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# A systematic study of the disposition and metabolism of mercury species in mice after exposure to low levels of thimerosal (ethylmercury)☆

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ABSTRACT

Thimerosal (TM) is an ethylmercury (etHg)-containing preservative used in some vaccines despite very limited knowledge on the kinetics and direct interaction/effects in mammals' tissues after exposure. Thus, this study aimed to evaluate the kinetics of Hg species in mice in a time course analysis after intramuscular injection of TM, by estimating Hg half-lives in blood and tissues. Mice were exposed to one single intramuscular dose of 20 μg of Hg as TM. Blood, brain, heart, kidney and liver were collected at 0.5 hour (h), 1 h, 8 h, 16 h, 144 h, 720 h and 1980 h after TM exposure ($n=4$). Hg species in animal tissues were identified and quantified by speciation analysis *via* liquid chromatography hyphenated with inductively coupled mass spectrometry (LC–ICP-MS). It was found that the transport of etHg from muscle to tissues and its conversion to inorganic Hg (inoHg) occur rapidly. Moreover, the conversion extent is modulated in part by the partitioning between EtHg in plasma and in whole blood, since etHg is rapidly converted in red cells but not in a plasma compartment. Furthermore, the dealkylation mechanism in red cells appears to be mediated by the Fenton reaction (hydroxyl radical formation). Interestingly, after 0.5 h of TM exposure, the highest levels of both etHg and inoHg were found in kidneys (accounting for more than 70% of the total Hg in the animal body), whereas the brain contributed least to the Hg body burden (accounts for $<1.0\%$ of total body Hg). Thirty days after TM exposure, most Hg had been excreted while the liver presented the majority of the remaining Hg. Estimated half-lives (in days) were 8.8 for blood, 10.7 for brain, 7.8 for heart, 7.7 for liver and 45.2 for kidney. Taken together, our findings demonstrated that TM (etHg) kinetics more closely approximates $Hg^{2+}$ than methylmercury (meHg) while the kidney must be considered a potential target for etHg toxicity.



## 1. Introduction

Thimerosal (TM), which contains ethylmercury (etHg), has been widely used as a preservative in a number of drug products, including vaccines, to help prevent life-threatening contamination with microbes (Tan and Parkin, 2000). However, the potential neurotoxic effects of organomercurial compounds, even at low exposures (Lebel et al., 1998; Berman et al., 2008; Delong, 2011; Bose et al., 2012; Petroni et al., 2012; Ida-Eto et al., 2013), have provoked concerns about the use of thimerosal in vaccines and other products (Clements et al., 2000; Ball et al., 2001).

The toxic properties of Hg compounds are directly related to the chemical form of the element. In general, exposure to organic forms of Hg is associated with nervous system damage, while inorganic forms are closely connected to renal damage (Clarkson and Magos, 2006). However, the toxicokinetics and potential toxic properties of TM (etHg) are mostly unknown (WHO, 2012).

Due to the lack of information about the behavior of TM in the mammalian body, the initial risk assessments for etHg were based on studies of oral methylmercury (meHg) toxicity. However, recent

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data indicate that the kinetics of tissue disposition and metabolism differ substantially between these two forms of organic Hg (Clarkson, 2002; Magos, 2003; Burbacher et al., 2005), indicating that meHg is not a suitable reference for risk assessment from exposure to TM-derived Hg. Therefore, the knowledge of the toxicokinetics of TM is mandatory to afford a meaningful assessment of the developmental effects of TM-containing vaccines.

Thus, the present study aimed to investigate the body burden disposition of TM in mice after a single low-dose exposure. Two forms of Hg found after the exposure (etHg and the dealkylated form (inoHg)) were measured in blood and animal tissues by using hyphenation of High Performance Liquid Chromatography to Inductively Coupled Plasma Mass Spectrometry (HPLC–ICP-MS) in a time course analysis to estimate Hg half-lives. It was also demonstrated that the mechanism of etHg dealkylation appears to be mediated by the Fenton reaction (hydroxyl radical formation).

## 2. Material and methods

### 2.1. Reagents

All reagents used were of analytical grade and the solutions were prepared using high purity water with a resistivity of 18.2 MΩ cm (Milli-Q Plus, Millipore, Bedford, MA, USA). A solution of 37% hydrochloric acid (Merck, Darmstadt, Germany) was doubly distilled in a quartz sub-boiling apparatus (Kürner Analysentechnik, Rosenheim, Germany). A clean laboratory and laminar-flow hood capable of producing class 100 were used for preparing solutions and samples for the HPLC–ICP-MS technique. All solutions used were stored in high-density polyethylene bottles. Materials were cleaned by soaking in 10% v/v $HNO_3$ for 24 h, rinsing five times with ultrapure water and dried in a class 100 laminar flow hood before use. All operations were performed on a clean bench. A 10 mg/l standard solution of inoHg was obtained from Perkin-Elmer (PerkinElmer, Norwalk, CT). A 1000 mg/l standard solution of meHg chloride ($CH_3HgCl$) and 1000 mg/l standard solution of etHg chloride ($CH_3CH_2HgCl$) in water were obtained from Alfa Aesar (MA, USA). Analytical calibration standards of Hg species were prepared daily over the range of 0.0–20.0 μg/l for the HPLC–ICP-MS by suitable serial dilutions of the stock solution.

