Determination of trimethylbismuth in the human body following ingestion of colloidal bismuth subcitrate

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Running title

Bismuth metabolism

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List of Abbreviations

AUC, area under the curve; Bi, bismuth; BiH3, bismuth trihydride; BiOCl, bismuth oxychloride; CBS, colloidal bismuth subcitrate; (CH3)3Bi, trimethylbismuth; (CH3)2BiH, dimethylbismuth hydride; CH3BiH2, methylbismuth dihydride; CRM, certified reference material; DMA, dimethylarsenic; EI, electron impact; EI-MS, electron impact mass spectrometry; FEP, fluorinated ethylene propylene; GC, gas chromatography; GC/EI-MS/ICP-MS, parallel EI-MS and ICP-MS detection after GC separation; HNO3, nitric acid; H2O2, hydrogen peroxide, IAC, inter-aggregate calibration; ICP-MS; inductively coupled
plasma mass spectrometry; IDMS, isotope dilution mass spectrometry; LD₅₀, lethal dose at which 50% of animals die; LT-GC/ICP-MS, low temperature-gas chromatography coupled with inductively coupled plasma-mass spectrometry; l.o.d., limit of detection; l.o.q., limit of quantification; M⁺, molecular ion; m/z, mass-to-charge ratio; na, not analyzed; PFA, perfluoroalkoxy; PTV, programmed temperature vaporisation; RSD, relative standard deviation; Tl, Thallium
Abstract

Biological methylation and hydride formation of metals and metalloids are ubiquitous environmental processes that can lead to the formation of chemical species with significantly increased mobility and toxicity. While much is known about the interaction of metal(loid)s with microorganisms in environmental settings, little information has been gathered on respective processes inside the human body as yet. Here, we studied the biotransformation and excretion of bismuth following ingestion of colloidal bismuth subcitrate (215 mg bismuth) to 20 male human volunteers. Bismuth absorption in the stomach and upper intestine was very low, as evidenced by the small quantity of bismuth eliminated via the renal route. Total bismuth concentrations in blood increased rapidly in the first hour following ingestion. Most of the ingested bismuth was excreted via feces during the study period. Trace levels of the metabolite trimethylbismuth ((CH$_3$)$_3$Bi) were detected via low temperature-gas chromatography/inductively coupled plasma-mass spectrometry (LT-GC/ICP-MS) in blood samples and in exhaled air samples. Concentrations were in the range of up to 2.50 pg/ml (blood) and 0.8-458 ng/m$^3$ (exhaled air), with high inter-individual variation being observed. Elimination routes of bismuth were exhaled air (up to 0.03 %), urine (0.03-1.2 %) and feces. The site of (CH$_3$)$_3$Bi production could not be identified in the present study, but the intestinal microflora seems to be involved in this biotransformation if accompanying ex vivo studies are taken into consideration.
Introduction

It is a well-known fact that the toxicity of metal(loid)s is essentially dependent on the chemical form, i.e. on the species of the element in question (Craig 2003; Hirner and Emons 2004; Dopp et al. 2004). In particular, alkylation often seems to considerably increase the toxic potential of metal(loid)s. Many studies have shown that in the environment methylated, and also in some cases hydride species, can be formed by different mechanisms and from a variety of metal(loid)s (Craig 2003). In particular, microorganisms, e.g. bacteria and fungi, have been reported to be involved in this specific kind of conversion (Thayer 2002).

In contrast to the considerable knowledge that has accumulated on the interaction of microorganisms with metal(loid)s in the environment, a paucity of information is currently available on the respective processes inside the human body. This lack of knowledge is particularly striking in view of the fact that certain segments of the digestive tract, viz. the oral cavity and the colon, are colonized by myriads of bacteria. The difficulty of analyzing metal(loid)organic compounds at trace and even ultratrace levels might at least partly account for this information gap.

