Pharmacokinetic Evaluation of the Equivalency of Gavage, Dietary, and Drinking Water Exposure to Manganese in F344 Rats

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ABSTRACT

Concerns exist as to whether individuals may be at greater risk for neurotoxicity following increased manganese (Mn) oral intake. The goals of this study were to determine the equivalence of 3 methods of oral exposure and the rate (mg Mn/kg/day) of exposure. Adult male rats were allocated to control diet (10 ppm), high manganese diet (200 ppm), manganese-supplemented drinking water, and manganese gavage treatment groups. Animals in the drinking water and gavage groups were given the 10 ppm manganese diet and supplemented with manganese chloride (MnCl₂) in drinking water or once-daily gavage to provide a daily manganese intake equivalent to that seen in the high-manganese diet group. No statistically significant difference in body weight gain or terminal body weights was seen. Rats were anesthetized following 7 and 61 exposure days, and samples of bile and blood were collected. Rats were then euthanized and striatum, olfactory bulb, frontal cortex, cerebellum, liver, spleen, and femur samples were collected for chemical analysis. Hematocrit was unaffected by manganese exposure. Liver and bile manganese concentrations were elevated in all treatment groups on day 61 (relative to controls). Increased cerebellum manganese concentrations were seen in animals from the high-manganese diet group (day 61, relative to controls). Increased (relative to all treatment groups) femur, striatum, cerebellum, frontal cortex, and olfactory bulb manganese concentrations were also seen following gavage suggesting that dose rate is an important factor in the pharmacokinetics of oral manganese. These data will be used to refine physiologically based pharmacokinetic models, extending their utility for manganese risk assessment by including multiple dietary exposures.

Key words: manganese; pharmacokinetics; oral exposure; dose rate

Manganese is an essential mineral that is found at low levels in virtually all diets (ATSDR, 2012). Ingestion is the principal route by which most people are exposed to manganese, although toxicologically significant inhalation exposures also occur. The gastrointestinal and hepatobiliary systems play an important regulatory role in maintaining tissue manganese concentrations within a relatively narrow physiologic range (Aschner and Aschner, 2005). For example, a moderate increase in dietary manganese intake is accompanied by a compensatory decrease in gut absorption, an increased liver manganese concentration, and an elevated biliary excretion level in order to maintain normal manganese concentrations in the brain and
other extrahepatic tissues (Aschner and Aschner, 2005; Dorman et al., 2001). Excessive exposure to manganese and/or hepatic injury can overwhelm these normal homeostatic controls, resulting in elevated tissue manganese and toxicity (Guillarte, 2010).

In humans, manganese-induced neurotoxicity is of primary concern and is thought to be the most sensitive endpoint. An extreme case of manganese neurotoxicity is known as managanism, which is usually caused by chronic inhalation of high levels of manganese. Hallmarks of managanism include behavioral changes, extrapyramidal motor dysfunction, and neurochemical and neuropathological changes in the basal ganglia and globus pallidus (Roels et al., 2012). Manganese-induced cognitive deficits have also been reported in workers with exposure to manganese-based welding fumes (Bowler et al., 2006; Chang et al., 2010; Ellingsen et al., 2008; Park et al., 2009). Affected brain regions following high-dose manganese exposure include the striatum, globus pallidus, and other dopaminergic and GABAergic acid-containing mid-brain structures that control motor functions (Guillarte, 2013).

In contrast to the numerous reports describing manganese toxicity following occupational inhalation exposure in humans, there are relatively few reports of managanism arising from water or dietary sources. In part, this trend is due to the relatively low levels of manganese found in these media. For example, most diets in North America result in a manganese intake below the current reference dose of 10 mg/day (Finley and Davis, 1999). Water concentrations of manganese typically range from 1 to 100 μg/l, with most values below 10 μg/l (Keen and Zidenberg-Cherr, 1994). On rare occasions, clinically apparent manganese toxicity can result from ingestion. For example, Kawamura et al. (1941) and Kondakis et al. (1989) documented outbreaks of managanism in Japan and Greece due to the ingestion of water from wells that were contaminated with extremely high levels of manganese (1.8–14 mg Mn/l). There has also been increasing concern regarding the role of environmental manganese exposure and children’s health (Zoni and Lucchini, 2013). For example, several community studies have suggested a link between manganese content in drinking water and a decrease in the IQ of children (Bouchard et al., 2011; Khan et al., 2012; Wasserman et al., 2006), although the toxicological significance of these studies remains to be established (Lucchini et al., 2009).