Additional reagents used for the speciation studies included methanol (99.9% v/v, HPLC-grade, Merck, Germany), mercaptoethanol (Sigma-Aldrich, USA) and L-cysteine (Fluka, Japan). Ammonium acetate (99.99%) was obtained from Aldrich Chemical Company (Milwaukee, USA) and formic acid, dimethyl sulfoxide (DMSO) and hydrogen peroxide ($H_2O_2$) were purchased from Sigma-Aldrich (USA).

### 2.2. Instrumentation and method

All measurements were carried out with a HPLC (Perkin-Elmer model L-200, a six-port injector Rheodyne 9725 and a reverse-phase column C8 Brownlee 3 μm, 33 mm × 4.6 mm) hyphenated to an ICP-MS (Elan DRCII PerkinElmer, Norwalk, CT, USA). Argon with a purity of 99.999% (White Martins, São Paulo, Brazil) was used throughout. A Vibracell VC 100 ultrasonic processor with a titanium probe controlled by USS-100 (Sonics & Materials, Danbury, CT, USA) was employed for Hg extraction from samples. The ultrasonic probe was set at an amplitude of 80%, power of 50 W and a frequency of 20 kHz. Samples volume injection was 100 μl. All separations were performed at room temperature under isocratic conditions. The isocratic mobile phase was 3% v/v methanol + 97% v/v (0.5% v/v 2-mercaptoethanol + 0.05% v/v formic acid). The flow rate was 1.2 ml/min. Data were evaluated using the software Chromera® (supplied with the instrument) and quantified by external calibration using a peak area. The complete description of experimental conditions for both ICP-MS and HPLC were provided by Souza et al. (2013). Data were validated by the analysis of Standard Reference Materials (SRM) 966 Toxic Metals in Bovine Blood from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) and Certified Reference Materials (CRMs) DOLT-3, DORM-3 and TORT-2 from the National Research Council Canada (NRCC). Found values were in adequate agreement with target values according to a *t* test at the 95% confidence level.

### 2.3. Experimental design

#### 2.3.1. In vivo study

Animals were handled and treated according to the guidelines of the Committee on the Care and Use of Experimental Animal Resources of the University of São Paulo, Brazil (Opinion number 12.1.1158.53.1, issued on the 14th of December of 2012). Male Swiss mice weighing approximately 25 g (sixth week of life) were obtained from the central animal facility. Animals were subjected to 12 h light/12 h dark cycles in an air-conditioned room (22–25 °C) with free access to food and water.

The animals ($n=28$) were exposed to 20 μg of Hg in the form of TM (sodium ethylmercury thiosalicylate, Sigma Chemical, St Louis, MO) through IM injection, which corresponded to approximately 0.8 mg Hg/kg. The dose was adapted to the mouse body weight and corresponded to a dose 20 times higher than that a child of 3.5 months of age receives in terms of Hg, only from vaccines (Clements et al., 2000). Animals were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg) to collect blood and organs (brain, heart, kidney and liver) after 0.5 hour (h), 1 h, 8 h, 16 h, 144 h (or 6 days (d)), 720 h (or 30 d) and 1980 h (or 80 d) after TM exposure (4 animals per time of study). The blood and organs were placed in metal-free Eppendorf tubes and kept at −80 °C until analysis. Plasma samples were not included in the present study due to the limitation to collect the necessary volumes from mice for Hg speciation.

The total mass of each Hg species accumulated in each sample was calculated, dividing the concentration found (μg/ml or μg/g) by the total volume of blood in the animal body (mice of 25 g, approximately 1.6 ml) or the mean mass obtained of the respective organ (in grams)—previously weighed in 3 control animals at approximately 25 g. This mass balance was performed due to the fact that Hg concentrations (as μg/ml or μg/g) do not reflect directly the absolute amount of Hg accumulated in tissues, *i.e.*, tissues with smaller sizes and lower Hg concentration may contain more Hg than tissues with higher Hg concentrations.

Moreover, along the time course, the total amount of Hg obtained at each moment—*i.e.*, the sum of Hg levels measured in all monitored tissues—was considered to be 100% (since most of the Hg is concentrated in these compartments). This assumption was made in order to better illustrate the contribution of each organ to Hg accumulation over time. Then, the percentages of inoHg and etHg were calculated in each organ at one time point assuming the whole amount of the exposure dose (20 μg) to be 100%.

#### 2.3.2. In vitro study

An *in vitro* experiment was conducted to evaluate the stability of TM and etHg in whole blood, plasma and erythrocytes. This part of the study was conducted in three different experiments to: (i) evaluate the stability of TM and etHg in whole blood, plasma and erythrocytes samples; (ii) and (iii) to elucidate the mechanism behind the dealkylation of etHg in blood. These experiments were performed on samples collected from two healthy human volunteers.