Following a pilot study with three volunteers (Hirner et al. 2004), we performed an ingestion experiment with bismuth, administering this element as a single oral dose to 20 male volunteers in the form of a therapeutically used colloidal bismuth subcitrate compound. Bismuth was chosen as the element of interest for a number of reasons:

i) Humans are exposed to bismuth via pharmaceutical products (Menge et al. 1992) and ubiquitous bismuth-containing cosmetics. For example, colloidal bismuth subcitrate and other bismuth compounds are used in the treatment of ulcers, and, more specifically, the eradication of *Helicobacter pylori*. Bismuth subsalicylate is present in relatively high concentrations (1-2 %) in “over-the-counter” indigestion remedies.

ii) Inorganic bismuth compounds have been shown to elicit low toxicity (e.g. the LD$_{50}$ of BiOCl is 22000 mg/kg (rat, oral)). Neurological disorders observed after the oral intake of
bismuth salts typically followed the ingestion of up to 20 g per day over a period of 20 days per month (Slikkerveer and de Wolff 1989; Sun et al. 2004). Nevertheless, toxic effects are rarely observed if bismuth-containing pharmaceuticals are taken in the recommended doses, which is probably due to the extremely low absorption of this metal (Sun et al. 2004).

iii) Bismuth metabolism in humans is as yet unknown. Some data on transport processes and bismuth protein interactions have been reported (Sun et al. 2004).

iv) Bismuth is known to be methylated by microorganisms in the environment as described above. The formation of trimethylbismuth and bismuth trihydride by *Methanobacterium formicicum* has been experimentally demonstrated in sewage sludge (Michalke et al. 2000; Michalke et al. 2002). Some of these microorganisms are known components of the human intestinal microflora.

v) Analysis of bismuth and its metabolites is facilitated by the fact that background concentrations of bismuth species in the human population are very low (Wiesmueller G, Environmental Specimen Bank, Muenster, Germany, personal communication).
Materials and Methods

Study population, study design and sampling. The study included 20 healthy, male volunteers (age = 27.2±2.4 years; weight = 82.5±11.6 kg; body mass index = 25±4 kg/m²), living in Essen, Germany, who were enrolled in this investigation. One of them participated twice, so that 21 data sets were obtained. No study participant took any form of medication and all were judged to be healthy based on their medical history, a physical examination, and a routine laboratory screening. The participants were non-smokers and had no history of metabolic disorder or disease of the gastrointestinal tract. Investigations were carried out at the Department of Hygiene and Occupational Medicine at the University Hospital of Essen, Germany. All participants gave informed written consent. The study was approved by the Ethics Committee of the University Hospital of Essen and was in accordance with the principles laid down in the Declaration of Helsinki.

At 9 a.m. of the first day two tablets of colloidal bismuth subcitrate (De-Noltab®, Yamanouchi Europe B.V., The Netherlands) containing a total of 215 mg bismuth were taken by each individual together with some tap water. They were asked not to chew the tablets.

Samples of exhaled air and blood were taken immediately before bismuth ingestion (t = 0) and 1, 2, 4, 8, 24, and 48 hours thereafter; blood was additionally collected 0.5, 32, and 56 hours after ingestion. Urine and feces samples were collected before the onset of the study and subsequently according to urinary/fecal urge during the study period. All participants were asked to note urine volume, fecal mass, as well as the time of sampling. Exhaled air was collected in 10 l Tedlar® bags (Supelco®, Taufkirchen, Germany), and then transferred to traps (custom made, borasilicate glass, length 20 cm, i.d. 8 mm, filled with 1.59 g 10 % SP™-2100 on 80/100 Supelcoport, Supelco, Bellefonte, USA and used after conditioning for 16 h at 280 °C) at -78 °C (ethanol/liquid nitrogen) and analyzed immediately as described below.

