and rat urine has been developed in our laboratory using a precolumn derivatization technique with mBB [11]. The mercapto group of BSH is specifically alkylated by mBB to generate a UV sensitive adduct which elutes at a reasonable retention time. Due to the specificity of the reaction, no interference from other non-thiol boron compounds is expected. The BSH–bimane conjugate is separated on a C_{18} column using reversed-phase ion-paired chromatography and detected by a spectrophotometric detector. The method has been found to be easy, specific and sensitive.

In the present study the further development of the reversed-phase HPLC method is reported for the specific and sensitive detection of BSH in different rat tissues. A modification of the early procedure was made to avoid interferences from endogenous components of the tissues. This method could be useful in measuring BSH concentrations in different tissues in the studies of targeted delivery of BSH.

2. Experimental

2.1. Materials and reagents

Sodium mercaptoundecahydrododecaborate was manufactured by Centronic (Croydon, UK) and was a generous gift from Neutron Technology Company (Atlanta, GA, USA). Monobromobimane (mBB) was purchased from Molecular Probes (Eugene, OR, USA). Tetrabutylammonium dihydrogen phosphate was purchased from Aldrich (Milwaukee, WI, USA) and tris-(hydroxymethyl)aminomethane from Bio-Rad (Richmond, CA, USA). HPLC grade methanol, potassium hydroxide, hexane and hydrochloric acid were obtained from J.T. Baker (Philipsburg, NJ, USA). Potassium phosphate, monobasic and acetonitrile was from EM Science (Gibbstown, NJ, USA). Tissue samples (liver, kidney and brain) were obtained from healthy Sprague–Dawley rats and were frozen at −20°C until analysis. These tissue samples were selected due to the likelihood of BSH uptake by these tissues in animals.

2.2. Preparation of buffers

Phosphate buffer (pH 7.0) was prepared from 0.2 *M* potassium monobasic phosphate by adjusting the pH to 7.0 with 2 *M* potassium hydroxide solution and subsequent dilution with water. Tris-HCl buffer (pH 8.8) was prepared by dissolving 12.1 g of tris(hydroxymethyl)aminomethane in 50 ml of water, adding sufficient amount of 5 *M* HCl to lower the pH to 8.8 and making the volume to 100 ml with water.

2.3. Preparation of tissue samples

Preparation of liver and kidney tissue samples

A stock solution of 5 mg/ml BSH in phosphate buffered saline (PBS) was prepared. Serial dilutions of the stock solution were made. Liver

and kidney tissue samples were homogenized in a 1:1 ratio (g/ml) of PBS containing varying amounts of BSH. The final concentrations of BSH ranged from 0.5 to 50 μg/ml. Four volumes of acetonitrile were added to deproteinize the tissue samples. The sample was centrifuged at 3172 *g* for 10 min. A 1-ml volume of the supernatant was removed, filtered through a sterile 0.22-μm filter unit (Millex-PF, Millipore, MA, USA) and dried under a gentle stream of nitrogen. The sample was reconstituted in 200 μl of tris-HCl buffer (pH 8.8). A 25-μl volume of 5 mg/ml of the thiol reactive monobromobimane (mBB) in acetonitrile was added for precolumn derivatization. The reaction mixture was vortex-mixed and allowed to stand in the dark for 4 h for completion of the reaction [11]. A 200-μl volume of the mixture was transferred into a 250-μl polypropylene autosampler vial and 50 μl was injected onto the HPLC system.

Preparation of brain tissue samples

Brain tissue samples were homogenized in a 1:1 ratio (g/ml) of PBS which contained varying amounts of BSH. The final spiked concentrations ranged from 0.5 to 50 μg/ml. Four volumes of acetonitrile were added to deproteinize the tissues. The sample was centrifuged at 3172 *g* for 10 min. A 2-ml volume of the supernatant was removed and 8 ml of hexane were added to defat the tissue samples. The sample was centrifuged again at ca. 3172 *g* for 5 min and 1 ml of the aqueous phase was removed and processed as described previously.

