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Report

Disposition of Bismuth in the Rat. I. Red Blood Cell and Plasma Protein Binding

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The plasma protein and red blood cell binding profile of bismuth was investigated as a function of bismuth concentration. The binding of bismuth to human serum albumin, bovine serum albumin, and human plasma was also evaluated by ultrafiltration and the data analyzed by nonlinear regression techniques. The binding of bismuth to plasma proteins was nonlinear and decreased as a function of incubation concentration and appeared to be limited by the number of ionized l-cysteine residues available for binding. In the concentration range studied, bismuth was associated primarily with the red blood cell fraction of whole blood obtained from male Sprague Dawley rats. The data indicated that binding to proteins was of moderate affinity, and in whole blood it was present primarily in the red blood cell compartment.

KEY WORDS: bismuth; protein binding; red blood cell.

INTRODUCTION

The disposition profile of bismuth has not been investigated in detail. This element is present in a number of pharmaceuticals including bismuth subsalicylate, the active ingredient in numerous antidiarrheal preparations. The absorption of bismuth from soluble and poorly soluble bismuth compounds has led to cases of severe poisoning and encephalopathies and is a cause for concern from a toxicity standpoint (1,2). The severity of these encephalopathies has ranged from general disorientation and speech and motor incoordination to death in several instances (3). Besides the use of bismuth compounds in the treatment of diarrhea, other bismuth salts have been used in cosmetics (4).

A systematic study of the protein binding profile of bismuth has not been reported in the literature. In a paper on the metabolism of radiobismuth, it was reported that 83% of the administered dose was present in serum (5). Other details such as the time after dosing when the measurement was made, number of binding sites, and binding constant were not given. The binding of bismuth to erythrocytes obtained from dog blood has been reported to be at the ratio of 1:1 (6). Russ *et al.* (6) reported that 17% of the administered dose of bismuth administered as ^{207}bismuth citrate is present in erythrocytes of mature female rats (5). These reported differences in bismuth binding could be interpreted as being species or salt related. In a study conducted by Feldman *et al.* (7), plasma concentrations of bismuth were not detectable following oral administration of 60 ml of Pepto-Bismol despite the fact that approximately 90% of the administered salicylate was recovered in urine. Bismuth may have been present in the red blood cell fraction, which was not analyzed for bismuth content.

The objective of the present study was to examine the protein binding and rat erythrocyte binding profile using bismuth nitrate as the model compound. The binding of bismuth to human serum albumin, bovine serum albumin, and human plasma was studied using ultrafiltration. Red blood cell binding was conducted using heparinized whole rat blood. Bismuth content was determined on a heated graphite furnace, using flameless atomic absorption spectroscopy. Because of the inability of this technique to differentiate between the various ionic species of bismuth, reference to bismuth in the publication refers to trivalent bismuth, which is the form present in bismuth nitrate.

MATERIALS AND METHODS

Analytical

The atomic absorption spectroscopic analysis of bismuth was carried out in a Perkin–Elmer Model 5000 spectrophotometer equipped with a heated graphite furnace (HGA 400). The temperature program was executed by a microprocessor attachment to the HGA 400, which provided for rapid and reproducible temperature control. Pyrrolitically coated graphite tubes were used for sample containment. The analytical procedure employed was a modified form of a previously published technique (8). Stock solutions of bismuth in 20% (v/v) glycerin in water were made at a concentration of 1 mg/ml. Glycerin was necessary to ensure aqueous solubilization of bismuth nitrate and the optimum amount was determined from an experiment where bismuth

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nitrate was incubated in solutions containing different ratios of glycerin to water. Appropriate dilutions were made and 10-μl aliquots were pipetted into 0.2 ml of either plasma, heparinized whole blood, or red blood cells to yield concentrations ranging from 0.01 to 5 μg/ml. The matrix was then diluted with an equal volume of deionized water. Bismuth was chelated with the addition of 0.2 ml of a 1% solution of sodium diethyl dithiocarbamate, and the sample vortexed for 5 sec. The mixture was allowed to stand for 5 min, following which 0.3 ml of methyl isobutyl ketone saturated with deioniozed water was added. The two phases were then mixed together by vortexing (Genie Vortex, Scientific Industries) for 30 sec. These samples were then centrifuged at 10,000g (Beckman Microfuge) for 5 min and the supernatant ketone layer separated. A 10-μl aliquot of the supernatant layer was placed in the graphite tube and analyzed for bismuth content. Samples containing bismuth from the ultrafiltrate in the protein binding experiments were analyzed directly. Aqueous standards were used to quantify bismuth in these samples because the ultrafiltrate was made up of an aqueous matrix and extractions were not necessary. The coefficient of variation for the assay ranged from 2.71% at 300 ng/ml to 7.03% at the 20 ng/ml concentration. The extraction efficiency was 99.4 ± 2.08%.