Whole blood (citrate stabilized, 10 ml S-Monovette®, SARSTEDT, Nuembrecht-Rommelsdorf, Germany), urine and feces samples were stored at -80 °C until analysis.
Total metal analysis. Bismuth concentrations in blood, urine, and feces were determined by ICP-MS following microwave-digestion of the samples (MARS 5, CEM, Kamp-Lintfort, Germany). Digestion was performed in closed PFA-vessels (XP 1500 Plus™, CEM, Kamp-Lintfort, Germany) which contained HNO₃ (65 %, sub-boiled) and 2 ml H₂O₂ (30 %, Suprapur®, Merck, Darmstadt, Germany). Sample volumes/weights and respective aliquot additions of HNO₃ were as follow: Blood 2 ml/3 ml; urine 4 ml/3 ml; feces 0.1–0.5 g/4 ml. The microwave parameters are presented as supplemental data (table 1). The digestate was diluted with ultrapure water (18.2 MΩ cm, Purelab Ultra, ELGA LabWater, Celle, Germany) to a final volume of 30 ml (blood and urine) or 40 ml (feces) prior to analysis. Feces samples with a high Bi-content were diluted to 1 in 100.

ICP-MS (Agilent 7500a, Agilent Technologies, Waldbronn, Germany) conditions such as radio frequency-power, ion lens voltages, gas flows, etc., were adjusted to obtain robust plasma conditions and to minimize matrix effects. Holmium (5 ppb) as internal standard was added and the solutions were delivered at 0.3 ml/min to a microflow nebulizer and routed through a double-pass Scott-type spray chamber (both from AHF Analysentechnik, Tuebingen, Germany) cooled to 2 °C. Quantification was performed by external calibration (blank, 0.02, 0.05, 0.2, 0.4, 0.7, 1, 4, 7, 10, 20, 30 and 40 µg/l bismuth) and validated by analyzing certified reference materials (CRM, Seronorm™ Trace Elements in Whole Blood, Levels 1 and 2 / Urine, SERO AS, Billingstad, Norway). In the case of feces samples there was no CRM available. The limits of detection and limits of quantification (3 σ-criterium and 10 σ-criterium) were determined daily (typically, blood 0.03 µg/l, 0.1 µg/l; urine 0.001 µg/l, 0.003 µg/l; feces 0.44 µg/kg, 1.46 µg/kg). All samples were measured in duplicate.

Identification of (CH₃)₃Bi. The (CH₃)₃Bi standard (VeZerf Laborsynthesen, Idar-Oberstein, Germany) was identified via GC/El-MS/ICP-MS as described elsewhere (Koesters et al. 2005). Gas chromatographic separation was performed on a 6890 N gas chromatographic system (Agilent Technologies, Waldbronn, Germany) which was equipped with a UNIS 2000
inlet system (Joint Analytical Systems, Moers, Germany) for programmed temperature vaporisation (PTV) after purge and trap sampling and a capillary column (HP-5MS, 0.25 mm x 30 m x 0.25 µm).

Two detection systems were used simultaneously: a 5973 N EI-MS and a 7500a ICP-MS (both from Agilent Technologies, Waldbronn, Germany). Parallel detection was realized with a post-column split inside the GC oven connecting the column with two uncoated capillary lines leading to the particular detectors. Operation parameters are listed in table 2 (supplemental data).

**Bismuth speciation analysis.** (CH$_3$)$_3$Bi present in exhaled air was determined via LT-GC/ICP-MS as described by Wickenheiser et al. (1998): A 3 l sub-sample (3 subsamples of 3 l per Tedlar$^\text{®}$ bag) was sucked slowly with a vacuum pump and a gas clock (Trommel-Gaszaehler TG 1/6, Ritter Apparatebau, Bochum, Germany) through a trap held at -78 °C. At the same time the chromatographic column temperature was maintained at -196 °C. After removal of the ethanol/liquid nitrogen mixture the analytes were thermally desorbed from the trap and separated via GC. The GC column was warmed up from -196 °C up to 5.8 °C at room temperature within five minutes and subsequently heated at a rate of 35 °C/min to a final temperature of 150 °C using a resistance wire (Transformator LTS 606, Thalheimer Transformatorenwerke, Thalheim, Germany). Transfer to the ICP-MS was accomplished via a fluorinated, ethylene propylene (FEP) tube (length 80 cm, i.d. 0.75 mm, o.d. 1/16″, CS-Chromatographie, Langerwehe, Germany). In order to assure a homogenous temperature distribution, the transfer line was inserted into a copper tubing (i.d. 1/16″, o.d. 1/8″) which was heated to 150 °C using a resistance wire (Power Supply, Typ 3252, Statron, Fuerstenwalde, Germany).