Many questions remain as to the conditions under which ingestion of manganese can result in an increased incidence of human neurological disease (Boyes, 2010). Experimental animal studies remain an important tool for the study of manganese pharmacokinetics and neurotoxicity (Dorman et al., 2012). Animal studies examining the pharmacokinetics and toxicity of ingested manganese often rely on 3 exposure methods: diet, drinking water, and gavage. Most dietary studies rely on the use of a defined rodent diet to which specified amounts of manganese have been added. Drinking water studies often use water mixed with manganese chloride (MnCl2), other soluble manganese salts, or insoluble manganese oxides (eg, MnO2). Gavage studies rely on the oral ‘injection’ of a bolus dose of manganese that is typically dissolved in an aqueous media. Both drinking water and dietary exposure studies more closely mimic adult human exposures, as they involve small, repetitive exposures to manganese throughout the day. Although these different methods are used, little data is available to assess the impact of either oral dose rate (eg, gavage versus drinking water) or manganese vehicle (eg, drinking water versus diet) on the pharmacokinetics of manganese exposure. The overall objective of this study was to determine whether dose rate influences the pharmacokinetics of manganese, and evaluate the equivalency of dietary and drinking water manganese intake with regards to tissue manganese concentrations.

**MATERIALS AND METHODS**

**Experimental design.** This study built on our laboratory’s previous research experience evaluating the pharmacokinetics of manganese following inhalation and oral exposures (reviewed in Dorman et al., 2012). Four treatment groups were used: control (AIN-93G diet containing 10 ppm Mn); high-dose dietary manganese (AIN-93G diet supplemented with 200 ppm Mn); high-dose drinking water manganese (AIN-93G diet containing 10 ppm Mn and drinking water to provide a daily manganese intake equivalent to the 200 ppm diet group); and high-dose manganese gavage (AIN-93G diet containing 10 ppm Mn and once-daily gavage to provide a daily manganese intake equivalent to the 200 ppm diet group). Exposures were conducted 7 days/week for 7 or 61 exposure days. Gavage exposures (MnCl2 in water; volume = 2 ml/kg body weight) occurred between 0800 and 1000 each day using manual restraint and a rodent gavage tube (18 gauge). A staggered start of 3 days was used to allow us to determine dietary manganese intake of the animals in the high-manganese diet group. Our intent was to provide similar weekly manganese intake rates across all 3 high-dose manganese exposure groups. This was accomplished by measuring daily feed consumption and water consumption for all rats. Throughout the study, the weekly average feed consumption in the high-dose diet manganese group was calculated (mg Mn/kg/day) and then used to determine the manganese concentration needed in the gavage solutions to provide a similar exposure (mg/Mn/day) based on the gavage dosing solution volume (2 ml/kg bw). Likewise, the weekly average feed consumption in the high-dose diet manganese group (mg Mn/kg/day) was used to determine the manganese concentrations needed in the drinking water to provide a similar exposure based on the drinking water group’s average weekly water consumption measured during the previous week. Our goal was to have a minimum of 8 rats per treatment group per time point available for tissue collection at the end of the study. To this end, the dietary and drinking water groups started with 9 rats/group/time point while the gavage group started with 11 rats/time point. The lot of feed (n = 1) and gavage solutions (n = 3) were analyzed for manganese content.

**Chemicals.** Manganese (Mn2+) chloride (MnCl2 • 4 H2O) was obtained from Sigma Aldrich Chemical Corporation (St. Louis, Missouri). The material is a crystalline powder that contains 27.3% manganese by weight and is relatively soluble in water.

