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Abstract

Thimerosal is a preservative that has been used in manufacturing vaccines since the 1930s. Reports have indicated that infants can receive ethylmercury (in the form of thimerosal) at or above the Environmental Protection Agency (EPA) guidelines for methylmercury (MeHg) exposure, depending on the exact vaccinations, schedule, and size of the infant. This study compared the systemic disposition and brain distribution of total and inorganic mercury in infant monkeys following thimerosal exposure with infants exposed to MeHg. Monkeys were exposed to MeHg (via oral gavage) or vaccines containing thimerosal (via i.m. injection) at birth and 1, 2, and 3 weeks of age. Total blood mercury (Hg) levels were determined 2, 4 and 7 days after each exposure. Total and inorganic brain Hg levels were assessed 2, 4, 7 or 28 days after the last exposure. The initial and terminal half-life of Hg in blood following thimerosal exposure was 2.1 and 8.6 days, which are significantly shorter than the elimination half-life of Hg following MeHg exposure at 21.5 days. Brain concentrations of total Hg were significantly lower by ~3-fold for the thimerosal-exposed infants when compared to the MeHg infants, while the average brain-to-blood concentration ratio was slightly higher for the thimerosal-exposed infants (3.5±1.0 vs. 2.5±0.6). A higher percentage of the total Hg in the brain was in the form of inorganic mercury for the thimerosal-exposed infants (34% vs 7%). The current study indicates that MeHg is not a suitable reference for risk assessment from exposure to thimerosal derived Hg. Knowledge of the toxicokinetics and developmental toxicity of thimerosal is needed to afford a meaningful assessment of the developmental effects of thimerosal-containing vaccines.

Introduction

Public perception of the safety and efficacy of childhood vaccines has a direct impact on immunization rates (Biroscak et al. 2003, Thomas et al. 2004). The current debate linking the use of thimerosal in vaccines to autism and other developmental disorders (IOM 2001, 2004) has led many families to question whether the potential risks associated with early childhood immunizations may outweigh the benefits (Blaxill et al. 2004; http://www.SafeMinds.org). Thimerosal is an effective preservative that has been used in the manufacturing of vaccines since the 1930s. Thimerosal is comprised of 49.6 % mercury by weight and breaks down in the body to ethylmercury and thiosalicylate (Tab and Parkin 2000). Recent reports have indicated that some infants can receive ethylmercury (in the form of thimerosal) at or above the Environmental Protection Agency (EPA) guidelines for methylmercury (MeHg) exposure, depending on the exact vaccinations, schedule, and size of the infant (Ball et al. 2001). Clements et al. (2000) calculated that children receive 187.5 micrograms of ethylmercury from thimerosal containing vaccines given over the first 14 weeks of life. According to the authors, this amount approaches or, in some cases, exceeds the EPA guidelines for MeHg exposure during pregnancy (0.1 μg/kg/day). Other estimates (Halsey 1999) have indicated that the schedule could provide repeated doses of ethylmercury from approximately 5 to 20 μg/kg over the first 6 months of life. Studies in preterm infants indicate that blood levels of mercury following just one vaccination (hepatitis B) increase by over 10-fold to levels above the EPA guidelines (Stajich et al. 2000).

EPA guidelines for MeHg are based on several decades of studies of humans and animal models of developmental toxicity (Burbacher et al. 1990; National Research Council 2000). Since little data exist for ethylmercury, the use of the MeHg guidelines would seem appropriate if the two compounds have similar toxicokinetic profiles and neurodevelopmental effects. The results from the few studies that have provided a direct comparison of these two compounds have been summarized recently in a review article by Magos (2003), who concluded that:

- Mercury clears from the body faster after the administration of ethylmercury than after the administration of MeHg
- The brain-to-blood mercury concentration ratio established for MeHg will

overestimate mercury in the brain after exposure to ethylmercury

- Because ethylmercury is decomposed faster than MeHg, the risk of brain damage is less for ethylmercury than for MeHg.

These conclusions are based on only a few studies, none of which included measurements of both blood and brain mercury levels in infant subjects.

The current study was initiated to provide a direct comparison of the blood and brain levels of mercury in infant nonhuman primates exposed orally to MeHg or via i.m. injections of vaccines containing thimerosal. Nonhuman primates have been used extensively in previous studies of MeHg toxicokinetics and developmental neurotoxicity (Stinson et al. 1989; Vahter et al. 1994, 1995; Burbacher et al. 1986, 1990; Gunderson et al. 1986, 1988; Rice and Gilbert 1982, 1990, 1995). The routes of administration (oral for MeHg and i.m. injection for thimerosal-containing vaccines) were chosen to mimic the two routes of mercury exposure for humans. The dosages and schedule of administration of mercury were chosen to be comparable to the current immunization schedule for human newborns, taking into consideration the faster growth (approximately 4 to 1) of the macaque infant (Gunderson and Sackett 1984). The results of this study provide important new information regarding the comparative toxicokinetics of these two compounds in newborns and infants.

Methods

Subjects: Forty-one infant Macaca fascicularis born at the Washington National Primate Research Center's Infant Primate Research Laboratory were used in the study. The birth weights of the infants were within the normal range for this species; the average birth weight was 341 grams (range 255 to 420 grams). Infants were weighed daily throughout the study and any clinical problems were recorded.

Mercury Dosing Schedule: The mercury-dosing schedule is shown in Table 1. Infants were assigned to 1 of 3 exposure groups at birth. Seventeen infants assigned to the thimerosal group were given the typical schedule of vaccines for human infants (see Table 1). Thimerosal (Omicron Quimica S.A.), dissolved in saline, was mixed with thimerosal-free vaccines to yield a final concentration of 4, 8, or 20 μg/ml mercury, depending on the vaccine and the age of the infant. The total dose of mercury

administered via the vaccines was 20 μg/kg on day 0 and at 7, 14, and 21 days of age. A dose of 20 μg/kg was chosen based on the range of estimated doses received by human infants receiving vaccines during the first 6 months of life.

Seventeen infants assigned to the MeHg group were given MeHg hydroxide (MeHgOH, 97% pure, Alfa Aesar, Johnson Matthey Co., Ward Hill, Massachusetts USA) dissolved in water to a concentration of 20 μg Hg/ml. MeHg was administered to infants via oral gavage at a dose of 20 μg/kg on their day of birth (day 0) and at 7, 14, and 21 days of age.

Seven infants were assigned to a control group. These infants did not receive any gavages or i.m. injections. Infants were assigned to the three groups on a semi-random basis, in order to balance gender ratios and average birth weights across groups.

Blood Draw Schedule: Blood was drawn from the saphenous vein of all infants at birth (prior to any Hg exposure). Blood was also drawn 2, 4, and 7 days after the initial Hg exposure (day 0) and after subsequent exposures on days 7 and 14. Depending on the sacrifice group (see below) blood was drawn up to 28 days after the final exposure on day 21 to further characterize the washout kinetics of Hg (see Table 1).

Sacrifice Schedule: Infants were sacrificed 2, 4, 7 or 28 days after their last Hg exposure on day 21 (see Table 1). Infants were sedated with an i.m. injection of ketamine (10mg/kg) and atropine (0.4 mg/kg) and then given an intravenous overdose of Nembutal (20 mg/kg). Autopsy personnel from the Primate Center drew blood and removed the brain and other organs for analysis. The autopsy typically lasted approximately 1 hour.

The number of infants at each sacrifice day for both the MeHg and thimerosal groups was Day 2=4, Day 4=4, Day 7=5, and Day 28=4. The 7 control infants were assigned sacrifice days as follows Day 2=3, Day 4=1, Day 7=2, and Day 28=1 (see Table 1). Infants were assigned to sacrifice groups at birth on a semi-random basis that balanced gender ratios and average birth weights across groups.