2.4. Instrumentation

The chromatographic system consisted of a Waters (Milford, MA, USA) Model 510 pump, a Waters Lambda-Max Model 481 variable-wavelength detector and a Hewlett-Packard (Avondale, PA, USA) Model 3396 A integrator. An Allcott (Norcross, GA, USA) Model 738 autosampler was also used.

2.5. Chromatographic conditions

Chromatographic separation was achieved on a C_{18} Econosphere ODS (5 μm, 150 × 4.6 mm I.D.) analytical column (Alltech Chromatography, Deerfield, IL, USA) preceded by a Hypersil ODS 10 × 4.6 mm I.D. guard column. The optimized mobile phase was methanol–0.02 *M* phosphate (pH 7.0) buffer (43:57, v/v) containing 0.01 *M* tetrabutylammonium dihydrogen phosphate as the ion-pairing agent. The flow-rate was set at 2.0 ml/min and the BSH–mBB adduct was monitored at 373 nm.

2.6. Calibration curves

Calibration curves were prepared for BSH in the different tissue samples, e.g. liver, kidney and brain. The absolute peak heights were plotted against the different BSH concentrations and the curves were fitted by least square linear regression analysis.

2.7. Reproducibility

The precision of the method was evaluated in terms of inter-day and intra-day variability. Three sets of calibration standards were prepared on three different days in each of the liver, kidney and brain tissues and the samples were injected in triplicate. The mean peak height was plotted vs. the BSH concentration and the coefficient of variation (C.V.) was calculated for the inter-day variation. The intra-day variation was calculated for the three injections and was expressed in terms of relative standard deviation (R.S.D.). Accuracy and precision of the assay method were determined by injecting unknown spiked concentrations along with the standard curve. The results of the unknown spiked samples were expressed as % bias.

2.8. Recovery

For the determination of relative recovery of BSH from different tissues, BSH standard solutions (in PBS) were prepared at two different concentrations (5 μg/ml and 25 μg/ml of BSH). The samples were then treated exactly as described previously for tissue samples. Relative recovery was determined by comparing the peak heights obtained from the different tissue homogenates (liver, kidney and brain) to peak

heights obtained from the BSH standard solutions.

3. Results and discussion

To select a suitable mobile phase, different ion-pairing reagents were tried. With tetramethylammonium chloride (50 m*M*) as the ion-pairing agent and 0.02 *M* phosphate buffer pH 3 (the conditions used in our earlier studies [11]) the BSH adduct showed a retention time of about 8.5 min. However, some endogenous components of the tissue appeared to react with the mBB and gave some peaks coeluting along with the drug peak. Tetrabutylammonium dihydrogen phosphate was then used to delay the elution of the BSH–mBB adduct. Consequently, the percentage of the organic modifier (methanol) was increased from 20% to 43% so that the BSH–adduct peak eluted at a reasonable retention time. Under these conditions, other peaks were shifted to the short-retention time positions and a cleaner background was obtained for the BSH adduct. However, the BSH adduct peaks became asymmetric and broad. The pH of the phosphate buffer was then optimized to give a more narrow BSH adduct peak. Best results were obtained when the pH of the phosphate buffer was adjusted to 7.0.

In our previous studies using tetramethylammonium chloride as the ion-pairing agent, although less than 10 min were sufficient to elute the BSH–mBB adduct, the run time for each sample had to be ca. 45 min. This was necessary to allow for the elution of unreacted reagent and its hydrolysis product at 39 and 31 min, respectively [11]. In the present studies with the longer chain ion-pairing agent tetrabutylammonium dihydrogen phosphate, and 43% methanol in the mobile phase the BSH–mBB adduct is eluted in less than 10 min. The unreacted reagent and its hydrolysis product elute with the solvent front. This enabled a run time for each sample of about 15 min.