**Animals.** This study was conducted under federal guidelines for the care and use of laboratory animals (National Research Council, 2011) and was approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee. All animal manipulations were performed in the NCSU Laboratory Animal Resource unit, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Male 5- to 6-week-old F344 rats (Charles River, Kingston, New York) were used; they were 7 weeks old when exposures began.
Animal husbandry. Animal rooms and exposure chambers were maintained at daily temperatures of 22 ± 4°C, relative humidity of 30–70%, and an air flow rate sufficient to provide 10–15 air changes per hour. Fluorescent lighting was controlled by automatic controls (lights on approximately 0700–1900). Rats were individually housed in polycarbonate cages. On arrival, rats were given their previous Purina 5001 diet (Purina Mills, St. Louis, Missouri) for the first week during the acclimation period. Afterwards, pelleted, semi-purified AIN-93G certified diet from Bio-Serv (Frenchtown, New Jersey) formulated to contain 10 ppm manganese and 35 ppm iron were fed to all animals. Diet and drinking water consumption was determined each day, beginning during the second week of the quarantine period. Assignment of animals to treatment groups was based on a weight randomization procedure that occurred at the end of the 2-week acclimation period. The day after randomization (study day 1), rats were either continued on the AIN-93G diet with 10 ppm manganese, or given a semi-purified AIN-93G certified diet from Bio-Serv that was formulated to contain 200 ppm manganese and 35 ppm iron. These diets were continued throughout the remainder of the study. A single lot of diet was used. It was stored under refrigeration, and feed was changed at least weekly. Food and water was available to all animals ad libitum throughout the study. Body weights were measured and recorded upon randomization, at least weekly throughout the study, and again on the day of necropsy. Animals were observed daily using cage side observations by experienced laboratory animal technical staff that were not blinded to treatment groups.

Necropsy and tissue collection. Necropsies were performed 4–6 hours following the gavage exposure (or equivalent) after 7 and 61 exposure days. Animals were anesthetized to effect with ketamine (80–100 mg/kg, ip) and xylazine (5–10 mg/kg, ip), and approximately 1-ml samples of bile and blood (serum) were collected using previously described methods (Dorman et al., 2001). Immediately afterwards, all rats were euthanized by exsanguination and the following tissues were collected: striatum, cerebellum, olfactory bulb, frontal cortex, liver, spleen, and proximal portion of the left femur for tissue manganese determination. Brain regions sampled are consistent with our prior experiments evaluating the pharmacokinetics of manganese and have been used in pharmacokinetic model development (Dorman et al., 2012). Plastic syringes fitted with a hypodermic needle were used for blood collection. Blood samples (approximately 1–2 ml) were placed into tubes (Becton Dickinson, Franklin Lakes, New Jersey). A small (approximately 50–80 μl) aliquot of blood was used to determine the packed cell volume using a standard clinical hematocrit centrifuge (Damon/IEC MB centrifuge, Needham, Massachusetts). Tissue samples were weighed and transferred to plastic vials, frozen in liquid nitrogen, and stored at approximately −80°C until manganese analyses were completed.

Chemical analyses. Liver (10–100 mg), food (150–300 mg), and gavage (25 μl) samples were thawed then digested in 1 ml 70% Optima grade nitric acid (Fisher Scientific, Pittsburgh, Pennsylvania) at 65°C for 2 h, then diluted 25-fold with MilliQ-purified water (EMD Millipore, Billerica, Massachusetts) and analyzed in triplicate by inductively coupled plasma atomic emission spectrometry (ICP-AES) using a JY2000 Ultrace spectrometer (Horiba Scientific, Edison, New Jersey). Serum and bile (0.1 ml samples) and spleen, femur, frontal cortex, striatum, olfactory bulb, and cerebellum (10–100 mg samples) were digested using a stepwise protocol: digestion in 0.2 ml 70% nitric acid and heating at 85°C until dry, resuspension in 0.2 ml 30% Optima grade hydrogen peroxide (Fisher Scientific) and heating at 85°C until dry, repeated resuspension in hydrogen peroxide and heating until dry, resuspension in 0.2 ml 70% nitric acid and heating until dry, then a final resuspension in 1 ml 2% nitric acid. Spleen, serum, and frontal cortex were analyzed by ICP mass spectrometry (ICP-MS) using an XSeries2 (Thermo Scientific, Waltham, Massachusetts) and femur, bile, striatum, olfactory bulb and cerebellum by graphite furnace atomic absorption spectrometry (GF-AAS) using an AAnalyst 600 spectrometer (Perkin Elmer, Waltham, Massachusetts). Reference standards were analyzed repeatedly during each run to ensure run consistency. ICP-AES and GF-AAS analyses were performed at the Environmental Chemistry Facility at Brown University using previously described methods (Herrera et al., 2014). ICP-MS analyses were performed in the Department of Earth, Environmental and Planetary Sciences at Brown University. Instrument availability was a major determinant in the selection of the analytic methods used. Analytic standards of manganese in solution were used to ensure comparability between different methods of analyses.

