ABSTRACT

This project developed data for the purpose of refining the human health risk of bromate (BrO₃⁻), a toxic water disinfection by-product. The results suggested that only a small fraction of BrO₃⁻ administered to rats would reach the systemic circulation as oral bioavailability was ~35%. BrO₃⁻ degrades in a multi-exponential manner following its administration as an IV bolus or oral gavage in female F344 rats. The rate of BrO₃⁻ reduction was higher compared to the rate of bromide (Br⁻) formation from BrO₃⁻. The deficits in total BrO₃⁻ recovery after its oral administration raises the possibility that some brominated biochemicals may be produced in vivo, which will slowly get metabolized and eliminated from the body.

Sex-dependent differences in BrO₃⁻ induced renal cell proliferation and nephrotoxicity after 125 or 400 mg/L KBrO₃ treatment in drinking water for 28 days, were accompanied by differential expression of several genes and proteins including
the cancer biomarker protein osteopontin, the cell cycle checkpoint p21 and kidney injury biomarker (Kim-1). Concentration-dependent increases in the genotoxic marker 8-hydroxyguanosine were also detected, but were similar in both males and females. These data support the hypothesis that both genotoxic and non-genotoxic mechanisms mediate BrO₃⁻-induced nephrotoxicity.

Concentration-and-sex-dependent increases in the expression of 3-bromo-tyrosine were also detected in rat kidneys after 15, 60, or 400 mg/L KBrO₃ treatment in drinking water for 28 days. These data suggest that BrO₃⁻ treatment results in the formation of reactive intermediates capable of brominating proteins. Thus, 3-bromo-tyrosine may play a role in mediating the sex-dependent differences after BrO₃⁻ treatment.

Data on the kinetics of BrO₃⁻ were subjected to Pharmacokinetic (PK) modeling. A pharmacokinetic model fitted with an individual animal data and weight² (1/Y²) weight scheme was chosen based on goodness of fit, and model diagnostics as a model of choice to estimate BrO₃⁻ pharmacokinetic parameters. BrO₃⁻ absorption occurs through the GI tract in with an absorption rate constant (Kₐ) ~0.16 min⁻¹, and undergoes extensive first pass before it reaches systemic circulation. BrO₃ distributes to the peripheral tissues following administration of ≥ 1 mg/kg KBrO₃ IV bolus, and eliminate rapidly from the body.

INDEX WORDS: Bromate, pharmacokinetics, kidney, sex-dependence, and mechanism of action
PHARMACOKINETICS (ADME), DOSE-RESPONSE AND PHARMACOKINETIC MODELING OF BROMATE IN F344 RATS

By

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A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

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MODELING OF BROMATE IN F344 RATS

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August 2012
DEDICATION

This is dedicated to my parents Sriramulu Kolisetty and Lakshmi Kolisetty, and to my family members, my brother Bajirao Kolisetty, sister in law Hemalatha Kolisetty, and most importantly to my beloved wife Chandrakala Yakkali.
ACKNOWLEDGEMENTS

First of all, I would like to thank Joseph Cotruvo & Associates LLC, for giving us the grants from the Water Research Foundation (AwwaRF) Project #4042 - International Ozone Assoc., Environment Agency of Abu Dhabi, Veolia Water, Metropolitan Water District of Southern Calif., Los Angeles Department of Water and Power, National Water Research Institute, Walkerton Clean Water Centre, Calleguas Municipal Water District, Long Beach Water Department, and in-kind contributions of the participants. I gratefully acknowledge and thank the Water Research Foundation (AwwaRF), and all of the sponsors for their financial, technical and administrative assistance in funding the project through which this information was discovered.

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in vivo studies, and Dr. Robert Arnold for use of his microscope for the immunohistochemistry studies.

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CHAPTER 1
INTRODUCTION

Water Disinfection

Water disinfection has long been practicing across the world, including the United States (since early 1900’s) (G. C. White, 1992). Water disinfection is a process that source or ground water goes through with a goal to reduce the level of risk from water borne diseases by killing, or inactivating, pathogenic microorganisms (Jolley, 1990). Source water disinfection can be achieved through application of various processes.

Water disinfectants are broadly categorized into two types; physical disinfectants and chemical disinfectants. Physical disinfectants include ultra violet (UV) light, electronic radiation, gamma rays, and heat (A. Acra, Karahagopian, Y., Raffoul, Z., Dajani, R., 1980; A. Acra, Raffoul, Z., Karahagopian, Y., 1984; Cheremissinoff, 1981; Ciochetti, 1984). Chemical disinfectants include chlorine (Cl₂), chlorine dioxide (ClO₂), hypochlorite (OCl⁻), ozone (O₃), bromine (Br₂), iodine (I), phenols, alcohols, soaps and detergents, Kwartair ammonium salts, hydrogen peroxide (H₂O₂), and several acids and bases.

The most commonly used chemical disinfectants are chlorine, ozone, chlorine dioxide and chloramines. The main advantages with these chemical disinfectants are that they are inexpensive, suitable for large scale water disinfection, they are able to kill or inactivate several types of pathogenic microorganisms present in the source water,
and can eliminate hydrogen sulfide (H$_2$S) and remove ammonia (NH$_3$) and other nitrogenous compounds (Morris, 1985).

Chlorination was the principle method of water disinfection that most treatment plants used across the world, including the United States, until early 1980’s. Introduction of filtration and chlorination in the United States resulted in the reduction of cholera incidence by 90%, typhoid by 80% and amoebic dysentery by 50% since its initial use (Pastore et al., 1998). For example, the number of people died from typhoid dropped from 185 to nearly zero per 100,000 population, between 1911 and 1915 in Niagara Falls, NY (S. C. White, Jernigan, & Venosa, 1986). In contrast, because chlorine is a powerful oxidant it can oxidize organic matter, anthropogenic contaminants, bromide/iodide (naturally present in many source waters such as lakes, rivers etc.), which leads to the production of disinfection by-products (DBPs) like chloroform, trihalomethanes (THMs) and halo acetic acids (HAAs) (Richardson & Postigo, 2012; Rook, 1974). These chlorinated DBPs are proven animal and human carcinogens and mutagens (ATSDR, 1997). This has caused a public health concern over using chlorine as a principle water disinfectant in the United States.

Greater than 600 DBPs are reported to be formed during the chemical disinfection process of drinking water (Richardson & Postigo, 2012). However, very few DBPs production are regulated by the Unites States Environmental Protective Agency (USEPA, 2006). Thus a significant gap in knowledge exists in identifying adverse health effects from unregulated water DBPs in humans. Table 1.1 and 1.2 list both regulated and unregulated DBPs formed during water disinfection.
Table 1.1 Select DBPs formed during drinking water disinfection

<table>
<thead>
<tr>
<th>DBP</th>
<th>Examples</th>
<th>Occurrence</th>
<th>Genotoxicity</th>
<th>Carcinogenicity</th>
<th>Regulated Level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THMs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>mid µg/L</td>
<td>-</td>
<td>+</td>
<td>80 µg/L</td>
</tr>
<tr>
<td></td>
<td>Bromo dichloro</td>
<td>low µg/L</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>methane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloro dibromo</td>
<td>low µg/L</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>methane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromoform</td>
<td>low µg/L</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>HAAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroacetic</td>
<td>sub µg/L</td>
<td>+</td>
<td>-</td>
<td>60 µg/L</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromoacetic</td>
<td>sub µg/L</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dichloro acetic</td>
<td>mid µg/L</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dibromo acetic</td>
<td>mid µg/L</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trichloro acetic</td>
<td>mid µg/L</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oxyhalides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromate</td>
<td>sub µg/L</td>
<td>+</td>
<td>+</td>
<td>10 µg/L</td>
</tr>
<tr>
<td></td>
<td>Chlorite</td>
<td>high µg/L</td>
<td>-</td>
<td></td>
<td>1 mg/L</td>
</tr>
</tbody>
</table>

Adapted and modified from (S.D. Richardson et al., 2007)
### Table 1.2 Select unregulated DBPs formed during drinking water disinfection

<table>
<thead>
<tr>
<th>DBP</th>
<th>Example(s)</th>
<th>Occurrence</th>
<th>Genotoxicity</th>
<th>Carcinogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halonitromethanes</td>
<td>Chloro nitro methanes</td>
<td>ng/L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Iodo-acids</td>
<td>Iodo acetic acid</td>
<td>sub µg/L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Other halo-acids</td>
<td>Bromo chloro acetic acid</td>
<td>low µg/L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Iodo-THMs</td>
<td>Iodoform</td>
<td>ng/L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Halofuranones</td>
<td>MX</td>
<td>sub µg/L</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Halo amides</td>
<td>Chloro acetamine</td>
<td>sub µg/L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Haloacetonitriles</td>
<td>Chloroacetonitrile</td>
<td>sub µg/L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Halopyrroles</td>
<td>2,3,5 – Tri bromo pyrrole</td>
<td>ng/L</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Nitro amines</td>
<td>N-nitroso pyrrolidine</td>
<td>ng/L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Formaldehyde</td>
<td>sub µg/L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other DBPs</td>
<td>Chlorate</td>
<td>high µg/L</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Adapted and modified from (S.D. Richardson et al., 2007)

**Disinfection Using Ozone**

Early application of ozone in the United States was primarily for non-disinfection purposes such as color removal, or taste and odor control (USEPA, 1999). Currently,
ozone, an oxidant, is being used as an alternative disinfectant to chlorine by most of the water treatment plants across the world to reduce the risk associated with the chlorinated DBPs formation such as THMs and HAAs in the drinking water (Sedlak, 2011).

Ozone can oxidize organic and inorganic micro pollutants present in the source water. Most importantly, ozone usage can reduce the chlorine demand in disinfecting the source water (USEPA, 1999). Unfortunately, ozonation also produces DBPs, the prominent of these being bromate (BrO$_3^-$), which is an animal carcinogen and possible human carcinogen (group 2B) (IARC, 1986).

**BrO$_3^-$ in Drinking Water**

Health concerns have been raised about BrO$_3^-$ as ozone can oxidize bromide (Br$^-$) if present, to BrO$_3^-$ (U. von Gunten & J. Hoigne, 1994). BrO$_3^-$ can also be present in hypochlorite and it is formed during the electrolysis of sodium chloride that contains Br$^-$. **Figure 1.1** shows the formation of BrO$_3^-$ during ozonation of Br$^-$ containing source water. The extent of BrO$_3^-$ formation during ozonation depends on several factors, such as the concentration of Br$^-$, pH, temperature, alkalinity, ammonia (NH$_3$), hydrogen peroxide (H$_2$O$_2$), amount of dissolved carbon in the source water and the level of ozonation (WHO, 2005).
**Figure 1.1:** Mechanism of $\text{BrO}_3^-$ formation during ozonation of $\text{Br}^-$ containing source water. Adapted with permission from von Gunten, U., & Hoigne, J. (1994): Bromate formation during ozonization of bromide-containing waters: Interaction of ozone and hydroxyl radical reactions. Copyright (2012) American Chemical Society.

**$\text{BrO}_3^-$ in Food**

$\text{BrO}_3^-$ may exist in a number of salt forms, but is most commonly found as potassium ($\text{KBrO}_3$) and sodium bromate ($\text{NaBrO}_3$). $\text{KBrO}_3$ is used as an oxidizer to mature flour during the baking process, and is also found in fish paste products etc. (IARC, 1999; JECFA, 1995; USFDA, 1994). Moreover, both $\text{NaBrO}_3$ and $\text{KBrO}_3$ are used as oxidizers in permanent wave neutralizing solutions and dyeing textiles using sulfur dyes (Mack, 1988). However, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1995) concluded that $\text{KBrO}_3$ usage is not appropriate during food processing to minimize the exposure of $\text{BrO}_3^-$ to humans.
**BrO$_3^-$ Environmental Levels and Human Exposure**

The annual mean BrO$_3^-$ contamination in the finished surface water is 2.9 μg/L with a range of < 0.2 to 25 μg/L in the USA (WHO, 2005). As of 2012, no data is available on BrO$_3^-$ concentrations from ground water treatment plants (WHO, 2005). Once BrO$_3^-$ is in the environment, it does not volatilize, and is only slightly absorbed into soils or sediment. Most likely BrO$_3^-$ converts to Br$^-$ by reacting with the organic matter (WHO, 2005).

**Toxic Effects of BrO$_3^-$ in Humans**

No long term studies have been conducted to identify the toxic effects of BrO$_3^-$ in humans. However, a few case studies of BrO$_3^-$ poisoning exist. These arise from either accidental or intentional ingestion of home permanent wave solutions (2% KBrO$_3$ or 10% NaBrO$_3$) in humans (USEPA, 2001). The toxic effects following ingestion included nausea, vomiting, abdominal pain, anuria and diarrhea, varying degrees of central nervous system depression, hemolytic anemia and pulmonary edema. Most of these toxic effects were irreversible (Lichtenberg, Zeller, Gatson, & Hurley, 1989; Watanabe et al., 1992). In addition, irreversible renal failure and deafness were also observed after acute exposure to 240-500 mg/kg KBrO$_3$ (185-385 mg of BrO$_3^-$/kg body wt.) in humans (Quick, Chole, & Mauer, 1975). The reported lethal dose of BrO$_3^-$ was estimated to be 150-385 mg BrO$_3^-$/kg body wt. in humans (Mack, 1988). Kidney is a primary target organ for BrO$_3^-$ in humans (Lichtenberg et al., 1989; Watanabe et al., 1992). No data is available to indicate whether children or adults are more susceptible to the effects of
BrO$_3^-$.

However, the limited acute data suggest that children and adults have equivalent responses to BrO$_3^-$ (USEPA, 2001).

**Non-Carcinogenic Effects of BrO$_3^-$ in Animals**

BrO$_3^-$ exposure can increase the concentration of serum enzymes such as glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, lactate dehydrogenase, alkaline phosphatase, cholinesterase and blood urea nitrogen, which are indicative of kidney toxicity, after treatment with $\geq$ 63 mg BrO$_3^-$/ kg body wt. /day, in drinking water for 13 weeks, in male and female rats (Kurokawa, Maekawa, Takahashi, & Hayashi, 1990). Other toxic effects include increase in organ to body weight ratio, basophilic regeneration and accumulation of eosinophilic droplets in the renal tubules of rats treated with $\geq$ 231 mg BrO$_3^-$/ kg body wt. as a single oral gavage (Kurata, Diwan, & Ward, 1992). BrO$_3^-$ can cause death by inducing proximal tubule (kidney) necrosis in animals dosed with $>500$ mg BrO$_3^-$/ kg body wt. as a single oral gavage (Kurata et al., 1992). No data are reported on BrO$_3^-$-induced toxicity at doses lower than 63 mg BrO$_3^-$/ kg body wt. /day in drinking water in animals.

**Carcinogenic Effects of BrO$_3^-$ in Animals**

BrO$_3^-$ is a proven animal carcinogen and suspected human carcinogen (IARC, 1986; USEPA, 2001). BrO$_3^-$ produces tumors at multiple sites in rats, including the kidney (adenomas and carcinomas), the thyroid gland (follicular cell adenomas and carcinomas) in both male and female rats, and the peritoneum (mesotheliomas) of male rats (DeAngelo, George, Kilburn, Moore, & Wolf, 1998; Kurokawa, Aoki, et al., 1986;
Kurokawa, Matsushima, Takamura, Imazawa, & Hayashi, 1987; Kurokawa, Takayama, et al., 1986). After treatment of male F344 rats with 0, 0.7, 1.3, 2.5, 5.6, 12 and 33 mg BrO₃⁻/kg body wt./day, in drinking water for 104 weeks (Kurokawa, Takayama, et al., 1986), the incidence of combined renal adenomas and carcinomas was 0%, 0%, 0%, 4%, 21%, 25% and 45%; the incidence of follicular tumors of the thyroid gland 0%, 0%, 0%, 4%, 0%, 15% and 37%; and the peritoneal mesotheliomas were 0%, 0%, 15%, 17%, 8%, 15% and 75%; respectively. Significant (P<0.05) increase in the incidence of renal tumors was observed at doses ≥ 5.6 mg BrO₃⁻/kg body wt./day, and for the thyroid tumors and mesotheliomas at 33 mg BrO₃⁻/kg body wt./day, compared to their controls (Kurokawa, Takayama, et al., 1986).

**Sex-Dependent Differences in BrO₃⁻-Induced Nephrotoxicity and Renal Tumors**

Sex-dependent differences exist with regard to formation of renal tumors. For example, tumors in male rat kidneys are induced at concentrations ≥ 125 mg/L KBrO₃ (≥ 7.3 mg BrO₃⁻/kg body wt./day) in drinking water after 2 years. In contrast, concentrations ≥ 250 mg/L KBrO₃ (≥ 16 mg/kg body wt./day) are needed to induce kidney tumors in female rats (Kurokawa, Takayama, et al., 1986). The incidence of renal tumors (adenomas and carcinomas combined) was 6%, 60% and 88%; and 0%, 56% and 80%; in male and female F344 rats respectively, after treatment with 0, 9.6 and 21.3 mg BrO₃⁻/kg body wt./day in drinking water for 2 yrs. Other studies (Kurokawa, Takayama, et al., 1986; T. Umemura et al., 2004), demonstrated that renal cell proliferation was induced in male rats at much lower concentrations than in female rat. This could be due to presence of a specific protein called alpha-2u-globulin in male
rat kidneys (T. Umemura et al., 2004; T. Umemura, K. Sai, A. Takagi, R. Hasegawa, & Y. Kurokawa, 1995). Others suggested that alpha-2u-globulin does not mediate the development of renal carcinomas in rats because renal tumors are produced in female rats, which do not express this protein (USEPA, 2001). Thus, some suggest that while alpha-2u-globulin does not mediate renal cell transformation, it may mediate the enhanced susceptibility of males to BrO₃⁻-induced renal carcinogenesis.

**Genotoxic and Mutagenic Effects of BrO₃⁻ in Animals**

BrO₃⁻ increases the levels of 8-hydroxy deoxy guanosine (8-OHdG), a marker for an oxidative DNA damage, in genomic DNA of male and female rat kidneys after treatment with ≥ 250 mg KBrO₃/L drinking water for 28 days (T. Umemura et al., 2004). Marginal increase in mutagenic effects in vivo was observed after treatment with 500 mg/L KBrO₃ in rats. Mutation frequency was increased in the gpt (glutamine pyruvate transaminase) in rats, but only with treatments at concentrations ≥ 250 mg KBrO₃/L for 3-4 weeks, and the mutation spectrum was inconsistent with 8-OH-dG-induced mutations (T. Umemura et al., 2006; T. Umemura et al., 2004; T. Umemura et al., 2009).

**Genotoxic and Non-genotoxic Mechanisms of Action of BrO₃⁻**

Studies have examined the mechanisms by which BrO₃⁻ causes renal toxicity and tumors both in vitro and in vivo. However, few studies have described the mechanisms of action of BrO₃⁻-induced tumor formation. BrO₃⁻ induces renal cell proliferation and tumors by genotoxic as well as non-genotoxic mechanisms (Kawanishi & Murata, 2006;
It is believed that BrO$_3^-$ causes cell proliferation in animals by inducing DNA damage, specifically by forming 8-OHdG lesions in DNA (Kawanishi & Murata, 2006; T. Umemura et al., 2004).

Oxidative stress could be another possible mechanism for BrO$_3^-$ induced genotoxicity in animals. However, the exact oxidizing moiety induced by BrO$_3^-$ is unknown. One possible explanation is that the cellular thiols, like glutathione (GSH), reduce BrO$_3^-$ to Br$^-$, which leads to the generation of free radicals such as bromine oxides (BrO$^-$ or BrO$_2^-$) in the body. These reactive free radicals may induce DNA damage by forming 8-OHdG (Kawanishi & Murata, 2006; Parsons & Chipman, 2000; T. Umemura & Kurokawa, 2006).

BrO$_3^-$ was proven to cause lipid peroxidation, as demonstrated by the increase in malondialdehyde levels in the kidneys of rats treated with BrO$_3^-$ (Chipman et al., 1998). However, 8-OHdG formation induced by BrO$_3^-$ did not correlate to lipid peroxidation (T. Umemura et al., 2004). Another hypothesis is that BrO$_3^-$ causes GSH depletion only at high concentrations, which is secondary to initial DNA oxidation, and 8-OHdG formation might result in a secondary oxidative stress (Chipman, Parsons, & Beddowes, 2006).

Recent in vitro studies suggest that non-genotoxic mechanisms mediate cell cycle arrest (Zhang et al., 2010). These same studies showed that BrO$_3^-$ exposure activated signaling pathways involved p21 independent of the formation of 8-OHdG (Zhang et al., 2010). cDNA array studies from other laboratories also suggest that BrO$_3^-$ can induce nephrotoxicity using non-genotoxic pathways (Ahlborn et al., 2009; Delker et al., 2006).
Cancer Risk Assessment of $\text{BrO}_3^-$

Based on the toxic, as well as carcinogenic effects, induced by $\text{BrO}_3^-$ in animals and humans, USEPA promulgated a maximum contaminant level (MCL) for $\text{BrO}_3^-$ at 10 µg/L drinking water (equivalent to ~0.3 µg $\text{BrO}_3^-$/kg body wt./day, as a bolus dose), with a maximum contaminant level goal (MCLG) of zero (USEPA, 2001). The estimated lifetime cancer risk from $\text{BrO}_3^-$ at 10 µg $\text{BrO}_3^-$/L drinking water, to humans is $0.5 \times 10^{-4}$ (5 in 100,000 individuals) (WHO, 2011). Whereas, the estimated cancer risk from $\text{BrO}_3^-$ by U.S. EPA is $2 \times 10^{-4}$ (2 in 10,000 individuals) additional cancers per lifetime in humans. These values were based upon linear extrapolation of combined kidney, testicular mesothelium, and thyroid tumor data in male rats; and the mechanisms of action, carcinogenesis and toxicity data from high concentrations (high mg $\text{BrO}_3^-$/L) used in the animal studies to the much lower concentrations (sub µg $\text{BrO}_3^-$/L) present in the drinking water.