Blood and Brain Hg Measurement: Blood samples were prepared for Hg analysis by diluting them with an equal volume of 1 % w/v NaCl solution. Aliquots were removed for mercury determination without digestion. One drop of antifoam reagent was added to the aliquot at the time of the analysis.

Half brain samples were fixed in formaldehyde prior to analysis. Samples of the fixative were analyzed to check for mercury content. The tissue was removed from the jar and blotted dry. A homogenate of the brain in 1% NaCl was prepared using a Polytron homogenyzer PT 10-35 (Brinkmann Instruments, Westbury, NY) while keeping the sample in an ice slurry. An aliquot of the homogenate was digested with 1 ml of 1% w/v cysteine and 2 ml of 45 % NaOH by heating at 95^0C for 10 to 15 minutes. Digest was allowed to cool and then diluted to volume by addition of 7 ml of 1% w/v NaCl. The digests were kept in an ice slurry until analysis. Aliquots were removed for mercury determination. One drop of antifoam was added to the aliquot at the time of the analysis.

Total Hg concentrations in blood and total and inorganic Hg concentrations in brain were measured using a procedure adapted from Greenwood et al. (1977). The method determines total mercury and its inorganic fraction (Magos and Clarkson 1972). Cadmium chloride in the presence of stannous chloride at high pH breaks the mercury-carbon bond with the subsequent reduction of Hg^{2+} to Hg^0, the latter is then measured by cold vapor atomic absorption at 254 nm with a Model #1235 mercury monitor from Laboratory Data Control (Thermo Separation Products). Inorganic mercury is determined by the addition of stannous chloride in the absence of cadmium chloride. Concentration of organic Hg was calculated from the difference between the measured total and inorganic Hg concentrations. The original concentration of $SnCl_2$ used for the Magos method (1972) was modified to prevent the decomposition of the ethylmercury during assay (Magos et al. 1985). To measure mercury in aqueous solution of thimerosal the amount of $SnCl_2$ was reduced from 100 µg to 50 µg per aliquot analyzed. For tissue homogenate samples, 500 µg of $SnCl_2$ was added to each aliquot. All reagents used for preparation and analysis of the samples were of analytical grade.

Quality control was assured by analysis of reference samples prior to each assay run. Fisher Mercury Reference Solutions (SM114-100, certified 1000 ppm + 1%) was used as a stock solution. Working standards of 30 and 10 ng Hg/ml were made daily from appropriate dilutions of the stock solution. In addition, the following certified reference materials were analyzed daily prior to analysis of the samples: Trace Elements in Whole Blood (Seronorm Trace Elements, Accurate Chemical & Scientific Corporation, Certified Reference Material #201605, 6.8-8.5 µg/L), and Trace Elements in Human Hair

(Commission of the European Communities, Certified Reference Material #397: 12 µg/g +/- 0.5). The detection limit of the instrument was estimated to be 0.75 ng Hg per aliquot used for analysis.

Data Analysis: The mean total blood Hg concentration data from both the oral MeHg and i.m. thimerosal groups (N=17 in each) were subject to analysis using the compartmental module of the pharmacokinetic modeling software SAAM II (SAAM Institute, Seattle, WA).

The accumulation and washout of total blood Hg concentration-time data from the MeHg infants were well described by a one-compartment model featuring a first-order absorption process. Regression fit of the data to the model yielded estimates of the absorption rate constant (k_a), elimination rate constant (K), and an apparent volume of distribution (V/F, F is the implicit bioavailability term). Half- lives ($T_{1/2}$) corresponding to each of the rate constants were calculated by dividing ln 2 by the rate constant estimate. Blood clearance (Cl/F) was derived from the product of K and V/F.

A one-compartment model failed to provide a satisfactory fit of the mean total blood Hg concentration-time data from the thimerosal infants. The model over-predicted the blood concentration during accumulation; at the same time, it under-predicted the blood concentration during washout rate (i.e., over-predicted washout rate). Further examination of a scatter-plot of the individual monkey data suggested a biphasic pattern in the washout of Hg from the blood following the last dose. Accordingly, a regression fit of the mean total blood Hg concentration data with a two-compartment model was attempted. This yielded a much better visual fit of the data, with minimal change in the objective function and Akaike Information Criterion (AIC**).** The two-compartment parameter estimates from the regression analysis included the absorption rate constant (k_a), rate constants for Hg transfer from the central to the peripheral compartment (k_{12}) and the return from the peripheral to the central compartment (k_{21}), the elimination rate constant from the central compartment (k_{10}), and the apparent volume of the central compartment (Vc/F). From these primary parameters, we further estimated the apparent distribution volume at steady-state (Vss/F), and the peripheral volume referenced to blood concentration (i.e., Vp=Vss – Vc). The initial and terminal rate constants and half-lives ($T_{1/2,\alpha}$ and $T_{1/2,\beta}$) for the biexponential decline of total blood Hg concentration were

estimated by standard formulae (Gibaldi and Perrier 1982). Blood clearance was computed by the product of Vc and k_{10}. For both the MeHg and thimerosal model fits, a fractional standard deviation of 0.1 was used as the weighting scheme.

The washout half-life of total and organic Hg in the brain of both the oral MeHg and i.m. thimerosal groups were estimated by regression fit to a monoexponential model using the WinNonlin software (Pharsight Corp., Mountain View, CA). One of the Day 28 brain samples from the MeHg exposure group had a spuriously high total Hg concentration; i.e., a concentration of 151 ng/g, which is more than 50% higher than the other samples obtained on Day 28 (71-90 ng/ml) and higher than those observed at the earliest sacrifice time at Day 2 (75 to 129 ng/g). The unreasonably high concentration is most likely due to contamination of the sample. Therefore, data from this brain and its corresponding blood were excluded from the regression analysis. The average brain-to-blood concentration ratio was also calculated using data from the earliest sacrifice duration (2 days). Because of different washout half-lives in blood and the brain, brain-to-blood concentration ratio is expected to vary with the duration of washout. Samples at Day 2 offered the best measure of the extent of uptake of Hg species into the brain that are least confounded by differences in their clearance rate.

Between-group statistical comparisons of the rate of washout of total Hg in blood, as well as total and organic concentrations in the brain, were accomplished through multiple regression analysis as implemented in the PROC GLM subroutine in SAS (version 9.1, SAS Institute, Gary NC). PROC GLM performs multiple regression within the framework of General Linear Models, and can accommodate missing data or sparse sampling and confounding from correlations between repeated measures. Hence, it is able to provide tests of hypotheses for the effects of time and group using blood and brain data obtained from sacrifice of individual animals at varying times during washout. Log transformed blood or brain Hg concentrations in animals from both the MeHg and thimerosal groups were entered as the dependent variable. The independent variables consisted of sampling time, group (MeHg=0, thimerosal=1), and a time-by-group interaction. Once the overall significance of the regression model was verified, the significant sources of variation (i.e., time, group and time-by-group) were identified. A difference in the rate of washout of Hg in blood or brain between groups was indicated by

a significant regression coefficient for time-by-group interaction. If there was no evidence for interaction, a significant decline in blood or brain Hg concentration over time for each group was assessed by the t-statistic associated with the estimated regression coefficient for time.

The following statistical comparisons of the washout rate of Hg were also undertaken: total Hg in blood versus total Hg in brain, total Hg in blood versus organic Hg in the brain, and total Hg versus organic Hg concentration in the brain. The difference between the pair of log transformed Hg concentrations for each animal sacrificed at the various times was calculated. Individual difference values in both groups were then entered as the dependent variable in the regression model. The independent variables were time, group and time-by-group interaction. A significant regression coefficient for the time variable indicates that the paired-log concentration difference (or the concentration ratio) varied with time; i.e., the two concentration measures (e.g. blood and brain) declined in parallel with time.

Results

Infant Growth and Health Status: The weights of infants during the study are shown in Figure 1. There were no significant differences in the weight gain across the 3 groups ($p>0.10$, all comparisons). The average weight gain during the first 23 days of life was 135 grams. The brain weights at sacrifice and brain-to-body weight ratios are shown in Table 2. There were no significant differences in the brain weights or brain-to-body weight ratios across the 3 groups ($p>0.10$, all comparisons). There were no serious medical complications for any of the infants.