Statistics. Individual data that appeared to be outliers were critically evaluated using a Dixon-type test for discordancy of an upper outlier (Barnett and Lewis, 1984). For comparing change in weight across treatment groups, a multivariate analysis of variance model (MANOVA) was used with treatment group as a fixed factor. Following an assessment for homogeneity of variance (Levene’s test), the tissue manganese concentrations at each time point were also inter-compared for the 3 manganese exposure groups using an analysis of variance (ANOVA) or with a Welch ANOVA for unequal variances followed by a Tukey’s honestly significant difference test to perform pairwise multiple comparisons. In addition to these analyses, we also used ANOVA to determine whether manganese exposure duration was a significant factor. Statistical analyses were performed using SAS Statistical Software (JMP, SAS Institutes, Inc., Cary, North Carolina). A probability value of .01 was used for Levene’s test, while < .05 was used as the critical level of significance for all other statistical tests. Unless otherwise noted, data presented are mean values ± SEM.

RESULTS

Clinical Effects. Clinical signs were not seen in any exposure group. One animal from the 61-day gavage group died due to gavage error. Terminal body weights (day 61 exposure group) were 256.8 ± 9.8, 244.3 ± 7.9, 233.7 ± 6.5, and 228.2 ± 7.1 g for the control, high-manganese diet, drinking water, and gavage exposure groups, respectively. This decrease was not statistically significant (P = .071, ANOVA). Growth curves for the 61-day exposure groups are presented in Figure 1. Repeated exposure (61 day) to elevated levels of manganese did not decrease body weight gain when compared with control animals given the 10-ppm manganese diet (P = .128, MANOVA). There were no treatment-related effects on terminal (day 61 exposure group) hematocrit or absolute liver or brain weights (data not shown). The collection of an adequate volume of bile from the younger rats (day 7) could not be obtained from 2, 1, 4, and 1 rat from the control diet, high-manganese diet (200 ppm), drinking water, and gavage groups, respectively.

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Mean water consumption rates during the 61 day study were 4.9 ± 0.4 g/d for the control and high-manganese diet, drinking water, and gavage exposure groups, respectively. Overall daily mean feed consumption rates during the 61 day study were 10.0 ± 0.6 g/d for the control, high-manganese diet, drinking water, and gavage exposure groups, respectively. No statistically significant difference was observed following high-dose manganese exposure (when compared with control animals). Equivalent daily manganese intakes (mg/kg/day) were used in the 200-ppm diet, drinking water, and gavage groups.

Estimated Oral Exposure
Nominal manganese and iron concentrations in control diet were 10 and 35 ppm, respectively while ICP-AES analysis revealed 202.4 and 35 ppm, respectively. Nominal manganese and iron concentrations in the high-manganese diet were 200 and 35 ppm, respectively while ICP-AES analysis revealed 12.2 and 44.8 ppm, respectively. Nominal manganese exposures (mg Mn/kg/day) in the high-manganese drinking water exposure groups were within 5% of the high-manganese exposures (mg Mn/kg/day) for the high-manganese diet group. Daily manganese intakes (mg/kg/day) were used in the following high-dose manganese exposure (when compared with control animals). Estimated oral exposures (mg Mn/kg/day) for the control, high-manganese diet, drinking water, and gavage exposure groups were within 5% of nominal concentrations. Overall daily mean food consumption rates during the 61 day study were 11.0 ± 0.5, 10.9 ± 0.4, 10.1 ± 0.3, and 10.0 ± 0.3 g/d for the control, high-manganese diet, drinking water, and gavage exposure groups, respectively. There was no effect of exposure group on daily feed or water consumption. Overall mean body weights were 213.1 ± 7.5, 206.1 ± 5.8, 197.6 ± 5.0, and 193.6 ± 5.6 g for the control, high-manganese diet, drinking water, and gavage exposure groups, respectively. Based on nominal dietary manganese concentrations and weekly mean body weight and feed consumption rates, rats in the control, high-dose manganese diet, drinking water, and gavage exposure groups, respectively. Daily manganese exposures (mg Mn/kg/day) for the high-manganese drinking water and gavage groups were within 5% of the high-dose manganese diet group.