(CH$_3$)$_3$Bi in whole blood samples was analyzed in a similar manner to the analytes in exhaled air. A three necked round bottom flask (100 ml) containing 1-4 ml of the sample, 10 ml of ultrapure water, and 100 µl of antifoam/ultrapure water (1 in 100 (v/v) (Antifoam 289, Sigma-
Aldrich, Taufkirchen, Germany), was purged with 60 ml/min helium (5.0, Air Liquide, Duesseldorf, Germany) for 2 minutes in order to remove oxygen. Then, blood samples were transferred from closed vessels through a septum (butyl-Teflon®-rubber, CS-Chromatographie, Langerwehe, Germany) into the flask *via* a syringe (5 ml, Becton Dickinson Discardit™ II) and disposable hypodermic needle (Sterican® 0.60 x 30 mm, Braun, Melsungen, Germany) in order to prevent a loss of the analyte. The blood gases were purged by 60 ml/min helium for 8 min and trapped quantitatively in custom-made U-tubes as described above, and analyzed by LT-GC/ICP-MS (Grueter et al. 2000). The quantitative transfer of blood gases was verified by repetitive purging (n = 2) of selected samples. All blood samples were measured in duplicate.

In both cases chromatography was carried out using a packed column (custom-made, borosilicate glass, length 80 cm, i.d. 8 mm, filled with 6.38 g 10% SP™-2100 on 80/100 Supelcoport, Supelco, Bellefonte, USA, and conditioned for 16 h at 280 °C). (CH₃)₃Bi was measured by ICP-MS at a mass-to-charge ratio of m/z 209 (corresponding to ²⁰⁹Bi). Quantification of (CH₃)₃Bi was achieved by integration of the gauss-type signal at a retention time of 307.1 ± 5 s and inter-aggregate calibration (IAC) (Feldmann 1997). IAC uses inorganic aqueous standards for species-independent quantification after separation of volatile species *via* GC and returns the effective nebulizer mass flow by differential weighting of the aspirated standard solution and the spray chamber drain. ²⁰⁵Tl added *via* the nebulizer was used as a continuous internal standard in order to compensate detector drift. This method was used because quantification procedures such as isotope dilution mass spectrometry (IDMS), or standard addition could not be applied for a variety of reasons (²⁰⁹Bi is monoisotopic and therefore IDMS is not possible; standards are unstable and therefore difficult to handle).

Limits of detection (l.o.d.) / limits of quantification (l.o.q.) of trimethylbismuth were determined according to the 3 σ- and 10 σ-criterium (typically, exhaled air 0.1 ng/m³, 0.3 ng/m³; blood 0.01 pg/ml, 0.04 pg/ml).
Creatinine measurement. Creatinine concentrations in urine were determined by an ADVIA® Chemistry System 1650 (Bayer Healthcare, Leverkusen, Germany) which is based on the reaction of creatinine with picric acid under alkaline conditions. The formation rate of the colored complex is proportional to the creatinine concentration (Tietz 1995).

Data expression and data analysis. Concentrations of the bismuth species in exhaled air, blood, urine and feces are presented as mean ± standard deviation (SD) or as ranges. Total bismuth concentrations in blood were fitted by an exponential curve and the effective half-live was taken from the rapid elimination phase.

The mass of exhaled bismuth was estimated based on the concentration-vs.-time curve and the average respiratory volume of 0.5 m³/h in rest. The AUC was calculated by creating a step function with the steps being ordered symmetrically between two sampling times.
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Results

Disposition and elimination of bismuth

Analysis of total bismuth concentrations in blood following uptake of colloidal bismuth subcitrate revealed similar profiles in all study participants. No bismuth was detected in pre-ingestion samples of the participants. The bismuth concentration typically increased to a maximum within the first hour following ingestion (figure 1), and, subsequently, decreased with half-lives of approximately $1.6 \pm 0.7 \text{ h}$. A significant variation in the maximum blood bismuth concentrations was observed between the individuals, with the maximum bismuth concentration ranging from 1.3 to 158.8 $\mu$g/l (supplemental data, table 3).