Oral MeHg Kinetics: The total blood Hg concentrations at 2 days (observed peak) following the first dose ranged from 8 to 18 ng/ml across the infants, i.e., a 2-fold variation. Progressive accumulation of total blood Hg was observed over the three subsequent doses of MeHg, such that the peak total blood Hg concentrations after the fourth dose were about 3-fold higher (30-46 ng/ml). The inter-animal variation in blood Hg concentrations remained at about 2-fold during accumulation. Blood Hg persisted through the entire period of washout, and was readily measurable in all 4 infants in the 28 day sacrifice group (16-21 ng/ml). This is consistent with previous reports of a greater

than 20 day elimination $T_{1/2}$ of methymercury in adult M. fascicularis (Stinson et al. 1989; Vahter et al. 1994, 1995) and explains the minimal decline (<20%) in blood mercury concentrations during the weekly intervals between MeHg doses.

The time course of total blood Hg was fitted to a one-compartment model. Figure 2 shows the excellent regression fit of the mean blood concentration-time data. Table 3 presents parameter estimates from the one-compartment model fit of the mean blood Hg concentration-time data. The distribution volume of total mercury following MeHg administration is estimated to be 1.7 L/kg, or about 20 times the blood volume (~8%). This means that only 1/20th of the body burden of mercury is confined to the vascular space. This is consistent with the known extensive extravascular distribution of Hg following methymercury exposure in primates and agrees with previous estimates of Hg distribution volume in adult M. fascicularis (Stinson et al. 1989). The elimination $T_{1/2}$ of total blood Hg is 21.5 days, which agrees with reported estimates in adult M. fascicularis (Stinson et al. 1989; Vahter et al. 1994, 1995). The blood clearance is estimated at 46.1 ml/day/kg, well within the range of clearance values observed earlier in adult M. fascicularis (Stinson et al. 1989). It appears that the systemic disposition kinetics of MeHg are the same between infant and adult M. fascicularis, i.e., no change during development.

A plot of the blood and brain total Hg concentration data from the infants sacrificed at various times during the washout period is shown in Figure 3. There was a significant decrease in total Hg from the blood during the washout period ($p< 0.01$). The apparent $T_{1/2}$ for total Hg in blood is 19.1±5.1 days (±standard error of regression estimate). The decrease in total Hg in the brain over time was marginally significant ($p< 0.07$), with an apparent $T_{1/2}$ of 59.5±24.1 days. The $T_{1/2}$ for total Hg in brain was significantly longer than the $T_{1/2}$ for total Hg in blood ($p= 0.05$) for the MeHg-exposed infants. The $T_{1/2}$ for total Hg in brain (59.5±24.1 days) is also longer than the previously reported washout $T_{1/2}$ from the brain for adult M. fascicularis (37 days, Vahter et al. 1994, 1995). It should be noted that the relatively high standard error of the half-life estimates for the brain reflects the large inter-animal variation in Hg concentrations at each sampling time, limited number of data points, and the short duration of sacrifice relative to the washout half-life. The concentration of total Hg in the brain is 1.7 to 3-

fold higher than in the blood (mean±SE = 2.5±0.3) 2 days after the last MeHg dose. This brain-to-blood concentration ratio increased as the duration between the last dose and the sacrifice lengthened. The ratio ranged from 3.9 to 7.4 at 28 days after the last exposure. The time-dependence for the brain-to-blood ratio is primarily due to the difference in the washout $T_{1/2}$ between total Hg in the blood and brain (p=0.06). The average brain-to-blood ratio for these infants at Day 2 after the last MeHg dose (2.5±0.3) is slightly lower than previously reported values (3 to 5) for adult macaque and squirrel monkeys over various durations of washout (Stinson et al. 1989; Vahter et al. 1994; Berlin et al. 1975). Although the cited differences in brain uptake and clearance of MeHg between adult and infant monkeys may be attributed to the effects of postnatal brain growth and development, it may also be related to variation in exposure regimen between studies.

A plot of the organic and inorganic Hg concentrations in the brain of MeHg-exposed infants sacrificed at various times during the washout period is shown in Figure 4. The decrease in organic Hg in the brain over time was not statistically significant (p= 0.17). The apparent $T_{1/2}$ for the washout of organic Hg from the brain was 58.4±25.0 days, close to the $T_{1/2}$ for total Hg. The concentration of inorganic Hg in the brain samples was below the quantifiable limit of the assay (7 ng/ml) in 8 of 17 MeHg-exposed infants. The average concentration of inorganic Hg for those infants with values above the detection limit (N=10) did not change significantly over 28 days of washout and was approximately 7 to 8 ng/ml (see Figure 5). Inorganic Hg represented only 6% to 10% of total Hg in the brain. These values are consistent with previously reported data in adult M. fascicularis (Vahter et al. 1994, 1995).

Intramuscular Thimerosal Kinetics: The initial total Hg concentrations in the day 2 blood samples, which ranged from 6 to 14 ng/ml, are comparable to the concentrations observed in the oral MeHg group. These blood levels are also similar to those reported in preterm infants receiving 12.5 μg of mercury from a hepatitis b vaccine (Stajich et al. 2000). Blood Hg concentrations declined relatively rapidly (by >50%) in between doses. As a result, there was minimal accumulation in blood Hg concentrations during weekly dosing. Also, blood Hg concentrations dropped below the detection limit of the assay in some animals by day 10 after the last vaccine injection.

The time course of total blood Hg concentrations was best described by a two-

compartment model; i.e., the disposition kinetics is biphasic, with a rapid initial phase followed by a slower terminal phase of clearance. Table 4 presents the parameter estimates derived from the two-compartment model analysis. A comparison of the model prediction and the observed blood concentration data are shown in Figure 5. The model predicted some accumulation in peak blood Hg concentrations, and minimal accumulation in trough concentrations. Since blood concentration data were not available before day 2, the predicted peak concentrations are extrapolations and should be viewed with caution. The initial volume of distribution in the central compartment was 1.7 L/kg, which is comparable to the overall distribution volume for oral MeHg. The initial and terminal blood half-life was 2.1 and 8.6 days, respectively. Mercury derived from thimerosal is eliminated much more rapidly than MeHg. The steady-state volume of distribution (i.e., Vss or the fully equilibrated volume) was estimated to be 2.5 L/kg, which is 50% larger than the initial distribution volume (i.e., Vc). Hence, the effective peripheral compartment volume at steady state is about 0.8 L/kg. Alternately, this means that, at steady state, partitioning of the body burden of Hg between the tissue regions associated with the central and peripheral compartments is about 2:1. The blood clearance of total Hg was estimated to be 248 ml/day/kg, which is 5.4-fold higher than the estimate for oral MeHg.

Figure 6 presents a scatter-plot of the blood and brain total Hg concentration data for infants sacrificed at various times during the washout. There was a significant decrease in total Hg concentration in the blood during the washout period ($p< 0.01$). The apparent $T_{1/2}$ for total Hg in blood is 6.9±1.7 days. There was also a significant decrease in total Hg concentration in the brain over time ($p< 0.01$), with an apparent $T_{1/2}$ of 24.2±7.4 days. The $T_{1/2}$ for total Hg in brain was significantly longer than the $T_{1/2}$ for total Hg in blood ($p< 0.01$) for the thimerosal-exposed infants. In addition, the $T_{1/2}$ for total Hg in blood and brain for these infants (6.9±1.7 days and 24.2±7.4 days) are significantly shorter ($p< 0.01$) than the $T_{1/2}$ for total Hg in blood and brain for the MeHg infants (19.1±5.1 days and 59.5±24.1 days). The concentration of total Hg in the brain of the thimerosal-exposed infants is 2.6 to 4.6-fold higher than in the blood (mean±SE = 3.5±0.5) 2 days after the last injection. Again, this ratio increased as the sacrifice was performed at longer durations from the last dose, primarily due to the difference in the

half-lives of total Hg in the blood and brain.