Red Blood Cell Binding

The binding of bismuth to red blood cells (RBC) was studied as a function of bismuth concentration. Whole blood was drawn into glass tubes containing 50 units of heparinized saline by cardiac puncture of male Sprague–Dawley rats lightly anesthetized with ether. A 10-μl aliquot of the appropriate bismuth solution was added to 1 ml of heparinized whole blood at concentrations ranging from 25 to 5000 ng/ml. These samples were incubated at 37°C for 2 hr with gentle agitation. This incubation period was sufficient for equilibrium to be achieved, as determined by repetitive sampling. Plasma and red blood cells were separated by centrifugation at 10,000g (at 37°C) and immediately frozen at −70°C (Revco Ultralow Freezer).

Protein Binding Studies

Human serum albumin, Fraction V powder (Sigma Chemical Company), and bovine serum albumin, Fraction V powder (Sigma Chemical Company), were used as received. The entire procedure was conducted at 37°C. Amicon Centrifree micropartition systems were used to separate bound from free solute. Pilot studies indicated less than 5% binding to these cartridges at the lowest concentrations studied. After the addition of bismuth to the protein solution or plasma at room temperature, the samples containing 25, 50, 100, 250, 500, and 1000 ng/ml of bismuth were incubated at 37°C for 30 min and then placed in the micropartition cartridges. The cartridges were centrifuged at 2000g and 37°C for 25 min. The ultrafiltrate was measured for bismuth content.

Data Analysis

The binding of bismuth to red blood cells was calculated as the fraction of bismuth in whole blood bound to red blood cells, by use of the following equation:

$$f_{\mathrm{rbc}} = \frac{C_{\mathrm{rbc}} \cdot H}{C_{\mathrm{rbc}} \cdot H + C_{\mathrm{p}} \cdot (1 - H)} \tag{1}$$

where f_{rbc} is the fraction of initial amount of bismuth incubated in red blood cells, C_{rbc} is the experimentally measured concentration in red blood cells, C_{p} is the concentration of bismuth measured in plasma, and H is the hematocrit.

Plasma protein binding parameters were estimated by fitting the binding data to the equation

$$r = \sum \frac{nK(D)}{1 + K(D)} \tag{2}$$

where r is the number of moles of bismuth bound per mole of protein, n is the number of binding sites, K is the equilibrium association constant of binding, and (D) is the concentration of free bismuth (moles/liter). A subroutine for a nonlinear curve fitting program (Nonlin 84) was written to analyze the data. Scatchard plots (9) were generated using the following transform of Eq. (2):

$$\frac{r}{(D)} = nK - rK \tag{3}$$

where the terms r, (D), n, and K are as defined before.

Data from the binding studies in human plasma were analyzed using the Klotz (10) and Scott (11) transformations of Eq. (2). The Klotz transformation is

$$\frac{1}{r} = \frac{1}{nK(D)} + \frac{1}{n} \tag{4}$$

The Scott transformation is as follows:

$$\frac{(D)}{r} = \frac{1}{n}(D) + \frac{1}{nK} \tag{5}$$

The terms in Eqs. (4) and (5) are as defined for Eq. (2).

RESULTS

In the concentration range studied bismuth exhibited nonlinear binding to human serum albumin and human plasma (Table I). The fraction of bismuth bound to 5% human serum albumin solution decreased from 0.926 ± 0.024 to 0.740 ± 0.025 in the 25–1000 ng/ml concentration range.

Table I. Bismuth Binding to Proteins: Effect of Concentration[a]

	Fraction bound		
Concentration	5% human serum albumin	5% bovine serum albumin	Human plasma
25	0.926 ± 0.024	0.985 ± 0.006	—[b]
50	0.921 ± 0.016	0.975 ± 0.005	0.976 ± 0.007
75	—[b]	0.973 ± 0.002	—[b]
100	0.904 ± 0.016	0.970 ± 0.004	0.967 ± 0.010
250	0.840 ± 0.028	0.906 ± 0.006	0.830 ± 0.005
500	0.780 ± 0.047	0.952 ± 0.010	0.700 ± 0.013
1000	0.740 ± 0.025	0.961 ± 0.060	0.730 ± 0.017

[a] Mean ± SD of four determinations.
[b] Not determined.