A total of 0.03-1.2 % (0.06–2.51 mg) of the ingested bismuth was eliminated renally during the study period. 68 ± 16 % of the absorbed bismuth were excreted in the first twelve hours after ingestion, mostly with the first urine after ingestion of the bismuth containing pharmaceutical. A high inter-individual variation in excretion maxima (height and time) was observed with maximum urinary bismuth concentrations being in the range from 0.02 to 12.8 mg/l. A record of the renally eliminated bismuth is presented in the supplemental data (table 3).

In contrast to the urinary data, the maxima of the fecal bismuth concentrations ranged from 0.06 to 2.36 g/kg (wet weight) amounting to a total excretion of typically more than 99 % of the ingested bismuth during the study period. As feces samples were not given at regular intervals by the volunteers, and as the respective quantities may not have been accurately measured and reported, the percentage of bismuth excreted via feces was calculated by subtraction of the exhaled and the renally eliminated bismuth from the totally ingested bismuth; the bismuth deposition in the body was neglected in this calculation. In another study Boertz has found that 91-93 % of the ingested bismuth is eliminated via the feces within five days after ingestion of colloidal bismuth subcitrate (Boertz 2008). This indicates that a few percent are stored in the human body or are eliminated via alternative pathways. In
summary, the analysis of total bismuth concentrations in urine and feces samples indicated that fecal excretion was the primary excretion route for bismuth compounds.

The standard deviations of the total bismuth concentrations in blood and urine were lower than 3%. In feces samples RSDs were in the range from 5 to 52%. A lack of homogeneity in some fecal samples is the likely cause of the larger RSDs observed.

The creatinine concentrations in the urine samples were in the range from 0.09 to 3.78 g/l. There was no obvious relationship between the creatinine concentrations and the respective bismuth levels.

**Identification and quantification of (CH$_3$)$_3$Bi**

A (CH$_3$)$_3$Bi standard was analyzed in parallel by ICP-MS and EI-MS after GC separation (GC/EI-MS/ICP-MS) (Koesters et al. 2005), and its identity was verified by the respective mass spectrum (supplemental data).

The EI mass spectrum of (CH$_3$)$_3$Bi (measured at 70 eV) shows four dominant signals at m/z = 254, 239, 224, and 209. While the signal at m/z = 254 represents the molecular ion, the others reflect the successive loss of three methyl groups. Simultaneously $^{209}$Bi was detected at a mass-to-charge ratio of m/z 209 in the ICP-MS component of this hyphenated technique.

The retention time of the standard compound was determined via LT-GC/ICP-MS. Finally, (CH$_3$)$_3$Bi in blood and exhaled air samples was identified on the basis of the retention time obtained (307.1 ± 5 s) via LT-GC/ICP-MS (figure 2). (CH$_3$)$_3$Bi could be determined in at least one sample of all participants. The number of participants who have (CH$_3$)$_3$Bi in exhaled air or blood at specific time intervals is shown in table 1.

A former pilot study (Hirner et al. 2004) indicated that the volatile bismuth species (CH$_3$)$_2$BiH, CH$_3$BiH$_2$ and BiH$_3$ may also be present in exhaled air. However, this observation could not be corroborated in the present study. (CH$_3$)$_3$Bi was the only volatile bismuth species detected in blood or breath samples.
Kinetics of (CH₃)₃Bi

In most samples of exhaled air (CH₃)₃Bi was detectable for the first time typically four hours after ingestion. The maximum concentrations were reached after eight hours in most of the participants exhibiting a high inter-individual variation which ranged from 0.8 to 458 ng/m³ (supporting information table 4). Up to 5.4 µg of the ingested bismuth (equivalent to 0.03 ‰) was eliminated via exhalation during the first 48 hours.

