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NIEHS Report on the Toxicity Studies of Nicotine Bitartrate Dihydrate (CASRN 6019-06-3) Administered in Drinking Water to Sprague Dawley Rats and Swiss Mice

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Foreword

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About This Report

Authors

Simona Panzacchi[a], Fiorella Belpoggi[a], Luciano Bua[a], John R. Bucher[b], Michelle C. Cora[b], Luana De Angelis[a], Laura Falcioni[a], Federica Gnudi[a], Daniele Mandrioli[a], Marco Manservigi[a], Isabella Manzoli[a], Scott A. Masten[b], Ilaria Menghetti[a], Esra Mutlu[b], Georgia K. Roberts[b], Kelly A. Shipkowski[b], Robert C. Sills[b], Matthew D. Stout[b], Valentina Strollo[a], Eva Tibaldi[a], J. Wren Tracy[c], Andrea Vornoli[a], Suramya Waidyanatha[b]

[a]Cesare Maltoni Cancer Research Center, Ramazzini Institute, Bologna, Italy

[b]Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

[c]ICF, Reston, Virginia, USA

Cesare Maltoni Cancer Research Center, Ramazzini Institute, Bologna, Italy
Subcontract to HHSN273201400015C
Simona Panzacchi, M.Phil., Study Director[1,2,3,8,9,10]
Fiorella Belpoggi, Ph.D., Principal Investigator (Retired)[1,3,5,6,10]
Daniele Mandrioli, M.D., Ph.D., Principal Investigator[1,3,5,6,9,10]
Luciano Bua, M.D.[3]
Luana De Angelis, L.T.[2,3]
Laura Falcioni, D.V.M.[3]
Federica Gnudi, Ph.D.[3]
Marco Manservigi, M.S.[3]
Isabella Manzoli, M.S.[2,3]
Ilaria Menghetti, M.S.[3]
Valentina Strollo, B.S.[3]
Eva Tibaldi, M.S.[3]
Andrea Vornoli, Ph.D.[3]

Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
John R. Bucher, Ph.D. (Retired)[1,9,10]
Michelle C. Cora, D.V.M.[9,10]
Scott A. Masten, Ph.D.[1,10]
Esra Mutlu, Ph.D. (currently at U.S. Environmental Protection Agency)[1,5,9,10]
Georgia K. Roberts, Ph.D.[1,10]
Kelly A. Shipkowski, Ph.D.[5,10]
Robert C. Sills, D.V.M., Ph.D.[3,10]
Matthew D. Stout, Ph.D.[1,5,10]
Suramya Waidyanatha, Ph.D.[1,5,9,10]

ICF, Reston, Virginia, USA
Contract GS00Q14OADU417 (Order No. HHSN273201600015U)
J. Wren Tracy, M.H.S.[9,10]

Contributors

Cesare Maltoni Cancer Research Center, Ramazzini Institute, Bologna, Italy
Subcontract to HHSN273201400015C
Annalisa Buscaroli, D.V.M.[3]
Fabiana Manservisi, Ph.D.[3]
Rita Montella, B.S.[3]
Giovanni Vecchi, D.V.M.[5]

Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Danica Andrews, B.S.[5]
Brian R. Berridge, D.V.M., Ph.D. (Retired)[3]
Milene L. Brownlow, Ph.D.[12]
Mark F. Cesta, D.V.M., Ph.D.[5]
Helen C. Cunny, Ph.D.[5]
Jennifer M. Fostel, Ph.D.[2,5]
Keith R. Shockley, Ph.D.[5]
Jason P. Stanko, Ph.D.[5]
Vicki L. Sutherland, Ph.D. (currently at Johnson & Johnson)[9]
Mary S. Wolfe, Ph.D.[12]

Experimental Pathology Laboratories, Inc., Research Triangle Park, North Carolina, USA
Contract HHSN273201500014C
Amy E. Brix, D.V.M., Ph.D.[3,9]

Battelle, Columbus, Ohio, USA
Contract HHSN273201400015C
Barney R. Sparrow, Ph.D., Principal Investigator[1,5,6]

Contract HHSN273201400027C
Brian L. Burback, Ph.D., Principal Investigator[5,6]
Kevin A. Carrico, B.A.[3]
Seth T. Gibbs, Ph.D.[3]
Elizabeth A. O'Dea, B.S.[3]
Jessica Pierfelice, B.S.[3]
Jeremy P. Smith, B.A.[3]
Billie Stiffler, Ph.D.[3]

Social & Scientific Systems, a DLH Company, Research Triangle Park, North Carolina, USA
Contract HHSN273201600011C
Sandra J. McBride, Ph.D., Principal Investigator[5,6]
Laura J. Betz, M.S.[2]
Shawn F. Harris, M.S.[2]

Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy
Teresa Gazzotti, Ph.D.[3]
Giampiero Pagliuca, Ph.D.[3]
Elisa Zironi, Ph.D.[3]

ASRC Federal, Research Triangle Park, North Carolina, USA
Contract HHSN316201200054W
Phyllis Brown, B.S.[2]
Hui Gong, M.S.[2]
Cari Martini, B.S.[2]
Christina Myers, M.S.[2]
Nicole Sayers, B.S.[2]