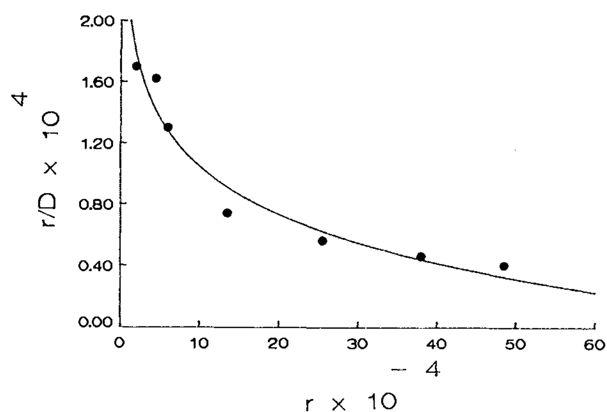

Fig. 1. Scatchard plot of bismuth binding to human serum albumin.

Trivalent bismuth binding to human plasma showed a similar nonlinear pattern, with the fraction bound decreasing from 0.976 ± 0.007 to 0.730 ± 0.017 in the 50–1000 ng/ml concentration range. Scatchard transformation of the binding data according to Eq. (2) suggested the presence of two classes of binding sites (Fig. 1). Table II presents the parameters obtained by computer fitting of the data for the protein binding of bismuth. The data indicate the fractional number of binding sites on each protein molecule. There is one binding site per 1493 molecules of human serum albumin in the primary class, while the secondary class contains one binding site per 53 molecules (assuming a molecular weight of 69000). Similar data were obtained for bismuth binding to bovine serum albumin (Table II). The Scott and Klotz transformations of the binding data to human plasma yielded one binding site per 357 and 323 molecules (Table III).

The results of the *in vitro* binding studies conducted using whole rat blood indicated that in the concentration range studied, bismuth was associated primarily with the red blood cell fraction (Table IV). While the data did not indicate evidence for saturation in the 25 to 5000 ng/ml concentration range, bismuth red blood cell-to-plasma concentration ratios ranged from 13.1 ± 4.5 at the 25 ng/ml concentration to 50.5 ± 9.7 at 5000 ng/ml. This indicates that the uptake of bisuth by red blood cells at low concentrations is hindered because of nonlinear protein binding when compared to the higher concentrations. However, the effect of protein binding on red blood cell uptake of bismuth in the rat is not significant as evidenced by the large amounts recovered from red blood cells when bismuth is incubated with whole blood. The nonlinearity in the red blood cell-to-plasma concentration ratio are also due in part to variations in the hematocrit of the blood samples, which ranged from 0.54 to 0.58.

Table II. Protein Binding Parameters for Bismuth Binding

Protein	n_1[a]	n_2[b]	K_1[c] (L/mol)	K_2[d] (L/mol)
Human serum albumin	0.00067	0.019	3.0×10^7	2.3×10^5
Bovine serum albumin	0.00094	0.014	8.7×10^7	2.8×10^7

[a] Number of binding sites in primary class.
[b] Number of binding sites in secondary class.
[c] Association constant for primary class.
[d] Association constant for secondary class.

Table III. Protein Binding Parameters in Human Plasma

Transformation	n[a]	K[b] (L/mol)
Scott	0.0031	1.3×10^7
Klotz	0.0028	2.7×10^7

[a] Number of binding sites.
[b] Association constant.

DISCUSSION

In vitro binding studies of bismuth to human plasma proteins indicated that the fraction bound to human serum albumin and human plasma was inversely related to the incubation concentration. It appears that the binding sites were saturated at the concentrations studied leading to a nonlinear binding profile. The profile for human albumin and human plasma was essentially similar.

Bismuth binding to plasma proteins may be of a specific nature. Heavy metal ions, such as lead and mercury, bind to metallothionines and other cysteine-containing proteins (13). Scatchard analysis of bismuth binding to human serum albumin revealed the presence of two classes of binding sites. The association constants for bismuth binding to proteins indicate that the binding is of a moderate affinity (Table II). The unusual feature of bismuth binding to plasma proteins is the low number of binding sites. This may be explained by considering the structure of the albumin molecule and the experimental conditions. Bismuth, like other heavy metal ions, shows a marked preference for sulfur containing amino acids (14). Methionine and cysteine are two such amino acids present in albumin. Bismuth does not bind to methionine as evidenced by the absence of a shift in the NMR spectrum of a solution containing bismuth and the amino acid (15). This leads to the speculation that bismuth may bind only to cysteine. An examination of the structure of the human serum albumin molecule indicates that there are 35 cysteine residues (16). Of the 35 cysteine residues, 34 are linked by a disulfide linkage to form cystine, with only 1 residue being present as cysteine. Since ionized cysteine residues are the reactive species, the ratio of ionized to nonionized cysteine residues could have affected the number of binding sites. At

Table IV. Fraction of Concentration of Bismuth Present in Red Blood Cells Following Incubation for 2 hr at 37°C[a]

Incubation concentration (ng/ml)	Percentage recovered in red blood cells
25	82.3 ± 5.0
50	98.5 ± 0.4
100	95.7 ± 2.6
250	95.8 ± 0.8
500	91.9 ± 6.7
1000	95.6 ± 1.8
2500	97.7 ± 0.7
5000	98.8 ± 0.4

[a] Mean ± SD of four determinations.