The major uncertainty in estimating $\text{BrO}_3^-$ risk to humans is the species, sex and dose differences observed for $\text{BrO}_3^-$-induced toxicity. For example, $\text{BrO}_3^-$-induces ototoxicity in humans, but not in animals (USEPA, 2001); and $\text{BrO}_3^-$-induces renal cell proliferation and tumors in male rats at lower concentrations compared to female rats (T. Umemura et al., 2004). Moreover, WHO and USEPA did not consider the $\text{BrO}_3^-$ chemical reactivity in the biological matrices, toxicokinetics (ADME), mechanism of action at low concentrations vs. high concentrations, sex-dependent differences in $\text{BrO}_3^-$ induced renal cell proliferation and death, and inter species variation etc., while calculating $\text{BrO}_3^-$ risk in humans. This highlights the necessity to refine the estimation of risk from $\text{BrO}_3^-$ to humans by filling these data gaps.
Hypothesis and Specific Aims

There are two general aims of this project: 1) to develop the data necessary to describe BrO$_3^-$ pharmacokinetics (ADME) at lower doses in animals, and 2) determine the extent to which the mode of action of BrO$_3^-$ at lower doses results from non-genotoxic mechanisms.

The intent is to provide data for refining the BrO$_3^-$ cancer risk assessment after its consumption through drinking water. These data will assist in the development of a physiological based pharmacokinetic (PBPK) model for BrO$_3^-$, which is necessary to estimate the BrO$_3^-$ risk in humans. The overall hypotheses for this project are

**Hypothesis I:** The reactivity of BrO$_3^-$ results in a substantial pre-systemic reduction to Br$^-$ at low doses, which leads to exhibition of non-linear pharmacokinetics in F344 rats.

**Hypothesis II:** Non-genotoxic events (non DNA damaging events) mediate the BrO$_3^-$ induced sex-differences in renal cell proliferation and death in F344 rats.

These hypotheses will be tested by the following Aims.

Specific Aims

1. Determine the pharmacokinetics (ADME) of BrO$_3^-$ at low doses in female F344 rats
2. Identify the molecular mechanisms controlling sex-dependent differences in BrO$_3^-$-induced nephrotoxicity in F344 rats
3. Develop the pharmacokinetic (PK) model for BrO$_3^-$ in female F344 rats
CHAPTER 2
MATERIALS AND METHODS

Materials

Potassium bromate (KBrO₃) (99+ %) was purchased from ACROS organics, (Fair Lawn, NJ, USA). Acetonitrile, xylene, ethanol (histological grade), ammonium hydroxide, Gill’s counter stain, 30% hydrogen peroxide and tissue cassettes were purchased from Fisher Scientific, (Hampton, NH, USA). Standard solutions of BrO₃⁻, and Br⁻ were purchased from Environmental Express (Mt. Pleasant, SC, USA). [¹⁸O]-BrO₃⁻ was purchased as a solution (2 mg/ml) from Icon Isotopes (Summit, NJ, USA). Cocktail anesthesia was made with a mixture of ketamine hydrochloride (100 mg/ml) (Fort Dodge Animal Health, Fort Dodge, IA, USA), Acepromazine maleate (10 mg/ml) (Boehringer-Ingelheim, Saint Joseph, MO, USA), and Xylazine hydrochloride (20 mg/ml) (Lloyd Laboratories, Shenandoah, IA, USA) in the ratio of 3:2:1 v/v prepared in our lab. Normal saline (0.9 % w/v NaCl) solution was prepared by dissolving 9 g NaCl (Fisher Scientific) in 1 L DI H₂O. Cannulae were prepared by creating a bend in PE 50 tube (0.58mm ID x 0.99mm OD) at one end (approximately 1 inch long) by immersion in hot water. Syringes (1, 6, 12 cc), and 21 gauge needles were purchased from Central Research Stores, UGA (Athens, GA, USA). ABC and DAB immunohistological staining kits were purchased from Vector Labs, (Burlingame, CA, USA). Carnoy’s fluid was prepared by mixing 60 parts of 200 proof (100%) ethanol, 30 parts chloroform and 10 parts of glacial acetic acid (Fisher Scientific). The bromodeoxyuridine (BrdU) antibody
was purchased from Fisher Scientific, and the 8-hydroxy deoxy guanosine (8-OHdG) antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). Antibodies against p21 and clusterin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against kidney injury molecule (Kim-1) and osteopontin were purchased from R&D Scientific (Flanders, NJ, USA) and AbCam (Cambridge, MA, USA), respectively. The TUNEL apoptosis detection kit was purchased from GenScript (Piscataway, NJ, USA). The 3-bromotyrosine antibody was supplied by the Pacific Northwest National Laboratories, (Richland, WA, USA). Liquid nitrogen and nitrogen (N₂) gas were purchased from Airgas (Athens, GA, USA).

**Methods**

**Animal Preparation**

Male and female F344 rats weighing between 160 – 240 grams were purchased from Charles River (Indianapolis, IN, USA). Animals were housed and maintained in accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol at University of Georgia (UGA), Athens, GA, USA; and in accordance with the U.S. Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Animals were housed in pathogen-free cages within a light and temperature controlled isolated room and provided with autoclaved rodent chow Picolab® Rodent Diet 20, 5053 (PMI Nutrition International LLC, Brentwood, MO, USA), and autoclaved DI water *ad libitum*. Temperature and humidity were maintained at 72ºF and 40% respectively. Animals were allowed to acclimate one week prior to BrO₃⁻ treatment. All
treatments and protocols were approved by an Institutional Animal Care and Use Committee (IACUC) at the UGA.

**Jugular Vein and Carotid Artery Cannulations in Female F344 Rats**

Rats were injected with cocktail anesthesia @ 20-25 μL /100 gram body wt. intra muscularly (IM). Hair was trimmed and shaved after anesthesia at the ventral thoracic region near the shoulder. Povidone iodine was used as an antiseptic. Jugular vein cannulation for oral gavage studies; and both jugular vein and carotid artery cannulations for IV bolus studies were performed on animals. Rats were kept in individual cages after surgeries for 24 hr. prior to BrO₃⁻ administration. All surgeries were performed in the morning (~ 9AM).

**28 Day Drinking Water Study**

Groups of 10 rats for each concentration and sex were treated with 0, 125 or 400 mg KBrO₃/L drinking water (tap water) for 28 days. Water, food consumption and body weight were measured 3 times a week throughout the study. Bromodeoxyuridine (BrdU) 100 mg/kg was injected intra peritoneal (IP) on day 26 and 27 to all the rats for assessing cellular proliferation. The BrdU solution was prepared by dissolving 100 mg of BrdU in 200 µL of ethanol, and 5 drops of 4N NaOH were added prior to dilution in 10 ml saline.

On day 28, rat body weights were recorded. Rats were sacrificed by cervical dislocation, and isolated the blood by closed cardiac puncture. Whole body perfusion was performed using saline (0.9% NaCl) for tissue collection. Liver, kidney, testes, and
thyroid organs were collected, weighed and fixed in Carnoy’s fluid for histopathology and immunohistochemistry. In addition, un-fixed kidney tissue collected from rats was flash frozen in liquid N$_2$, and stored at -80 °C for further analysis.

In another independent 28 day drinking water study, groups of 6 rats for each concentration and sex were treated with 0, 15, 60 or 400 mg KBrO$_3$ / L drinking water (tap water) for 28 days. All the procedures were followed as mentioned above except that BrdU was not administered to rats.

**Preparation and Administration of BrO$_3^-$ for Acute Dosing Studies**

BrO$_3^-$ (KBrO$_3$) solution was prepared by dissolving an appropriate quantity of KBrO$_3$ per ml of de ionized water, followed by sterile filtering prior to use. If the administered BrO$_3^-$ dose was less than LAW (least amount weighed, < 120 mg), aliquots were prepared. BrO$_3^-$ was administered via jugular vein for the IV bolus studies; and orally for the oral gavage studies using a 2 mm ball tipped 2” curved needle in rats.

**Sample Analysis of Animal Diet and Drinking Water**

Rat chow samples were crushed and homogenized. One gram of crushed diet pellets was weighed into a 15 ml polypropylene vial, and then 10 ml of reagent water was added. The sample was mixed thoroughly before sonication for 30 min. at room temperature. The resulting suspension was centrifuged at 5,000 g for 10 min, and the supernatant transferred and processed with 0.45 μm filter and $^{18}$C cartridge. The first 3 ml was discarded and the remaining solution was collected for analysis. The chow samples were analyzed using ion-chromatography with inductively-coupled plasma
mass spectrometry (IC-ICP-MS). BrO$_3^-$ concentrations in rat chow were found to be below the limit of quantitation (LOQ) 4.2 μg/kg and the Br$^-$ concentration was 745 μg/kg. The drinking water samples were analyzed using liquid chromatography with tandem electron spray ionization mass spectrometry (LC-ESI-MS). The concentration of BrO$_3^-$ in the drinking water of control animals was < 0.045 μg/L, the isotope dilution LC/MS limit of detection. The sample analysis methods are discussed below.

**Samples from Acute Dosing Studies**

Blood samples (300 μL) were collected into micro centrifuge tubes containing 10 μL [$^{18}$O]-BrO$_3^-$ as an internal standard (250μg/L) from the carotid artery of treated female rats at 5, 10, 20, 40, 60, 120, 240, 480 and 720 min. Blood samples were centrifuged at 14,000 g for 30 sec to separate RBC and plasma. The plasma was removed and extracted twice with 1 ml of acetonitrile into a 15 ml conical tube. The resulting extracts were dried under N$_2$ gas; and then sealed and shipped for the analysis of BrO$_3^-$ using LC-ESI-MS at Southern Nevada Water Authority (SNWA), NV, and USA.

**LC-ESI-MS**

BrO$_3^-$ in blood was extracted and analyzed by liquid chromatography (LC) with tandem mass spectrometry detection (MS/MS). Extract volumes of 10 ml were separated using a 250 x 4.6 mm Synergi Max-RP C12 column with a 4 μm pore size (Phenomenex, Torrance, CA). An Agilent (Palo Alto, CA) G1312A binary pump, an HTC-PAL auto sampler (CTC Analytics, Zwingen, Switzerland) and a gradient consisting of 0.1% formic acid (v/v) in water (A) and 100% methanol (B), at a flow rate
of 700 μL/min, were used for separation. Mass spectrometry was performed using an API 4000 triple-quadrupole mass spectrometer (Applied Bio systems, Foster City, CA). The ESI negative mode was employed, as it was found to be most sensitive and selective of the various ionization techniques/polarities. Optimal compound-dependent parameters were determined, and source-dependent parameters optimized for LC and ionization conditions.

BrO$_3^-$ values in sample extracts from rat blood were determined by isotope dilution using $[^{18}\text{O}]$-BrO$_3^-$, incorporated prior to extraction, at final extract concentration of 250 μg/L to correct for decay and matrix suppression of BrO$_3^-$. The established limit of quantitation (LOQ) for BrO$_3^-$ was 0.5 μg/L, accounting for dilution at extraction. Further, an additional transition containing the m/z 81 isotope of bromine was used to qualitatively confirm peaks obtained using the most abundant m/z 79 isotope.

Blood and/or plasma sample extracts from animals administered with BrO$_3^-$ were received dry in 15 ml PFTE vials, and then reconstituted in 1 ml of 10% acetonitrile in water, then mixed by vortexing. This liquid was transferred into calibrated 15 ml vials and evaporated under a gentle N$_2$ stream to approximately 900 μL and brought to a final volume of 1.5 ml with de-ionized water. The aqueous samples were then applied to an On Guard II Ba cartridge followed by application to an On Guard II H cartridge (Dionex, Sunnyvale, CA) in series. The in-series cartridges were prepared by flushing with 10 ml of de-ionized water. Samples were eluted at a flow rate of 0.5 ml/min, with the first 0.75 ml discarded, and the subsequent 500 μL collected in an auto sampler vial for analysis. The analytical method employed was adapted from existing methodology for oxy halide
analysis (Snyder et al., 2005) with the incorporation of an internal standard correction using $^{18}$O-Bro$_3^-$, a shorter analytical column and elution schemes exclusive for BrO$_3^-$.

The method detection limit (MDL) for BrO$_3^-$ was calculated from average results of eight reagent water samples fortified with analyte and internal standard, extracted and analyzed as described. A conservative instrument reporting limit of detection (LOD) was established at 0.1 μg/L and used as the lowest calibration point. The calibration range and the limit of quantitation (LOQ) for BrO$_3^-$ analysis in this study were 0.1 to 50 μg/L, and 0.5 μg/L, respectively.

**Samples from In vitro Metabolism Studies**

Fresh blood (200 μL) from female F344 rats was added to 2 ml micro centrifuge tubes. BrO$_3^-$ solution (10 μL, containing the desired BrO$_3^-$ concentration) was spiked immediately into the blood, and then incubated for 1, 2, 5 and 10 min. Acetonitrile (600 μL) was added to the reaction mixture to stop the reaction at the end of incubation period. Blood samples were then extracted twice with acetonitrile (600 μL each).

BrO$_3^-$ degradation at time zero was measured by the addition of 600 μL acetonitrile to the whole blood prior to the BrO$_3^-$ spike. Samples were then immediately extracted twice with acetonitrile (600 μL each). The spiked BrO$_3^-$ concentrations in blood were established by spiking the same BrO$_3^-$ concentrations into distilled water. Similar extraction procedures were followed as mentioned above.

All the extracted blood and water samples were dried under N$_2$ gas, and then sealed and shipped for the analysis of BrO$_3^-$ and Br$^-$ in the same sample using the IC-ICP-MS, at Center for Advanced Water Technology (CAWT), Singapore. Similar
Experimental procedures were followed for studying the BrO$_3^-$ metabolism studies in both liver and kidney homogenates of female F344 rats.

**IC-ICP-MS**

BrO$_3^-$ and Br$^-$ in blood and urine were also extracted and analyzed by ion-chromatography with inductively-coupled plasma mass spectrometry (IC-ICP/MS). A Dionex (Sunnyvale, CA) DX 500 ion chromatography (IC) system equipped with a GP50 gradient pump was connected to a 250 mm × 4 mm (Ion Pac® AS9-HC) analytical column and 4 mm (AG-9-HC) guard column. A 15-min isocratic elution with 9 mM sodium bicarbonate was used for chromatographic separations. A Dionex AS40 autosampler with 0.5 mL cartridge was used for the injection of 250 µL of sample into the IC column. For detection, an inductively-coupled plasma mass spectrometer (ICP/MS) from Agilent Technologies (model 7500c, Palo Alto, CA) was used under hot plasma conditions for elemental analysis. The eluent from the IC column was introduced directly into the spray chamber of the ICP/MS. A time resolved elemental signal was obtained for bromine masses $m/z$ 79 and $m/z$ 81; however, $m/z$ 79 was used for quantitation since it yielded a higher signal to noise ratio and improved sensitivity. The time resolved signal was converted into chromatographic data and analytes quantified from extrapolation of peak areas against those of calibration standards. The limit of quantitation (LOQ) for both BrO$_3^-$ and Br$^-$ in rat blood and tissue samples was 2.1 µg/L (Quiñones, Snyder, Cotruvo, & Fisher, 2006).
**Samples from BrO₃⁻ and Br⁻ Oral Gavage Studies**

Male and female F344 rats after BrO₃⁻ or Br⁻ or DI water dosing by oral gavage were kept in an individual metabolism cages throughout the experiment. Total urine volume was measured at each time point (every 6 hr. for the first 24 hr.), and then every 24 hr. after dosing. Urine (300 μL) was collected at each time point into a 1.5 ml micro centrifuge tubes. Samples were then centrifuged at 14,000 g for 30 sec to separate out any precipitation present, and the supernatant was collected and extracted twice with 1ml of acetonitrile. The extracted samples were dried under N₂ gas, and then sealed and shipped for the analysis of BrO₃⁻ and Br⁻ using IC-ICP-MS at Center for Advanced Water Technology (CAWT), Singapore.

Blood samples were collected by closed cardiac puncture at the end of experiment. All the blood samples were extracted as described above for BrO₃⁻ in the acute dosing studies in rats, and analyzed using IC-ICP-MS.

**Urine Samples from the 28 Day Drinking Water Study**

On day 27, urine volumes were measured and recorded for every 24 hr. All the urine samples were extracted and analyzed as described above in the BrO₃⁻ and Br⁻ oral gavage studies for BrO₃⁻ and Br⁻ using IC-ICP-MS; and total organic bromine (TOBr) using ICP-MS at Center for Advanced Water Technology (CAWT), Singapore.

**Total Organic Bromine (TOBr) Analysis using ICP-MS**

Urine samples were sent to the Center for Advanced Water Technology (CAWT) Singapore for Total Organic Bromine (TOBr) analysis. The method used was adapted
from (Hua, 2006; ISO, 2004; USEPA, 1997). It involved the adsorption of organic halogen onto activated cartridges, pyrolysis of organic halogen at 800°C to produce hydrogen halide, which is absorbed by de ionized (DI) water, and the quantitation of total bromine in the resulted solution by ICP-MS.

Urine samples (400 or 500 µL each) were received and stored at -18°C. When ready for analysis, the sample tubes were allowed to reach room temperature, and 50ml DI water was added (in several increments) to dissolve the samples, which was transferred to a 100-ml bottle. The sample solution was diluted with 5 ml nitrate stock solution, and then was aspirated at 3 ml/min to pass through two activated carbon cartridges (in series). Afterward, 25 ml of nitrate wash solution was used to rinse the cartridges at 3 ml/min. The two cartridges for a specified sample were placed in the auto X36 auto sampler, and were combusted under pure oxygen (99.995%) at 800°C. DI water (10 ml) was placed into the sulfuric acid vessel to absorb pyrolysis products (mainly hydrogen halides) from organic halogen. The resulted solution was tested for total bromine by ICP-MS at m/z =79, with bromide standard solution as the calibration standard.

**Hematoxylin and Eosin (H&E) staining**

Collected tissues were weighed, sliced and placed in Carnoy’s fluid for 24 hr. and then preserved in 70% ethanol (v/v) at 4°C. Preserved tissues were placed in tissue cassettes, and slides were prepared at the Poultry Diagnostic Research Center, University of Georgia, which also conducted the H&E staining.
**Immunohistochemistry**

Slides were incubated in xylene 5 min each for 2 times, followed rehydration using gradients of histological grade ethyl alcohol (100%, 95% and 70%) for 6 min each. Slides were then rinsed in PBS. Later, slides were incubated in 30% H$_2$O$_2$ for 30 min.; followed by incubation with 2% normal horse serum for 20 min. to block nonspecific binding. Sections then were washed two times with PBS and incubated with primary antibodies against BrdU (5 µg/ml), clusterin (10 µg/ml), Kim-1 (15 µg/ml) or osteopontin (10 µg/ml) for 24 hours, or antibodies against 8-OHdG (9.75 µg/ml) for 18 hr., or p21 (10 µg/ml) for 3 hr., or 3-bromotyrosine (10 µg/ml) at room temperature. Following incubation in the primary antibody, samples were washed three times in PBS for 5 min and incubated in PBS containing a universal secondary antibody and Elite Reagent (ABC Kit, Vector labs) for 30 min each, followed by incubation with 3, 3’diaminobenzena-dine (DAB) reagent, and a counter stain (Gills Haematoxyllin). Slides were then bathed in ammonium hydroxide (10% v/v), dehydrated, and fluoromount was applied followed by cover slips. Visualization of staining was done using a Nikon AZ100 fluorescence microscope. Six pictures were taken randomly from each slide for each antibody per tissue. If the staining was nuclear, total nuclei as well as positive nuclei were counted, and the results were expressed as % nuclei in a given field. If the staining was cytoplasmic, a score of 0-4 was given based on percent tubules stained in a given field, and a score of 0 was given for no staining, 0.5 for ≤ 10%, 1 for 11-25%, 2 for 26-50 %, 3 for 51-75 %, and 4 for 76-100% staining in a given field.
**TUNEL Staining**

Slides were incubated in xylene 5 min. each for 2 times, followed by rehydration with gradient of ethyl alcohol histological grade (100%, 95% and 70%) for 6 min. each. Then slides were washed in PBS. Slides were placed in 0.1 M citrate buffer (pH 6) and micro waved for 1 min at 750W, cooled with water and then rinsed in PBS for 5 min. Later, slides were incubated with blocking solution (3% H₂O₂ in methanol) for 10 min., and 100 µL DNase I Solution for 10 min. (for the positive control slides only), and 50 µL TUNEL reaction mixture for 60 min., 50 µL Streptavidin-HRP Solution for 30 min., followed by incubation with DAB reagent and a counter stain (Gills). Slides were then bathed in ammonium hydroxide (10% v/v), dehydrated, and fluoromount was applied followed by cover slips. Visualization of staining was done using a Nikon AZ100 fluorescence microscope. Six pictures were taken randomly from each slide for each antibody per tissue. For nuclear staining, total nuclei as well as positive nuclei were counted, and the results were expressed as % nuclei in a given field.