CSS Corporation, Research Triangle Park, North Carolina, USA
Contract HHSN273201500006C
Steven Brecher, Ph.D., Principal Investigator[5,6,11]
Sudha Iyer, B.S.[11]
Varghese Tharakan, D.V.M.[11]

ICF, Reston, Virginia, USA
Contract GS00Q14OADU417 (Order No. HHSN273201600015U)
Dave Burch, M.E.M., Principal Investigator[5,6]
Kathleen A. Clark, B.A.[10]
Sarah K. Colley, M.S.P.H.[10]
Katherine S. Duke, Ph.D.[10]
Hannah J. Eglinton, B.A.[10]
Lindsey M. Green, M.P.H.[12]
Tara Hamilton, M.S.[10]
Pamela A. Hartman, M.E.M.[10]
Cary E. Haver, M.P.H.[5]
Aishwarya Javali, M.S.[12]
Rachel C. McGill, B.S.[10]
Margaret E. McVey, Ph.D.[10]
Kevin O'Donovan, B.A.[10]
Ashley E. Peppriell, Ph.D.[10]
Lisa M. Prince, Ph.D.[10]
Karen E. Setty, Ph.D.[9]
Samantha J. Snow, Ph.D.[10]
Jessica A. Wignall, M.S.P.H.[5,6]

Author and Contributor Roles and Definitions[a]

No.	Role	Definition
1	Conceptualization	Ideas; formulation or evolution of overarching research goals and aims
2	Data Curation, Formal Analysis, and Software	Management activities to annotate (produce metadata), scrub, and maintain research data (including software code, when it is necessary for interpreting the data) for initial use and later reuse or Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data or Programming and software development; design of computer programs; implementation of computer code and supporting algorithms; testing of existing code components
3	Investigation	Conduct of the research/investigation process, specifically the performance of experiments or the collection of data/evidence
4	Methodology	Development or design of methodology; creation of models
5	Project Administration	Management and coordination responsibility for research planning and execution
6	Resources for Study Conduct	Provision of study materials, reagents, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools
7	Validation	Verification, whether as a part of the activity or separately, of the overall replication/reproducibility of results/experiments and other research outputs
8	Visualization	Preparation, creation, and/or presentation of the published work, specifically visualization/data presentation
9	Writing: Original	Preparation, creation, and/or presentation of the published work, specifically the writing of the initial draft (including substantive translation)
10	Writing: Review and Editing	Preparation, creation, and/or presentation of the published work by those from the original research group, specifically provision of substantive critical review, commentary, or revision—including pre- or post-publication stages
11	Quality Assessment	Conduct of independent assessments of accuracy, consistency, and completeness of various aspects of research products and their components, including data; identification of areas in the conduct and documentation of studies that merit correction or improvement of the description of methodologies
12	Peer Review and Production	Coordination and management of external peer review and publication, including identification of experts, conflict-of-interest screening, correspondence with reviewers, preparation of review documents, and publication activities

[a]Developed using the Contributor Roles Taxonomy (CRediT) framework.[1]

Peer Review

The Division of Translational Toxicology (DTT) at the National Institute of Environmental Health Sciences (NIEHS) conducted an external peer review of the draft *NIEHS Report on the Toxicity Studies of Nicotine Bitartrate Dihydrate (CASRN 6019-06-3) Administered in Drinking Water to Sprague Dawley Rats and Swiss Mice* by letter in February 2024 by the experts listed below. Reviewer selection and document review followed established DTT practices. The reviewers were charged to:

(1) Peer review the draft *NIEHS Report on the Toxicity Studies of Nicotine Bitartrate Dihydrate (CASRN 6019-06-3) Administered in Drinking Water to Sprague Dawley Rats and Swiss Mice*.

(2) Comment on DTT's interpretations of the data.

DTT carefully considered reviewer comments in finalizing this report.

Peer Reviewers

Alison Holloway, Ph.D.
Associate Chair
McMaster University
Hamilton, Ontario, Canada

Kamisha Johnson-Davis, Ph.D.
Associate Professor
University of Utah
Salt Lake City, Utah, USA

Wentai Luo, Ph.D.
Senior Research Associate
Portland State University
Portland, Oregon, USA

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Abstract

Nicotine contributes nearly all the alkaloid content of tobacco plants. Historically, human exposures were associated with smoking, chewing, or sniffing various forms of tobacco, and abundant literature details the adverse effects of chronic exposures to these tobacco products. Less attention has been given, however, to understanding the specific role nicotine plays in these adverse outcomes. Although recognized to possess potent pharmacological and addictive properties, nicotine is now delivered in electronic cigarettes and is a common ingredient in over-the-counter products designed to reduce or stop smoking, including gum and dermal patches.