A plot of the organic and inorganic Hg concentrations in the brain of thimerosal-exposed infants sacrificed at various times during the washout period is shown in Figure 7. There was a significant decrease in organic Hg in the brain over the washout period ($p< 0.01$). The apparent $T_{1/2}$ for the washout of organic Hg from the brain was 14.2±5.2 days, which is significantly shorter than the $T_{1/2}$ for total Hg in brain ($p< 0.01$). The inorganic form of Hg was readily measurable in the brain of the thimerosal-exposed infants. The average concentration of inorganic Hg did not change across the 28 days of washout and was approximately 16 ng/ml (see figure 9). This level of inorganic Hg represented 21 % to 86% of the total Hg in the brain (mean±SE = 70±4%), depending on the sacrifice time. These values are considerably higher than the inorganic fraction observed in the brain of MeHg infants (6% to10%).

Discussion

There are notable similarities and differences in the kinetics of Hg following oral administration of MeHg and i.m. injection of thimerosal in vaccines. The absorption rate and initial distribution volume of total Hg appear to be similar between i.m. thimerosal and oral MeHg. This means approximately equal peak total blood Hg levels following a single exposure to either MeHg or thimersoal or following episodic exposures that are apart by longer than four elimination half-life (i.e., >80 days for MeHg or >28 days for thimerosal). Studies in preterm and term human infants have reported similar results (Stajich et al. 2000). Infants receiving 12.5 ug of mercury from a single hepatitis b vaccine had blood mercury levels at 48 to 72 hours consistent with what would be anticipated after an equal dose of MeHg.

While the initial distribution volume of total Hg is similar for the 2 groups, a biphasic exponential decline in total blood Hg is observed only following i.m. injections of thimerosal. This suggests continual distribution into and localization in tissue sites over time. It is relevant to note that the kidney-to-blood concentration gradient of total Hg is much higher in the thimerosal infants than in the MeHg infants (mean±SE 95.1±10 vs 5.8±0.6). The second slower phase of washout could also represent the gradual biotransformation of ethylmercury (the presumed principal organic form of Hg after thimerosal administration) to Hg-containing metabolites that have a different tissue

distribution or are more slowly eliminated. Further investigations of the disposition fate of thimerosal-derived mercury should address these issues.

Total Hg derived from i.m. thimerosal is cleared from the infant M. fascicularis much more quickly than MeHg. The washout $T_{1/2}$ of total blood Hg following i.m. injections of thimerosal in vaccines is much shorter than the $T_{1/2}$ of MeHg (6.9 vs. 19.1 days). These results support the earlier conclusion of Magos (2003) that mercury is cleared from the body faster after the administration of ethylmercury than after the administration of MeHg. More interestingly, the washout blood Hg $T_{1/2}$ in the thimerosal-exposed infant macaques is remarkably similar to the blood mercury $T_{1/2}$ of approximately 7 days in human infants injected with thimerosal-containing vaccines reported by Pichichero et al. (2002).

An important consequence of the difference in blood half-lives is the remarkable accumulation of blood Hg during repeated exposure to MeHg. While the initial blood Hg concentration (at 2 days after the first dose) did not differ between the MeHg and thimerosal groups, the peak blood Hg concentration in the MeHg-exposed infants rose to a level nearly 3 times higher than in the thimerosal infants after the 4th dose. Furthermore, the blood clearance of total Hg is 5.4-fold higher after i.m. thimerosal than after oral MeHg exposure. This means that for an equivalent level of chronic exposure, the area under the curve (AUC) of total blood mercury concentrations in infants receiving repeated i.m. injections of thimerosal-containing vaccines will be significantly lower than infants exposed chronically to MeHg via the oral route.

A much lower brain concentration of total Hg was observed in the thimerosal infants compared to the MeHg infants, i.e., a 3- to 4-fold difference for an equivalent exposure of Hg. Moreover, total Hg is cleared much more rapidly from the brain after thimerosal than after methymercury exposure (24 vs 60 days). It appears that the difference in brain Hg exposure between thimerosal and MeHg is largely driven by their differences in systemic disposition kinetics (i.e., the blood level). The average brain-to-blood partitioning ratio of total Hg in the thimerosal group was slightly higher than that in the MeHg group (3.5 ± 0.5 vs 2.5 ± 0.6, t-test, $p=0.11$). Thus, the brain-to-blood mercury concentration ratio established for MeHg will underestimate the amount of mercury in the brain after exposure to thimerosal.

The large difference in the blood Hg half-life compared to the brain half-life for the thimerosal-exposed infants (6.9 days vs 24 days) indicates that blood Hg may not be a good indicator of risk of adverse effects on the brain, particularly under conditions of rapidly changing blood levels such as those observed following vaccinations. The blood concentrations of the thimerosal-exposed infants in the current study are within the range of those reported for human infants following vaccination (Stajich et al 2000). Data from the current study predicts that while little accumulation of Hg in the blood occurs over time with repeated vaccinations, accumulation of Hg in the brain of infants will occur. Thus, conclusion regarding the safety of thimerosl drawn from blood Hg clearance data in human infants receiving vaccines may not be valid, given the significantly slower half-life of Hg in the brain as observed in the infant macaques.

There was a much higher proportion of inorganic Hg in the brain of thimerosal infants than MeHg infants (up to 71% vs. 10%). Absolute inorganic Hg concentrations in the brains of the thimerosal-exposed infants were approximately twice that of the MeHg infants. Interestingly, the inorganic fraction in the kidneys of the same cohort of infants was also significantly higher following i.m. thimerosal than oral MeHg exposure (0.71±0.04 vs. 0.40±0.03). This suggest that the dealkylation of ethylmercury is much more extensive than that of MeHg.

Previous reports have indicated that the dealkylation of mercury is a detoxification process that helps to protect the CNS (Magos et al. 1985; Magos 2003). These reports are largely based on histology and histochemistry studies of adult rodents exposed to mercury for a short period of time. The results of these studies indicated that damage to the cerebellum was only observed in MeHg treated animals who had much lower levels of inorganic mercury in the brain than animals comparably treated with ethylmercury. Moreover, the results did not indicate the presence of inorganic mercury deposits in the area where the cerebellar damage was localized (granular layer).
In contrast, previous studies of adult M. fascicularis monkeys exposed chronically to MeHg have indicated that demethylation of mercury occurs in the brain over a long period of time following MeHg exposure and that this is not a detoxification process (Vahter et al. 1994, 1995; Charleston et al. 1994, 1995, 1996). Results from these studies indicated higher inorganic Hg concentrations in the brain 6 months after MeHg exposure

had ended while organic Hg had cleared from the brain. The estimated half-life of organic Hg in the brain of these adult monkeys was consistent across various brain regions at approximately 37days (similar to the brain half-life in the present infant monkeys). The estimated half-life of inorganic Hg in the brain in the same adult cohort varied greatly across some regions of the brain, from 227 days to 540 days. In other regions, the concentrations of inorganic Hg remained the same (thalamus) or doubled (pituitary) 6 months after exposure to MeHg had ended (Vahter et al. 1994, 1995). Stereologic and autometallographic studies on the brains of these adult monkeys indicated that the persistence of inorganic Hg in the brain was associated with a significant increase in the number of microglia in the brain, while the number of astrocytes declined. Notably, these effects were observed 6 months after exposure to methymercury ended, when inorganic Hg concentrations were at their highest levels, or in animals solely exposed to inorganic Hg (Charleston et al. 1994, 1995, 1996). The effects in the adult macaques were associated with brain inorganic Hg levels approximately 5 times higher than those observed in the present group of infant macaques. The longer-term effects (greater than 6 months) of inorganic Hg in the brain have not been examined. In addition, whether similar effects are observed at lower levels in the developing brain is not known. It is important to note that a recent publication has demonstrated "an active neuroinflammatory process" in brains of autistic patients, including a marked activation of microglia (Vargas et al. 2005).