**BrO₃⁻ Pharmacokinetic Data Analysis and Modeling**

Non-compartmental analysis (NCA) of plasma BrO₃⁻ time-concentration profile following administration of KBrO₃ as an IV bolus or oral gavage in female F344 rats was performed using a linear regression program (WinNonlin Version 5.2, Pharsight Corporation, Cary, NC). Constants such as elimination rate constant (Kₑ), apparent volume of distribution (Vₑ), and total body clearance (CLₑ) were estimated. Parameters such as plasma BrO₃⁻ area under curve (AUC) and terminal half-life (t₁/₂) were estimated using the log-trapezoidal method, and by 0.693/Kₑ, respectively. Variables such as
BrO$_3^-$ maximum plasma concentration ($C_{\text{max}}$), and time at maximum plasma concentration ($T_{\text{max}}$) were taken directly from the graph.

Metabolism parameters such as $K_m$ and $V_{\text{max}}$ for BrO$_3^-$ degradation, and Br$^-$ formation from BrO$_3^-$ in female F344 rat blood were calculated based on the raw data from the BrO$_3^-$ in vitro metabolism studies in rat blood (Bull et al., 2012) using PRISM 5.0.

Raw data from (Bull et al. 2012) was utilized to develop a pharmacokinetic (PK) model for BrO$_3^-$ in female F344 rats. PK modeling was performed using a nonlinear regression analysis program (WinNonlin Version 5.2, Pharsight Corporation, Cary, NC, USA). The best PK model to describe BrO$_3^-$ disposition was selected based on the data fit and the model diagnostics such as Akaike's information criterion (AIC), Schwarz's Bayesian criterion (SBC), percent co-efficient of variation (%CV), and the goodness of fit. The resulting PK model was used to estimate the pharmacokinetic parameters for BrO$_3^-$.

**Statistical Analysis**

All the data were reported as the mean ± standard error of the mean (SEM) of 4 to 10 rats. The pharmacokinetic (PK) parameters of BrO$_3^-$ for all the doses administered as either IV bolus or oral gavage were analyzed using one way ANOVA. Individual means were compared using Tukey's test with $P < 0.05$ considered as indicative of a statistically significant difference between the mean values. The individual means of change in percent organ to body weights were compared using paired t-test with $P <$
0.05 considered as indicative of a statistically significant difference between the mean values. Immunohistochemistry and TUNEL apoptosis raw data were analyzed by using two way ANOVA followed by a Holm-Sidak test for the multiple comparisons with $p < 0.05$ considered as indicative of a statistically significant difference between the mean values.
CHAPTER 3

PHARMACOKINETICS OF BrO$_3^-$ IN FEMALE F344 RATS

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Abstract

Bromate (BrO$_3^-$), a toxic by-product formed during ozonation of ground or source water containing bromide (Br$^-$). The reactivity of BrO$_3^-$ with the biological reductants (especially thiols) results in a substantial reduction to Br$^-$ at low doses, which leads to exhibition of non-linear pharmacokinetics in F344 rats. To test this hypothesis, BrO$_3^-$ acute dosing studies (0.1 to 20 mg/kg KBrO$_3$) administered as either IV bolus or oral gavage were conducted in female F344 rats. Analysis of BrO$_3^-$ pharmacokinetic parameters revealed that disproportionate increase in peak concentration (C$_{max}$ or C$_0$) and the area under curve (AUC) of plasma BrO$_3^-$ were in correlation with the increased plasma BrO$_3^-$ clearance for the doses 0.5 and 1 mg/kg, compared to 0.1 mg/kg KBrO$_3$. BrO$_3^-$ was eliminated from the body in a first order fashion with an elimination rate constant (K$_e$) of 0.03 min$^{-1}$, and plasma half-life of ~23 min. The oral bioavailability (F) of BrO$_3^-$ is ~35 %, indicates the occurrence of high pre systemic reduction of BrO$_3^-$ in rats. The plasma half-life for BrO$_3^-$ after oral gavage is ~30 min. for doses up to 2.5 mg/kg KBrO$_3$. However, the increases in plasma BrO$_3^-$ half-life following the oral administration of 20 mg/kg KBrO$_3$ was in correlation with the decreased elimination rate constant (K$_e$) from 0.03 to 0.016 min$^{-1}$ and increased volume of distribution (V$_d$) from 0.87 to 1.46 L/kg. The rate of BrO$_3^-$ reduction was higher compared to the rate of Br$^-$ formation from BrO$_3^-$ both in vitro and in vivo. The incubation of 0-2840 μM BrO$_3^-$ in rat blood in vitro showed that BrO$_3^-$ degrades in a rapid initial and slow secondary manner. Loss of BrO$_3^-$ during both phases was accompanied by increases in Br$^-$ concentrations indicating that the BrO$_3^-$ loss was due to its reduction. A very small amount ~6% was recovered as BrO$_3^-$ in urine following administration of a single dose of 8.1 mg KBrO$_3$/kg as an oral
gavage. Br\textsuperscript{-} elimination from BrO\textsubscript{3}\textsuperscript{-} over the first 48 hours was 18% lower than it was eliminated from an equi molar single dose of Br\textsuperscript{-}, 5 mg KBr/kg (15.5 ± 1.6 vs. 18.8 ±1.2 μmol/kg, respectively). The cumulative excretion of Br\textsuperscript{-} from KBr vs. KBrO\textsubscript{3} was equivalent 72 hours after administration. Moreover, the deficits in total BrO\textsubscript{3}\textsuperscript{-} recovery raises the possibility that some brominated biochemicals may be produced in vivo, which slowly gets metabolized and eliminated from the body.

**Introduction**

Bromate (BrO\textsubscript{3}) is a proven animal carcinogen and probable human carcinogen (group 2B) (IARC, 1986). Health concerns have been raised as it is a by-product of the ozonation of ground or source water.

BrO\textsubscript{3} is a reactive compound, as reduction of BrO\textsubscript{3} to bromide (Br\textsuperscript{-}) occurs, some extent in the human gastric juice (Jason D. Keith, Gilbert E. Pacey, Joseph A. Cotruvo, & Gilbert Gordon, 2006; J.D. Keith, G.E. Pacey, J.A. Cotruvo, & G. Gordon, 2006). This is due to the presence of reducing agents such as H\textsubscript{2}S, sulfite ions, glutathione and/or cysteine in the gastric juice. The in vitro half-life of BrO\textsubscript{3} in the human gastric juice containing typical concentration of H\textsuperscript{+}, CL\textsuperscript{-}, H\textsubscript{2}S and other reducing agents is 10-15 min (Jason D. Keith et al., 2006; J.D. Keith et al., 2006).

BrO\textsubscript{3} pharmacokinetics (ADME) is rapid, as it is quickly absorbed, distributed, and eliminated from the body in animals (Fujii et al., 1984). It is widely distributed to kidney, pancreas, stomach, small intestine and red blood cells. This might be due to the involvement of sodium iodide transporter (NIS) in transporting BrO\textsubscript{3} across the cell membrane and into the tissues (Fisher & Bull, 2006). The major organs for BrO\textsubscript{3}
metabolism are RBC, liver and the kidney (Tanaka et al., 1984). The extent of BrO$_3^-$ degradation is approximately stoichiometric to the amount thiol (-SH) containing compounds such as glutathione, cysteine and ergothioneine in the blood and the other tissues (Moriarty-Craige & Jones, 2004; Tanaka et al., 1984). The urinary excretion of BrO$_3^-$ was evidenced for the doses ≥2.5 mg/kg KBrO$_3$ oral gavage in animals (Fujii et al., 1984).

Based on toxic, as well as carcinogenic, effects induced by BrO$_3^-$ in animals and humans, USEPA promulgated a maximum contaminant level (MCL) for BrO$_3^-$ at 10 µg/L drinking water (equivalent to ≈0.3 µg BrO$_3^-$/kg body wt./day, as a bolus concentration), with a maximum contaminant level goal (MCLG) of zero (USEPA, 2001). But, this value was estimated based on very limited preclinical and/or clinical BrO$_3^-$ pharmacokinetics (ADME) data in animals and humans (Cotruvo, 2010; Fisher & Bull, 2006; Fujii et al., 1984; Tanaka et al., 1984).

The intent of the current study is to provide BrO$_3^-$ pharmacokinetic data for the purpose of refining the BrO$_3^-$’s cancer risk assessment in humans. This generated data will assist in the development of a physiological based pharmacokinetic (PBPK) model for BrO$_3^-$, which is necessary to estimate the BrO$_3^-$ risk in humans.

Our hypothesis is that reactivity of BrO$_3^-$ with the biological reductants (especially thiols) results in a substantial reduction to Br$^-$ at low concentrations, which leads to exhibition of non-linear pharmacokinetics in F344 rats. The specific aims of this chapter are to determine the rates of BrO$_3^-$ clearance from the body after IV bolus and oral gavage; explore the extent to which BrO$_3^-$ is reduced to Br$^-$ in vitro and in vivo; and
compare the urinary excretion of BrO$_3^-$ and Br$^-$ following oral administration of equi molar dose of KBrO$_3$ or KBr in female F344 rats.

Results

**BrO$_3^-$ Acute Dosing (IV bolus) Studies in Female F344 Rats**

The mean plasma BrO$_3^-$ time-concentration profile following the administration of 0.1, 0.5, 1, or 2.5 mg/kg KBrO$_3$ as an IV bolus in female F344 rats are shown in (Figure 3.1). BrO$_3^-$ pharmacokinetic (PK) parameters estimated by non-compartmental analysis (NCA) in WINNONLIN, V 5.2 are shown in (Table 3.1). Plasma BrO$_3^-$ concentrations were 10% or less of $C_0$ within 60 min. Greater than 95% of the administered BrO$_3^-$ was eliminated from the body with in 120 min. (Figure 3.1). The peak concentration ($C_{max}$ or $C_0$) and the area under curve (AUC) of BrO$_3^-$ in plasma did not increase in proportion with the BrO$_3^-$ dose, for the doses 0.5 and 1 mg/kg KBrO$_3$. This was in correlation with the increased plasma BrO$_3^-$ clearance for these doses, compared to 0.1 mg/kg KBrO$_3$. BrO$_3^-$ was eliminated from the body in a first order fashion with an elimination rate constant ($K_e$) of 0.03 min$^{-1}$, and the plasma half-life of ~23 min. The apparent volume of distribution ($V_d$) of BrO$_3^-$ was not significantly changed with the BrO$_3^-$ dose.

**BrO$_3^-$ Acute Dosing (oral gavage) Studies in Female F344 Rats**

The mean plasma BrO$_3^-$ time-concentration profile following administration of 0.5, 2.5, or 20 mg/kg KBrO$_3$ oral gavage in female F344 rats are shown in (Figure 3.2). BrO$_3^-$ pharmacokinetic (PK) parameters estimated by non-compartmental analysis (NCA) in WINNONLIN, V 5.2 were showed in (Table 3.2). The time ($T_{max}$) to reach BrO$_3^-$
peak concentration \( (C_{\text{max}}) \) in plasma was 10 min.; and greater than 95% of the administered \( \text{BrO}_3^- \) was eliminated within 2 hr. after administration of \( \text{BrO}_3^- \). The peak concentration \( (C_{\text{max}} \text{ or } C_0) \) and the area under curve (AUC) of \( \text{BrO}_3^- \) in plasma were increased in proportion with the \( \text{BrO}_3^- \) dose. This was in correlation with the similar plasma \( \text{BrO}_3^- \) clearance (~20 ml/min/kg) for the oral doses ranging from 0.5 to 20 mg/kg \( \text{KBrO}_3 \). The oral bioavailability (F) of \( \text{BrO}_3^- \) is ~35 %, indicating the occurrence of high pre systemic reduction of \( \text{BrO}_3^- \) in animals. The plasma half-life for \( \text{BrO}_3^- \) was ~30 min., and the elimination rate constant \( (K_e) 0.03 \text{ min}^{-1} \) for the doses up to 2.5 mg/kg.

However, the plasma half-life for \( \text{BrO}_3^- \) was increased to 43 min. following the oral administration of 20 mg/kg \( \text{KBrO}_3 \). This was in correlation with the decreased elimination rate constant \( (K_e) \) from 0.03 to 0.016 min\(^{-1}\) and the increased volume of distribution from 0.87 to 1.46 L/kg. The mean absorbance time (MAT) for \( \text{BrO}_3^- \) to reach systemic circulation is 14-18 min.

**Metabolism of \( \text{BrO}_3^- \) in vivo**

Plasma \( \text{BrO}_3^- \) and \( \text{Br}^- \) time-concentration profile following administration of 0.5 or 2.5 mg/kg \( \text{KBrO}_3 \) oral gavage in rats are shown in (Figure 3.3). Plasma \( \text{BrO}_3^- \) concentration was increased significantly after 10 min., and totally cleared within 4 hr. from the body (Figure 3.3A). This was is in correlation with the time- and- concentration dependent increase in plasma \( \text{Br}^- \) concentration (Figure 3.3B). A significant time- and-concentration-dependent increase in plasma \( \text{Br}^- \) (2.02 to 3.59 mg/L) compared to controls was observed following the administration of 2.5 mg/kg \( \text{KBrO}_3 \) (Figure 3.3B).
However, the rate of BrO\textsuperscript{3−} reduction was higher compared to the rate of Br\textsuperscript{−} formation from the BrO\textsuperscript{3−} in the rat blood.

**Metabolism of BrO\textsuperscript{3−} in vitro**

The extent of BrO\textsuperscript{3−} degraded immediately upon addition to fresh rat blood (time zero) was depicted in (Figure 3.4A). The difference of BrO\textsuperscript{3−} measured in water and blood immediately after BrO\textsuperscript{3−} spike is the amount of BrO\textsuperscript{3−} degraded at time zero (< 30 sec samples processing time). This is the amount of BrO\textsuperscript{3−} degraded even after denaturation of proteins by the acetonitrile at time zero. Greater than 90% of BrO\textsuperscript{3−} degradation for the concentrations 0-66 μM BrO\textsuperscript{3−}, and ≤ 25% for the concentrations 320-2840 μM BrO\textsuperscript{3−} was observed at time zero. In addition to initial rapid BrO\textsuperscript{3−} degradation, a slower secondary degradation rate of <10%/min for BrO\textsuperscript{3−} was also observed for all the tested BrO\textsuperscript{3−} concentrations in rat blood (Table 3.3).

Figure 3.4B and C display the time and concentration dependent degradation of BrO\textsuperscript{3−}, and formation of Br\textsuperscript{−} from BrO\textsuperscript{3−} for up to 10 minutes in the rat blood in vitro. The secondary rate of loss ~10%/min appears consistent in samples with BrO\textsuperscript{3−} spiked at concentrations ≥66 μM in rat blood. Similar experiments were conducted with liver and kidney homogenates. These results also indicated that BrO\textsuperscript{3−} degradation occurs very rapidly than the Br\textsuperscript{−} formation from BrO\textsuperscript{3−} (data not shown).

Table 3.3 provides the data on approximate rates of initial and secondary BrO\textsuperscript{3−} degradation in the rat blood. Despite, very high initial loss of BrO\textsuperscript{3−} in rat blood, the secondary loss of BrO\textsuperscript{3−} proceeds at rates more or less proportional to the residual concentration measured after the initial rapid reduction of BrO\textsuperscript{3−}. The recovery of Br\textsuperscript{−}
during the initial loss of BrO$_3^-$ was proportional to the loss of BrO$_3^-$ and the recovery of BrO$_3^-$ as Br$^-$ confirms that the degradation was due to rapid and essentially complete reduction of the added BrO$_3^-$. 

**Excretion of BrO$_3^-$ and Br$^-$ in Urine**

Excretion of both BrO$_3^-$ and Br$^-$ was observed in rat urine after administration of KBrO$_3$ oral gavage in animals. The cumulative % dose recovery of BrO$_3^-$ and Br$^-$ after oral administration of 8.1 mg/kg KBrO$_3$, and Br$^-$ from 5 mg/kg KBr was shown in (Figure 3.5). A very small amount ~6% of the dose was recovered as the parent compound (BrO$_3^-$) in urine for the first 12 hours after KBrO$_3$ dosing and no excretion of BrO$_3^-$ was observed 12 hr. after dosing. Approximately 45% and 6% of administered BrO$_3^-$ was recovered as Br$^-$ in urine and blood respectively, 4 days after dosing. Approximately, 90% of administered Br$^-$ was recovered in urine in first 15 days after dosing in animals. The remaining amount of Br$^-$ was recovered in the blood at the end of the study. Br$^-$ elimination from BrO$_3^-$, 8.1 mg KBrO$_3$/kg, over the first 48 hours was 18% lower than it was eliminated from an equi molar single dose of Br$^-$, 5 mg KBr/kg (15.5 ± 1.6 vs. 18.8 ±1.2 μmol/kg, respectively). The cumulative excretion of Br$^-$ from KBr vs. KBrO$_3$ was equivalent 72 hours after administration. The deficit in recovery of the administered BrO$_3^-$ raises the possibility that some brominated biochemicals might be produced in vivo and slowly gets metabolized and eliminated from the body.
Background Levels of BrO$_3^-$ and Br$^-$ in Female F344 Rat Blood and Urine

The background levels of BrO$_3^-$ in female F344 rat blood ranged between 1 – 8.5 µg/L (Figure 3.6A and B). These blood samples were collected just before the animals dosed with KBrO$_3$ by either IV bolus (Jugular vein) or oral gavage. The background levels of Br$^-$ in female F344 rat blood was ~2000 µg/L. No significant difference in intraday, inter day, and inter individual variation in the background levels of Br$^-$ was observed. No excretion of BrO$_3^-$ was observed in the control rat’s urine. The background level of Br$^-$ excretion in control rat’s urine is 0.65 µM/kg/24 hours (Figure 3.6C).
Table 3.1: Summary of pharmacokinetic parameters for BrO$_3^-$ administered via intravenous (IV) route to female F344 rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>KBrO$_3$ (mg/kg)</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_0$</td>
<td>μg/L</td>
<td></td>
<td>370$^a$ ± 19</td>
<td>1350$^a$ ± 60</td>
<td>2360$^a$ ± 420</td>
<td>8590$^b$ ± 1420</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$</td>
<td>min·μg/L</td>
<td></td>
<td>7400$^a$ ± 1490</td>
<td>22800$^b$ ± 1270</td>
<td>37200$^b$ ± 5400</td>
<td>139400$^c$ ± 11000</td>
</tr>
<tr>
<td>CL</td>
<td>ml/min/kg</td>
<td></td>
<td>15$^a$ ± 2.0</td>
<td>22$^b$ ± 0.0</td>
<td>28$^b$ ± 3.0</td>
<td>18$^a$ ± 1.0</td>
</tr>
<tr>
<td>$V_d$</td>
<td>L/kg</td>
<td></td>
<td>0.55$^a$ ± 0.1</td>
<td>0.64$^a$ ± 0.1</td>
<td>0.92$^a$ ± 0.10</td>
<td>0.57$^a$ ± 0.06</td>
</tr>
<tr>
<td>$K_e$</td>
<td>min$^{-1}$</td>
<td></td>
<td>0.03$^a$ ± 0.0</td>
<td>0.03$^a$ ± 0.0</td>
<td>0.03$^a$ ± 0.00</td>
<td>0.03$^a$ ± 0.00</td>
</tr>
<tr>
<td>Half-life</td>
<td>min</td>
<td></td>
<td>24.5$^a$ ± 6.0</td>
<td>20$^a$ ± 2.0</td>
<td>23$^a$ ± 1.50</td>
<td>22$^a$ ± 1.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM of 4-6 different animals.