(CH₃)₃Bi was also detected in blood samples, viz. in concentrations of up to 2.5 pg/ml. The concentration-vs.-time profiles of (CH₃)₃Bi in blood were similar to the corresponding profiles of (CH₃)₃Bi in exhaled air. Two typical profiles representing the curve shape in most participants are shown in figure 3a and 3b. In 7 out of 20 volunteers the (CH₃)₃Bi concentrations in blood were below the limit of detection at all sampling times (supplemental data, table 4).

In contrast, no obvious relationship was found between the total bismuth concentrations in blood and the respective (CH₃)₃Bi concentrations in blood and exhaled air samples (figure 4a and 4b). Blood samples taken after 8 h and later showed maxima of (CH₃)₃Bi concentrations for most participants; during the first two hours after ingestion no maxima was observed.
Discussion

This work represents the first in vivo study on bismuth biodisposition in humans which includes the analysis of a volatile bismuth species. In addition to data on total bismuth uptake and elimination, which basically confirmed the results of previous studies on bismuth biodisposition (Slikkerveer and de Wolff 1989; Sun et al. 2004; Klotz 2000), data on the identification and quantification of (CH₃)_3Bi in blood and exhaled air samples are given.

Biodisposition and elimination of total bismuth

After ingestion of colloidal bismuth subcitrate containing tablets, bismuth levels in blood increased rapidly to a maximum. This is in line with previous reports. The early maxima of bismuth levels in blood suggest that some bismuth absorption may occur in the stomach and the upper sections of the intestine. Nwokolo and co-workers observed the persorption (transmucosal penetration) of bismuth particles in the gastric antral mucosa, which would explain the minor but rapid absorption of the ingested bismuth (Nwokolo et al. 1992). Though every effort was made to ensure the standardization of the sampling and analytical procedures, the maxima of total bismuth concentration in blood varied by more than two orders of magnitude. These differences in bismuth absorption are not well understood, but factors such as variations in eating patterns prior to the study and the pH of the stomach may be implicated (Sun et al. 2004). Animal studies have shown that the simultaneous oral administration of bismuth salts and thiolates increase the bismuth level in blood plasma (Williams 1977; Chaleil 1981). Increased bismuth levels in blood have also been reported following a rise of intragastric pH by the application of ranitidine or omeprazole (Nwokolo et al. 1991; Treiber et al. 1994): The solubility of colloidal bismuth subcitrate (CBS) ranges from less than 1 mg/ml at acidic pH to several hundred mg/ml in pure water (Sun et al. 2004).

The elimination routes of total bismuth were exhaled air (up to 0.03 ‰ as (CH₃)₃Bi), urine (0.03-1.2 ‰) and feces. Most of the bismuth was excreted via the fecal route, exposing the metal to the microflora of the intestine. Interestingly, this is not the case for arsenic which is
also an element of group 15 of the periodic system. Apart from the higher toxic potential of its inorganic forms, arsenic is predominantly eliminated via urine (Craig 2003). As a consequence of the predominant elimination of bismuth via feces, this metal is an ideal element to investigate intestinal biomethylation.

Identification and quantification of \((\text{CH}_3)_3\text{Bi}\)

\((\text{CH}_3)_3\text{Bi}\) in exhaled air and blood was identified unambiguously in samples of all study participants via GC/ICP-MS using a commercially available compound as a reference. This analytical approach was completed by the characterization of the reference compound by GC/EI-MS/ICP-MS (Koesters et al. 2005). The use of this novel but robust technique allowed the reliable monitoring of \((\text{CH}_3)_3\text{Bi}\) concentrations in exhaled air and blood samples, which provided strong evidence for the occurrence of \((\text{CH}_3)_3\text{Bi}\) in human matrices following bismuth ingestion. Quantification of \((\text{CH}_3)_3\text{Bi}\) was performed using an inter-aggregate calibration (IAC) method as described by Feldmann (1997) and as further validated by Diaz-Bone and Hitzke (2008).