In 1999, the American Academy of Pediatrics and the Public Health Service published a joint statement that urged "all government agencies to work rapidly toward reducing children's exposure to mercury from all sources." The statement recommended that thimerosal be removed from vaccines as soon as possible as part of this overall process (American Academy of Pediatrics 1999). Between 1999 and 2001, vaccines currently recommended for children 6 years of age and under were made available in thimerosal-free formulations in the United States (CDC 2001). Exposures to thimerosal through pediatric vaccines, however, still occur in other countries where multiple-dose vials are used to maintain childhood immunization programs and the control of preventable disease (Knezdvic et al. 2004).

Recent publications have proposed a direct link between the use of thimerosal

containing vaccines and the significant rise in the number of children being diagnosed with autism, a serious and prevalent developmental disorder (for review, see IOM 2001). Results from an initial Institute of Medicine (IOM) review of the safety of vaccines found that there was not sufficient evidence to render an opinion on the relationship between ethylmercury exposure and developmental disorders in children (IOM 2001). The IOM review did, however, note the possibility of such a relationship and recommended further studies be conducted. A recently published second IOM review (IOM 2004) appears to have abandoned the earlier recommendation as well as back away from the American Academy of Pediatrics goal. This approach is difficult to understand, given our current limited knowledge of the toxicokinetics and developmental neurotoxicity of thimerosal, a compound that has been (and will continue to be) injected in millions of newborns and infants.

The key findings of the current study are the differences in the disposition kinetics and demethylation rates of thimerosal and MeHg. Consequently, MeHg is not a suitable reference for risk assessment from exposure to thimerosal derived Hg. Knowledge of the biotransformation of thimerosal, the chemical identity of the Hg-containing species in the blood and brain, and the neurotoxic potential of intact thimerosal and its various biotransformation products, including ethylmercury are urgently needed to afford a meaningful interpretation of the potential developmental effects of immunization with thimerosal-containing vaccines in newborns and infants. This information is critical if we are to respond to public concerns regarding the safety of childhood immunizations.

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Table 1. Study Design and Schedule

	Age (Days)																		
	Birth Age (0)	*2*	*4*	*7*	*9*	*11*	*14*	*16*	*18*	*21*	*23*	*25*	*28*	*31*	*35*	*38*	*42*	*45*	*49*
Mercury Dose[1] (Oral MeHg)	20			20			20			20									
Mercury Dose[2] (I.M. Thimerosal in Vaccine)	OPV-0 HB-20			OPV-0 HB-4 DTP-8 Hib-8			OPV-0 DTP-10 Hib-10			OPV-0 HB-4 DTP-8 Hib-8									
Blood Draws[3]	0	2	4	7	2	4	7	2	4	7	2	4	7	10	14	17	21	24	28
Sacrifice Day[4]											2	4	7						28

[1]Dose of MeHg in µg/kg

[2]Dose of ethylmercury in µg/kg

[3]Days after most recent dose

[4]Days after last (4^{th}) dose

Table 2. Mean (SE) body and brain weight (grams) and brain to body ratio at sacrifice for controls, MeHg exposed and thimerosal exposed animals.

Exposure Group	**Body Weight**	***Brain Weight***	***Brain to Body Ratio***
Controls (n=9)	509.3 (52.0)	52.1 (2.5)	0.107 (0.009)
MeHg Exposed (n=17)	499.1 (17.5)	51.1 (1.1)	0.103 (0.003)
Thimerosal Exposed (n=17)	529.1 (25.4)	52.7 (1.2)	0.102 (0.003)

Table 3. Parameter estimates derived from a one-compartment analysis of the mean blood total Hg concentration for the oral methylmercury group (N=17).

Model Parameters	**Mean ± SD**
V/F (L/kg)	1.67 ± 0.07
k_a (day^{-1})	2.07 ± 1.04
K (day^{-1})	0.0276 ± 0.0024
$T_{1/2}$ (days)	21.5
Cl/F (ml/day/kg)	46.1

Table 4. Parameter estimates derived from a two-compartment analysis of the mean blood total Hg concentration for the i.m. thimerosal group (N=17).

Model Parameters	**Mean ± SD**
k_a (day^{-1})	3.24 ± 3.00
k_{12} (day^{-1})	0.081 ± 0.076
k_{21} (day^{-1})	0.177 ± 0.138
k_{10} (day^{-1})	0.148 ± 0.024
$T_{1/2, \alpha}$ (day)	2.13
$T_{1/2, \beta}$ (day)	8.62
Vc/F (L/kg)	1.68 ± 0.30
Vss/F (L/kg)	2.45
Vp (L/kg)	0.77
Cl/F (ml/day/kg)	248

Figure 1. Weight gain of infants during study

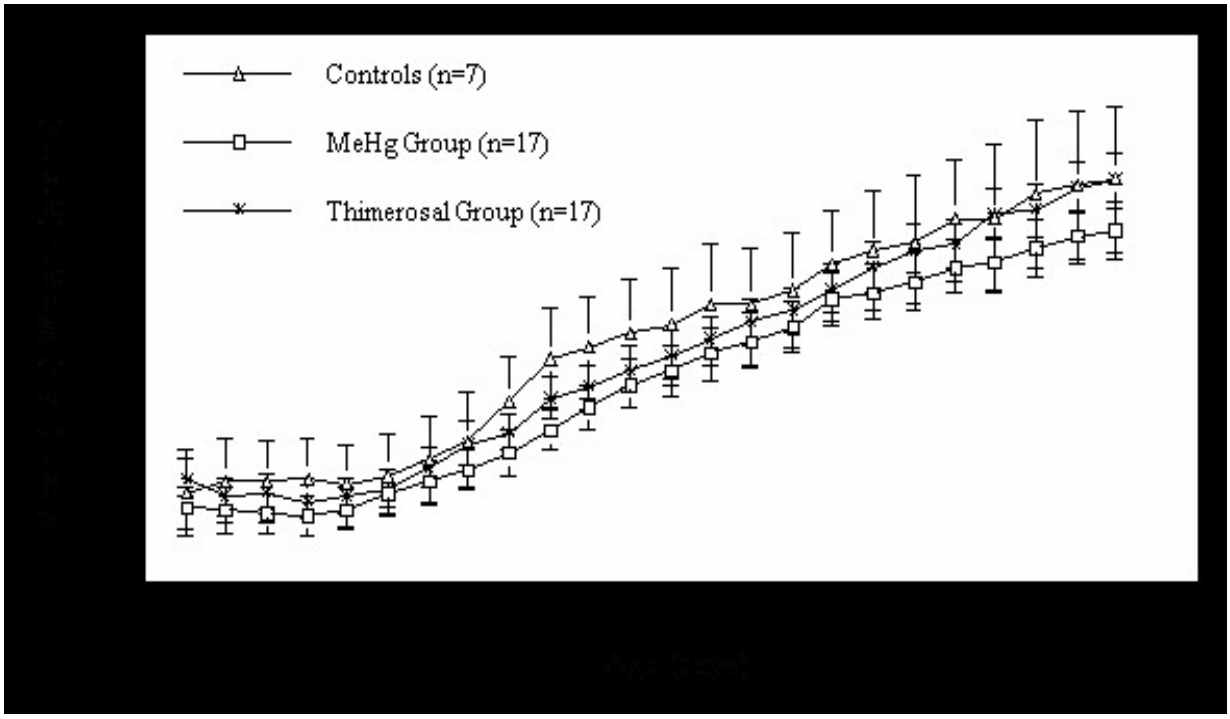


Figure 2. Comparison of model predicted and observed mean blood total Hg concentration during and after four weekly oral doses (20 μg/kg) of methylmercury.