Values denoted with different superscripts are significantly different (p<0.05) from each other for the indicated pharmacokinetic parameter.
Table 3.2: Summary of pharmacokinetic parameters for BrO$_3^-$ administered via oral gavage to female F344 rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>0.5</th>
<th>2.5</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$</td>
<td>µg/L</td>
<td>$140^a \pm 15$</td>
<td>$750^a \pm 100$</td>
<td>$4350^b \pm 50$</td>
</tr>
<tr>
<td>AUC$_{\text{0-inf}}$</td>
<td>min·µg/L</td>
<td>$8200^a \pm 1140$</td>
<td>$39640^a \pm 8290$</td>
<td>$380200^b \pm 18670$</td>
</tr>
<tr>
<td>$F$</td>
<td>%</td>
<td>$36^a$</td>
<td>$30^a$</td>
<td>$35^a$</td>
</tr>
<tr>
<td>$\text{CL}$</td>
<td>ml/min/kg</td>
<td>$23^a \pm 3$</td>
<td>$20^a \pm 4$</td>
<td>$22^a \pm 4$</td>
</tr>
<tr>
<td>$K_e$</td>
<td>min$^{-1}$</td>
<td>$0.025^a \pm 0.01$</td>
<td>$0.03^a \pm 0.0$</td>
<td>$0.016^a \pm 0.0$</td>
</tr>
<tr>
<td>Half-life</td>
<td>min</td>
<td>$28^a \pm 8$</td>
<td>$23^a \pm 2.4$</td>
<td>$43^a \pm 8.8$</td>
</tr>
<tr>
<td>$V_d$</td>
<td>L/kg</td>
<td>$0.87^a \pm 0.01$</td>
<td>$1.05^b \pm 0.01$</td>
<td>$1.46^b \pm 0.01$</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>min</td>
<td>$10^a \pm 0$</td>
<td>$8.33^a \pm 1.67$</td>
<td>$20^b \pm 0$</td>
</tr>
</tbody>
</table>

*Data are presented as the mean ± the SEM of 4-6 different animals.*

Values denoted with different superscripts are significantly different (p<0.05) from each other for the indicated pharmacokinetic parameter.
Table 3.3: Initial and secondary loss of BrO$_3^-$ upon addition to the rat blood \textit{in vitro}

<table>
<thead>
<tr>
<th>BrO$_3^-$ µM</th>
<th>Initial loss µM</th>
<th>% loss</th>
<th>Residual µM</th>
<th>2º Loss µM/min</th>
<th>%/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.02</td>
<td>0.02</td>
<td>100</td>
<td>0</td>
<td>0.06</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>3.7</td>
<td>92.5</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>66</td>
<td>60</td>
<td>91</td>
<td>6</td>
<td>23.7</td>
<td>7.4</td>
</tr>
<tr>
<td>320</td>
<td>80</td>
<td>25</td>
<td>240</td>
<td>47.2</td>
<td>6.64</td>
</tr>
<tr>
<td>710</td>
<td>175</td>
<td>25</td>
<td>535</td>
<td>83.1</td>
<td>5.6</td>
</tr>
<tr>
<td>1470</td>
<td>230</td>
<td>16</td>
<td>1240</td>
<td>100.6</td>
<td>3.5</td>
</tr>
<tr>
<td>2840</td>
<td>380</td>
<td>13</td>
<td>2460</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: Time course changes in plasma BrO$_3^-$ concentration following administration of KBrO$_3$ as IV bolus in rats. 0.1, 0.5, 1, or 2.5 mg/kg KBrO$_3$ was administered as an IV bolus in female F344 rats. Blood samples at each time point were collected through carotid artery and analyzed for BrO$_3^-$ using LC-ESI-MS (LOQ 0.5 µg/L). Data are presented as the mean ± SEM of 4-6 different animals.
Figure 3.2: Time course changes in plasma BrO₃⁻ concentration following oral administration of KBrO₃ in rats. 0.5, 2.5 or 20 mg/kg KBrO₃ was administered as an oral gavage in female F344 rats. Blood samples at each time point were collected through carotid artery and analyzed for BrO₃⁻ using LC-ESI-MS (LOQ 0.5 µg/L). Data are presented as the mean ± SEM of 4-6 different animals.
Figure 3.3: Time concentration profile of BrO$_3^-$, and Br$^-$ from BrO$_3^-$, following oral administration of KBrO$_3$ in rats. 0.5 or 2.5 mg/kg KBrO$_3$ was administered as oral gavage in female F344 rats. Blood samples at each time point were collected by closed
cardiac puncture and analyzed for BrO₃⁻ and Br⁻ in the same sample using IC-ICP-MS (LOQ 2.1 µg/L for BrO₃⁻ and Br⁻). This graph shows the time concentration profile of BrO₃⁻ (A), Br⁻ from BrO₃⁻ (B) following oral administration of KBrO₃. Data were presented as the mean ± SEM of 4-6 different animals.
A.

\[ \text{BrO}_3^- \text{ Concentration (M)} \]

B.

\[ \text{BrO}_3^- \text{ Concentration (M)} \]

\[ \text{Time (min)} \]
Figure 3.4: Time-dependent changes in BrO$_3^-$, and Br$^-$ from BrO$_3^-$, concentrations in rat blood *in vitro*. **A.** The difference of BrO$_3^-$ concentrations measured in the water and rat blood at each spiked BrO$_3^-$ concentration is the amount of BrO$_3^-$ degraded in the blood at time zero. **B.** Time-dependent changes in BrO$_3^-$ concentration in rat blood *in vitro*. **C.** Time-dependent changes in Br$^-$ formation from BrO$_3^-$ in rat blood *in vitro*. Data were presented as the mean ± SEM of N=3, with duplicates.
Figure 3.5: The cumulative urinary excretion of BrO$_3^-$ and Br$^-$ after oral administration of equimolar doses of KBrO$_3$ or KBr in rats. Equimolar dose of BrO$_3^-$ (8.1 mg/kg KBrO$_3$) or Br$^-$ (5 mg/kg KBr) was administered as an oral gavage in female F344 rats. Urine samples were collected and analyzed for both BrO$_3^-$ and Br$^-$ in the same sample over the first 96 hours following administration of 8.1 mg/kg KBrO$_3$; and over a 15 day period following administration of 5 mg/kg KBr. All the samples were analyzed using IC-ICP-MS (LOQ 2.1 µg/L for BrO$_3^-$ and Br$^-$). Data were presented as the mean ± SEM of 4-6 different animals.
Figure 3.6: The background levels of BrO$_3^-$ and Br$^-$ in rat blood and urine. The background levels of BrO$_3^-$ were measured in female F344 rats before its administration as an IV bolus (A), or as oral gavage (B); and the amount of Br$^-$ excreted/kg/24 hr. in control female F344 rat urine (C). Blood samples were collected through carotid artery and analyzed by LC-ESI-MS (LOQ 0.5 µg/L). Urine samples were analyzed by IC-ICP-MS (LOQ 2.1 µg/L for BrO$_3^-$ and Br$^-$). Data are presented as the mean ± SEM of 4-6 different animals.
Discussion

The findings of the current study are consistent with earlier results (Fujii et al., 1984). However, the current study represents an advancement over the previous studies (Fujii et al., 1984), by conducting ADME studies at very low doses of BrO$_3^-$.

This makes these results more relevant to human exposure levels and more useful for estimating the risk from BrO$_3^-$ in humans.

Accumulation of alpha-2u-microglobulin in the kidney is a mechanism known to contribute to carcinogenesis specifically in the male rat after exposure to BrO$_3^-$, and other chemicals (IARC, 1999). This mode of action does not occur in other species or in the female rat. Therefore, we have chosen to collect systemic time course data for BrO$_3^-$ in female F344 rats because it appears that the female rat is the most appropriate model for purposes of extrapolating carcinogenic risk to humans.

The initial plasma BrO$_3^-$ concentration ($C_0$) for the IV bolus studies has been estimated by non-compartmental analysis (NCA) in WINNONLIN, V 5.2. The difference between $C_0$ and the concentrations measured at 5 min. after BrO$_3^-$ administration should be the amount of BrO$_3^-$ (approximately 40% of the $C_{max}$), that was either degraded in the blood; or distributed to the tissues. Similar degree of BrO$_3^-$ loss was observed for the doses up to 2.5 mg/kg KBrO$_3$ IV bolus. The disproportionate increases in peak concentration ($C_{max}$ or $C_0$) and the area under curve (AUC) of BrO$_3^-$ in plasma for the doses 0.5 and 1 mg/kg KBrO$_3$ was due to increased plasma BrO$_3^-$ clearance, compared to 0.1 mg/kg KBrO$_3$.

The thiol containing compounds like cysteine, cysteinylglycine, glutathione, homocysteine have total concentrations of about 580 μM in normal humans.
(Himmelfarb, McMonagle, & McMenamin, 2000; Pastore et al., 1998). The reaction of
BrO$_3^-$ with these reductants could reduce about 1.5 mM of BrO$_3^-$, and the plasma
reductants will be completely depleted if the plasma concentration of BrO$_3^-$ exceeds 1.5
mM in the body.

The low bioavailability (~35%) of BrO$_3^-$ after oral gavage reflects high pre
systemic breakdown of BrO$_3^-$ in the stomach and liver (Cotruvo, 2010; Jason D. Keith et
al., 2006; J.D. Keith et al., 2006; Tanaka et al., 1984). Therefore, gastric and hepatic
degradation appear to be a substantive barrier for the low doses of BrO$_3^-$ to enter the
systemic circulation in rats. Br$^-$ is the final metabolic product formed from the result of
BrO$_3^-$ degradation in the body. A rapid increase in plasma Br$^-$ 10 minutes following
administration of 2.5 mg/kg KBrO$_3$ oral gavage confirms that a substantial portion of the
administered BrO$_3^-$ is rapidly reduced to Br$^-$ following oral administration. Breakdown of
BrO$_3^-$ is very rapid and occurs by direct reaction with reductants in the body, particularly
thiols (Murata et al., 2001). Earlier works found that occurrence of BrO$_3^-$ degradation is
significantly less in plasma than in red blood cells (Tanaka et al., 1984). This is most
likely because of the much smaller concentrations of thiols in plasma relative to
intracellular concentrations. (Tanaka et al., 1984), were able to prevent some
degradation by heat treatment, suggesting that at least part of the degradation was
indirectly dependent upon metabolism.

BrO$_3^-$ degradation studies in rat blood $in vitro$ indicated that a large rapid
reduction of BrO$_3^-$ occurred immediately upon contact with blood (time zero). In addition
to this, secondary BrO$_3^-$ degradation continued at a rate of about 10%/minute was
observed in the rat blood. This value was more or less consistent with rates of systemic
elimination *in vivo*. To correct for losses that would occur during sample preparation, an internal spike of $[^{18}\text{O}]$-BrO$_3^-$ was immediately added to the freshly drawn blood. Measurement of the loss of the $[^{18}\text{O}]$-BrO$_3^-$ provided a means of correcting for the losses that occur during preparation of plasma (approximately 30s). Once acetonitrile was added to the plasma, the remaining BrO$_3^-$ was stable for months.

An approximate deficit of 20% was observed following administration of 8.1 mg/kg KBrO$_3$ (recovered as both BrO$_3^-$ and Br$^-$), compared to the recovery of an acute equi molar dose of KBr in the urine. These data suggests the possibility that organic by-products might be formed in conjunction with the degradation of BrO$_3^-$.

Very high inter day and individual variability in background levels of BrO$_3^-$ were observed in animals. This could be due to the involvement of many factors regulating endogenous level of bromate but, the exact reasons for this variability were unknown. Further studies are needed in assessing the factors responsible for the variability in background levels of BrO$_3^-$ in female F344 rats. This allowed us to analyze the BrO$_3^-$ plasma time-concentration data on an individual basis rather than average. But, it became apparent as experiments progressed that BrO$_3^-$ concentrations in plasma of untreated animals were consistently above the limit of quantitation (LOQ) prior to administration of BrO$_3^-$ and this was also true in the terminal phase of sampling.

In conclusion, our data suggested that only a small fraction of the administered BrO$_3^-$ would reach the systemic circulation as oral bioavailability was ~35% in rats. The rate of BrO$_3^-$ reduction was higher compared to the rate of Br$^-$ formation from BrO$_3^-$ both *in vitro* and *in vivo*. BrO$_3^-$ degraded in rapid initial and slow secondary manner in the rat blood *in vitro*. Moreover, the deficit in total recovery of BrO$_3^-$ following its administration
as an oral gavage raised the possibility that some brominated biochemicals might be produced \textit{in vivo}, which will slowly get metabolized and eliminated from the body.
CHAPTER 4

SEX-DEPENDENT DIFFERENCES IN THE MOLECULAR MECHANISMS OF BrO₃⁻-INDUCED NEPHROTOXICITY IN F344 RATS

Abstract

Bromate (BrO$_3^-$), a water disinfection by-product, induces nephrotoxicity in male rats at much lower concentrations than in female rats. The mechanisms controlling sex-dependent differences in BrO$_3^-$-induced nephrotoxicity are not well understood. To advance our understanding of the molecular mechanisms of BrO$_3^-$-induced nephrotoxicity, we performed microarray analysis on kidneys isolated from rats treated for 28-days and quantitated changes in mRNA expression using qPCR. We also assessed spatial changes in the expression of several biomarkers of renal injury using immunohistochemistry. Microarray analysis of male and female rats orally exposed to BrO$_3^-$ at concentrations within the carcinogenic range (0, 125 and 400 mg/L KBrO$_3$) showed sex differences in renal gene expression. The greatest differences were seen in genes encoding for cellular differentiation, followed by those encoding for apoptosis, ion transport and cell proliferation. Sex-dependent differences were especially prominent for the cell cycle checkpoint gene p21, the renal injury protein Kim-1, and the kidney injury marker and cancer biomarker protein osteopontin. Concentration-related nephrotoxicity, as assessed by H&E staining, was greater in males, compared to female rats, as was cellular proliferation, as assessed by bromodeoxyuridine staining. In contrast, no sex-dependent differences were detected in the fraction of proximal renal cells with elevated 8-hydroxydeoxyguanosine (8-OHdG) the proposed genotoxic mediator of BrO$_3^-$-induced renal carcinogenesis. Immunohistochemistry showed concentration-dependent increases in the expression of osteopontin in male rats only, which localized to the proximal tubules. Similarly, BrO$_3^-$ treatment increased clusterin and Kim-1 staining in the proximal tubules; however, staining for these proteins did not differ greatly between
male and female. These data demonstrate that sex-dependent differences exist in the molecular mechanisms of BrO$_3^-$-induced nephrotoxicity in rats and that these differences likely contribute to greater renal carcinogenesis at low concentrations in the males compared to the female rats.
Introduction

Health concerns have been raised about bromate (BrO$_3^-$) as it is a by-product of the disinfection of ground/source water to make water safe for human consumption. Ozone and hypochlorite (oxidants) are the common disinfectants used by water treatment plants to disinfect water by killing or inactivating microorganisms (USEPA, 1999). Ozone can oxidize bromide (Br$^-$) if present, to BrO$_3^-$ (Urs von Gunten & Juerg Hoigne, 1994). BrO$_3^-$ can also be present in hypochlorite when it is formed by the electrolysis of sodium chloride that contains some Br$^-$. The extent of BrO$_3^-$ formation (~0.2 – 25 µg/L drinking water) (WHO, 2005) during ozonation depends on several factors, such as the concentration of Br$^-$, pH, alkalinity, amount of dissolved carbon in the source water, and the level of ozonation (USEPA, 1999).

BrO$_3^-$ is an animal carcinogen and possible human carcinogen (group 2B) (IARC, 1986). The target organs for BrO$_3^-$-induced carcinogenesis are the kidney and thyroid in male and female rats and testicular mesothelium in male rats (Kurokawa, Hayashi, Maekawa, Takahashi, & Kokubo, 1982; Kurokawa et al., 1983; Kurokawa et al., 1990). Within in the kidney the renal proximal tubular region is the primary target site for BrO$_3^-$ (Sai et al., 1994). Rats are more sensitive to the carcinogenic effects of BrO$_3^-$ than mice (by approximately 5-fold), followed by hamsters, which are much less sensitive than rats (by about 1/60th) (Gold, Slone, Manley, Garfinkel, & B.N., 2012).

The mechanisms involved in BrO$_3^-$-induced carcinogenesis and nephrotoxicity are not fully understood (Kurokawa et al., 1985; T. Umemura, Takagi, Sai, Hasegawa, & Kurokawa, 1998). BrO$_3^-$ does induce oxidative DNA damage in the kidney of both male and female rats at high concentrations, which is reflected in the specific formation of 8-
hydrodeoxyguanosine (8-OHdG) in genomic DNA (T. Umemura et al., 2004). The specificity of the oxidative damage to guanine (Ballmaier & Epe, 2006; Chipman et al., 2006), as opposed to other bases, is attributed to catalytic activity involving cellular thiols (Kawanishi & Murata, 2006).

The extent to which differing mechanisms contribute to BrO$_3^-$-induced renal cancer and toxicity in male and female rats has yet to be established. At the only two concentrations in which males and females have been studied together (250 and 500 mg KBrO$_3$/L), the incidence of renal tumors are essentially identical (Kurokawa et al., 1983). On the other hand, BrO$_3^-$ causes renal cell proliferation at concentrations of $\geq$ 30 mg/L (KBrO$_3$) in male, but not in female rats (T. Umemura et al., 2004). These lower concentrations of BrO$_3^-$ are associated with the accumulation of alpha-2u-globulin (a protein produced in very high amounts in male, but not female rats) in the proximal renal tubule cells of male F344 rats (Takashi Umemura, Kimie Sai, Astuya Takagi, Ryuichi Hasegawa, & Yuji Kurokawa, 1995). However, the role of alpha-2u-globulin has been discounted because accumulation of alpha-2u-globulin does not associate with BrO$_3^-$-induced renal tumors in female rats (EPA, 2002).

Recent *in vitro* studies from our laboratory suggest that non-genotoxic mechanisms mediate cell cycle arrest and demonstrate that BrO$_3^-$ treatment activated signaling pathways involving p21, independently of formation of 8-OHdG (Zhang et al., 2010). The main goal of this current study was to determine if similar molecular mechanisms were involved in differences in BrO$_3^-$-induced toxicity in the kidney of male and female rats after *in vivo* treatment. A concentration of 125 mg/L KBrO$_3$ concentration was selected for study as it was the highest concentration that failed to
increase cell replication in the proximal renal tubules of female F344 rats, but a substantial increase in males in previous studies (T. Umemura et al., 2004). This concentration was also carcinogenic in males in two independent studies (DeAngelo et al., 1998; Kurokawa, Aoki, et al., 1986). The 400 mg/L KBrO₃ concentration was selected as it was in the range of concentrations that produced cell proliferation in both males and females (although at a lower rate in females) (T. Umemura et al., 2004) and kidney tumors in both sexes (Kurokawa, Aoki, et al., 1986).

Results

Effect of KBrO₃ treatment on gene expression in the kidney

147 and 506 genes were differentially expressed in male and female rat kidneys respectively after exposure them to 125 mg/L KBrO₃ (14.5 mg/kg/day in males; 16 mg/kg/day in females) in drinking water for 28 days; compared to their controls (Appendix Figure 1A). Exposure of rats to 400 mg/L KBrO₃ (47.4 mg/kg/day in males; 49 mg/kg/day in females) resulted in the differential expression of 530 and 507 genes in male and female rat kidneys respectively; compared to their controls (Appendix Figure 1B). Fifteen differentially expressed genes were in common between males and female rats at 125 mg/L KBrO₃ (Appendix Figure 1A), and eighty genes were commonly expressed in males and female rats exposed to 400 mg/L KBrO₃ (Appendix Figure 1B). The top 25 differentially expressed genes in male and female rat kidney are presented in (Appendix Table 1). Most notable changes in male rats were seen in transcripts associated with kidney injury and inflammation including kidney injury molecule 1 (Havrc1), osteopontin (Spp1), and interleukin 1β (Il1β). In contrast, female
rats showed up regulation of multiple xenobiotic metabolizing enzymes including sulfotransferases (Sult2a1 and Sult2a2) and cytochrome P450s (Cyp2c7 and Cyp2c40). Principal component (PC) analysis of all differentially expressed genes in male and female rats identified a subset of genes whose transcription was altered at the 400 mg/L concentration in both male and female rats and only in the 125 mg/L concentration in male rats (Appendix Figure 2A). The heat map displayed in (Appendix Figure 2B) illustrates the marked difference in gene expression patterns in male and female rats treated with the 125 mg/L concentration of KBrO₃. Here, sex had a reduced influence on differential gene expression in the 400 mg/L concentration, compared to the 125 mg/L concentration. In particular, the number of genes involved in cell differentiation, ion transport, apoptosis, and cell proliferation were very similar at the 400 mg/L concentration. In contrast, at the lower concentration changes in genes involved in cell differentiation, apoptosis, and cell proliferation were only seen in the male kidney.

**Effect of KBrO₃ treatment on mRNA expression in the kidney**

Several changes in gene expression were previously identified in studies of male rats treated with BrO₃⁻, including insulin growth factor binding protein 1 (Igfbp1), activating transcription factor 5 (Atf5), solute carrier family 26, member 4 (Slc26a4, pendrin), asparagine synthase (Asns), cyclin G1 (Ccng1) and clusterin (Apo lipoprotein J) (Ahlborn et al., 2009; Delker et al., 2006). In the present study, we focused on genes whose expression demonstrated a high degree of sex bias after exposure to KBrO₃ and genes that represent biomarkers of renal injury. Based on the initial microarray data, quantitative real-time PCR (qPCR) was performed on three transcripts of particular
interest; cyclin-dependent kinase inhibitor 1A (Cdkn1a, p21), kidney injury molecule 1 (Havcr1) and osteopontin (Spp1).

p21 mRNA transcript levels were significantly up-regulated in both male (3-fold) and female (5-fold) rat kidney after exposure to 400 mg/L KBrO₃, compared to controls (Appendix Figure 3A). Both sexes had similar basal levels of p21 mRNA transcript. No sex-dependent differences were observed after treatment with 125 mg/L KBrO₃. In contrast, females had higher levels of expression compared to males at 400 mg/L KBrO₃.

Kim-1 mRNA levels were also significantly increased approximately 3- and 11-fold in male rats after exposure to 125 and 400 mg/L KBrO₃ respectively (Appendix Figure 3B). No increases were detected in Kim-1 mRNA levels in female rats after exposure to KBrO₃.

The level of osteopontin mRNA was significantly increased in both male (2 to 3-fold) and female (1.5 to 2-fold) rat kidney after exposure to both 125 and 400 mg/L KBrO₃ (Appendix Figure 3C). Males had higher levels of osteopontin mRNA transcript compared to females at both concentrations.

**Effect of KBrO₃ on renal pathology and proliferation**

H&E staining demonstrated that exposure of rats to 400 mg/L KBrO₃ increased tubular damage in male rats, but not in female rats (Figure 4.1C and F). Little evidence of tubular damage was detected in either male or female kidneys exposed to 125 mg/L KBrO₃ (Figure 4.1B and D). Exposure of rats to KBrO₃ also altered the morphology of the thyroid in male and female rats, and appeared to increase cell proliferation in both
sexes (Figure 4.2A to F). Similar results were seen in the testes of male rats, which also showed increases in the thickness of the testicular mesothelium after exposure to KBrO₃ (Figure 4.2G to I).