Tissue Manganese Concentrations
Tables 1 and 2 show mean tissue manganese concentrations for the 7- and 61-exposure day groups, respectively. Increased manganese exposure was not associated with changes in either serum or spleen manganese concentrations when compared with controls for either the 7 or 61 exposure day groups. Because no treatment effect was noted we pooled serum and spleen manganese concentrations for additional analyses. Serum manganese concentrations were 1.12 ± 0.13 and 0.56 ± 0.08 µg Mn/g for the 7 and 61 exposure day groups, respectively. Spleen manganese concentrations were 0.20 ± 0.01 and 0.14 ± 0.01 µg Mn/g for the 7 and 61 exposure day groups, respectively. Serum and spleen manganese concentrations were significantly lower at day 61 when compared with values obtained in younger animals after 7 exposure days.

High-dose drinking water manganese exposure resulted in increased (relative to controls) liver (day 7) and bile (day 61) manganese concentrations. High-dose dietary manganese exposure resulted in increased (relative to controls) cerebellum (day 7 and 61), liver (day 7 and 61), and bile (day 7 and 61) manganese concentrations. High-dose gavage manganese exposure resulted in increased (relative to controls) cerebellum (day 7 and 61), olfactory bulb (day 7 and 61), striatum (day 7 and 61), frontal cortex (day 61), femur (day 7 and 61), liver (day 7 and 61), and bile (day 61) manganese concentrations. Gavage exposure also resulted in higher manganese (when compared with either the high-manganese diet or drinking water exposure groups) concentrations in the cerebellum (day 7 and 61), olfactory bulb (day 7 and 61), striatum (day 7 and 61), frontal cortex (day 61), femur (day 61), liver (day 7), and bile (day 61). Olfactory bulb, frontal cortex, femur, and liver manganese concentrations were not affected by exposure duration. Cerebellum, striatum, and bile manganese concentrations were dependent upon exposure duration and manganese treatment conditions. Bile manganese concentrations in rats given the control diet were significantly higher at day 7 when compared with rats in the 61 exposure group.

DISCUSSION
The overall goals of this study were to determine whether the exposure method (eg, diet vs drinking water) or dose rate (eg, drinking water vs gavage) of ingested manganese affect tissue manganese concentrations. This experiment used 3 methods of oral manganese administration (diet, drinking water, and gavage) and was designed to produce similar manganese intake levels between these methods. This was accomplished by using a staggered start to the exposures that allowed us to initially determine the daily dietary intake of animals on a 200-ppm manganese diet. Daily manganese exposures (mg Mn/kg/day) in the high-manganese drinking water and gavage groups were then adjusted to be within 5% of the high-dose manganese exposure group's average daily exposure.

During the course of the study, animals in the 200-ppm manganese diet group ingested approximately 11.1 mg Mn/kg body weight/day. The dietary manganese exposures used in this study are quite high when compared with typical dietary intake in people. For example, Yamada et al. (2014) reported that adult Japanese men and women ingested 4.9–5.1 mg Mn/day or approximately 0.08–0.09 mg Mn/kg/day. Dietary intake levels reported by Yamada are somewhat higher than those seen in Western populations which range from 0.9 to 5.0 mg Mn/day for women and 1.0–5.2 mg Mn/day for men (Bautista et al., 2005; Nkwenkeu et al., 2002; Patterson et al., 1984). Based on data reported by Yamada et al., (2014) we estimate that the daily manganese intake rate (mg/kg/day) for rats receiving the 200-ppm manganese diet in our study were approximately 130-fold higher than those seen in adult humans on a Japanese diet. Manganese intake from drinking water is normally substantially lower than intake from food. In the United States, a median drinking water manganese concentration of 10 µg/l results in intake of manganese of 20 µg/day for an adult consuming 2 l of water per day (WHO, 2011).
The manganese exposures in this study more closely mimic those used in certain subchronic experimental animal toxicology studies (Dorman et al., 2000; Lazrishvili et al., 2009; Roels et al., 1997). Our study provides an important insight into whether an oral exposure to manganese could be used as a surrogate for an inhalation exposure, which is a more relevant route for occupational or environmental human exposure (Lucchini and Zona, 2015). In our study, we showed that subchronic high dose (approximately 11 mg Mn/kg body weight/day) gavage exposure could result in an approximate doubling of brain manganese concentrations. The magnitude of this change is very similar to those observed in animals following certain subchronic inhalation exposure. For example, adult rats exposed to manganese sulfate at 0.1 mg Mn/m^3 for 65 exposure days developed an approximate doubling in olfactory bulb and striatal manganese concentrations (Dorman et al., 2004). Likewise, rhesus monkeys exposed to manganese sulfate at 0.06 mg Mn/m^3 for 65 exposure days also had an approximate doubling in olfactory bulb and globus pallidus manganese concentrations (Dorman et al., 2006).