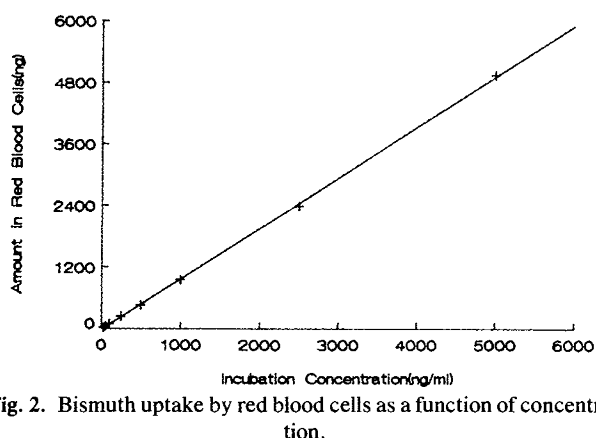

Fig. 2. Bismuth uptake by red blood cells as a function of concentration.

the pH at which the binding studies were conducted (pH 6.5), this ratio was approximately 1.6×10^{-3} (buffers were not used because bismuth is complexed by phosphate and citrate buffers), thus reducing the availability of binding sites. Consideration of the structure of albumin and examination of the number of binding sites leads to a possible interpretation of the protein binding data. The differences in the number of binding sites for human plasma could be due to the fact that while the 5% solution had a pH of 6.5, human plasma had a pH of 7.4, leading to a lower fraction of ionized cysteine residues. The Klotz and Scott plots yielded an average number of binding sites for the two classes because of its inability to distinguish between the two classes of binding sites.

The nonlinear nature of bismuth binding to plasma proteins indicates that at low plasma concentrations there would be a smaller fraction available for uptake by rat erythrocytes than at high concentrations. Although definite conclusions are not possible in view of the species differences, an indication that this could be happening is seen in Table IV, where at a concentration of 25 ng/ml, 82% was associated with red blood cells, while at the higher concentrations, more than 90% was recovered from red blood cells. The extensive binding of bismuth to red blood cells has important implications because pharmacokinetic parameters and body burden have hitherto been determined in plasma. The red blood cell to plasma concentration ratios ranged from 28.1 for the 25 ng/ml to 50.5 for the 5000 ng/ml concentrations. This indicated that bismuth was preferentially bound to red blood cells when present in whole blood. When the data were evaluated using amounts instead of concentrations by correcting for the hematocrit, the apparent nonlinearity was not noticed. Bismuth uptake by red blood cells increased in proportion to the incubated amount (Fig. 2). However, the data were not strictly linear and deviations from linearity were noticed at the low concentrations. The reason for the deviations could have been due to nonlinear protein binding at the low concentrations. At the low concentrations (25–100 ng/ml), the fraction bound decreased only slightly, from 0.926 to 0.904, while at the concentrations between 100 and 1000 it decreased to 0.74. This could lead to impaired red blood cell binding at lower blood concentrations, while at higher blood concentrations a greater free plasma fraction would lead more extensive binding to red blood cells. The assay was more variable at the lower concentrations (CV 7.03% at 20 ng/ml vs 2.71% at 300 ng/ml), which could have contributed to the nonlinearity at the lower blood concentrations. Definitive proof of this would be obtained when protein binding of bismuth is studied in the presence of red blood cells. The high percentage of bismuth bound to erythrocytes has pharmacokinetic implications and would suggest that pharmacokinetic studies be conducted using whole blood. Utilization of plasma concentrations versus whole blood concentrations would lead to erroneous conclusions about the fate of bismuth. Studies by D'Souza *et al.* have shown that bismuth elimination from the body depends to a limited extent on the turnover rate of erythrocytes (12). The perturbation of oxidative metabolism (4) could also be expected in red blood cells if bismuth were to accumulate in red blood cells.

In conclusion, it appears that bismuth is associated chiefly with the red blood cell fraction of rat blood. Its protein binding appears to be nonlinear in nature, with an extremely small number of binding sites on the albumin molecule. These findings have significant implications for the toxicity and pharmacokinetics of bismuth.

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