BrdU staining was measured to verify increases in cell proliferation in the kidney (Figure 4.3A to D). BrdU staining increased in a concentration-dependent manner in male rat kidney, but not in female rat kidney (Figure 4.3E), as compared to controls. BrdU staining was also detected in the thyroid of male and female rats exposed to KBrO₃, but there appeared to be no differences in staining between males and females (Figure 4.4A to F). It should be noted that there were also no differences in serum thyroid hormone (T₃, T₄ or TSH) levels in either male or female rats after exposure to 125 or 400 mg/L KBrO₃ (data not shown). BrdU staining also increased in in the testes (Figure 4.4G to I). In contrast to the kidney, thyroid, and testes, little evidence of proliferation was seen in the liver of rats exposed to KBrO₃ (data not shown). Alterations in hematoxylin and eosin (H&E) staining and increases in BrdU staining in male kidneys correlated with an increase in the organ to body weight ratios (Table 4.1). The organ to body weight ratio was unchanged in the livers of either male or female rats, consistent with the lack of BrO₃⁻ effect on the liver in chronic studies (Kurokawa et al., 1982).

**Sex differences in the genotoxicity of KBrO₃**

8-OHdG staining was measured to study sex-dependent differences in KBrO₃ induced genotoxicity in the kidney (Figure 4.5A to D). Treatment of male and female rats to KBrO₃ increased 8-OHdG staining in the renal proximal tubules (Figure 4.5E). No statistical significant difference in 8-OHdG staining was observed between male and
female rats. 8-OHdG staining was also studied in thyroid and testes to determine if sex difference in these markers were organ specific. Male thyroid showed increases in 8-OHdG staining, which appeared to be higher than that seen in females (Figure 4.6A to F). KBrO₃ also appeared to increase 8-OHdG staining in the testes (Figure 4.6G to I).

**Sex-dependent differences in the expression of osteopontin (OPN) after exposure to KBrO₃**

OPN is a recently discovered biomarker of renal injury reported to be as sensitive as Kim-1 to nephrotoxicants (Rached et al., 2008). The localization of OPN staining in male rat kidneys appeared to be in damaged tubular cells (Figure 4.7A to D). Treatment of rats with KBrO₃ increased OPN staining at concentrations ≥ 125 mg/L in male rat kidneys compared to controls. In contrast, increases in OPN staining were not detected in female rat kidneys compared to controls (Figure 4.7 E). The expression of OPN did not correlate with its mRNA expression in the kidney. OPN staining was not studied in either the thyroid or the testes as its expression has only been reported to occur in either bone or in carcinomas of the kidney or prostate (Briese et al., 2010).

**Sex-dependent differences in the expression of p21 after exposure to KBrO₃**

In agreement with our recent in vitro findings (Zhang et al., 2010), exposure of rats to KBrO₃ resulted in concentration-dependent increases in p21 cytoplasmic staining. Increases were detected in both male and female rat kidneys compared to controls (Figure 4.8A to D). Even though, males and females had similar basal levels, males displayed higher levels of p21 staining after treatment with 125 mg KBrO₃/L
(Figure 4.8E). This is in contrast to the lack of increases in p21 mRNA expression at the same concentration in both sexes (see above). The expression of p21 was also increased in thyroid (Figure 4.9A to F) and testicular tissues (Figure 4.9G to I) of rats exposed to KBrO₃, and there did not appear to be a difference in expression between male and females for the thyroid.

**Sex-dependent differences in the expression of clusterin after exposure to KBrO₃**

Clusterin expression was also studied as the microarray results, and prior studies (Geter et al., 2006), indicated that KBrO₃ exposure increases clusterin mRNA. Clusterin is another possible biomarker of kidney injury. In agreement with prior studies, exposure of rats to KBrO₃ increased clusterin expression in a concentration-dependent manner in both males and females (Figure 4.10A to D). There was no significant difference in clusterin expression between males and female in the kidney (Figure 4.10E). Exposure of rats to KBrO₃ also increased clusterin expression in the thyroid (Figure 4.11A to F) and testes (Figure 4.11G to I), but no increases were observed in the thyroid of female rats.

**Sex-dependent differences in the expression of Kim-1 after exposure to KBrO₃**

Treatment of rats with KBrO₃ induced concentration-dependent increases in Kim-1 expression in both male and female rats (Figure 4.12A to D); however, the level of Kim-1 staining in female kidneys was higher compared to males at 400 mg/L (Figure 4.12E). This finding is in contrast to the increases in mRNA expression in male rats, but not in female rats. This difference may arise from the fact the analysis of mRNA was
performed on total cortical tissue, which contains several different types of cells. In contrast, immunohistochemistry for Kim-1, as well as p21 and osteopontin, was assessed on a cellular basis. Kim-1 expression was not studied in either the thyroid or the testes as this protein is typically associated only with nephrotoxicity.
<table>
<thead>
<tr>
<th>KBrO₃ (mg/L)</th>
<th>0</th>
<th>125</th>
<th>400</th>
</tr>
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<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
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<td>3.94 ± 0.09</td>
<td>3.75 ± 0.15</td>
</tr>
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<tr>
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<td>1.09* ± 0.05</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
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<td>3.60 ± 0.06</td>
<td>3.48 ± 0.07</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.40 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
</tbody>
</table>

*Data are presented as the mean ± the SEM of 4-6 different animals

*Statistically significant from control, p < 0.05 based on paired t-test
**Figure 4.1:** Sex-dependent alterations in renal pathology after KBrO$_3$ exposure. Male (A-C) and female (D-F) F344 rats were exposed to 0 (A and D), 125 (B and E) or 400 (C and F) mg/L KBrO$_3$ in drinking water for 28 days prior to pathological examination using hematoxylin and eosin (H&E) staining. Black arrows in C indicate damage to the tubular epithelium in male rat kidneys. The scale is shown in the top right corner. The slides are representative of 4-6 separate animals.
**Figure 4.2:** Effect of KBrO$_3$ on rat thyroid and testicular pathology. F344 rats were exposed to 0 (A, D and G), 125 (B, E, H) or 400 (C, F and I) mg/L KBrO$_3$ in drinking water for 28 days prior to isolation of male (A-C) and female (D-F) thyroid or testicular (G-I) tissue for pathological examination using hematoxylin and eosin (H&E) staining. The black arrows in figures G and I point the mesothelioma in testes. The slides are representative of 4-6 separate animals and the scale is shown in the top right corner.
Figure 4.3: Sex-dependent alterations in BrdU staining in rat kidney after KBrO₃ exposure. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 26 days and injected with BrdU on days 27 and 28 prior to isolation of kidneys and analysis of cell proliferation using immunohistochemistry. Data are representative of 4-6 separate animals. Black arrows in B and D denote positive staining in nuclei. The scale is shown in the top right corner. Data in E denotes the quantification of BrdU staining based on the number of positive nuclei per field and is presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while # indicates a
statistically significant difference (P < 0.05) between males and females at the indicated concentration of KBrO₃.
Figure 4.4: Effect of KBrO₃ on BrdU staining in rat thyroid and testicular tissues. F344 rats were exposed to 0 (A, D and G), 125 (B, E and H) or 400 (C, F and I) mg/L KBrO₃ in drinking water for 28 days prior to isolation of male (A-C) and female (D-F) thyroid or testicular (G-I) tissue for determination of cellular proliferation using BrdU staining. Black arrows represent the nuclear staining of BrdU. The slides are representative of 4-6 separate animals and the scale is shown in the top right corner.
**Figure 4.5:** 8-OHdG staining in rat kidney after KBrO₃ exposure. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis for DNA damage using immunohistochemistry. Black arrows in B and D denote positive staining in nuclei. The scale is shown in the top right corner. Data in E denotes the quantification of 8-OHdG staining based on the number of positive nuclei per field and is presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Figure 4.6: Effect of KBrO₃ on 8-OHdG staining in rat thyroid and testicular tissues. F344 rats were exposed to 0 (A, D and G), 125 (B, E, H) or 400 (C, F and I) mg/L KBrO₃ in drinking water for 28 days prior to isolation of male (A-C) and female (D-F) thyroid or testicular (G-I) tissue for determination of DNA damage using 8-OHdG staining. Black arrows represent the nuclear staining of 8-OHdG. The slides are representative of 4-6 separate animals and the scale is shown in the top right corner.
**Figure 4.7:** Sex-dependent alterations in osteopontin staining in rat kidney after KBrO₃ exposure. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis of osteopontin expression using immunohistochemistry. Black arrows in B denote positive staining of brush border in the proximal tubules. Data in E denotes the quantification of osteopontin expression based on cytoplasmic staining and is presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as
compared to control, while # indicates a statistically significant difference (P < 0.05) between males and females at the indicated concentration of KBrO$_3$. 
**Figure 4.8:** Sex-dependent alterations in p21 staining in rat kidney after KBrO$_3$ exposure. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO$_3$ in drinking water for 28 days prior to isolation of kidneys and analysis of p21 expression using immunohistochemistry. White arrows in B and D denote cytosolic staining. The scale is shown in the top right corner. Data in E denotes the quantification of p21 expression based on cytoplasmic staining and is presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while # indicates a statistically significant difference (P < 0.05) between males and females at the indicated concentration of KBrO$_3$. 

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Figure 4.9: Effect of KBrO₃ exposure on p21 staining in rat thyroid and testicular tissues. F344 rats were exposed to 0 (A, D and G), 125 (B, E, H) or 400 (C, F and I) mg/L KBrO₃ in drinking water for 28 days prior to isolation of male (A-C) and female (D-F) thyroid or testicular (G-I) tissue for determination of p21 staining using immunohistochemistry. Black arrows represent the nuclear staining of p21, while white arrows denote cytosolic staining. The slides are representative of 4-6 separate animals and the scale is shown in the top right corner.
Figure 4.10: Sex-dependent alterations in clusterin staining in rat kidney after KBrO₃ exposure. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO₃ for 28 days prior to isolation of kidneys and analysis of clusterin expression using immunohistochemistry. White arrows in B and D denote cytosolic staining. The scale is shown in the top right corner. Data in E denotes quantification of clusterin expression based on cytoplasmic staining and is presented as the average ± the SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Figure 4.11: Effect of KBrO₃ exposure on clusterin staining in rat thyroid and testicular tissues. F344 rats were exposed to 0 (A, D and G), 125 (B, E, H) or 400 (C, F and I) mg/L KBrO₃ in drinking water for 28 days prior to isolation of male (A-C) and female (D-F) thyroid or testicular (G-I) tissue for determination of clusterin staining using immunohistochemistry. White arrows denote cytosolic staining in the tissue. The slides are representative of 4-6 separate animals and the scale is shown in the top right corner.
Figure 4.12: Sex-dependent alterations in Kim-1 staining in rat kidney after KBrO$_3$ exposure. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO$_3$ in drinking water for 28 days prior to isolation of kidneys and analysis of Kim-1 expression using immunohistochemistry. White arrows in B and D denote cytosolic staining. The scale is shown in the top right corner. Data in E denotes quantification of Kim-1 expression based on cytoplasmic staining and are presented as the average ± the SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while # indicates a statistically significant difference (P < 0.05) between males and females at the indicated concentration of KBrO$_3$. 
Discussion

BrO$_3^-$ is an oxidant found in waters that have been chlorinated or ozonated for the purpose of making them safe for consumption. (S. D. Richardson, M. J. Plewa, E. D. Wagner, R. Schoeny, & D. M. Demarini, 2007). As a side effect, this type of disinfection can oxidize organic and inert elements into a variety of organic by-products. BrO$_3^-$ is one of these by-products and is a possible human carcinogen and proven rodent carcinogen. One proposed mechanism of action for BrO$_3^-$ has been the specific formation of 8-OHdG in DNA, at least in the kidney (Kawanishi & Murata, 2006). Total tumor production (kidney, thyroid, and mesothelium) in the male rat has been used by EPA to set the maximum contaminant limit (MCL) at 0.1 µg/L for BrO$_3^-$ (CEPA, 2009).

Recent in vitro studies from our laboratories suggest that BrO$_3^-$ mechanism of action is not solely mediated by 8-OHdG. In fact, data suggest that treatment of renal cells in vitro induces damage via both DNA-dependent and DNA-independent mechanisms (Zhang et al., 2010). The DNA-dependent mechanism were characterized by the formation of 8-OHdG at high concentrations, while the DNA-independent mechanisms appeared to be mediated by oxidative injury, characterized by activation of numerous signaling proteins, including p21, which occurred at lower concentrations.

Previous studies demonstrate that KBrO$_3$ exposure alters gene expression in the kidney (Geter et al., 2006); however, these studies did not further investigate mechanisms of action that explain sex-dependent differences in toxicity. In particular, detailed studies have been limited to male rats. To focus our studies, we performed
microarray analysis of RNA isolated from male and female rat kidneys exposed to 125 and 400 mg/L KBrO$_3$ in drinking water for 28 days.

The sex-dependent increases in gene transcripts that mediate cell proliferation in males suggest that male rats are likely to be more sensitive to BrO$_3^-$-induced cancer than females. This difference is not apparent in previous cancer bioassays because of the very limited data in female rats. As previously shown by (T. Umemura et al., 2004), we confirmed that elevated rates of cell replication were higher in male than female rats at all concentrations tested. The increased renal cell proliferation with accumulation of alpha-2u-microglobulin in males treated with BrO$_3^-$ concentrations, and the increased cell replication at concentrations as low as 30 mg/L KBrO$_3$ may be a key mediator in the formation of renal carcinogenesis at lower concentrations in males (T. Umemura et al., 2004). Some studies have associated BrO$_3^-$-induced renal carcinogenicity with increases in 8-OHdG in nuclear DNA at concentrations as low as 250 mg KBrO$_3$/L in both male and female rats (T. Umemura & Kurokawa, 2006). However, studies (Arai, Kelly, Minowa, Noda, & Nishimura, 2002) show that knocking out 8-OHdG glycosidase activity in mice did not increase renal neoplasia relative to wild type liter mates, despite a large increase in 8-OHdG adducts. Results from the current study show no difference in 8-OHdG adducts between male and females and that increases in 8-OHdG staining did not correlate to differences in proliferation. In contrast, increases in others markers of kidney injury (Kim-1, osteopontin, clusterin, etc.) occurred at lower concentrations in male than female rats and were clearly associated with increased proliferation.

One major difference between male and female rats was in the expression of osteopontin (OPN) in kidneys. OPN is a glycoprotein, commonly associated with bone
remodeling. It is reported to be up regulated in tubular segments after kidney injury (Camacho L, 2011; Xie Y, 2001), and over express in papillary renal cell tumors (Matusan, Dordevic, Stipic, Mozetic, & Lucin, 2006). The increase in OPN expression in males, compared to females, may decrease cellular apoptosis and increase cell regeneration (Xie et al., 2001). This could contribute to the increase in cell proliferation seen in male rats. The inability of BrO$_3^-$ to increase OPN in females, even at concentrations as high as 400 mg/L, despite similar increases in 8-OHdG, raises questions about the hypothesis that 8-OHdG is the main driver of carcinogenicity in the male rat kidney, as 8-OHdG staining was similar in both males and females at both concentrations.

p21 is known to have roles in oxidative stress and to be activated after DNA damage (Abbas & Dutta, 2009). We recently demonstrated that p21 expression is increased in renal cell lines after KBrO$_3$ exposure, which correlated to alterations in cell cycle. Increases in cytosolic p21 expression are reported to be pro–proliferative and anti-apoptotic (Rossig et al., 2001). The exact role of p21 in the mechanisms of action of BrO$_3^-$ remains a topic of future studies. It is possible that p21 is acting synergistically with clusterin to promote renal tumor formation. This hypothesis is supported by studies showing that clusterin mediates cell protection, lipid recycling, cell aggregation and cell attachment by acting as an anti-apoptotic protein during kidney injury (F. Dieterle et al., 2010). Studies of human colon cancer suggest a conversion from the nuclear form of clusterin to the cytoplasmic form may promote tumor progression (Pucci S, 2004).

The increases in Kim-1 expression after BrO$_3^-$ exposure agrees with studies using other nephro-toxicants in rodents and humans (Camacho L, 2011). However, to
our knowledge, this is the first time that BrO$_3^-$ exposure has been shown to increase Kim-1 expression. The higher levels of Kim-1 protein expression in females at 400 mg/L, relative to males, are in sharp contrast with the microarray and qPCR data. This is actually not that surprising as Kim-1 can be shed from the kidney as a consequence of the nephrotoxicity and be present in the urine (Vaidya, Ramirez, Ichimura, Bobadilla, & Bonventre, 2006). These data suggest that there is a component of cytotoxicity and reparative hyperplasia in the response of females to BrO$_3^-$-treatment (at higher concentrations) as well.

In conclusion, we demonstrated that sex-dependent differences in the nephrotoxicity of BrO$_3^-$ are accompanied by differential expression of several genes and proteins including osteopontin and p21. We also demonstrated that BrO$_3^-$ induces the expression of osteopontin and p21 in sex- and concentration-dependent manner. Finally, these data, combined with prior studies of other investigators, support the hypothesis that both genotoxic and non-genotoxic mechanisms are operable with BrO$_3^-$ treatment and confirm that the difference in non-genotoxic mechanisms may predominate in determining sex based differences in BrO$_3^-$-induced nephrotoxicity.
CHAPTER 5

BrO$_3^-$ INCREASES 3-BROMOTYROSINE EXPRESSION IN MALE RAT KIDNEYS

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Abstract

Bromate (BrO₃⁻), a toxic disinfection by-product formed during ozonation of source water, induces renal tumors at lower concentrations in male rats than in female rats. Our previous studies showed that differences in non-genotoxic mechanisms may mediate sex-based differences in BrO₃⁻-induced nephrotoxicity. However, the exact non-genotoxic mechanisms are not well defined. To address this data gap, male and female F344 rats were treated with 0, 15, 60, or 400 mg/L potassium bromate (KBrO₃) in drinking water for 28 days. The kidney tissue was isolated and analyzed for cell proliferation and apoptosis using immunohistochemistry. BrO₃⁻ treatment induced concentration-dependent increase in the expression of cytosolic 3-bromotyrosine in male rat kidneys, but not in females, at concentrations as low as 15 mg/L KBrO₃. This correlated to the excretion of total organic bromine (TOBr) in the urine. TUNEL staining, a marker for an apoptosis was higher in female rat kidneys, compared to males, at lower concentrations of KBrO₃, but increased in male rat kidneys at higher concentrations. In contrast, DNA damage, identified by 8-OHdG-staining, was similar in both male and female rat kidneys. The sex-dependent expression of the renal injury marker and cancer protein biomarker osteopontin was observed at concentrations ≥ 60 mg/L KBrO₃. Expression of the cell cycle checkpoint p21 was greater in females than males at 15 mg/L KBrO₃, but these differences were lost as the concentration increased. Increases in 3-bromotyrosine staining and urinary TOBr indicate the formation of reactive intermediates capable of brominating proteins. The sex-dependent expression of 3-bromotyrosine correlated with the non-genotoxic mechanisms of BrO₃⁻. Thus, 3-bromotyrosine may be a novel biomarker for reactive intermediates resulting...
from the reduction of BrO$_3^-$ in vivo, and may mediate the sex-dependent differences of BrO$_3^-$-induced nephrotoxicity.

**Introduction**

One of the proposed mechanisms for the increased susceptibility of male rats to BrO$_3^-$-induced renal cell proliferation than females is the accumulation of alpha-2$_u$-globulin in the proximal tubules (T. Umemura et al., 2004; Takashi Umemura, Sai, Takagi, Hasegawa, & Kurokawa, 1993). Significant increases in alpha-2$_u$-globulin accumulation in the kidney were seen after treatment of male rats with concentrations $\geq$ 125 mg/L KBrO$_3$ in drinking water after 28 days (T. Umemura et al., 2004). In contrast, BrO$_3^-$-induced the increases in renal cell proliferation in male rat kidneys after treatment with concentrations $\geq$ 30 mg/L KBrO$_3$ in drinking water after 28 days (T. Umemura et al., 2004). Others have suggested that alpha-2$_u$. globulin does not mediate the development of renal carcinomas after BrO$_3^-$ treatment because renal tumors are produced in female rats, which do not express this protein (Takashi Umemura et al., 1993). This raises questions on the involvement and the role played by alpha-2$_u$-globulin in the susceptibility of male rat kidneys to BrO$_3^-$-induced tumors. The US EPA (USEPA, 2001) concluded that although alpha-2$_u$-globulin does not mediate renal cell transformation; it may mediate the enhanced susceptibility of males to BrO$_3^-$-induced renal carcinogenesis.

Alpha-2u globulin is a well-characterized low molecular protein, synthesized mainly in the liver (Klaassen, 2007). Once synthesized alpha-2u globulin secretes into plasma, and then is transported to the kidneys through renal arteries. It is freely filtered
through the glomerulus (Roy, 1966), and is partially taken up by the proximal tubules, with the remaining protein excreted into the urine (W. Neuhaus, and Lerseth, D. S., 1979; W. Neuhaus, Flory, W., Biswas, N., and Hollerman, C.E., 1981).