Nicotine bitartrate dihydrate (NBD) salt was selected for rodent toxicity studies because it is used in nicotine-containing consumer products and is more stable than pure freebase (-)-nicotine. Additionally, no significant differences were observed between the salt and freebase forms in the evaluation of toxicokinetic parameters following oral exposure of rats. Following palatability testing, Sprague Dawley rat dams were exposed to NBD in drinking water ad libitum from gestation day (GD) 6 through lactation day (LD) 21. Upon weaning, pups were exposed to the same drinking water concentrations as their respective dams for 4 weeks or 3 months. Young adult male and female Swiss mice also were exposed to NBD in drinking water ad libitum for 4 weeks or 3 months.

Two-week Palatability Studies in Rats and Mice

Initial studies were conducted to evaluate the palatability of NBD to Sprague Dawley rats and Swiss mice exposed to 0, 6.25, 12.5, 25, 50, or 100 mg nicotine/L (mg/L) in drinking water for 14 days. In rats, the highest two exposure concentrations (50 and 100 mg/L) were considered unpalatable due to severely reduced body weight and water consumption for both sexes. In mice, water consumption was significantly decreased at 100 mg/L for both sexes, although it remained within animal welfare guidelines and was therefore selected as the highest exposure concentration for mice in the 4-week study.

Perinatal and Four-week Dose Range-finding Study in Rats

Beginning on GD 6, groups of F_0 time-mated female rats were exposed to NBD in drinking water throughout gestation and lactation at exposure concentrations of 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L. Groups of 10 F_1 rats per sex continued on study after weaning and were provided drinking water with the same respective NBD concentrations as their dams for 4 additional weeks. Although feed and water consumption by dams was reduced at the higher NBD concentrations, there were no significant effects of NBD exposure on pregnancy status, gestation, or parturition. However, during late lactation, a cluster of pup mortality was observed in exposed groups at NBD concentrations ≥3.12 mg/L. At necropsy, pups terminated early often presented with gross lesions in the intestine, which correlated histopathologically with moderate diffuse inflammation. In total, 42 pups died in the exposed groups at NBD concentrations ≥3.12 mg/L, whereas no mortality was observed in the control groups and in the group exposed to 1.56 mg/L during the same period (postnatal days [PNDs] 19–24). Therefore, exposure was suspended in all exposed groups approximately 4 days before weaning, after which the remaining pups survived to study termination. After resumption of initial exposures following weaning, no mortality occurred during the remainder of the study.

During the 4-week exposure period, body weights of male and female F_1 rats in the 12.5 and 25 mg/L groups were decreased by >10% relative to the control groups. Water consumption was

less than that of control rats for most exposed groups, with the 25 mg/L groups consuming on average <60% (females) and <70% (males) as much water as respective control groups. Although there were some organ weight changes, no histopathological correlation with those changes was observed.

While not significant, mild peritubular/focal kidney inflammation was observed in exposed males and females, as was minimal intestine inflammation in females and degenerative lesions of the testes in males in the 25 mg/L groups compared to control rats, the only groups other than early-death pups examined for histopathology.

Perinatal and Three-month Study in Rats

Beginning on GD 6, groups of F_0 dams were exposed to NBD in drinking water throughout gestation at exposure concentrations of 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L. During lactation, all exposed groups were administered 1.56 mg/L to avoid the late-lactation pup mortality observed in the perinatal and 4-week study. After weaning, groups of 10 F_1 rats/sex/exposure group were continued on study and were given drinking water containing the same respective concentrations as their dams for an additional 3 months.

There were no significant effects of NBD exposures on pregnancy status, gestation, or parturition, although water consumption by F_0 dams in the 25 mg/L group was below that of the control dams, and at GD 21 was 58% of that of the control group. No abnormal pup mortality occurred preweaning, and no mortality occurred postweaning. During lactation, body weights of male and female pups in most exposed groups were somewhat lower than the control pups. At the end of the 3-month study, body weights of male and female rats were again within 10% of control groups, although water consumption by the 25 mg/L groups was approximately 60% of that of the control groups.

Histopathological evaluation showed that minimal to mild inflammation in the kidney was significantly increased in males at 12.5 mg/L. While not significant, minimal inflammation of the intestine was observed in greater numbers in some exposed groups of males and females. The incidence of minimal chronic reactive mandibular lymphadenitis was significantly increased in males at 25 mg/L. A statistically significant increased trend of the total number of degenerative lesions of the testes and animal bearing lesions were observed in males. Degenerative lesions of the testes, a rare finding in this strain at this age, were observed only in the 12.5 and 25 mg/L groups, whereas no degenerative lesions were observed in the control groups and at the lowest exposures. No increases in lesions with clear toxicological significance were attributed to the NBD exposures.

Four-week Dose Range-finding Study in Mice

Groups of 10 male and 10 female mice were exposed to NBD in drinking water for 4 weeks at exposure concentrations of 0, 6.25, 12.5, 25, 50, or 100 mg/L. Additional groups of 10 male and 10 female mice were exposed to 0 or 100 mg/L in drinking water and then monitored for a 2-week recovery period after exposure. All exposure concentrations were well tolerated with no reductions in body weight or feed and water consumption. There were no significant differences in organ weights. Histopathological evaluation of mice exposed to the highest concentration (100 mg/L) showed higher incidences of minimal focal inflammation in the kidney in males and Harderian gland in females, although the incidences were not significant. Exposed female mice also had higher, nonsignificant incidences of minimal focal necrosis with inflammation in the liver compared to control mice.