Our study also showed that the rate at which tissue manganese concentrations reached apparent steady state varied following gavage exposure. For example, olfactory bulb manganese concentrations following 7 exposure days were statistically equivalent to levels seen after 61 exposure days suggesting that this tissue compartment rapidly reached peak concentrations under these high-dose exposure conditions. In contrast, other tissues (e.g., striatum) more slowly reached apparent steady state concentrations following gavage manganese exposure. These observations are similar to the results of our study with nonhuman primates which showed that olfactory bulb, but not globus pallidus, manganese concentrations following 15 exposure days of manganese sulfate inhalation at 1.5 mg Mn/m^3 were statistically equivalent with those seen after 33 or 65 exposure days (Dorman et al., 2006). One explanation that has been proposed to account for these differences relate to differences in the tissue storage capacity for this metal and the rate at which saturation is reached (Nong et al., 2008).

A number of physiological mechanisms exist to maintain relatively constant tissue manganese concentrations despite wide fluctuations in oral manganese intake (Schroeder et al., 1966). When dietary manganese levels are high, adaptive changes often include reduced gastrointestinal absorption of manganese, enhanced manganese liver metabolism, and increased biliary and pancreatic excretion of manganese (reviewed in Aschner et al., 2005). Thus, the fraction of ingested manganese retained by the body is tightly regulated in order to maintain normal tissue manganese concentrations under different dietary conditions. The 200-ppm manganese diet was selected with the intent of altering manganese homeostasis as evidenced by changes in liver and bile manganese concentrations without producing overt signs of toxicity. Although oral manganese intakes in our study were extremely elevated, the animals did not develop outward clinical signs or statistically significant changes in body weight gain, feed consumption, or water consumption. We did observe changes in liver manganese concentrations that were unaffected by the duration of the exposure but were dependent on the manganese exposure. Animals with elevated manganese intake (approximately 11 mg Mn/kg/day) by gavage developed a statistically significant

### Table 1. Mean (± SEM) Tissue Manganese Concentrations (µg Mn/g) Following 7 Exposure Days

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Diet</th>
<th>Water</th>
<th>Gavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>0.42 ± 0.01 (9)</td>
<td>0.50 ± 0.04 (9)</td>
<td>0.47 ± 0.01 (9)</td>
<td>0.59 ± 0.03 (11)*</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>0.42 ± 0.02 (9)</td>
<td>0.51 ± 0.03 (9)</td>
<td>0.48 ± 0.02 (9)</td>
<td>1.52 ± 0.30 (9)*</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.31 ± 0.01 (9)</td>
<td>0.35 ± 0.01 (9)</td>
<td>0.34 ± 0.01 (9)</td>
<td>0.55 ± 0.08 (11)*</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.39 ± 0.07 (8)</td>
<td>0.35 ± 0.02 (9)</td>
<td>0.36 ± 0.01 (9)</td>
<td>0.48 ± 0.02 (10)</td>
</tr>
<tr>
<td>Bile</td>
<td>6.61 ± 1.57 (7)</td>
<td>231.35 ± 78.42 (7)</td>
<td>61.90 ± 16.83 (5)</td>
<td>142.94 ± 43.83 (8)</td>
</tr>
<tr>
<td>Serum</td>
<td>0.74 ± 0.14 (9)</td>
<td>1.35 ± 0.26 (8)</td>
<td>1.10 ± 0.15 (6)</td>
<td>1.55 ± 0.29 (10)</td>
</tr>
<tr>
<td>Femur</td>
<td>0.35 ± 0.02 (9)</td>
<td>0.42 ± 0.05 (9)</td>
<td>0.38 ± 0.02 (9)</td>
<td>0.53 ± 0.04 (11)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.19 ± 0.03 (9)</td>
<td>0.19 ± 0.01 (8)</td>
<td>0.19 ± 0.01 (9)</td>
<td>0.24 ± 0.02 (11)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.45 ± 0.06 (9)</td>
<td>3.31 ± 0.12 (9)</td>
<td>3.04 ± 0.07 (9)</td>
<td>4.11 ± 0.27 (10)*</td>
</tr>
</tbody>
</table>

Number in parentheses = number of samples (n).
Bold text: *P < .05; versus controls, significant ANOVA or Welch ANOVA followed by Dunnett’s test.
*Increased when compared with all other groups (P < .05, Welch ANOVA and Tukey’s HSD).