The accumulation of alpha-2u-globulin can occur by forming bromotyrosines through interaction of its tyrosine residues with chemicals like hypobromous acid (HOBr), hydrogen peroxide (H$_2$O$_2$) or bromide (Br$^-$) in the kidney proximal tubules (Kloss, 1985). Accumulated alpha-2u-globulin protein in the proximal tubules of the kidney can induce both cell proliferation and damage, which eventually leads to the formation of renal tumors (Swenberg, 1993). Alpha-2u-globulin induced nephropathy is characterized by the accumulation of protein droplets in the proximal tubule, resulting in single cell necrosis, the formation of granular casts at the junction of the proximal tubule and the thin loop of Henley, and the presence of regenerative tubules in the kidney (Swenberg, 1993).

**Bromotyrosine Formation**

Bromotyrosines can form by several possible mechanisms. Eosinophils secrete peroxidase (EPO) during oxidative tissue injury induced by inflammation, cancer or and asthma (Wu, 1999). EPO amplifies the oxidative damage potential of H$_2$O$_2$ by utilizing Br$^-$ as a co-substrate to form bromotyrosines with proteins present in the body. In particular, HOBr, formed from H$_2$O$_2$ and Br$^-$, or generated by the inflammatory cells, is very active in brominating various endogenous biochemicals, and tyrosine residues (Senthilmohan, 2006; Wu, 1999). HOBr can also form bromohydrins by reacting with phospholipids present in the body (Panasenko, Vakhrusheva, Tretyakov, Spalteholz, &
Arnhold, 2007). These brominating products may be useful tools to identify the site of oxidative tissue injury in the body (Wu, 1999).

Alpha-2u-globulin contains seven tyrosine residues which are amenable to bromination (Unterman, 1981). Bromination of these tyrosines will prevent the degradation of α-2u-globulin, which leads to accumulation in the proximal tubules, and could contribute to the carcinogenic effects of BrO₃⁻ in animals (Short, 1989). Among agents that produce accumulation of alpha-2u-globulin in the male rat kidney, BrO₃⁻ is unique. The difference may lie with the types of products that might arise in the reduction of BrO₃⁻.

Our previous studies tested the hypothesis that both genotoxic and non-genotoxic mechanisms are operable with BrO₃⁻ treatment, and suggested that both mechanisms may mediate BrO₃⁻-induced nephrotoxicity (chapter 4). However, the exact non-genotoxic mechanisms are not well defined. To address this data gap, male and female F344 rats were treated with 0, 15, 60, or 400 mg/L KBrO₃ in drinking water for 28 days. A concentration range of 15-400 mg/L KBrO₃ was selected to see if bromotyrosine formation induced after KBrO₃ exposure, and if its expression correlated to other markers of nephrotoxicity in male and female rats.

Results

Sex-dependent differences in the excretion of BrO₃⁻, Br⁻ and Total organic bromine (TOBr)

The excretion of BrO₃⁻, Br⁻ and Total organic bromine (TOBr) in the urine of male and female F344 rats after treatment with 0, 15, 60 or 400 mg/L KBrO₃ in drinking water
for 27 days was normalized to the daily consumed dose of BrO₃⁻/kg body weight (Table 5.1). The amount of BrO₃⁻ excreted was ~10% of the BrO₃⁻ intake in both males and females. Most of the administered BrO₃⁻ was reduced to Br⁻, as the amount of Br⁻ eliminated was 5-8 times greater than BrO₃⁻.

TOBr was increased in the urine of both male and female rats following BrO₃⁻-treatment for 27 days (Table 5.1). It is notable that there is a significant background of TOBr in control animals reflecting a background of halogenation. There was a significant increase in the levels of TOBr in the urine of male and female F344 rats after exposure to concentrations as low as 60 mg KBrO₃/L drinking water. A further increase was seen as the concentrations were increased to 400 mg KBrO₃/L, but with a reduced slope. TOBr in urine represents a small fraction of the bromine excreted in urine (1% of the increment in Br⁻ concentrations in males and 0.7% in females). Given that there is a 20% deficit in total bromine excreted, it is probable that a significant retention of brominated biochemicals could occur in the body of the rat. The total bromine recovery increased in a concentration-dependent manner, and approximated 75–90% of the administered concentration in male rats. In contrast, total bromine recovery was more variable in females, decreasing from 88% at 60 mg/L to 61% at 400 mg/L. This deficit in recovery of total bromine suggested that some bromine may be distributed and retained in the tissues.
Sex-dependent differences in the expression of 3-bromotyrosine after KBrO₃ treatment

Treatment of male rats with KBrO₃ increased the staining of bromotyrosine in the kidney after 28 days (Figure 5.1A-D). Lower levels of 3-bromotyrosine staining were detected in renal proximal tubules in control male rats than the females. A concentration-dependent increase in 3-bromotyrosine staining was detected in the renal proximal tubules of male rats beginning at the 15 mg KBrO₃/L treatment (Figure 5.1I). No increases in 3-bromotyrosine staining were observed after treatment of female rats with BrO₃⁻ at any concentration (Figure 5.1E-H). 3-Bromotyrosine staining was not observed in the testicular mesothelium, but increased staining was observed in Sertoli cells and spermatocytes after treatment with 15 and 60 mg/L KBrO₃ compared to controls (Appendix Figure 4 A-D). No changes in staining for 3-bromotyrosine were observed in the thyroid gland of male and female rats after BrO₃⁻ treatment (Appendix Figure 5).

Sex-dependent differences in the BrO₃⁻ induced nephrotoxicity

TUNEL and 8-OHdG staining was used to assess sex-dependent differences in BrO₃⁻-induced nephrotoxicity and genotoxicity, respectively (Figures 5.2 and 5.3). Treatment of both male and female rats with KBrO₃ induced concentration-dependent increase in TUNEL staining (Figure 5.3A-H). TUNEL staining was higher in female rat kidneys, compared to males, after treatment with 15 mg/L KBrO₃, but this difference was not seen after treatment with 60 mg/L (Figure 5.3I). In contrast, treatment of rats
with 400 mg/L KBrO$_3$ increased TUNEL staining in male rats, but resulted in decreased staining in female rats compared to 60 mg/L (Figure 5.2I).

As previously reported, treatment of rats with KBrO$_3$ resulted in concentration-dependent increases in 8-OHdG in both male and female rat kidneys (Figure 5.3A-H). Similar levels of staining were observed in both male and female rat kidneys (Figure 5.3I). Significant increase in 8-OHdG staining, compared to control were detected at concentrations $\geq$ 60 mg/L KBrO$_3$ in male rat kidneys. These data suggest that there is not a significant sex difference in oxidative DNA damage induced by BrO$_3$ in the kidney, which agrees with previous published studies (T. Umemura et al., 2004).

**Sex-dependent differences in the expression of osteopontin (OPN) after KBrO$_3$ treatment**

Treatment of male and female F344 rats with BrO$_3$ resulted in significant concentration-dependent increase in osteopontin staining at concentrations as low as 60 mg/L KBrO$_3$ in male rat kidneys (Figure 5.4I). In contrast, osteopontin staining was not increased in female kidneys at any concentration of KBrO$_3$ tested (Figure 5.4I). The localization of osteopontin staining in male rat kidneys appeared to be in damaged tubular cells (Figure 5.4D).

**Sex-dependent differences in the expression of p21 after KBrO$_3$ treatment**

Treatment of rats with KBrO$_3$ resulted in concentration-dependent increases in p21 staining in both male and female F344 rat kidneys (Figure 5.5A-H). Increase in the frequency of p21 staining, compared to controls, were seen at concentrations as low
as 15 mg/L, whereas no increases were detected in male rats at this same concentration (Figure 5.5I). In contrast, the frequency of p21 staining increased to similar levels in male and female rats treated with 60 and 400 mg/L KBrO₃ (Figure 5.5I).

**Sex-dependent differences in the expression of Kim-1 after KBrO₃ treatment**

Treatment of male and female F344 rats with KBrO₃ resulted in concentration-dependent increase in Kim-1 staining in both male and female rat kidneys (Figure 5.6A-H). Kim-1 expression was increased at concentrations as low as 15 mg/L, compared to controls, and was similar in males and females at 15 and 60 mg/L, but slightly higher in females after treatment with 400 mg/L (Figure 5.6I).

**Sex-dependent differences in the expression of clusterin after KBrO₃ treatment**

Treatment of rats with KBrO₃ resulted in concentration-dependent increases in the frequency of clusterin staining in both male and female rat kidneys (Figure 5.7A-H). Increases in clustering staining, above control levels, were seen at concentrations as low as 15 mg/L. There did not appear to be a significant difference in clusterin staining between males or females at any concentration tested (Figure 5.7I).
Table 5.1: Recovery of BrO$_3^-$, Br$^-$, and ToBr in male and female rats' urine after exposed to KBrO$_3$ for 27 days

A.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Intake (µmol)</th>
<th>Recovery in Male Rat Urine (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBrO$_3$ (BrO$_3^-$)</td>
<td>BrO$_3^-$</td>
<td>BrO$_3^-$</td>
</tr>
<tr>
<td><strong>Con</strong></td>
<td>0</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>15 (11.5)</td>
<td>2.67 ± 0.04</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>60 (46)</td>
<td>10.6 ± 0.7</td>
<td>0.70 ± 0.15</td>
</tr>
<tr>
<td>400(308)</td>
<td>61.4 ± 7.5</td>
<td>6.5 ± 1.74*</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Intake (µmol)</th>
<th>Recovery in Female Rat Urine (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBrO$_3$ (BrO$_3^-$)</td>
<td>BrO$_3^-$</td>
<td>BrO$_3^-$</td>
</tr>
<tr>
<td><strong>Con</strong></td>
<td>0</td>
<td>0.00 ± 0.0</td>
</tr>
<tr>
<td>15 (11.5)</td>
<td>1.77 ± 0.09</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>60 (46)</td>
<td>8.02 ± 0.77</td>
<td>0.72 ± 0.09</td>
</tr>
<tr>
<td>400(308)</td>
<td>42.2 ± 5.4</td>
<td>4.79 ± 0.91*</td>
</tr>
</tbody>
</table>

*Statistically significant from control, p < 0.05 based on one way ANOVA followed by tukey’s test.

All the intake and recovery values are presented as mean ± SEM of 6 animals.
Figure 5.1: Effect of KBrO₃ treatment on 3-bromotyrosine staining in rat kidney. Male (A to D) and female (E to H) F344 rats treated with 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and the analysis of staining using immunohistochemistry. Arrows in C show the cytoplasmic staining in the proximal tubules of the kidney. Panel 5.1I shows the quantification of 3-bromotyrosine staining.
was based on cytosolic staining. The data are presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Figure 5.2: Effect of KBrO₃ treatment on TUNEL staining in rat kidney. Male (A-D) and female (E-H) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry.
Black arrows in D and H indicate the positive staining in nuclei. Panel 5.2I shows the quantitation of staining based on the number of positive nuclei per field. Data are presented as the mean ± SEM of the mean of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while *# indicates a statistically significant difference between male and females.
Figure 5.3: Effect of KBrO₃ treatment on 8-OHdG staining in rat kidney. Male (A and B) and female (C and D) F344 rats were exposed to 0 to 400 mg/L KBrO₃ for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry. Panel 5.3I represents the quantitation of staining based on the number of positive nuclei per field. Black arrows indicate positive stained nuclei in D and H. Data are presented as
the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Figure 5.4: Effect of KBrO₃ treatment on osteopontin staining in rat kidney. Male (A to D) and female (E to H) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry. Panel 5.4I shows the quantitation of osteopontin staining. Black arrows in D demonstrate the localization of osteopontin staining to the damaged proximal tubular epithelium. Data are presented as the mean ± SEM of 4-6 different
animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while *# indicates a statistically significant difference between male and females.
Figure 5.5: Effect of KBrO₃ treatment on p21 staining in rat kidney. Male (A to D) and female (E to H) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry. Panel 5.5I represents the quantitation of cytosolic p21 staining. Arrows in D and H point the cytosolic p21 staining in the proximal tubules of the kidney. Data are presented as
the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while *# indicates a statistically significant difference between male and females.
Figure 5.6: Effect of KBrO₃ treatment on Kim-1 staining in rat kidney. Male (A to D) and female (E to H) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry. Panel 5.6I shows the quantitation of KIM-1 staining. Arrows in D and H indicate Kim-1
staining in the proximal tubules of the kidney. Data are presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control, while *# indicates a statistically significant difference between male and females.
**Figure 5.7:** Effect of KBrO₃ treatment on clusterin staining in rat kidney. Male (A to D) and female (E to H) F344 rats were exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry. Panel 5.7I shows the quantitation of clusterin staining. Arrows in
D and H indicate clusterin staining in the proximal tubules of the kidney. Data are presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Discussion

Sex-dependent difference in the formation of renal tumors and biomarkers of nephrotoxicity has been reported in rats after treatment with BrO$_3^-$ (DeAngelo et al., 1998; Kurokawa, Takayama, et al., 1986). Our previous studies tested the hypothesis that both genotoxic and non-genotoxic mechanisms mediate nephrotoxicity after BrO$_3^-$ treatment (chapter 4). However, the exact non-genotoxic mechanisms involved are not well defined. To address this data gap, male and female F344 rats were treated with 0, 15, 60, or 400 mg/L potassium bromate (KBrO$_3$) in drinking water for 28 days. This concentration range was selected as the lower concentration does not induce tumor formation in either male or female rats, but does induce cellular proliferation in male rats (T. Umemura et al., 2004). In addition, the concentration of 400 mg/L induced renal tumors in both male and female rats.

Few studies have directly linked difference in nephrotoxicity to differences in the level of BrO$_3^-$ and its metabolites. In this study, sex-dependent differences were not observed in BrO$_3^-$ excretion in the urine of male and female F344 rats after treatment with 0-400 mg/L KBrO$_3$ in drinking water for 27 days. This indicates the extent of BrO$_3^-$ reduction in the body was similar in both sexes. However, the amount of Br$^-$ excreted in urine was significantly lower in female rats compared to males, raising the possibility that sex-dependent differences existed in the formation of reactive intermediates from BrO$_3^-$. Furthermore, the decreasing ratio of the increment of Br$^-$ relative to the BrO$_3^-$ degradation in both sexes indicates that a lower fraction of BrO$_3^-$ is systemically reduced to Br$^-$ at higher concentrations of BrO$_3^-$. This could be due to either BrO$_3^-$ being
distributed and accumulated in the tissues, or formation of brominated products which will slowly get metabolized and excreted from the body (Bull et al.).

Even though BrO$_3^-$, ToBr and Br$^-$ urine excretion followed similar patterns in male and female rats, sex differences were observed in ToBr excretion, with higher levels in males compared to females at concentrations $\geq$ 60 mg/L KBrO$_3$. ToBr is a measure of all brominated species including altered macromolecules, such as proteins and lipids. Increased levels of ToBr correlated with BrO$_3^-$ induced renal apoptosis in male rats, suggesting that ToBr may be a better indicator of BrO$_3^-$ exposure and toxicity than BrO$_3^-$ or Br$^-$ themselves. The increase in TOBr excretion in rat urine with higher BrO$_3^-$ concentrations suggests that a halogenation intermediate is formed during the reduction of BrO$_3^-$ . These results are consistent with our previous studies (Bull et al.) and imply that a pool of Br$^-$ might be formed from BrO$_3^-$ in the body that is not reflected by either BrO$_3^-$ or Br$^-$ excretion.

The overall level of TOBr represented a small fraction of the Br$^-$ excreted in urine (1% and 0.7% increment in Br$^-$ concentrations in males and females, respectively). Given that there is a 20% deficit in total Br$^-$ excreted, it is probable that a significant retention of brominated biochemicals could occur in the rat. This hypothesis is supported by the formation of organic bromine-containing by-products as confirmed by measurement of increases in TOBr in urine.

BrO$_3^-$ is capable of selectively interacting with the abundant sulfhydryl groups present in mammalian systems to form intermediate reduction products such as BrO$_2$, BrO, and Br$^-$ (Murata et al., 2001). The most probable organic by-products are bromamines formed with free amino groups (Hawkins & Davies, 2005), but some more
stable brominated compounds (Carr, Decker, Park, & Frei, 2001) are likely to be formed and have sufficient stability to account for the increases in TOBr. Hypobromous acid (HOBr), the intermediate formed from BrO$_3^-$, can react with unsaturated lipids to form bromohydrins (Carr et al., 2001), and with proteins to form bromotyroisines (Pattison & Davies, 2004).

Based on the documented formation of brominated tyrosines, we sought evidence of increased 3-bromotyroisine formation in target organs for BrO$_3^-$-induced cancer in rats. The higher basal levels of 3-bromotyroisine in the renal proximal tubules of female rats, compared to males, could be the result of the halogenations that occur in normal animals. A striking result from these studies is the increase in cytosolic 3-bromotyroisine staining in male rats, but not females. Moreover, a significant increase in the formation of 3-bromotyroisine compared to controls were observed at concentrations as low as 15 mg/L KBrO$_3$, again only in male rat kidneys. This suggests that bromotyroisines might play a role in mediating sex-dependent differences in the BrO$_3^-$ induced cell proliferation in rat kidneys (T. Umemura et al., 2004). The increased accumulation of bromotyroisines could be a result of the interaction of reactive intermediates (especially HOBr) formed from the BrO$_3^-$, with the tyrosine residues of alpha-2u-globulin or the other proteins present in the kidney. This may lead to accumulation of alpha-2u-globulin in the proximal tubules, which may increase the susceptibility of male rats to BrO$_3^-$ induced cell proliferation at low concentrations (T. Umemura et al., 2004).

Increases in TUNEL staining in females, but not males, at lower concentrations of BrO$_3^-$ (15 mg/L KBrO$_3$) suggest that BrO$_3^-$ induces renal cell apoptosis in females
kidneys at lower concentrations compared to males. It’s important to point out that this sex bias is lost as the concentration of BrO$_3^-$ increases, suggesting either the activation of additional toxic pathways, or the depletion of defense mechanisms in males. Additionally, alterations in cell proliferation may account for these changes (T. Umemura et al., 2004). Thus, the pathways of toxicity activated at higher concentrations of KBrO$_3$ may be dependent on 8-OHdG formation.

Increase in 8-OHdG staining indicates oxidative DNA damage (Seki et al., 2002). Further, 8-OHdG is an established marker of BrO$_3^-$-induced oxidative DNA damage (Kasai, Nishimura, Kurokawa, & Hayashi, 1987; Kurokawa et al., 1990). Exposure of male rats to BrO$_3^-$ increased 8-OHdG staining only at concentrations of 60 mg/L KBrO$_3$ and higher. Similar levels of staining were observed in both male and female rats, with slightly lower levels being detected in females after exposure to 400 mg/L. These data suggest that there is no sex difference in oxidative DNA damage induced by BrO$_3^-$ in the kidney.

Osteopontin (OPN), a kidney injury and cancer biomarker protein, is reported to be highly expressed in papillary renal cell tumors, and its expression correlates to poor prognosis in clear cell types of renal carcinoma (Matusan, Dordevic, Mozetic, & Lucin, 2005). Osteopontin expression in the kidney was evident in the damaged tubular epithelium. This pattern of osteopontin expression most accurately represents the sex-dependence of renal tumors after BrO$_3^-$ treatment based on previous chronic bioassays (T. Umemura et al., 2004; T. Umemura et al., 1998). As the increased expression of OPN may decrease cellular apoptosis and increase cell regeneration (Xie et al., 2001),
this might be another non-genotoxic mechanism that increases cell proliferation in males rats at lower concentrations of BrO₃⁻.

Another sex-dependent difference was observed in the expression of cytosolic p21. Increases in cytosolic p21 expression are reported to be pro–proliferative and anti-apoptotic (Rossig et al., 2001). The significant concentration- and sex-dependent increases in cytosolic p21 expression in female F344 rat kidneys after treatment with 15 mg/L KBrO₃ may counter the effect of apoptosis induced by BrO₃⁻ at low concentrations in female rat kidneys.

The increase in Kim-1 expression after BrO₃⁻ treatment agrees with our and others previous studies in rodents (Camacho L, 2011). The significant increase in the expression of Kim-1 in both male and female rat kidneys suggest that BrO₃⁻ can induce the renal injury at concentrations as low as 15 mg/L KBrO₃. The higher levels of Kim-1 protein expression in females at 400 mg/L, relative to males is actually not that surprising as Kim-1 can be shed from the kidney as a consequence of the nephrotoxicity and be present in the urine (Vaidya et al., 2006). These data suggest that there is a component of cytotoxicity and reparative hyperplasia in the response of females to BrO₃⁻-treatment (at higher concentrations) as well.