Kinetics of \((\text{CH}_3)_3\text{Bi}\)

In comparison to the amount of bismuth ingested, only low concentrations of \((\text{CH}_3)_3\text{Bi}\) were measured in breath and blood. The courses of \((\text{CH}_3)_3\text{Bi}\) concentrations in both matrices were similar (figure 3), which indicates a close relationship between the two compartments with respect to this volatile bismuth species. The occurrence of \((\text{CH}_3)_3\text{Bi}\) in exhaled air two hours after bismuth ingestion points to a relatively rapid methylation process.

The big differences in AUCs observed in the individuals are a reflection of their different potential to methylate bismuth. The amount of exhaled bismuth was calculated from an average respiratory volume of 0.5 m$^3$/h and the AUC. This value was used to obtain an estimate of eliminated bismuth via exhaled air.

In a few study participants all \((\text{CH}_3)_3\text{Bi}\) blood levels were below the limit of detection, whereas the accompanying \((\text{CH}_3)_3\text{Bi}\) concentrations in exhaled air were measurable. The
variation coefficients of the measurements were generally in the range from 3 to 10 %, the larger variations may be partly due to (CH\textsubscript{3})\textsubscript{3}Bi decomposition during sample preparation. Most maxima of (CH\textsubscript{3})\textsubscript{3}Bi concentrations were reached in the interval from 8 to 24 hours after bismuth ingestion. Here, a differentiation between slower and faster methylation was observed (figure 4). As the number of sampling intervals was limited, precise concentration maxima, elimination patterns and half-lives of (CH\textsubscript{3})\textsubscript{3}Bi could not be determined.

The high variability observed in bismuth methylation may be either due to a gene polymorphism similar to that found for arsenic methylation in humans (Marnell et al. 2003; Drobna et al. 2004; Aposhian and Aposhian 2006) or to a varying composition of the intestinal microflora which has been shown to methylate bismuth \textit{ex situ} (Michalke et al. 2007 and 2008).

**Origin of (CH\textsubscript{3})\textsubscript{3}Bi**

Two scenarios are plausible for bismuth methylation in the human body: A microbial pathway with participation of microorganisms present in the intestine and an endogenous pathway as described for arsenic and other elements (Aposhian and Aposhian 2006; Thayer 2002).

Anaerobic incubation of feces samples obtained from volunteers following ingestion of bismuth demonstrated that intestinal microorganisms are able to methylate bismuth \textit{ex vivo} (Hirner et al. 2004, Michalke et al. 2007 and 2008). Finally, a strong indication that microbial methylation takes place \textit{in vivo} was the detection of significant amounts of trimethylbismuth in freshly collected feces (Boertz, 2008).

These observations in combination with the fact that bismuth is mainly excreted \textit{via} feces are strong indications that methylation of bismuth takes place in the human intestine. After microbial volatilization of (CH\textsubscript{3})\textsubscript{3}Bi in the colon, this species diffuses into the blood and is then transferred to the lungs, from where it is exhaled.
Nevertheless, by the current data the participation of an abiotic or endogenous enzymatic pathway, in particular in the human liver, cannot be ruled out. Abiotic methylation of bismuth by methyl-cobalamine has been demonstrated by Michalke et al. (2002).

A possible indication of the endogenous contribution to the methylation of bismuth is the detection of small amounts of trimethylbismuth as early as two hours after ingestion. Since the transport into the intestine normally requires a longer time period, it is unlikely that intestinal microorganisms account for \((\text{CH}_3)_3\text{Bi}\) production during this early period. Moreover, similar time profiles as observed in the present study for trimethylbismuth has been found for the methylated arsenic derivatives which are formed in the liver (Yu 1999; Styblo et al. 1999). Further studies will be necessary to differentiate the relative contribution of endogenous and microbial methylation of bismuth.