Three-month Study in Mice

Groups of 15 male and 15 female mice were exposed to NBD in drinking water for 3 months at exposure concentrations of 6.25, 12.5, 25, or 50 mg/L, and groups of 30 male and 30 female mice were exposed to 0 or 100 mg/L. No mortality was observed during the 3-month study. All exposure concentrations were well tolerated, with slightly lower water consumption by the highest exposed group (100 mg/L). During histopathological evaluation, minimal inflammation was diagnosed in several organs in exposed and control mice; however, none of these lesions were considered of toxicological significance.

Summary

Under the conditions of these studies, other than reductions in feed and water consumption and resulting lower body weight gains at the higher NBD exposure concentrations, there were no clinical findings considered to be of clear toxicological significance for the NBD exposures in Sprague Dawley rats or Swiss mice. However, there was a high rate of mortality of rat pups exposed to NBD at concentrations ≥3.12 mg/L during the late-lactation phase of the perinatal and 4-week study that was unexpected and may be related to inhibition of normal cholinergic neural development by prenatal nicotine exposures, whereas no mortality was observed in the control groups and in the group exposed to 1.56 mg/L during the same period. This finding prompted a change in the design of the perinatal and 3-month study in rats to provide all groups of exposed lactating dams the same lowest NBD exposure concentration (1.56 mg/L), which effectively prevented pup mortalities during this apparently vulnerable developmental period. There were no histopathological findings considered to be of clear toxicological significance.

Synonyms: nicotine tartrate dihydrate; (S)-3-(1-methyl-2-pyrrolidinyl)pyridine (2R,3R)-2,3-dihydroxybutanedioate dihydrate

Summary of Findings Considered Toxicologically Relevant in Male and Female Rats and Mice Exposed to Nicotine Bitartrate Dihydrate in Drinking Water for Three Months

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Male Swiss Mice	Female Swiss Mice
Concentrations in Drinking Water	Gestation and postweaning: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L Lactation: 0 or 1.56 mg/L (all exposed groups)	Gestation and postweaning: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L Lactation: 0 or 1.56 mg/L (all exposed groups)	0, 6.25, 12.5, 25, 50, or 100 mg/L	0, 6.25, 12.5, 25, 50, or 100 mg/L
Survival Rates	10/10, 10/10, 10/10, 10/10, 10/10, 10/10	10/10, 10/10, 10/10, 10/10, 10/10, 10/10	30/30, 15/15, 15/15, 15/15, 15/15, 30/30	30/30, 15/15, 15/15, 15/15, 15/15, 30/30
Body Weights	F_1 generation: *Lactation:* ↓ Pup mean body weight from PND 17 to PND 28 (1.56, 3.12, 6.25, and 12.5 mg/L groups ≤90% of the control group) *Study termination:* Exposed groups within 10% of the control group	F_0 generation: *Gestation:* No effect *Lactation:* No effect F_1 generation: *Lactation:* ↓ Pup mean body weight from PND 17 to PND 28 (1.56, 3.12, and 6.25 mg/L groups ≤90% of the control group) *Study termination:* Exposed groups within 10% of the control group	Exposed groups within 10% of the control group	Exposed groups within 10% of the control group
Clinical Observations	None	None	None	None
Organ Weights	None	None	None	None
Nonneoplastic Effects	None	None	None	None
Clinical Pathology	None	None	None	None

PND = postnatal day; None = no effects for this endpoint.

Introduction

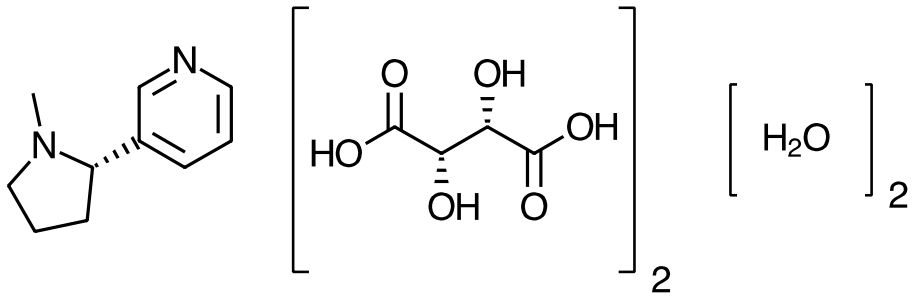

Figure 1. Nicotine Bitartrate Dihydrate (CASRN 6019-06-3; Chemical Formula: $C_{10}H_{14}N_2$•$2C_4H_6O_6$•$2H_2O$; Molecular Weight: 498.4 g/mol)

Synonyms: nicotine tartrate dihydrate; (S)-3-(1-methyl-2-pyrrolidinyl)pyridine (2R,3R)-2,3-dihydroxybutanedioate dihydrate.