Clusterin expression was increased in both male and female kidneys after BrO₃⁻ treatment. Clusterin can mediate cell protection, lipid recycling, cell aggregation and cell attachment by acting as an anti-apoptotic protein during kidney injury (Dieterie et al., 2010). The presence of clusterin may reduce apoptosis and act synergistically with cytosolic p21 on BrO₃⁻ induced apoptosis in female rat kidneys.
In conclusion, tissue 3-bromotyrosine and urinary TOBr were increased with the 
BrO₃⁻ in F344 rats. This indicates the formation of reactive intermediates capable of brominating proteins. The sex-dependent expression of 3-bromotyrosine is in correlation with the non-genotoxic mechanisms of BrO₃⁻, rather than the genotoxic mechanisms. Thus, 3-bromotyrosines may represent a novel biomarker for reactive intermediates resulting from the reduction of BrO₃⁻ in vivo and may play a role in mediating the sex differences of BrO₃⁻ induced renal cell proliferation and death in animals.
CHAPTER 6

PHARMACOKINETIC MODELING OF BrO₃⁻
Abstract

Bromate (BrO$_3^-$), a toxic water disinfection by-product formed during ozonation of source water containing bromide (Br$^-$). Data gaps are exist in the knowledge of the pharmacokinetics of BrO$_3^-$ at low doses in animals. To fill these data gaps, we developed a pharmacokinetic (PK) model for BrO$_3^-$ to better understand its absorption and disposition in rats. Raw data from our recent study on the BrO$_3^-$ absorption, and disposition in female F344 rats (Bull et al. 2012) was used for the development of a pharmacokinetic model. Our results demonstrated that BrO$_3^-$ disposition after IV bolus was best described by using 1-compartmental pharmacokinetic model for the doses up to 0.5; and by using 2- compartmental pharmacokinetic model for the doses ranging from 1- 2.5 mg/kg potassium bromate (KBrO$_3$). BrO$_3^-$ disposition after its administration as an oral gavage for the dose range 0.5 to 20 mg/kg KBrO$_3$ was best described by using 1-compartmental pharmacokinetic model. A pharmacokinetic model fitted with an individual animal data and weight$^{-2}$ ($1/Y^2$) weight scheme was chosen based on goodness of fit, and model diagnostics such as Akaike's information criterion (AIC), Schwarz's Bayesian criterion (SBC), percent co-efficient of variation (%CV) values as a model of choice to estimate the pharmacokinetic parameters of BrO$_3^-$ in rats. This model suggests that BrO$_3^-$ absorption occurs through the GI tract in a first order manner with an absorption rate constant ($K_a$) $\sim$0.16 min$^{-1}$, and undergoes extensive first pass in the stomach and liver before it reaches systemic circulation. BrO$_3^-$ distribution to the peripheral tissues can be seen following administration of $\geq$ 1 mg/kg KBrO$_3$ as an IV bolus, and eliminate rapidly from the body.
Introduction

Bromate (BrO$_3^-$), a toxic water disinfection by-product formed during ozonation. It is a proven animal carcinogen and probable human carcinogen (group 2B) (IARC, 1986). Health concerns have been raised as it is a by-product of the ozonation of ground or source water.

There is limited data describing BrO$_3^-$ uptake, distribution and elimination in male rats and none in females (Fisher & Bull, 2006). BrO$_3^-$ is a reactive compound and it is probable that substantial amount of BrO$_3^-$ undergoes pre-systemic reduction to Br$^-$ in the humans (Jason D. Keith et al., 2006; J.D. Keith et al., 2006). It is reported that even high doses of BrO$_3^-$ are rapidly degraded in blood and tissues in vitro (Tanaka et al., 1984).

Based on the toxic, as well as carcinogenic, effects induced by BrO$_3^-$ in animals and humans, USEPA promulgated a maximum contaminant level (MCL) for BrO$_3^-$ at 10 µg/L drinking water (equivalent to ~0.3 µg BrO$_3^-$ /kg body wt. /day, as a bolus concentration), with a maximum contaminant level goal (MCLG) of zero (USEPA, 2001). However, this value was estimated based on very limited preclinical and/or clinical BrO$_3^-$ pharmacokinetics (ADME) data in animals and humans (Cotruvo, 2010; Fisher & Bull, 2006; Fujii et al., 1984; Tanaka et al., 1984).

Data gaps exist in the knowledge of the pharmacokinetics of BrO$_3^-$ at low doses in animals. This propels the necessity to develop a pharmacokinetic (PK) model that describes the BrO$_3^-$ disposition following administration of KBrO$_3$ IV bolus or oral gavage in rats. Recently, our laboratory published data on the absorption and disposition of BrO$_3^-$ in female F344 rats. The main purposes of the current study are to use the raw
data of the (Bull et al., 2012) to relate the BrO$_3^-$ dose, and dosing frequency to its concentration in the body. We also used the data to explore structure and the behavior of the system and to aid in predicting the physiological phenomenon involved in the disposition of BrO$_3^-$ at low doses. Such a model will be useful in predicting PK parameters for the various concentrations of KBrO$_3$. Moreover, this model can be used as a preliminary PK model for BrO$_3^-$, before the development of physiological based pharmacokinetic (PBPK) model, which is necessary to estimate the BrO$_3^-$ risk in humans.

Results

**Compartmental Modeling of Plasma BrO$_3^-$ Time-Concentration Data**

The observed plasma BrO$_3^-$ time-concentration data from our recent studies (Bull et al., 2012) displayed a multi-exponential decay following its administration in female F344 rats. However, the best data fit was observed using a 1-compartmental pharmacokinetic (PK) model for the doses 0.1 and 0.5 mg/kg KBrO$_3$, and 2-compartmental PK model for the doses 1 and 2.5 mg/kg KBrO$_3$ administered as an IV bolus (Figure 6.1). This suggests that BrO$_3^-$ might display a non-linear mechanism as dose increases by distributing to the peripheral tissues. The best data fit for the doses 0.5, 2.5 and 20 mg/kg KBrO$_3$ administered as an oral gavage was observed using a 1-compartmental PK model (Figure 6.2).
Plasma $\text{BrO}_3^-$ concentration-time profile after KBrO$_3$ IV bolus fitted by a 1-compartment linear PK model described by the following explicit equation

$$C_t = C_0 \cdot e^{-kt} \quad \text{Equation 1.}$$

Where $C_t$ is the Plasma $\text{BrO}_3^-$ concentration at time $t$ min., $C_0$ is the Plasma $\text{BrO}_3^-$ concentration at time zero, $k$ is the elimination rate constant, and $t$ is the time in min.

Plasma $\text{BrO}_3^-$ concentration-time profile after KBrO$_3$ IV bolus fitted by a 2-compartment linear PK model described by the following explicit equation

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad \text{Equation 2.}$$

where $C$ is the drug plasma concentration at the time $t$ min., $A$ and $B$ are the mass constant that represent the ordinate intercepts for the distribution and elimination phases; $\alpha$ and $\beta$ are the slopes of the distribution and elimination phases.

Plasma $\text{BrO}_3^-$ concentration-time profile after KBrO$_3$ oral gavage fitted by a 1-compartment linear pharmacokinetic model described by the following explicit equation

$$C_t = \left\{ \frac{(F^* \text{Dose}^*K_a)}{V} \cdot (K_a - K_{el}) \right\} \cdot \left[ \exp (-K_{el} * t) - \exp (-K_a * t) \right] \quad \text{Equation 3.}$$

Where $C_t$ is the Plasma $\text{BrO}_3^-$ concentration at time $t$ min., $F$ is the $\text{BrO}_3^-$ oral bioavailability, $K_a$ is the oral absorption rate constant for $\text{BrO}_3^-$, $V$ is the apparent volume of distribution, $K_{el}$ is the elimination rate constant, and $t$ is the time in min.
Compartmental PK Model Fit of Individual, Average and Pooled Animal Data

The individual, naïve average, or naïve pooled animal plasma BrO\textsubscript{3} time-concentration profile after KBrO\textsubscript{3} IV bolus or oral gavage were fitted to the compartmental PK model. The best data fit and the model diagnostics such as Akaike’s information criterion (AIC), Schwarz’s Bayesian criterion (SBC), percent coefficient of variation (%CV), and correlation coefficient (r\textsuperscript{2}) were observed for the PK models fitted with the individual animal data from the BrO\textsubscript{3} IV bolus (Figure 6.3), (Table 6.1) and oral gavage studies (Figure 6.4), (Table 6.2) in female F344 rats.

Assessment of Goodness of fit

The best fit between the observed and the model predicted plasma BrO\textsubscript{3} time-concentration profile after IV bolus (Figure 6.5) or oral gavage (Figure 6.6) was observed after application of weight\textsuperscript{2} (1/Y\textsuperscript{2}) weighting scheme to the 1- or 2-compartmental PK model, compared to the uniform, and weight\textsuperscript{1} (1/Y) weighting schemes. PK models fitted with uniform and 1/Y weighting schemes under predicted the plasma BrO\textsubscript{3} concentrations in the terminal phase of the BrO\textsubscript{3} time-concentration curve. Moreover, the percent co-efficient of variation (%CV) associated with the parameters was <15% for the models fitted with the 1/Y\textsuperscript{2} weight scheme compared to the >25%, and 20% for the uniform and 1/Y weight schemes, respectively. Similar type of data fit was observed for all the tested doses of BrO\textsubscript{3} administered as either IV bolus or oral gavage.
The plots of weighted predicted (y-axis) and observed plasma BrO₃⁻ concentration showed that the data points scattered randomly around the line of identity (45° line) and distributed evenly throughout the data for the model with 1/Y² weight scheme (Figure 6.7C). The residual plots of weighted residual and weighted predicted plasma BrO₃⁻ concentration showed that residuals distributed randomly for the model with 1/Y weight scheme. Residuals followed a cone shaped trend for the model fitted with uniform weight (Figure 6.8A), and the “run” for the 1/Y² weight scheme (Figure 6.8C). The residual plots of weighted predicted and time showed that residuals distributed randomly for the models fitted with uniform or 1/Y weight scheme (Figure 6.9A and B), but followed a cone shaped trend for the 1/Y² weight scheme (Figure 6.9C). Similar type of residuals distribution was observed for all the tested doses of BrO₃⁻ administered as either IV bolus or oral gavage.

A pharmacokinetic model fitted with an individual animal data and weight² (1/Y²) weight scheme was chosen based on goodness of fit (visual), and model diagnostics such as Akaike’s information criterion (AIC), Schwarz’s Bayesian criterion (SBC), percent co-efficient of variation (%CV) values as a model of choice to estimate the pharmacokinetic parameters of BrO₃⁻ in rats. PK parameters were estimated with the selected model, following administration of KBrO₃ as an IV bolus (Table 6.3) or oral gavage (Table 6.4). These parameters suggests that BrO₃⁻ absorption occurs through the GI tract in a first order manner with an absorption rate constant (Kₐ) ~0.16 min⁻¹, and undergoes extensive first pass in the stomach and liver before it reaches systemic circulation. BrO₃⁻ distribution to the peripheral tissues can be seen following administration of ≥ 1 mg/kg KBrO₃ as an IV bolus, and eliminates rapidly from the body.
Table 6.1: Comparison of the model diagnostics for the models fitted with individual or average or pooled animal data of the KBrO₃ IV bolus studies in rats

<table>
<thead>
<tr>
<th>Diagnostic</th>
<th>Individual</th>
<th>Average</th>
<th>Pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Co-efficient of Variation (% CV)</td>
<td>&lt;15%</td>
<td>&gt;20%</td>
<td>&lt;10-40%</td>
</tr>
<tr>
<td>Correlation co-efficient ($r^2$) (obs, pre)</td>
<td>0.99</td>
<td>0.99</td>
<td>0.97</td>
</tr>
<tr>
<td>Akaike’s information criterion (AIC)</td>
<td>7.09</td>
<td>34.6</td>
<td>136.84</td>
</tr>
<tr>
<td>Schwarz’s Bayesian criterion (SBC)</td>
<td>6.87</td>
<td>34.92</td>
<td>140.40</td>
</tr>
<tr>
<td>Condition Number</td>
<td>0.5173 E+06</td>
<td>0.4368E+06</td>
<td>0.6998E+06</td>
</tr>
</tbody>
</table>
Table 6.2: Comparison of the model diagnostics for the models fitted with individual or average or pooled animal data of the KBrO₃ oral gavage studies in rats

<table>
<thead>
<tr>
<th>Diagnostic</th>
<th>Individual</th>
<th>Average</th>
<th>Pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Co-efficient of Variation (% CV)</td>
<td>&lt;15%</td>
<td>~10%</td>
<td>~10%</td>
</tr>
<tr>
<td>Correlation co-efficient ($r^2$) (obs, pre)</td>
<td>0.98</td>
<td>0.98</td>
<td>0.81</td>
</tr>
<tr>
<td>Akaike’s information criterion (AIC)</td>
<td>28.48</td>
<td>37.07</td>
<td>221.52</td>
</tr>
<tr>
<td>Schwarz’s Bayesian criterion (SBC)</td>
<td>27.85</td>
<td>36.45</td>
<td>225.05</td>
</tr>
<tr>
<td>Condition Number</td>
<td>332</td>
<td>476</td>
<td>428</td>
</tr>
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Table 6.3: Pharmacokinetic parameters for BrO$_3^-$ administered as IV bolus

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>KBrO$_3$</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>min$^{-1}$</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>0.12$^a$ ± 0.03</td>
<td>0.2$^a$ ± 0.01</td>
</tr>
<tr>
<td>beta</td>
<td>min$^{-1}$</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>0.03$^a$ ± 0</td>
<td>0.03$^a$ ± 0</td>
</tr>
<tr>
<td>$K_{10}$</td>
<td>min$^{-1}$</td>
<td>0.03$^a$ ± 0.02</td>
<td>0.04$^a$ ± 0</td>
<td>0.07$^a$ ± 0.01</td>
<td>0.08$^b$ ± 0.02</td>
<td></td>
</tr>
<tr>
<td>$K_{12}$</td>
<td>min$^{-1}$</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>0.03$^a$ ± 0</td>
<td>0.07$^a$ ± 0.02</td>
</tr>
<tr>
<td>$K_{21}$</td>
<td>min$^{-1}$</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>0.04$^a$ ± 0</td>
<td>0.08$^a$ ± 0.02</td>
</tr>
<tr>
<td>$AUC_{0-\text{inf}}$</td>
<td>min.µg/L</td>
<td>7470$^a$ ± 1300</td>
<td>23100$^b$ ± 1440</td>
<td>46500$^c$ ± 4000</td>
<td>146270$^d$ ± 1620</td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>µg/L</td>
<td>460$^a$ ± 100</td>
<td>1500$^a$ ± 110</td>
<td>2500$^a$ ± 250</td>
<td>11770$^b$ ± 1500</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>ml/min/kg</td>
<td>11$^a$ ± 2.0</td>
<td>21$^b$ ± 0.0</td>
<td>28$^b$ ± 3.0</td>
<td>18$^a$ ± 1.0</td>
<td></td>
</tr>
<tr>
<td>$V_1$</td>
<td>L/kg</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>0.42$^a$ ± 0.05</td>
<td>0.25$^a$ ± 0.05</td>
</tr>
<tr>
<td>$V_2$</td>
<td>L/kg</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>0.23$^a$ ± 0.06</td>
<td>0.38$^a$ ± 0.01</td>
</tr>
<tr>
<td>$V^{SS}$</td>
<td>L/kg</td>
<td>0.43$^a$ ± 0.05</td>
<td>0.48$^a$ ± 0.04</td>
<td>0.65$^a$ ± 0.01</td>
<td>0.63$^a$ ± 0.01</td>
<td></td>
</tr>
<tr>
<td>$V_d$</td>
<td>L/kg</td>
<td>0.43$^a$ ± 0.05</td>
<td>0.48$^a$ ± 0.04</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

*NA indicates parameter is not applicable for the corresponding dose*
Table 6.4: 1-compartmental model pharmacokinetic parameters for BrO$_3^-$ administered as oral gavage

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>$0.5$</th>
<th>$2.5$</th>
<th>$20$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{01}$</td>
<td>min$^{-1}$</td>
<td>$0.14^a ± 0.01$</td>
<td>$0.16^a ± 0.02$</td>
<td>$0.17^a ± 0.02$</td>
</tr>
<tr>
<td>$K_{10}$</td>
<td>min$^{-1}$</td>
<td>$0.02^a ± 0$</td>
<td>$0.03^a ± 0$</td>
<td>$0.01^a ± 0$</td>
</tr>
<tr>
<td>AUC</td>
<td>min. µg/L</td>
<td>$8112^a ± 1300$</td>
<td>$37395^a ± 8000$</td>
<td>$440382^a ± 5629$</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>µg/L</td>
<td>$121^a ± 13$</td>
<td>$867^a ± 160$</td>
<td>$4529^b ± 59$</td>
</tr>
<tr>
<td>AUC$_{0-inf}$</td>
<td>min-µg/L</td>
<td>$8200^a ± 1140$</td>
<td>$39640^a ± 8290$</td>
<td>$380200^b ± 18670$</td>
</tr>
<tr>
<td>CL</td>
<td>ml/min/kg</td>
<td>$23^a ± 3$</td>
<td>$20^a ± 4$</td>
<td>$22^a ± 4$</td>
</tr>
</tbody>
</table>
A. 0.1 mg/kg KBrO$_3$

B. 0.5 mg/kg KBrO$_3$

C. 1 mg/kg KBrO$_3$
Figure 6.1: Compartmental pharmacokinetic model fit of BrO\(_3\)\(^-\) plasma concentration-time profile after IV bolus in female F344 rats. The observed BrO\(_3\)\(^-\) plasma concentration-time profile in an individual animal after administration of A. 0.1, B. 0.5, C. 1, D. 2.5 mg/kg KBrO\(_3\) as an IV bolus was fitted into 1- or 2- compartment pharmacokinetic (PK) model for the best data fit using WinNonlin, V5.2. Red open circles denote the observed data, and blue solid line denotes the model predicted data. Weight\(^{-2}\) \((1/Y^2)\) weighting scheme was applied to all the models.
A. 0.5 mg/kg KBrO₃

B. 2.5 mg/kg KBrO₃

C. 20 mg/kg KBrO₃
**Figure 6.2:** Compartmental pharmacokinetic model fit of BrO$_3^-$ plasma concentration-time profile after oral gavage in female F344 rats. The observed BrO$_3^-$ plasma concentration-time profile in an individual animal after oral administration of A. 0.5, B. 2.5, and C. 20 mg/kg KBrO$_3$ was fitted into 1 compartment PK model for the best data fit using WinNonlin, V5.2. Red open circles denote the observed data, and blue solid line denotes the model predicted data. Weight$^{-2}$ weighting scheme was applied to all the models.
A. 1 mg/kg KBrO₃ Individual

B. 1 mg/kg KBrO₃ Naïve Average

C. 1 mg/kg KBrO₃ Naïve Pooled
**Figure 6.3**: 2-compartment model fit of individual, naïve average, and naïve pooled animal plasma BrO$_3^-$ time-concentration profile following administration of 1 mg/kg KBrO$_3$ IV bolus in rats. An individual (A) (N=1), naïve average (B) (N=4), and naïve pooled data (C) (N=4), of plasma BrO$_3^-$ time-concentration profile in rats following administration of 1 mg/kg KBrO$_3$ IV bolus in female F344 rats was fitted into 2-compartment model using WinNonlin, V5.2. Red open circles denote the observed data, and blue solid line denotes the model predicted data. Weight$^2$ ($1/Y^2$) weighting scheme was applied to all the models. Similar type of fit for the individual, naïve average and naïve pooled data was observed for the doses ranging from 0.1 to 2.5 mg/kg KBrO$_3$. 
A. 2.5 mg/kg KBrO₃ Individual

B. 2.5 mg/kg KBrO₃ Naïve Average

C. 2.5 mg/kg KBrO₃ Naïve Pooled
Figure 6.4: 1-compartment model fit of individual, naïve average, and naïve pooled animal plasma BrO$_3^-$ time-concentration profile following administration of 2.5 mg/kg KBrO$_3$ as an oral gavage in rats. The individual (A) (N=1), naïve average (B) (N=4), and naïve pooled data (C) (N=4), of plasma BrO$_3^-$ time-concentration profile in rats following administration of 2.5 mg/kg KBrO$_3$ oral gavage in female F344 rats was fitted into 1-compartment model using WinNonlin, V5.2. Red open circles denote the observed data, and blue solid line denotes the model predicted data. Weight$^{-2}$ ($1/Y^2$) weighting scheme was applied to all the models. Similar type of fit for the individual, naïve average and naïve pooled data was observed for the doses ranging from 0.5 to 20 mg/kg KBrO$_3$. 
A. Uniform Weight

B. Weight$^1$

C. Weight$^2$
Figure 6.5: Effect of weight on the pharmacokinetic model fit of KBrO₃ IV bolus time-concentration profile. The individual animal plasma BrO₃⁻ concentration-time profile data following administration of 1 mg/kg KBrO₃ IV bolus in rats was fitted into 2-compartment PK model with uniform (A), Weight⁻¹ (1/Y) (B), and Weight⁻² (1/Y²) (C) weighting schemes. Red open circles denote the observed data, and blue solid line denotes the model predicted data. Similar type of weight effect was observed for the doses ranging from 0.1 to 2.5 mg/kg KBrO₃.
A. Uniform Weight

B. Weight$^1$

C. Weight$^2$
Figure 6.6: Effect of weight on the pharmacokinetic model fit of KBrO$_3$ oral gavage time-concentration profile. The individual animal plasma BrO$_3^-$ concentration-time profile data following administration of 2.5 mg/kg KBrO$_3$ oral gavage in rats was fitted into 1-compartment PK model with uniform (A), Weight$^{-1}$ (1/Y) (B), and Weight$^{2}$ (1/Y$^2$) (C) weighting schemes. Red open circles denote the observed data, and blue solid line denotes the model predicted data. Similar type of weight effect was observed for the doses ranging from 0.5 to 20 mg/kg KBrO$_3$. 
A. Uniform Weight

B. Weight$^1$

C. Weight$^2$
Figure 6.7: Observed vs. weighted predicted plasma BrO₃⁻ concentration after 1 mg/kg KBrO₃ IV bolus. Data points scatter should lie close to the line of identity (45° line). A. Uniform, B. weight⁻¹ (1/Y), and C. weight² (1/Y²) weighting schemes. Similar type of distribution around the line of identity (45° line) was observed for all the tested doses and the pharmacokinetic models.
A. Uniform Weight

B. Weight$^1$

C. Weight$^2$
Figure 6.8: Weighted predicted plasma BrO$_3^-$ concentration vs. weighted residual plasma BrO$_3^-$ concentration after 1 mg/kg KBrO$_3$ IV bolus. Error distribution should be randomly modeled throughout the range of the data. A. Uniform, B. weight$^{-1}$ (1/Y), and C. weight$^{-2}$ (1/Y$^2$) weighting schemes. Similar type of residuals distribution was observed for all the tested doses and the pharmacokinetic models.
Figure 6.9: Time vs. weighted residual plasma BrO$_3^-$ concentration after 1 mg/kg KBrO$_3$ IV bolus. The error distribution should be randomly modeled across the range of the time. **A. Uniform, B. weight$^1$ (1/Y), and C. weight$^2$ (1/Y$^2$) weighting schemes.** Similar type of residuals distribution was observed for all the tested doses and the pharmacokinetic models.
Discussion

Pharmacokinetic (PK) models are a type of mathematical models that relates drug or toxin concentration, and dosing frequency to concentrations in the body. They may be used to predict the time-concentration profile of a chemical in blood, and tissues based on given observed data (Bonate, 2011). The observed plasma BrO$_3^-$ time-concentration profile can be best described by using a 1-compartmental pharmacokinetic (PK) model for the doses 0.1 and 0.5 mg/kg KBrO$_3$, and 2-compartmental PK model for the doses 1 and 2.5 mg/kg KBrO$_3$ administered as an IV bolus. This suggests that BrO$_3^-$ might display a non-linear mechanism as dose increases by distributing to the peripheral tissues. The best data fit for the doses 0.5, 2.5 and 20 mg/kg KBrO$_3$ administered as an oral gavage was observed using a 1-compartmental PK model.