### Table 2. Mean (±SEM) Tissue Manganese Concentrations (µg Mn/g) Following 61 Exposure Days

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Diet</th>
<th>Water</th>
<th>Gavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>0.46 ± 0.01 (9)</td>
<td>0.56 ± 0.03 (9)</td>
<td>0.52 ± 0.02 (9)</td>
<td>0.68 ± 0.02 (10)*</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>0.49 ± 0.03 (9)</td>
<td>0.49 ± 0.01 (9)</td>
<td>0.50 ± 0.05 (9)</td>
<td>1.12 ± 0.13 (10)*</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.36 ± 0.01 (9)</td>
<td>0.43 ± 0.01 (9)</td>
<td>0.44 ± 0.01 (9)</td>
<td>0.75 ± 0.07 (10)*</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.25 ± 0.02 (8)</td>
<td>0.29 ± 0.01 (9)</td>
<td>0.27 ± 0.02 (9)</td>
<td>0.73 ± 0.08 (9)*</td>
</tr>
<tr>
<td>Bile</td>
<td>1.48 ± 0.20 (8)</td>
<td>34.49 ± 5.68 (8)</td>
<td>38.63 ± 11.74 (9)</td>
<td>67.38 ± 7.91 (9)*</td>
</tr>
<tr>
<td>Serum</td>
<td>0.66 ± 0.27 (9)</td>
<td>0.73 ± 0.16 (9)</td>
<td>0.42 ± 0.04 (9)</td>
<td>0.46 ± 0.03 (10)</td>
</tr>
<tr>
<td>Femur</td>
<td>0.32 ± 0.01 (9)</td>
<td>0.40 ± 0.02 (8)</td>
<td>0.40 ± 0.03 (9)</td>
<td>0.66 ± 0.07 (10)*</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.13 ± 0.02 (9)</td>
<td>0.16 ± 0.01 (9)</td>
<td>0.13 ± 0.01 (9)</td>
<td>0.15 ± 0.02 (10)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.62 ± 0.11 (8)</td>
<td>3.30 ± 0.17 (9)</td>
<td>2.94 ± 0.05 (9)</td>
<td>3.36 ± 0.09 (10)</td>
</tr>
</tbody>
</table>

Number in parentheses = number of samples (n).
Bold text: *P < .05; versus controls, significant ANOVA or Welch ANOVA followed by Dunnett’s test.
*Increased when compared with all other groups (P < .05, Welch ANOVA and Tukey’s HSD).
18.2–47.4% increase in liver manganese concentrations when compared with rats given a control diet that provided approximately 0.5 mg Mn/kg/day. Bile manganese concentrations were dependent upon both exposure duration and concentration. Statistically significant increases in bile manganese concentrations were seen after 7 exposure days. For example, rats fed the 200-ppm manganese diet developed a statistically significant increase in bile manganese concentration that was approximately 35-fold higher than that seen in control animals given a diet with a 20-fold lower manganese concentration. Increased biliary manganese elimination persisted throughout the study; albeit at a lower absolute concentration as the exposure duration increased. Rats given the 200-ppm manganese diet for 61 exposure days developed a statistically significant 23-fold increase in bile manganese concentrations when compared with control animals. Biliary excretion of manganese also demonstrated a dose-rate dependency since rats given approximately 11 mg Mn/kg/day by gavage developed a statistically significant 1.7- to 2-fold increase in bile manganese concentrations when compared with animals receiving similar amounts of manganese by either diet or drinking water. Interestingly, we also observed elevated biliary manganese excretion in 8-week-old rats maintained on the low manganese control diet when compared with their 16-week old counterparts. Other investigators have shown that the rat biliary tract undergoes histologically apparent modification in endocrine cell number between 49 and 91 days of age (Park and Bendayan, 1993). Cuesta de Juan et al. (2007) showed that the expression of a number of rat liver enzymes involved in bile acid homeostasis also change between 8 and 36 weeks of age suggesting that this time period is subject to ongoing alterations in the hepatobiliary system. The mechanisms involved in bile biliary excretion remain poorly understood; therefore, it is unknown whether our observation of an age-dependent change in manganese excretion could be related to these or other factors.