Chemical and Physical Properties

Nicotine (CASRN 54-11-5) is a naturally occurring alkaloid and insecticide found in the solanaceous (nightshade) family of plants, which includes tobacco plants.[2] In tobacco leaves, nicotine contributes approximately 95% of the total alkaloid content.[3] While nicotine is a major component of cigarettes, it is also used therapeutically for smoking cessation. Nicotine in the tobacco plant exists mainly in the S-isomer form, with <1% of nicotine occurring as the R-isomer form. It is an oily, colorless to pale yellow liquid with a boiling point of 247°C and a molecular weight of 162.23 g/mol.[4] It is soluble in water (log P 1.17) below 60°C and is soluble in organic solvents (e.g., alcohol, chloroform, oils).[2; 5] When exposed to light or air, nicotine gradually turns brown, and when heated to decomposition, it emits toxic fumes (e.g., nitrogen oxide, carbon monoxide).[6; 7] Nicotine, in its tartrate salt form as bitartrate dihydrate (Figure 1; CASRN 6019-06-3), is a hygroscopic, white powder. It has a melting point of 97°C–100°C and a molecular weight of 498.4 g/mol.[8; 9]

Production, Use, and Human Exposure

Nicotine is primarily isolated from the leaves of two species of tobacco plants in the nightshade plant family, *Nicotiana tabacum* and *N. rustica*.[2] Human use of and exposure to nicotine generally comes from tobacco products, which contain different amounts of nicotine.[10] People are exposed while processing and extracting tobacco (green tobacco sickness), storing and applying certain insecticides (now rare), and using any tobacco product.[11] Nicotine constitutes one of the main compounds in tobacco products and electronic cigarettes and in chewing gums and other smoking cessation products.[12-14] Almost 77% of adult smokers started smoking before 18 years of age, and every day in the United States, nearly 2,300 youth smoke their first cigarette.[15] The global population of smokers is 1.1 billion, and approximately 80% live in low- and middle-income countries.[16] A person smoking 25 cigarettes per day will absorb approximately 0.43 mg nicotine/kg body weight (mg/kg) and reach a nicotine blood concentration in the range of 4–72 ng/mL (0.025–0.444 μM).[17; 18]

Concentrations of cotinine, the most abundant metabolite of nicotine in exposed mammals,[19] in active smokers are generally >10 ng/mL in plasma and >200 ng/mL in urine.[20] High

concentrations of cotinine, comparable to the ones found in active smokers, have been found in the urine of electronic cigarette users.[21]

Regulatory Status

Nicotine regulation is strongly connected with legislation affecting tobacco products and the emerging market for electronic cigarettes. In the United States, the U.S. Food and Drug Administration (FDA) has regulated cigarettes, smokeless tobacco, and roll-your-own tobacco since 2009 and recently finalized a rule to regulate all tobacco products, including electronic cigarettes, cigars, and hookah and pipe tobacco.[22] On July 28, 2017, FDA announced a new comprehensive plan that places the issue of nicotine addiction at the center of the Agency's tobacco regulation effort.[23] In Europe, tobacco products are regulated by Directive 2014/40/EU.[24]

Absorption, Distribution, Metabolism, and Excretion

The metabolism of nicotine is species specific. In humans, nicotine is extensively and rapidly metabolized by the liver to six primary metabolites and several minor metabolites. It is excreted, unchanged to a small degree (on average, approximately 5%), by the kidney. Quantitatively, the most abundant metabolite in mammalian species is cotinine; in humans, approximately 70%–80% of nicotine is converted to cotinine. Many mammalian species (e.g., mice, dogs, rabbits, monkeys) primarily metabolize nicotine to cotinine, as do humans, whereas rats and guinea pigs form as much nicotine-N′-oxide as cotinine and 3′-hydroxycotinine because of differences in the predominant cytochrome P450 enzymes.[19] In all species, the liver provides considerable first-pass metabolism of nicotine before it enters systemic circulation from the gastrointestinal tract following oral or intraperitoneal exposure. Nicotine plasma half-life in rodents is generally shorter than in primates (45 minutes in rats and 6–7 minutes in mice versus 2 hours in humans and nonhuman primates).[19] Therefore, absorption, distribution, metabolism, excretion and underlying kinetics are important processes to consider when designing animal experiments to achieve internal nicotine doses comparable to those expected in humans.[19]

Pharmacology and Associated Toxicities

The primary pharmacological action of nicotine results when it binds to presynaptic nicotinic cholinergic receptors in the central and peripheral nervous system and neuromuscular junctions. Nicotine is readily taken up from circulation into the brain, where exposure activates the receptor and where persistent exposure can desensitize or competitively block interaction with acetylcholine, the natural ligand. Binding affects activity of corticobasal ganglia-thalamic brain circuits, initiating a secondary cascade of neurotransmitter release—including dopamine, norepinephrine, acetylcholine, serotonin, γ-aminobutyric acid (GABA), glutamate, and endorphins—in the prefrontal cortex, thalamus, and visual system, leading to a myriad of physiological and behavioral effects.[25]