Even though, similar data fits were observed with the individual animal, average and pooled animal data, the model diagnostics such as Akaike’s information criterion (AIC), Schwarz’s Bayesian criterion (SBC), percent coefficient of variation (%CV), and correlation coefficient ($r^2$) were better for the PK models fitted with the individual animal data from the BrO$_3^-$ IV bolus and oral gavage studies in female F344 rats.

% CV measures the relative error associated with parameter values. The lower the %CV, the less errors associated with the parameter, and the better the model (Bonate, 2011). % CV was similar in all the models tested. Akaike’s criterion (AIC) and Schwarz’s Bayesian Criterion (SBC) are useful diagnostics in selecting better models among the tested models (Bonate, 2011). However, SBC selects the simpler model than AIC; and AIC selects a more complex model than the SBC. This is one reason why
it’s typically better to choose both values in selecting the model. The low values of AIC and SBC in a compartmental PK model fitted with an individual animal data, compared to other models fitted with either average or pooled animal data indicates that the amount of information lost, or the error that is unexplainable in the model, is less compared to the true model. The correlation co-efficient ($r^2$) between observed vs. predicted concentration was higher in the models fitted with the average data, compared to the individual animal data. This is not uncommon.

Different weight schemes were applied to the observed individual animal data in order to achieve the most consistent fit to low and high values. Weighting schemes account for the heterogeneity in the variance of data (Weiner, 2000). The best model among the tested models fitted with uniform, or Weight$^{-1}$ $(1/Y)$, and Weight$^{-2}$ $(1/Y^2)$ weight schemes, was selected by conducting goodness of fit, and analyzing the model diagnostics.

Residuals analysis is one of the criterions that used in assessing model goodness of fit. It aids in determining if the model assumptions are wrong or whether a different structural model should be used. Residuals are the difference between observed and model predicted values. They follow the normal distribution with a mean zero and variance. Essentially, residual is an error that is unexplained by the model. A model with randomly distributed residuals has a better fit compared to the models following a particular trend (Bonate, 2011). Analysis of distribution of the residuals was analyzed by plotting the weighted residual plasma BrO$_3^-$ concentration against the weighted predicted plasma BrO$_3^-$ concentration, or time. The residuals followed a cone shaped distribution in a model fitted with uniform weight. This indicates that weighting
scheme was not ideal, and should change to $1/Y$. In addition, the residuals were distributed randomly in a model fitted with $1/Y$ weighting scheme. The models fitted with $1/Y^2$ weighting scheme under predicted the observations in the early phase, and over predicted in the terminal phase, as evidenced by the “run”, as all the residuals were positive in the initial phase, and are negative in the terminal phase. Based on the residual plots, the $1/Y$ weighting scheme is the best fit for these data.

Analysis of residual distribution by plotting against time showed that residuals followed a cone shaped distribution in a model fitted with $1/Y^2$ weighting scheme. However, the residuals were distributed randomly throughout the data in a model fitted with the $1/Y$ weighting scheme. The higher the value of weighted residual sums of squares (WRSS), the larger the error among the observations. The models fitted with $1/Y^2$ weighing scheme had the lower WRSS compared to the models fitted with the other weight schemes, indicating that the error among observations will be less with the $1/Y^2$ weighting scheme.

In summary, BrO$_3^-$ disposition occurs in a multi-phasic manner. The observed plasma BrO$_3^-$ time-concentration profile data was best described by using a 1-compartmental pharmacokinetic (PK) model for the doses 0.1 and 0.5 mg/kg KBrO$_3$, and 2-compartmental PK model for the doses 1 and 2.5 mg/kg KBrO$_3$ administered as an IV bolus. The best data fit for the doses 0.5, 2.5 and 20 mg/kg KBrO$_3$ administered as an oral gavage was observed using a 1-compartmental PK model. The model fitted with the individual animal data had better model diagnostics compared to others. The residuals were distributed randomly throughout the data for the models fitted with the $1/Y$ weight scheme compared to the models fitted with uniform or $1/Y^2$ weight schemes.
A pharmacokinetic model fitted with an individual animal data and weight\(^{-2}\) \((1/Y^2)\) weight scheme was chosen as a model of choice to estimate the pharmacokinetic parameters of BrO\(_3^-\) in rats. PK parameters were estimated with the selected model, following administration of KBrO\(_3\) as an IV bolus or oral gavage. These parameters suggest that BrO\(_3^-\) absorption occurs through the GI tract in a first order manner with an absorption rate constant \((K_a) \sim 0.16 \text{ min}^{-1}\), and undergoes extensive first pass in the stomach and liver before it reaches systemic circulation. BrO\(_3^-\) distribution to the peripheral tissues can be seen following administration of ≥ 1 mg/kg KBrO\(_3\) as an IV bolus, and eliminates rapidly from the body.
CHAPTER 7

SUMMARY

This project developed data for the purpose of refining the human health risk of BrO$_3^-$, a toxic water disinfection by-product. The overall hypotheses for this project were;

*Hypothesis I:* The reactivity of BrO$_3^-$ results in a substantial pre-systemic reduction to Br$^-$ at low concentrations, which leads to exhibition of non-linear pharmacokinetics in F344 rats, and *Hypothesis II:* Non-genotoxic events (non DNA damaging events) mediate BrO$_3^-$ induced sex-differences in renal cell proliferation and death in F344 rats.

We tested these hypotheses with the following specific aims.

1. We determined the pharmacokinetics (ADME) of BrO$_3^-$ at low concentrations in female F344 rats

2. We identified the molecular mechanisms controlling sex-dependent differences in BrO$_3^-$-induced nephrotoxicity in F344 rats

3. We developed a pharmacokinetic (PK) model for BrO$_3^-$ in female F344 rats

The pharmacokinetic (ADME) studies of KBrO$_3$ (0.1-2.5 mg/kg, IV bolus) in female F344 rats demonstrated that $>95\%$ of the administered BrO$_3^-$ was eliminated within 120 min. The disproportionate increase in peak concentration ($C_{\text{max}}$ or $C_0$) and the area under curve (AUC) of plasma BrO$_3^-$ were in correlation with the increased plasma BrO$_3^-$ clearance for the doses 0.5 and 1 mg/kg, compared to 0.1 mg/kg KBrO$_3$. BrO$_3^-$ was
eliminated from the body in a first order fashion with an elimination rate constant (K_e) of 0.03 min^{-1}, and plasma half-life of ~23 min. The oral bioavailability (F) of BrO_3^{-} is ~35 %, indicates the occurrence of high pre systemic reduction of BrO_3^{-} in rats. The plasma half-life for BrO_3^{-} after oral gavage is ~30 min. for doses up to 2.5 mg/kg KBrO_3. However, the increases in plasma BrO_3^{-} half-life following the oral administration of 20 mg/kg KBrO_3 was in correlation with the decreased elimination rate constant (K_e) from 0.03 to 0.016 min^{-1} and increased volume of distribution (V_d) from 0.87 to 1.46 L/kg. A significant increase in the plasma Br^{-} from BrO_3^{-} was observed only for oral doses ≥ 2.5 mg/kg KBrO_3. The rate of BrO_3^{-} reduction was greater compared to the rate of Br^{-} formation from the BrO_3^{-} \textit{in vivo}. BrO_3^{-} degraded in a multiphasic manner with an initial rapid degradation, and a slower secondary degradation in rat blood \textit{in vitro}. Br^{-} elimination from BrO_3^{-}, 8.1 mg KBrO_3/kg, over the first 48 hours was 18% lower than that from an equi molar single dose of Br^{-}, 5 mg KBr/kg. The cumulative excretion of Br^{-} from KBr vs. KBrO_3 was equivalent 72 hours after administration. The deficit in total recovery of BrO_3^{-} raises the possibility that some brominated biochemicals may be produced \textit{in vivo}, which may slowly get metabolized and eliminated from the body. A high inter individual variation in the background levels of BrO_3^{-} (1 – 8.5 µg/L) was observed in female F344 rat blood.

We studied the molecular mechanisms controlling sex-dependent differences in BrO_3^{-}-induced nephrotoxicity in F344 rats. Male and female F344 rats were treated with 0-400 mg/L KBrO_3 in drinking water for 28 days. Our results demonstrated that BrO_3^{-} induced similar levels of genotoxicity in both sexes, identified by 8-OHdG staining (Figure 7.1). In contrast, it induced higher levels of renal cell apoptosis in female rat
kidneys, identified by TUNEL staining, at low concentrations (15 mg/L KBrO₃); and in male rat kidneys at high concentrations (400 mg/L KBrO₃) (Figure 7.1). Sex-dependent differences in BrO₃⁻-induced nephrotoxicity were accompanied by differential expression of several genes and proteins. BrO₃⁻ induced the expression of osteopontin and p21 in a sex- and concentration-dependent manner (Figure 7.2). Finally, these data, combined with the prior studies of other investigators, support the hypothesis that both genotoxic and non-genotoxic mechanisms are operable after BrO₃⁻ treatment and suggest that differences in non-genotoxic mechanisms may predominate in determining sex-based differences in BrO₃⁻-induced nephrotoxicity.

We also studied sex-dependent differences in BrO₃⁻-induced nephrotoxicity and compared these to differences in the disposition of BrO₃⁻ and its metabolites following 0-400 mg/L KBrO₃ treatment in drinking water for 28 days in F344 rats. Sex-dependent differences were observed in ToBr excretion, with higher levels in males compared to females at concentrations ≥ 60 mg/L KBrO₃. Increased levels of ToBr correlated with BrO₃⁻-induced renal apoptosis in male rats, suggesting that ToBr may be a better indicator of BrO₃⁻ exposure and toxicity than BrO₃⁻ or Br⁻ their self. Based on the documented formation of brominated tyrosines, we studied the expression of 3-bromotyrosine in male and female rat kidneys. A significant increases in the formation of 3-bromotyrosine compared to controls were observed at concentrations as low as 15 mg/L KBrO₃, only in male rat kidneys (Figure 7.3). This suggests that bromotyrosines might play a role in mediating sex-dependent differences in the BrO₃⁻-induced cell proliferation in rat kidneys. Moreover, the sex-dependent expression of 3-bromotyrosine correlated with the non-genotoxic mechanisms of BrO₃⁻, rather than the genotoxic
mechanisms. Thus, 3-bromotyrosine may represent a novel biomarker for reactive intermediates resulting from the reduction of BrO\textsuperscript{-} in vivo and may play a role in mediating the sex differences in BrO\textsuperscript{-} induced renal cell proliferation and cell death in rats.

Data on the pharmacokinetics of BrO\textsuperscript{-} were subjected to PK modeling. A pharmacokinetic model fitted with an individual animal data and weight\textsuperscript{-2} (1/Y\textsuperscript{2}) weight scheme was chosen based on goodness of fit, and model diagnostics such as Akaike’s information criterion (AIC), Schwarz’s Bayesian criterion (SBC), percent co-efficient of variation (%CV) values as a model of choice to estimate the pharmacokinetic parameters of BrO\textsuperscript{-} in rats. Analysis of BrO\textsuperscript{-} PK parameters following its oral administration, revealed that BrO\textsuperscript{-} absorption occurs through GI tract in a first order manner with a rate constant (K\textsubscript{a}) of \(\sim 0.16\ \text{min}^{-1}\). BrO\textsuperscript{-} undergoes extensive first pass in the stomach and liver before it reaches systemic circulation, which results in low bioavailability \(\sim 35\%\). BrO\textsuperscript{-} distribution to the peripheral tissues can be seen following administration of \(\geq 1\ \text{mg/kg KBrO}_3\) as an IV bolus. Approximately 90\% of the orally administered BrO\textsuperscript{-} metabolized to Br\textsuperscript{-} in the body including blood, liver, and the kidney etc., and the remaining BrO\textsuperscript{-} excretes through kidneys at 0.002 L/min/kg (Figure 7.4).

Collectively, these data demonstrate that BrO\textsuperscript{-} pharmacokinetics is rapid and substantial pre systemic reduction of BrO\textsuperscript{-} to Br\textsuperscript{-} occurs at low doses. Both genotoxic and non-genotoxic mechanisms are operable with BrO\textsuperscript{-} treatment. The difference in non-genotoxic mechanisms may predominate in determining sex based differences in BrO\textsuperscript{-}-induced nephrotoxicity. Reactive metabolites of BrO\textsubscript{3} such as HOBr and BrO\textsuperscript{-} are capable of brominating proteins, which leads to the formation of 3-bromotyrosine in
rat kidneys. The sex-dependent expression of 3-bromotyrosine correlated with the non-genotoxic mechanisms of BrO$_3^\cdot$. Pharmacokinetic modeling of BrO$_3^\cdot$ revealed that extensive reduction of BrO$_3^\cdot$ occurs in the liver, and blood, which leads to a very low amount of BrO$_3^\cdot$ excreted through kidneys.

The US EPA estimated BrO$_3^\cdot$'s risk to humans based upon linear extrapolation of combined kidney, testicular mesothelium, and thyroid tumor data in male rats. However, the presence of alpha-2u-globulin; a male rat kidney specific protein mediates the enhanced susceptibility of male rats, may have skewed these data compared to female rats, especially with regards to cellular proliferation (Umemura et al., 1995; 1998; 2004). In addition, mechanisms of toxicity mediated by alpha-2u-globulin do not occur in humans, or female rat or the other species. Hence, data presented in this dissertation are more appropriate for assessing the risk of BrO$_3^\cdot$ to humans because of its use of female rats for the purposes of extrapolating BrO$_3^\cdot$ carcinogenic risk to the humans.

The US EPA also estimated the risk of BrO$_3^\cdot$ to humans in the absence of complete toxico/pharmacokinetics (ADME) parameters. Data presented in this dissertation on the ADME of BrO$_3^\cdot$ provides the relationship between the dose exposed and the relevant internal exposure (systemic circulation) in female rats. Our studies showed that BrO$_3^\cdot$ undergoes extensive pre-systemic reduction to Br$^\cdot$, a non-toxic stable metabolite, at lower doses. This results in low oral bioavailability of ~35%.

Studies in this dissertation also filled the data gaps in understanding the sex-dependent mechanisms involved in BrO$_3^\cdot$-induced nephrotoxicity in rats. Our studies showed that both genotoxic as well as non-genotoxic mechanisms are operable with the BrO$_3^\cdot$ treatment, and BrO$_3^\cdot$ induces similar levels of oxidative DNA damage in both male
and female rat kidneys at higher concentrations (carcinogenic doses). We also demonstrated that the occurrence of sex-dependent non-genotoxic mechanisms after exposure to low concentrations of \( \text{BrO}_3^- \) might predominate in determining sex based differences in \( \text{BrO}_3^- \)-induced nephrotoxicity. This is in contrast to the assumption made by USEPA that the occurrence of oxidative DNA damage induced by \( \text{BrO}_3^- \) is linear in rat kidneys.

Data presented in this dissertation suggest that \( \text{BrO}_3^- \) risk to humans needs to be reassessed. The studies presented in this dissertation provide extensive pharmacokinetics (ADME), dose-response, and mechanism of action data that was not available to the EPA during its first review of \( \text{BrO}_3^- \) in 2001. Thus, these data should allow EPA to refine the risk of this disinfection by-product to humans.
Figure 7.1: Sex-dependent differences of BrO$_3^-$-induced nephrotoxicity in rats. Male and female F344 rats were exposed to 0 to 400 mg/L KBrO$_3$ for 28 days prior to isolation of kidneys and analysis of staining using immunohistochemistry. This graph shows the quantitation of TUNEL and 8-OHdG staining, a marker for an apoptosis, and DNA damage, respectively, based on the number of positive nuclei per field. Data are presented as the mean ± SEM of 4-6 different animals.
**Figure 7.2:** Summary of expression of renal biomarkers in male and female F344 rats after treatment with KBrO₃. Male and female F344 rats exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and the analysis of expression of biomarkers using immunohistochemistry. This graph shows the quantification of the expression of various renal biomarkers in males (A) and females (B). The data are presented as the mean ± SEM of 4-6 different animals.
Figure 7.3: Effect of KBrO₃ treatment on 3-bromotyrosine staining in rat kidney. Male and female F344 rats exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of kidneys and the analysis of expression of 3-bromotyrosine using immunohistochemistry. This graph shows the quantification of the expression of 3-bromotyrosine in males and female rat kidneys. The data are presented as the mean ± the mean of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Figure 7.4: Pharmacokinetic Model for BrO₃⁻ in Female F344 Rats
APPENDIX

Appendix Table 1

1A. Top 25 genes differentially expressed in male rat kidney after KBrO₃ treatment for 28-days in drinking water

<table>
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<th>400 mg/L</th>
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### 1B. Top 25 genes differentially expressed in female rat kidney after KBrO$_3$ treatment for 28-days in drinking water

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Appendix Figure 1: Sex-dependent alterations in gene expression in rat kidney after KBrO$_3$ exposure. F344 male and female rats were exposed to 0, 125 and 400 mg/L KBrO$_3$ for 28 days prior to isolation of total RNA. The number of common and uncommon genes that were significantly different between male and female rats were calculated and presented in Venn diagram by concentration, 125 mg/L (1A) and 400 mg/L (1B).
Appendix Figure 2: Principal component analysis (PCA) and heat map of differentially expressed genes in male and female rat kidney after KBrO₃ exposure.

Appendix Figure 2A: Principal component analysis (PCA) of differentially expressed genes in male and female rat kidney after KBrO₃ exposure. Gene transcripts differentially expressed (fold change ≥ 1.5, and p-value < 0.005) in at least one or more treatment groups in male and/or female rat kidney were used for PCA. Gene log₂ ratios, each individual animal compared to the mean of control animals, were used to produce...
eigenvalues and the PCA plot of the first two components (PC1 = x axis, PC2 = y axis) is shown.

**Appendix Figure 2B:** shows data presented as a heat map of fifteen down-regulated and nineteen up-regulated genes taken from principal component 2 that include molecular markers of tissue injury and cell proliferation as clustered by treatment group. Hierarchical clustering was performed by Euclidian distance using log$_2$ ratios. Red color denotes increase in gene expression and green color indicates decrease in gene expression.
Appendix Figure 3: Sex-dependent alterations in mRNA expression in rat kidney after KBrO₃ exposure. Male and female F344 rats were exposed to 0, 125 or 400 mg/L KBrO₃ for 28 days prior to isolation of total renal RNA and analysis of selected mRNA transcripts including p21 (A), Kim-1 (B) and osteopontin (C) as determined by qPCR. Data in A-C are presented as the mean ± S.E.M. of at least 6 different animals.
*Denotes a statistically significant difference (P < 0.05) as compared to control, while # indicates a statistically significant difference (P < 0.05) between males and females at the indicated KBrO₃ concentration.
Appendix Figure 4: Effect of KBrO₃ treatment on 3-bromotyrosine staining in rat testes. Male (A to D) F344 rats exposed to 0 to 400 mg/L KBrO₃ in drinking water for 28 days prior to isolation of testes and the analysis of staining using immunohistochemistry. Panel E shows the quantification of 3-bromotyrosine staining was based on cytosolic staining. The data are presented as the mean ± SEM of 4-6 different animals. *Denotes a statistically significant difference (P < 0.05) as compared to control.
Appendix Figure 5: Effect of KBrO$_3$ treatment on 3-bromotyrosine staining in rat thyroid. Male (A to D) and female (E to H) F344 rats exposed to 0 to 400 mg/L KBrO$_3$ in drinking water for 28 days prior to isolation of thyroid and the analysis of staining using immunohistochemistry. Panel 5I shows the quantification of 3-bromotyrosine staining
was based on cytosolic staining. The data are presented as the mean ± SEM of 4-6 different animals.
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