**Toxicological implication of bismuth volatilization**

Only low concentrations of the volatile \((\text{CH}_3)_3\text{Bi}\) were detected in blood and exhaled air after ingestion of the bismuth-containing pharmaceutical. It has to be emphasized that a bismuth containing-species with increased toxicity was formed from a bismuth-containing drug by this metabolic step. Based on LD$_{50}$ values available for CBS (7047 mg/kg, mice, oral, (Zhang et al. 2006)) and for \((\text{CH}_3)_3\text{Bi}\) (484 mg/kg, rabbit, oral (Craig 2003)) the acute toxicity increased by more than one order of magnitude. It should also be noted that the lipophilic potency of inorganic bismuth increases by methylation, which facilitates the crossing of membranes such as the blood-brain barrier. It may be speculated that the encephalopathies diagnosed in the 1970s in French and Australian patients were associated with the formation of the volatile toxic \((\text{CH}_3)_3\text{Bi}\) species (Michalke et al. 2002). These patients had taken bismuth salts for the treatment of duodenal ulcers for extended periods of time (Lowe 1974; Martin-Bouyer 1978).
Outlook

In view of the continuing use of bismuth compounds as pharmaceuticals and the widespread exposure of people to bismuth-containing cosmetics there is a need for further studies on bismuth metabolism in the human body. In these studies one should elucidate the influence of food on absorption and methylation of bismuth, the involvement of the intestinal microflora in the metabolic activity of a metal compound used as a drug, and the identification of the microorganisms which are responsible for bismuth methylation. Alternative bismuth methylating pathways, if these are present, should also be investigated in detail, e.g. by characterizing the methylating enzyme systems in human liver cells following incubation with bismuth-containing compounds.
References


Footnote to the title

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Legend for Figures

Figure 1  Profile of total bismuth in blood samples of 20 volunteers taken in the first 12 hours following ingestion of a bismuth containing pharmaceutical. In all pre-ingestion samples bismuth concentration was below the limit of detection. Subsequent to t = 12 hours, there was little change in the blood bismuth concentration.

Figure 2 (CH₃)₃Bi was detected at m/z = 209 for the ²⁰⁹Bi trace in GC/ICP-MS after 310.1 s (average 307.1 ± 5 s). Here a comparison between a trimethylbismuth containing sample (exhaled air, left y-axis) and a trimethylbismuth standard (right y-axis) is shown.

Figure 3 (CH₃)₃Bi concentrations in exhaled air and blood of two male volunteers. (a) A rapid production of (CH₃)₃Bi, and (b) a slower production of (CH₃)₃Bi.

Figure 4 Total bismuth concentrations in blood and (CH₃)₃Bi concentrations in exhaled air of two male volunteers. No relationship was found between (a) rapid and (b) slower (CH₃)₃Bi production and total bismuth absorption.
Table 1 Detection of \((\text{CH}_3)_3\text{Bi}\) versus number of volunteers at specific time intervals. \((\text{CH}_3)_3\text{Bi}\) was found in samples of all study participants.

<table>
<thead>
<tr>
<th>Time after ingestion (h)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
<th>32</th>
<th>48</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of volunteers with ((\text{CH}_3)_3\text{Bi}) in blood samples</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>13</td>
<td>14</td>
<td>13</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Number of volunteers with ((\text{CH}_3)_3\text{Bi}) in exhaled air samples</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>7</td>
<td>17</td>
<td>17</td>
<td>20</td>
<td>-</td>
<td>15</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 2

Counts per second (sample of exhaled air) vs Retention time (h) for Standard and Sample.

- Dashed line represents the Standard.
- Solid line represents the Sample.

The peak at 310.1 s corresponds to the isotope 209 Bi.
Figure 3b

(b) volunteer 113

\[
\text{(CH}_3)_3\text{Bi as Bi in exhaled air (mg/m}^3) \quad \text{pg (CH}_3)_3\text{Bi as Bi)/ml blood}
\]

Time after ingestion (h)

\[
0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 60
\]
Figure 4b

(b) volunteer 113

- - - Exhaled air

- - - Blood

Bi in blood (μg/l)

(CH₃)₃Bi as Bi in exhaled air (ng/m³)

Time after ingestion (h)