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Liquid chromatographic determination of sodium mercaptoundecahydrododecaborate in rat urine and plasma after precolumn derivatization

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the determination of disodium mercaptoundecahydrododecaborate (BSH) in biological fluids. Monobromobimane was used as a precolumn derivatizing agent. A stable derivative was obtained. The derivative was separated on a C_{18} column using reversed-phase ion-pairing chromatography and detected by a spectrophotometric detector at 373 nm. The detection limit was 200 ng/ml (0.1 ppm boron). Calibration curves were prepared for rat urine and plasma samples. The calibration curves were linear in the range of 1 μg/ml to 100 μg/ml for urine samples and 0.2 μg/ml to 50 μg/ml for plasma samples.

1. Introduction

Disodium mercaptoundecahydrododecaborate ($Na_2B_{12}H_{11}SH$), also known as BSH, is one of the most useful agents for boron neutron capture therapy (BNCT). The therapy is based on neutron irradiation of the tumor cells after being selectively bound with ^{10}B enriched compounds. The high-energy alpha particles that are generated by the nuclear reaction between neutrons and boron atoms are capable of destroying cells within 10 μm of the reaction site [1,2].

The analytical methods used for determination of the boron level in biological samples include colorimetric analysis, lower limit 0.5 ppm boron [3], prompt gamma-spectrometry, lower limit 1 ppm boron [4], atomic emission, lower limit 0.05 ppm boron [5], inductive coupled plasma atomic emission spectrometry (ICP-AES), lower limit 0.05 ppm boron [6], and isotachophoretic analysis, lower limit 75 ppm boron [7]. These methods, however, measure the concentration of total boron and can not be used to specifically measure BSH concentration. The methods also involve time-consuming sample preparation procedures, and/or the use of sophisticated equipment which is not widely available in clinical or research laboratories. Recently, a method using FTIR spectroscopy has been developed [8–10]. The lower limit of detection is ca. 2.5 ppm boron. Although sample preparation in the FTIR method is much easier, the method is still not specific enough for measurement of BSH

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since the determination is based on the B–H band.

Due to the wide availability of HPLC and the high sensitivity and specificity of this technique, it seems that development of an HPLC method for the determination of BSH in biological samples is attractive. However, there are several problems associated with that. Because of the highly hydrophilic nature of BSH, the compound can not be separated on hydrophobic reversed-phase HPLC columns. More importantly, BSH does not have prominent UV absorption properties and thus its measurement in biological samples using a UV detector is limited due to the extremely low signal-to-noise ratio. Recently, we have developed an HPLC method for BSH determination in biological samples using a pre-column derivatization technique. The method is easy, sensitive and specific for BSH. It is, thus, useful for pharmacokinetic and metabolic studies of BSH. A thiol reactive compound, monobromobimane (mBB), has been used to react with BSH prior to introduction of the sample into the HPLC column. The derivative has a reasonable retention time on the reversed-phase C_{18} column. It also has high absorption properties in the relatively clean visible region and hence improves the sensitivity of the spectrophotometric detection of BSH.

2. Experimental

2.1. Materials and reagents

BSH, manufactured by Centronic (Croydon, UK), was generously provided by Neutron Technology (Atlanta, GA, USA). Monobromobimane (mBB) was purchased from Molecular Probes (Eugene, OR, USA). Tetramethylammonium chloride was from Aldrich (Milwaukee, WI, USA), and tris(hydroxymethyl)aminomethane from Bio-Rad (Richmond, CA, USA). Methanol (HPLC grade), acetonitrile, potassium monobasic phosphate, phosphoric acid and hydrochloric acid were from J.T. Baker (Philipsburg, NJ, USA). Urine and plasma were obtained from male and female Sprague–Dawly rats which had never been exposed to BSH or any other drugs.

2.2. HPLC equipment and chromatographic conditions

The chromatographic system consisted of a Waters 510 pump, Waters Lambda-Max 481 L-C spectrophotometer (Waters, Milford, MA, USA), Hewlett-Packard 3396A integrator (HP, Avondale, PA, USA) and Alcott 738 autosampler (Alcott, Norcross, GA, USA) equipped with a 100-μL loop. Chromatographic separation was achieved on a Hypersil ODS (5 μm, 150 × 4.6 mm I.D.) analytical column (Alltech Chromatography, Deerfield, IL, USA) preceded by a 10 × 4.6 mm I.D. guard column filled with the same packing material. The mobile phase was methanol–0.05 *M* tetramethylammonium chloride in 0.02 *M* phosphate buffer, pH 3.0 (1:4, v/v). The flow-rate was maintained at 2.0 ml/min and the BSH–mBB adduct was detected at 373 nm.