Under the experimental conditions used, the BSH adduct eluted at about 6.5 min. Fig. 2a,b shows typical liquid chromatograms in a blank liver tissue sample and a liver tissue sample spiked with BSH, respectively. The chromatograms obtained from kidney showed similar results. However, the brain tissue was treated with hexane to defat the tissue. Fig. 3a,b shows typical liquid chromatograms from a blank brain sample and a brain sample spiked with BSH, respectively, after hexane treatment. The BSH-adduct peak was well separated and showed no interference from any endogenous components of the tissues.

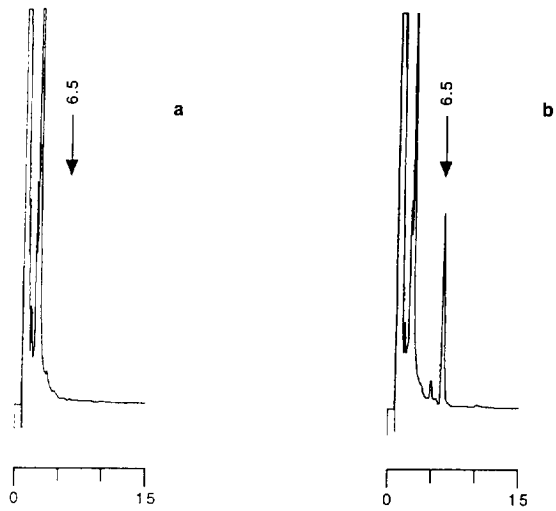

Fig. 2. Typical chromatograms in rat liver. (a) Blank liver, (b) liver spiked with 25 μg/ml of BSH. Mobile phase: methanol–0.02 *M* phosphate buffer (pH 7) (43:57, v/v) containing 0.01 *M* tetrabutylammonium dihydrogen phosphate; flow-rate 2 ml/min.

Table 1 shows the results of the relative recovery from the different tissues. The recovery of BSH at the lower concentration (5 μg/ml) varied from 64.1% in the brain to 86.9% in the kidney. At higher concentration (25 μg/ml) the recovery ranged form 61.1 to 78.1% for the different tissues. The recovery of BSH was the highest in kidney and the lowest in the brain tissue samples. This might be due to the fact that BSH binds to the brain tissue tighter than to the other tissues, or due to the irreversible covalent binding between the mercapto group of BSH and the thiol group of some biological compounds in the brain. The calibration curve parameters of BSH extracted from the different tissues are

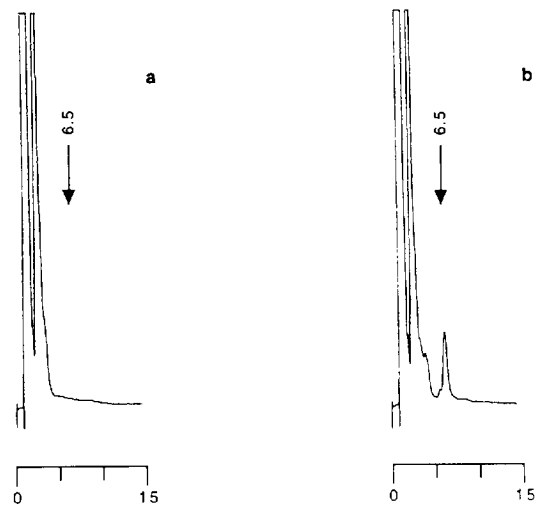

Fig. 3. Typical chromatograms in rat brain. (a) Blank brain, (b) brain spiked with 15 μg/ml of BSH. Mobile phase: methanol–0.02 *M* phosphate buffer (pH 7) (43:57, v/v) containing 0.01 *M* tetrabutylammonium dihydrogen phosphate; flow-rate 2 ml/min.

shown in Table 2. The correlation coefficients (r^2) in different tissues were found to be greater than 0.997.