Physiological and toxic responses to nicotine are complex and dependent on dose. In low doses, nicotine increases heart rate, blood pressure, respiratory rate, and alertness. As doses increase, symptoms include headache, dizziness, confusion, anxiety, and tremors and can lead to lethargy, convulsions, and coma. A variety of cardiac dysrhythmias have been reported, and severe intoxications can result in respiratory paralysis.[26]

Nicotine's effects on the body also differ across lifespan. As reviewed in Ren et al.[27] nicotine can cross the placental barrier, leading to in utero exposure. Nicotine exposure in utero can affect fetal hemodynamics, which can lead to tissue hypoxia, restrict intrauterine and postnatal growth, and increase the risks of fetal morbidity and mortality. Testosterone production is inhibited in males, and both sexes exhibit an increase in motor, sensory, cognitive, and behavioral deficits at infant and toddler stages. Studies in rodents have demonstrated specific effects of nicotine on brain development in areas controlling cardiac and respiratory functions at levels below those resulting in general growth inhibition, and smoking during pregnancy is thought to be a major cause of sudden infant death syndrome, possibly from nicotine exposure.[28; 29]

Early exposure to nicotine results in increased reward responses to nicotine and other drugs of abuse in adolescence, along with learning and memory deficits and emotional dysregulation that can persist into adulthood. In contrast, the aging brain's exposure to nicotine is largely considered neuroprotective, delaying onset of Alzheimer's dementia and Parkinson's disease, and positively affecting attention, learning, and memory, as well as improving mood and reducing stress.[27] Linker et al.[30] demonstrated that nicotine affects brain microglia, which are involved in brain remodeling, differently in adolescent and adult rats. Microglia activation after nicotine exposure in adolescence results in a proinflammatory state, which has been suggested to restructure synaptic pruning patterns in reward-encoding brain regions. Nicotine exposure suppresses reactive microglia in the adult brain, however.

Risks for chronic cardiovascular and respiratory diseases and cancers are elevated for smokers, but the specific contribution of nicotine to these well-recognized smoking sequelae is unclear.[27] Data on the potential carcinogenicity of nicotine are sparse. Most nicotine studies in the AMES *Salmonella* assay (including urine of rats exposed to nicotine) were negative; however, nicotine induced DNA damage in the *Escherichia coli* pol A+/pol− test.[31] There is inadequate evidence of an association between nicotine exposure and carcinogenic effects in human studies.[32] In animal studies, Haussmann and Fariss concluded there was limited evidence suggesting an association between long-term nicotine exposure and a "lack of complete carcinogenic effects,"[32] and the International Agency for Research on Cancer does not classify nicotine as a carcinogen.[33] Tang et al.[34] more recently reported that electronic cigarette smoke was carcinogenic in FVB/N mice, causing lung adenocarcinomas, but no rodent cancer bioassays of a traditional design have been reported.

Study Rationale

Increasing human exposure to nicotine (in the absence of other tobacco smoke components) from electronic cigarettes and smoking cessation therapies prompted interest in more comprehensive rodent toxicology and cancer studies than was available in the existing literature. Nicotine bitartrate dihydrate (NBD) was selected as the test article because of its solubility, palatability, and use in nicotine-containing consumer products. Additionally, preliminary toxicokinetic information suggested that it results in systemic exposure similar to freebase (-)-nicotine. Lunell et al.[35] showed that the primary site of nicotine absorption from a nicotine inhaler was the oral mucosa; therefore, drinking water was selected as the route of administration to model the episodic human exposure from smoking or vaping. To consider the potential for exposure to nicotine during pregnancy, a perinatal component was included in the rat study. While human exposure likely includes nicotine exposure prior to conception, this study focused on post-implantation outcomes.

Materials and Methods

Toxicokinetic Studies for Test Article Selection

Limited toxicokinetic studies of freebase (-)-nicotine and nicotine bitartrate dihydrate (NBD) were performed using a single gavage administration to male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats, and systemic nicotine exposure was assessed to aid in test article selection. The in-life portion of the toxicokinetic studies was conducted at Battelle (West Jefferson, OH). Bulk chemical analysis, formulation preparation and analysis, and biological sample analyses were performed at Battelle (Columbus, OH). Rats received a single gavage administration of freebase (-)-nicotine or NBD in ASTM Type I water at a dose of 0.5 mg/kg. Because the tartrate salt crystallizes as the dihydrate, a monoisotopic mass of 498.2 g/mol was used for calculating freebase (-)-nicotine concentrations. Freebase (-)-nicotine represents 32.56% of the monoisotopic mass of the NBD compound. Additional information on the toxicokinetic studies is provided in Appendix B.

Procurement and Characterization of Freebase (-)-Nicotine

Nicotine as freebase (-)-nicotine was obtained in a single lot (1515L008) from Siegfried (Zofingen, Switzerland) via Interchem Corporation (Paramus, NJ). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Battelle (Columbus, OH) (Appendix A). Reports on analyses performed in support of these studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

Freebase (-)-nicotine (lot 1515L008), a colorless to yellow or brownish liquid, was identified using infrared (IR) spectroscopy, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, ^{13}C NMR spectroscopy, and high-performance liquid chromatography (HPLC) with mass spectrometry (MS). The IR spectrum was consistent with a reference spectrum, the NMR spectra were consistent with the predicted spectra of freebase (-)-nicotine, and the HPLC/MS spectra and fragmentation were consistent with nicotine. Elemental analysis was also performed by Galbraith Laboratories (Knoxville, TN) to confirm the composition.