2.3. Preparation of the buffers

Phosphate buffer, pH 3.0, was prepared from 0.2 *M* potassium monobasic phosphate by titrating to pH 3.0 with phosphoric acid 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 5 *M* HCl to bring the pH to 8.8 and then adjusting the volume to 100 ml with water.

2.4. Effect of incubation time on the reaction

To determine the adequate reaction time for the BSH–mBB reaction, 1 ml of a 50 μg/ml solution of BSH in Tris-HCl buffer was placed in a 1.5-ml amber glass autosampler vial. A 100-μl volume of a 5 mg/ml solution of mBB in acetonitrile was added. The vial was vortex-mixed, wrapped with aluminum foil and placed in the autosampler. Samples (50 μl) were taken at 5 min, 20 min, 1 h, then hourly up to 10 h and injected onto the HPLC system. Peak heights

detected at 373 nm were recorded. The procedure was repeated three times and the mean percentage of maximum response was plotted versus time.

2.5. Preparation of the calibration standards

A stock solution of 5 mg/ml BSH in water was prepared. For preparation of calibration standards of rat urine, 10–1000 μg/ml BSH standard solutions were made by serial dilutions of the stock solution with water. Aliquots of 450 μl of rat urine were placed each in a glass disposable culture tube and spiked with 50 μl of the appropriate BSH standard solution to provide urine concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 μg/ml. For the preparation of calibration standards of rat plasma, standard BSH solutions of concentrations ranging from 1 to 500 μg/ml were prepared from the 5 mg/ml BSH stock solution by serial dilution with water. Aliquots (120 μl) of rat plasma were placed each in a disposable polypropylene microcentrifuge tube. To each tube 30 μl of the appropriate BSH standard solution was added to provide BSH plasma concentrations of 0.2 to 100 μg/ml.

2.6. Assay procedure

Rat urine samples

Each urine calibration standard was diluted with two volumes of 1 *M* tris-HCl buffer, pH 8.8, filtered through a 0.22-μm membrane using a Millex-PF syringe filter unit (Millipore, Bedford, MA, USA) prior to derivatization with mBB. Aliquots (500 μl) were placed in polypropylene disposable microcentrifuge tubes and to each tube 50 μl of a 5 mg/ml solution of mBB in acetonitrile was added. The reaction mixture was vortex-mixed and allowed to stand in the dark for 4 h, then 200 μl were transferred into a 250-μl polypropylene autosampler vial and a 50-μl aliquot was injected onto the HPLC system.

Rat plasma samples

To 150 μl of plasma calibration standard in a polypropylene disposable microcentrifuge tube, 600 μl of acetonitrile was added to precipitate the plasma proteins. The tube was vortex-mixed and subsequently centrifuged at 1650 *g* for 5 min. A 600-μl volume of the supernatant was placed into a disposable glass culture tube and evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was dissolved in 100 μl of Tris-HCl buffer, pH 8.8. After addition of 20 μl of a 5 mg/ml solution of mBB in acetonitrile, the tube was vortex-mixed for 30 s and allowed to stand in the dark for 4 h. The reaction mixture was transferred into a polypropylene autosampler vial and a 50-μl volume was injected onto the chromatographic system.

2.7. Calibration curves

Calibration curves for both urine and plasma were prepared. The absolute peak heights were plotted versus BSH concentrations and the curves were fitted by least square linear regression analysis.

2.8. Reproducibility

Four sets of calibration standards were prepared both in urine and plasma. For each set, urine calibration standards were injected in triplicate, and plasma calibration standards were injected in duplicate. The mean peak height was plotted versus BSH concentration. The standard error and coefficient of variation of each point was calculated.

To determine the intra-assay variation in plasma, a 200-μl plasma standard was prepared, derivatized with monobromobimane and injected in triplicate. The relative standard deviation from the mean peak height of each plasma standard was calculated. The intra-assay variation in urine was also estimated from the relative standard deviation of the peak heights of each urine calibration standard.

2.9. Stability of the derivative in biological fluids

To test the stability of the BSH–mBB adduct a 50 μg/ml solution of BSH in plasma calibration

standard and a 100 μg/ml urine calibration standard were prepared, derivatized with monobromobimane and kept in the dark at room temperature. The samples were injected onto the HPLC after 4 h, 24 h and 48 h.

3. Results and discussion

Monobromobimane has been widely used for high sensitivity HPLC determination of biologically interesting thiols, such as cystine, N-acetylcystine, homocystine, glutathione, 6-mercaptopurine, and coenzyme A [11–16]. The thioether bonds formed by the reaction between monobromobimane and organic thiols are extremely stable and resist cleavage except under drastic chemical conditions.