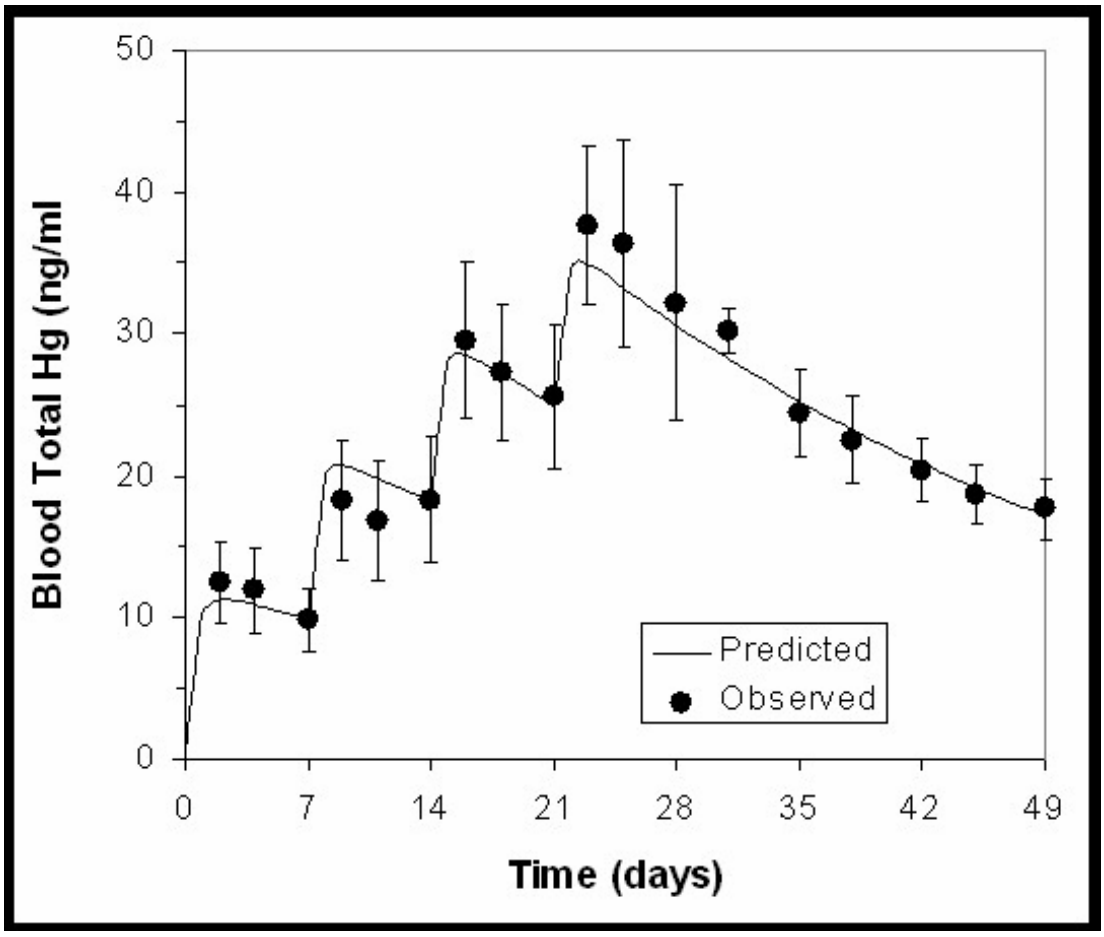


Figure 3. A semi-logarithmic plot of washout of total Hg in blood (○) and the brain (●) after 4 weekly oral doses (20 μg/kg) of methylmercury. The data were collected from groups of infants sacrificed at 2, 4, 7 and 28 days following the last dose. The lines represent nonlinear regression fit of the data to a monoexpoential model. The regression estimate (± standard error) of $T_{1/2}$ is shown along with the correlation coefficient (r).

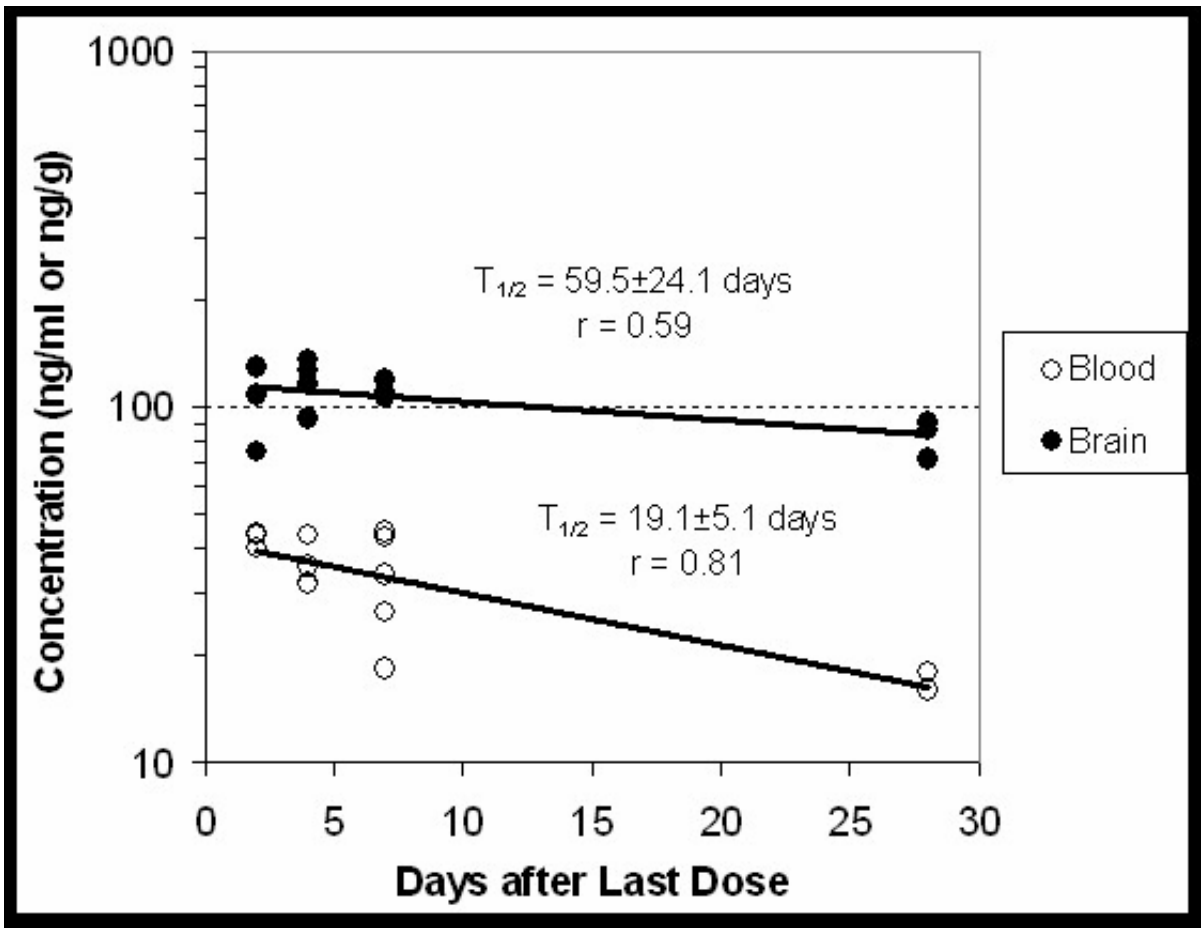


Figure 4. A semi-logarithmic plot of the washout of organic (♦) and inorganic (◊) Hg in the brain after 4 weekly oral doses (20 μg/kg) of methylmercury. The data were collected from groups of infants sacrificed at 2, 4, 7 and 28 days following the last dose. The lines represent nonlinear regression fit of the data to a monoexpoential model. The regression estimate (± standard error) of $T_{1/2}$ for organic Hg is shown along with the correlation coefficient (r). The half-life of inorganic Hg is too long (>120 days) to be accurately estimated from the present data (i.e., r is not significantly different from 0).