The standard curves were linear over the concentration range 0.5–50 μg/ml. The lower limit of detection was found to be 0.5 μg/ml (about 0.25 ppm of boron) in kidney, brain and liver. The signal-to-noise ratio at this sensitivity level was 2. The intra-day and inter-day reproducibility data are summarized in Tables 3 and 4. In the intra-day study the average relative standard deviation for the assay of BSH in the different spiked tissues varied from 1.0 to 5.1%. Moreover, the inter-day data indicated good reproducibility with the average coefficient of variation ranging from 2.1 to 9.3%. Absolute peak heights were used in the construction of the calibration curves since better correlation was obtained with peak height rather than peak area. The accuracy and precision of the method were judged by the validation data obtained for unknown spiked concentrations of BSH in different tissues. The data is tabulated in Table 5. The % bias varied between 2.6 and 6% in the different tissues. It should be noted that no internal standard was used in the studies. Although a number of internal standards were considered, no suitable compound was found. The difficulty in using an internal standard in the present study

Table 1
Extraction recovery of BSH from different rat tissues

Tissue	Extraction recovery of BSH (%)
Concentration 5 μg/ml	
Liver	66.7
Kidney	86.9
Brain	64.1
Concentration 25 μg/ml	
Liver	71.0
Kidney	78.1
Brain	61.1

Table 2
Equations of calibration curves for the analysis of BSH in different tissues

Tissue	Curve fit	Correlation (r^2)
Liver	$y = -7468.6 + 2848.5x$	0.998
Kidney	$y = -3976.3 + 2931.5x$	0.999
Brain	$y = -4922.1 + 2425.7x$	0.997

Overall curves of the concentration range 0.5–50 μg/g of tissues; $n = 3$; detection limit, 0.5 μg/g of tissue.

Table 3
Intra-day variation in the different tissues ($n = 3$)

Sample	Spiked concentration (μg/ml)	Relative standard deviation (R.S.D.) (%)
Liver	5	3.9
	10	3.9
	25	2.2
	50	2.5
Kidney	5	3.9
	10	5.1
	25	2.9
	50	1.2
Brain	5	1.4
	10	1.6
	25	1.0
	50	2.0

Table 4
Inter-day variation in the different tissues ($n = 3$)

Sample	Spiked concentration (μg/ml)	Coefficient of variation (%)
Liver	5	9.3
	10	4.6
	25	4.7
	50	4.2
Kidney	5	3.7
	10	2.1
	25	3.7
	50	4.2
Brain	5	3.5
	10	5.5
	25	4.0
	50	2.1

is that it would compete with BSH for the mBB, which could present a potential problem especially in the analysis of unknown samples. Moreover, an internal standard, though desirable, is not essential in the method as described. Since external standards could be prepared along with the unknown samples and subsequently treated in parallel with the latter, an internal standard may not be necessary. Based on our experiments, the correlation was satisfactory and there appeared to be no need for an internal standard.

4. Conclusions

An HPLC method was developed for the determination of BSH in tissues. The method involves an ion-pairing chromatographic technique using mBB as a precolumn derivatization agent. The method was found to be simple, specific and sensitive. A modification in the ion-pairing agent and the percentage of methanol in the mobile phase was made to give a clean background for the separation of the BSH adduct. This modification also enabled the run time to be shortened to about 15 min as compared to the 45-min run time of our previous method. This assay method could be used to specifically evaluate the BSH concentrations in different tissues in targeted delivery of BSH.

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Table 5
Accuracy and precision data for BSH assay in tissues

Sample	Spiked concentration (μg/ml)	Predicted concentration[a] (μg/ml)	Bias[b] (%)
Liver	10	9.7	3.0
	25	24.3	2.6
Kidney	10	9.4	6.0
	25	24.5	2.2
Brain	10	10.3	3.0
	25	23.3	3.2

[a] Results are mean of three runs.
[b] Bias = (predicted concentration − actual concentration)/(actual concentration) · 100.

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