In our studies, BSH reacts with monobromobimane under alkaline conditions, in a manner which appears similar to that of the organic thiols, to give a fairly stable BSH–mBB adduct. The mercapto group of BSH is specifically alkylated by the reagent to generate a UV-sensitive adduct. The chemical reaction is illustrated in Fig. 1. Due to the specificity of the reaction, no interference from other non-thiol boron compounds is expected. The derivatization results in a largely enhanced absorption of BSH at a longer wavelength. This approach improves the detection limit at an adequate signal-to-noise ratio and therefore eliminates the necessity to extract BSH from the biological materials. It should also be noted that the adduct is fluorescent and can thus be detected with a fluorescent detector. The excitation maximum is 315 nm and an emission maximum occurs between 370 and 390 nm. However, in this study a spectrophotometric detector was used because it has the advantage of wide availability.

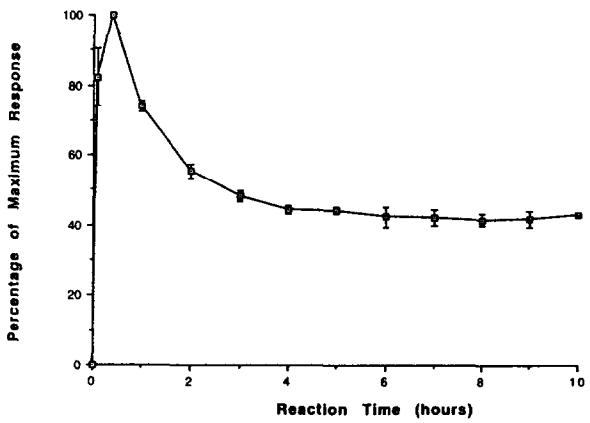

Fig. 2. Reaction–time profile of BSH and mBB. Measured response is the peak height of the BSH–mBB adduct. Vertical bars indicate standard deviation ($n = 3$).

Experiments were carried out to determine the reaction time necessary for completion of the derivatization. The results are shown in Fig. 2. It can be seen that reaction occurs immediately and proceeds to a maximum in ca. 20 min. The BSH–mBB peak height then starts to decrease gradually. After 4 h, no significant change was observed in the height of the BSH derivative peak. It seems that the BSH–mBB adduct is

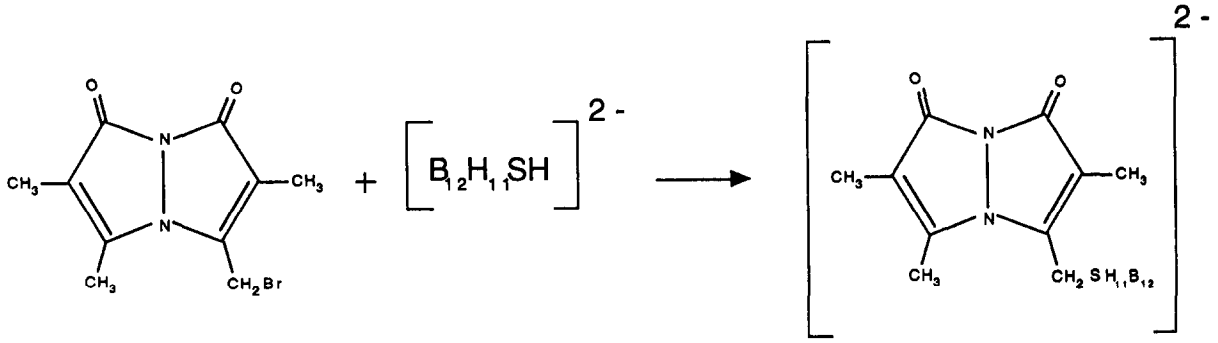

Fig. 1. Chemical reaction of BSH and monobromobimane in Tris-HCl buffer, pH 8.8.

involved in a second reaction that probably reaches equilibrium in 4 h. The result indicates that at least a reaction time of 4 h is required before the reaction mixture can be injected onto the HPLC system.

Under the experimental conditions used in our studies, the BSH–mBB adduct was eluted after ca. 8.5 min. Typical chromatograms for rat urine and plasma can be seen in Figs. 3 and 4, respectively. The peak of the BSH–mBB adduct was apparently well separated from other peaks, including the peaks of plasma thiols, such as cystine, which are known to react with monobromobimane [11,15]. A relatively clean blank at the BSH retention time can be seen both in Fig. 3 and Fig. 4.

The sensitivity of the assay is 200 ng/ml (ca. 0.1 ppm of boron). The signal-to-noise ratio at this sensitivity level is two. The lowest concentration that could be detected was 1 μg/ml (0.5 ppm boron) in urine and 0.2 μg/ml (0.1 ppm boron) in plasma.