Another goal of this project was to determine whether the rate at which manganese is ingested could influence systemic delivery of this metal to the brain and other tissues. In particular, we were interested in determining whether oral gavage could result in altered systemic delivery of manganese when compared with drinking water or dietary exposure. The toxicokinetic profiles of several chemicals have been shown to differ substantially in animals treated via gavage versus administration via other oral routes. Gavage administration in rodents bypasses the highly absorptive buccal surface membranes and in some cases (eg, with the drug donepezil) leads to reduced systemic delivery (Atcha et al., 2010). In contrast, Yuan et al. (1995) showed that gavage administration of benzyl acetate to rats was associated with higher benzoic acid plasma concentrations when compared with those seen following similar dietary administration. Similarly, oral gavage resulted in higher tissue concentrations of sulindac and lovastatin in rats when compared with animals receiving comparable dietary exposure (Kapetanovic et al., 2006; Martín-Jiménez et al., 2008). These types of observations have led to concerns being raised regarding the appropriateness of gavage administration for the testing of chemicals for endocrine disruptor effects (Vandenberg et al., 2014). In our study, we found multiple examples where tissue manganese concentrations in animals from the gavage treatment group were statistically higher than those seen in the high-dose diet and drinking water exposure groups. For example, gavage exposure in the 61-day exposure group was associated with bile, striatal, olfactory bulb, femur, and liver manganese concentrations that were (respectively) approximately 2.0-, 1.6-, 2.6-, 1.4-, and 1.1-fold higher when compared with animals receiving similar amounts of manganese by either diet or drinking water. These results suggest that bolus gavage administration of manganese could be associated with higher brain manganese concentrations, and as a consequence, higher risk of neurotoxicity.

Our study also shows that equivalent dietary and drinking water exposure of a high dose of manganese (approximately 11 mg Mn/kg/day) resulted in very few significant differences in tissue manganese concentrations. Ingestion of a high-dose manganese diet was associated with statistically higher bile manganese concentrations after 7 exposure days when compared with animals given excess manganese by drinking water. However, this difference was no longer seen following 61 exposure days. High-dose dietary, but not high-dose drinking water, manganese exposure was associated with elevated cerebellum manganese concentrations when compared with animals receiving the 10-ppm manganese control diet for 61 exposure days. It is unknown why this 1 brain region would be affected by a drinking water exposure to a high concentration of manganese. Likewise, liver manganese concentrations and bile manganese concentrations following 61 days of exposure were also statistically equivalent between the 200-ppm diet and high-dose manganese drinking water groups. Our findings suggest that drinking water and dietary exposures result in toxicologically comparable tissue manganese concentrations, especially in the striatum, a known target for manganese neurotoxicity (Guilarte, 2010). This observation could prove useful to toxicologists considering whether dietary studies may help inform the human health risk assessment of manganese in drinking water.

An important limitation of this study is the use of a single high dose of manganese for the 3 different types of oral exposures. One future goal of our research team is to use the data from this study to help refine existing human physiologically based pharmacokinetic (PBPK) models (Andersen et al., 1999; Schroeter et al., 2011; Yoon et al., 2011) to more thoroughly include oral exposure to manganese. A significant strength of PBPK models is that they can be used to extrapolate between exposure doses and durations (Andersen et al., 2010), thus reducing the impact of this experimental limitation. Increased consideration of oral exposure in PBPK models is also important from a risk assessment perspective. Current human PBPK models predominantly focus on inhalation exposures, with secondary consideration of oral routes of administration (Taylor et al., 2012). Refined PBPK models developed with data obtained from this study could be used to explore the biological plausibility of any purported effects of manganese in drinking water, and could therefore play an important role as regulatory agencies reexamine manganese drinking water standards. As noted earlier, the gavage exposures used in this study resulted in changes in brain manganese concentrations that were qualitatively similar to those seen by others following repeated 6-h inhalation exposure to approximately 0.1 mg/m3. The results of this study will also further support the use of PBPK models to evaluate conditions under which route-dependent changes in manganese pharmacokinetics could occur.

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