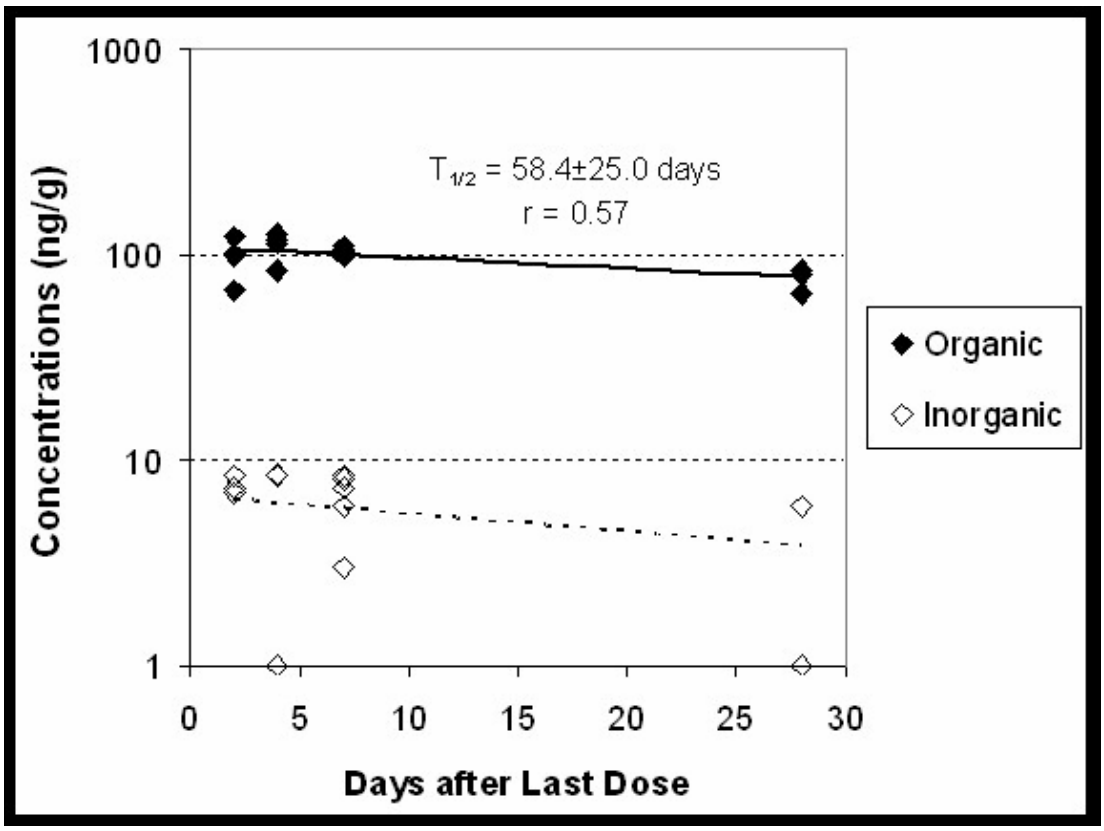


Figure 5. Comparison of model predicted and observed mean blood total Hg concentration during and after four weekly i.m. injection of vaccine containing thimerosal at 20 μg/kg of Hg.

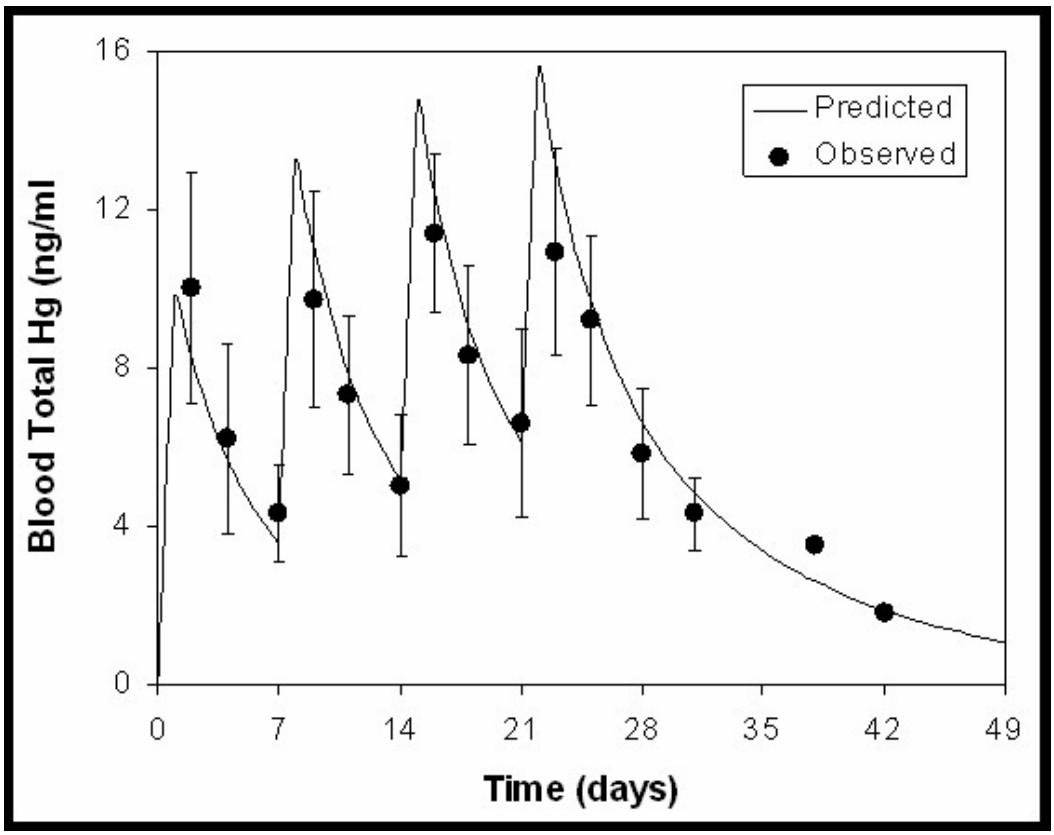


Figure 6. A semi-logarithmic plot of washout of total Hg in blood (○) and the brain (●) after four weekly i.m. injections of vaccine thimerosal at 20 μg/kg of Hg. The data were collected from groups of infants sacrificed at 2, 4, 7, 10, 17 and 21 days following the last dose. The lines represent nonlinear regression fit of the data to a monoexpoential model. The regression estimate (± standard error) of $T_{1/2}$ is shown along with the correlation coefficient (r).