It should be noted that although 10 min is enough to elute the BSH–mBB adduct, the run time necessary for each sample is ca. 45 min to allow for the elution of unreacted reagent and its hydrolysis products that eluted at 39 and 31 min, respectively. Absolute peak heights were used for the construction of the calibration curves since better correlation was obtained with peak height rather than with peak area. Calibration curves were linear in the range of 1 μg/ml to 100 μg/ml in urine with a slope of 0.67 ($\pm$0.03) and an intercept of -0.698 ($\pm$0.24), and 0.2 μg/ml to 50 μg/ml in plasma with a slope of 2.28 ($\pm$0.13) and an intercept of 0.96 ($\pm$0.23), respectively. The correlation coefficients (r^2) were 0.998 for the urine data and 0.999 for the plasma data. The inter-assay variations of the calibration standards at different BSH concentrations in both urine and plasma are shown in Table 1. The intra-assay variation of urine samples varied between 1.2% and 2.7%, and the intra-assay variation of plasma samples varied between 1.9% and 3.3%.

The BSH–mBB adduct was found to be stable at room temperature for a reasonable time which allows for long unattended runs. No significant change in peak height was noted when urine and

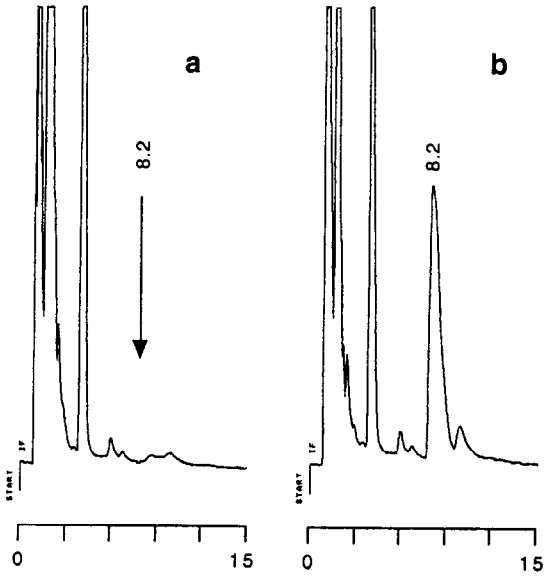

Fig. 3. Typical chromatograms in rat urine; (a) blank urine, (b) urine spiked with 100 μg/ml BSH. Mobile phase: methanol–0.05 *M* tetramethylammonium chloride in 0.02 *M* phosphate buffer, pH 3.0 (1:4, v/v); flow-rate, 2 ml/min.

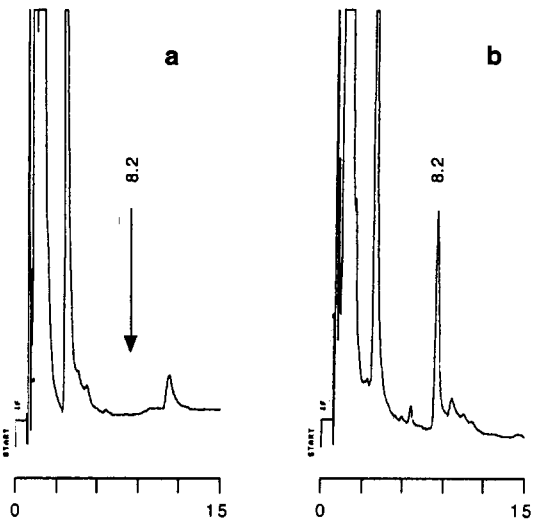

Fig. 4. Typical chromatograms in rat plasma; (a) blank plasma, (b) plasma spiked with 20 μg/ml BSH. Mobile phase: methanol–0.05 *M* tetramethylammonium chloride in 0.02 *M* phosphate buffer, pH 3.0 (1:4, v/v); flow-rate, 2 ml/min.

Table 1
Inter-assay variation in rat urine and plasma ($n = 4$)

Rat urine		Rat plasma	
BSH concentration (μg/ml)	Coefficient of variation (%)	BSH concentration (μg/ml)	Coefficient of variation (%)
1	14.88	0.2	9.96
2	9.19	0.5	8.81
5	2.86	1	6.77
10	7.36	4	5.19
20	5.63	10	4.76
50	3.15	20	5.80
100	3.33	50	3.78

plasma calibration standards were kept at room temperature for 48 h.

4. Conclusion

Disodium mercaptoundecahydrododecaborate (BSH) reacts with monobromobimane under alkaline conditions to form a stable adduct. The adduct can be separated by reversed-phase ion-pairing chromatography coupled with spectrophotometric detection at 373 nm for the quantitative measurement of BSH. The method is easy, sensitive and specific, and it can be applied to BSH determination in biological samples such as urine and plasma.

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