Purity was determined using HPLC with ultraviolet (UV) detection, differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and gas chromatography with flame ionization detection (GC/FID). Moisture content was measured by Karl Fischer titration performed by Galbraith Laboratories, and the specific rotation and water analyses were performed at Exova Inc. (Santa Fe Springs, CA).

Karl Fisher titration yielded a water content of 0.18%. The specific rotation analysis indicated an average of −133.2°C, and the water analysis indicated 0.23% water content (Appendix A). DSC measured the boiling point as 244.9°C. One impurity, likely ethanol, identified in the ^{1}H NMR spectrum and quantified using GC/FID, was approximately 0.3% of the total area. Purity evaluation by HPLC/UV detection identified two impurity peaks representing 0.4% of the total area detected. The overall purity of lot 1515L0008 was determined to be >99% (Table 1).

Accelerated stability studies were conducted on the bulk chemical using HPLC/UV to measure purity after storage at approximately −20°C, 5°C, room temperature, and 60°C while protected

from light. Stability was maintained for at least 2 weeks at or below room temperature; therefore, the bulk test article was stored at 1°C–8°C and protected from light.

Table 1. Summary of Chemical Purity in the Toxicokinetic Study of Freebase (-)-Nicotine

Lot Number	Water (%)		Purity		
	KF	SRA	% Overall	Number of Detected Impurities	% Impurity
1515L008	0.18	0.23	>99	2	0.4

KF = Karl Fisher titration; SRA = specific rotation analysis.

Procurement and Characterization of Nicotine Bitartrate Dihydrate

NBD was obtained in a single lot (1531H012) from Siegfried (Zofingen, Switzerland) via Interchem Corporation (Paramus, NJ). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Battelle (Columbus, OH) (Appendix A). Reports on analyses performed in support of the NBD studies are on file at NIEHS.

The chemical identity of NBD (lot 1531H012), a white or almost-white powder, was confirmed using IR spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and HPLC/MS. The IR spectrum agreed with the structure of NBD, and NMR spectra were consistent with the predicted spectra of NBD. The HPLC/MS spectra and fragmentation were consistent with nicotine. Elemental analysis was also performed by Galbraith Laboratories (Knoxville, TN) to confirm the composition of NBD.

Purity was determined using HPLC with UV detection, DSC, and TGA. Moisture content was measured for the lot by Karl Fischer titration performed by Galbraith Laboratories, and the specific rotation and water analyses were performed at Exova Inc. (Santa Fe Springs, CA).

Karl Fisher titration yielded a water content of 8.97%. The specific rotation analysis indicated an average of +23.0°C, and the water analysis indicated 7.2% water content (Appendix A). DSC was attempted, but the nature of NBD (a dihydrate salt complex) resulted in broad duplicate overlapping peaks, and accurate purity and melting points could not be determined. No impurities were detected using HPLC/UV or TGA (Table 2).

Accelerated stability studies were conducted on the bulk chemical using HPLC/UV to measure purity after storage at approximately −20°C, 5°C, room temperature, and 60°C while protected from light. Stability of NBD was maintained when sealed, protected from light, and stored for 2 weeks at ≤60°C. Thus, the bulk test article was stored at room temperature and protected from light.

Table 2. Summary of Chemical Purity in the Toxicokinetic, Four-week, and Three-month Studies of Nicotine Bitartrate Dihydrate

Lot Number	Water (%)		Purity		
	KF	SRA	% Overall	Number of Detected Impurities	% Impurity
1531H012	8.97	7.2	100	0	<0.1

KF = Karl Fisher titration; SRA = specific rotation analysis.

Preparation and Analysis of Drinking Water Formulations for the Palatability, Four-week, and Three-month Studies

Dose formulations for the 14-day (palatability) and 4-week studies were prepared at the Ramazzini Institute (Bologna, Italy) similarly to those for the 3-month studies but were not analyzed. The drinking water formulations for the 3-month studies were prepared weekly at the Ramazzini Institute (Bologna, Italy) by mixing NBD with tap water to give the desired concentrations for each species. All concentrations are given in mg nicotine/liter (mg/L). For the rat study, formulations were prepared at concentrations of 0, 1.56, 3.12, 6.25, 12.5, and 25 mg/L. For the mouse study, formulations were prepared at concentrations of 0, 6.25, 12.5, 25, 50, and 100 mg/L. Drinking water formulations were stored at room temperature in high-density polyethylene (HDPE) plastic bottles covered with black plastic foil to avoid light exposure and dispensed in dark glass bottles daily to the animals. Aliquots (from three different time points) of each preadministration formulation dose were frozen and sent in an insulated container to Battelle (Columbus, OH) for analysis, where they were stored at −30°C to −15°C until analyzed.