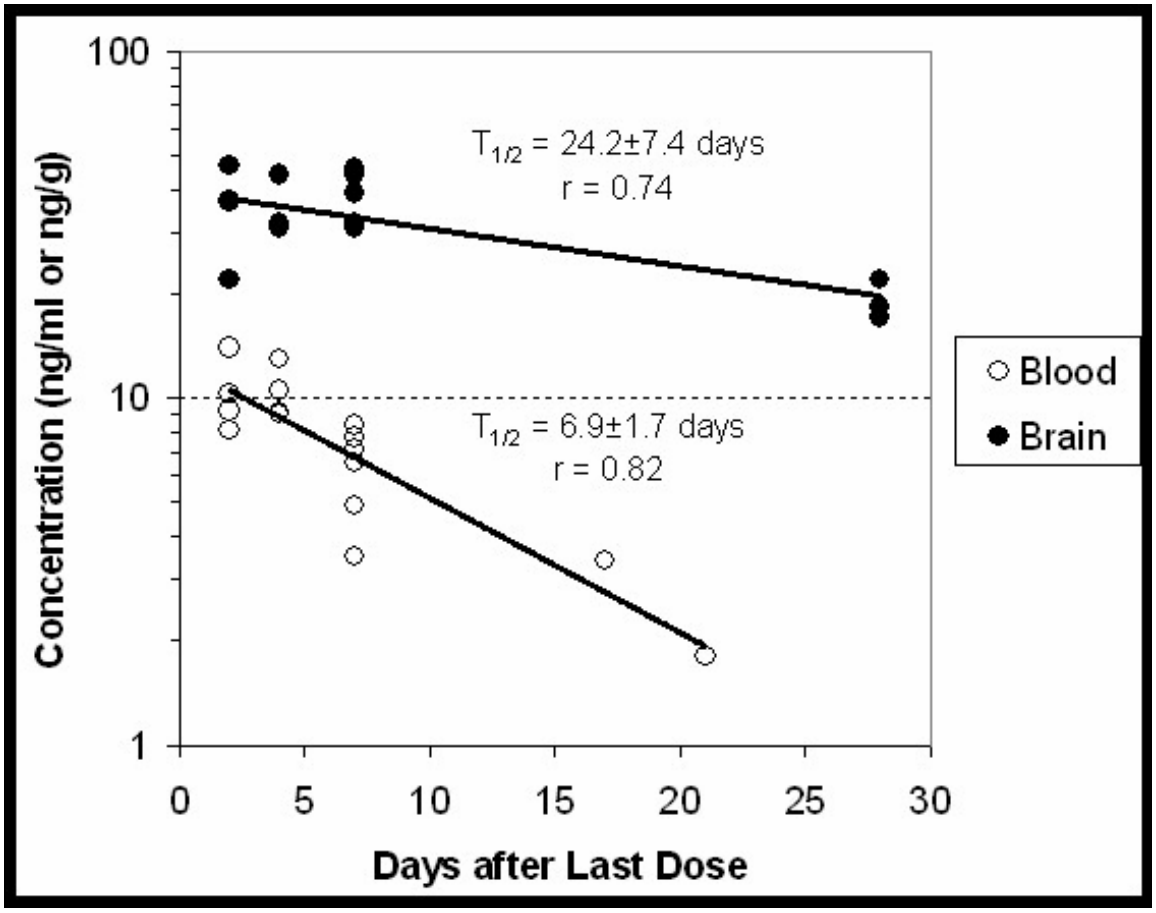
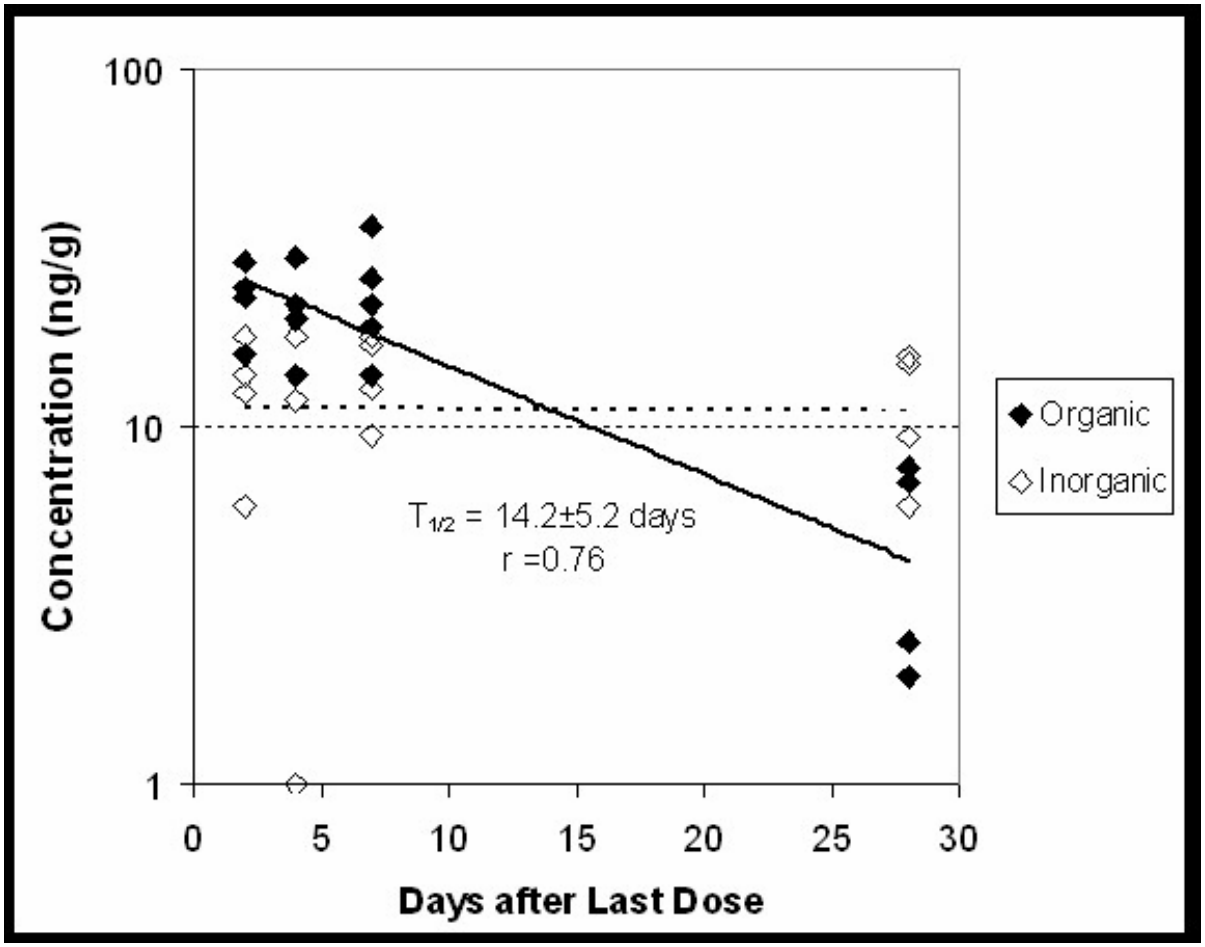


Figure 7. A semi-logarithmic plot of washout of organic (♦) and inorganic (◊) Hg in the brain after 4 weekly i.m.injection of vaccines containing thiomerosal at 20 μg/kg of Hg. The data were collected from groups of infants sacrificed at 2, 4, 7 and 28 days following the last dose. The lines represent nonlinear regression fit of the data to a monoexpoential model. The regression estimate (± standard error) of $T_{1/2}$ for organic Hg is shown along with the correlation coefficient (r). The half-life of inorganic Hg is too long (>120 days) to be accurately estimated from the present data (i.e., r is not significantly different from 0).



List of abbreviations

MeHg- methylmercury

EPA- Environmental Protection Agency

Hg- mercury

Hg^{2+}- mercuric mercury

Hg^{0}- elemental mercury

i.m.- intramuscular

µg/kg/day- micrograms per kilogram per day

µg/ml- micrograms per milliliter

µg/L- micrograms per liter

L/kg- liters per kilogram

ml/day/kg- milliliters per day per kilogram

mg/kg- micrograms per kilogram

NaCl- sodium chloride

NaOH- sodium hydroxide

$SnCl_2$-stannous chloride

ng Hg/ml- nanograms mercury per milliliter

ng/ml- nanograms per milliliter

ng/g- nanograms per gram

ppm- parts per million

K- elimination rate constant

V/F-volume of distribution (F is the implicit bioavailability term)

k_{a-} absorption rate constant

$T_{1/2}$- half-lives

Cl/F- blood clearance

AIC- Akaike Information Criterion

k_{12}- rate constants for Hg transfer from the central to the peripheral compartment

k_{21}- return from the peripheral to the central compartment

k_{10}- elimination rate constant from the central compartment

Vc- initial distribution volume

Vc/F- apparent volume of the central compartment

Vss- fully equilibrated volume

Vss/F- apparent distribution volume at steady-state

Vp=Vss – Vc- peripheral volume referenced to blood concentration

SE- standard error

AUC- area under the curve

CNS- central nervous system

IOM- Institute of Medicine