Select NBD formulations from the perinatal and 3-month rat study and the 3-month mouse study were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a qualified method. The results of preadministration formulation analyses for the 3-month studies are shown in Table A-6 (rats) and Table A-7 (mice). No postadministration samples were analyzed. The concentrations of the NBD formulations analyzed prior to exposure were within 10% of the target concentrations, except for the 1.56 mg/L formulations from the perinatal and 3-month rat study received on April 27, 2017, and May 25, 2017, which were 59.0% and 15.7% above target, respectively. All rat formulations had relative standard deviation (RSD) values within 5%, except for the 6.25 mg/L formulation from the first shipment, which had an RSD value of 6.6%.

Multiple formulations had results outside the acceptance criteria for accuracy and precision (average concentration within 10% of the target concentration and RSD values within 5%). Several mouse formulations used in the study had RSD values outside 5% (ranging from 6.2% to 9.2%). These deviations were not considered to significantly affect the studies.

Animal Source and Welfare

Male and female Sprague Dawley rats and Swiss mice were obtained from the Cesare Maltoni Cancer Research Center colony (Bologna, Italy). Sprague Dawley rats and Swiss mice were selected for these studies based on familiarity and historical information by the study laboratory, and the authors consider them to be good models for general toxicology evaluations including a perinatal design. Animal care and use were in accordance with the Italian law regulating the use and humane treatment of animals for scientific purposes (Decreto Legislativo N. 26, 2014, which adopts European Union Directive 2010/63/EU). Study protocols were examined, approved, and formally authorized by the Italian Ministry of Health.

Palatability Study and Exposure Concentration Selection Rationale

For the palatability study in male and female Sprague Dawley rats and Swiss mice, selection of 0, 6.25, 12.5, 25, 50, or 100 mg/L exposure concentrations (as NBD) for up to 14 days was

informed by literature on the range of nicotine and cotinine concentrations in serum of heavy smokers or electronic cigarette users. Smoking one cigarette per day yields about 1 mg of absorbed nicotine; therefore, a 70 kg person would take in 0.0143 mg nicotine/kg/day.[36] Using five exposure concentrations and threefold spacing, the concentrations selected were 0.0143, 0.0429, 0.143, 0.429, and 1.287 mg freebase (-)-nicotine/kg (equivalent to 1, 3, 10, 30, and 90 cigarettes) based on a 70 kg person.

The palatability study provided preliminary information for the subsequent 4-week study in Sprague Dawley rats and Swiss mice exposed to NBD in drinking water. There were no exposure-related deaths or gross or microscopic findings in male or female rats in the palatability study at exposure concentrations of 6.25, 12.5, 25, or 50 mg/L. The highest exposure concentration of 100 mg/L was unpalatable for rats. In addition, exposure to 50 mg/L induced a significant reduction in final mean body weight and an approximately 50% reduction in final mean water consumption; therefore, it was not considered palatable. All other exposure concentrations (6.25, 12.5, and 25 mg/L) were considered palatable for rats. Therefore, the exposure concentrations selected for the perinatal and 4-week study in rats were 0, 1.56, 3.12, 6.25, 12.5, and 25 mg/L. All exposure concentrations (0, 6.25, 12.5, 25, 50, and 100 mg/L) were considered palatable for mice. Exposure concentrations of NBD equivalent to 0, 6.25, 12.5, 25, 50, and 100 mg/L of drinking water were selected for the 4-week study in mice.

Four-week Dose Range-finding Studies

F_0 female Sprague Dawley rats were 17 weeks old when the experiment started. They were mated and quarantined for 7 days and were approximately 20 weeks old on the first day of exposure. Gestation day (GD) 0 was defined as the first day with evidence of mating. Siblings were allocated to separate groups, and groups were normalized to attain similar group average body weights before mating. Randomization was performed using a random number generator. Beginning on GD 6, groups of five F_0 female rats were provided NBD in drinking water throughout gestation and lactation at the following concentrations: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L; tap water served as the control. Due to high pup mortality observed during lactation, administration of NBD was interrupted, and all animals were given tap water instead of NBD in drinking water from approximately 4 days before weaning until the start of the 4-week study. Lactation day (LD) 21 was the last lactation day all animals were exposed. Feed and dosed water were available ad libitum.

F_0 female rats were housed individually during gestation and with their respective litters during lactation. The day of parturition was considered LD 0 for dams and postnatal day (PND) 0 for pups. F_0 female rats were weighed on GDs 5, 7–12, 15, 18, and 21 and LDs 1, 4, 7, 14, and 21. Feed and water consumption were also measured on these days. Pup body weights by sex and litter were recorded on PNDs 4, 7, 14, and 21.

Due to increased litter mortality, pups were weaned between PND 26 and PND 29 starting on PND 26 of the last litter that was born. Weaning marked the beginning of the 4-week study. Beginning on the day the last rat litter reached PND 30, the F_1 rats were provided NBD in drinking water for 4 weeks at the following concentrations: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L; tap water served as the control. The control and high exposure groups (0 and 25 mg/L, respectively) consisted of 20 male and 20 female rats each. All other exposed groups (1.56, 3.12, 6.25, and 12.5 mg/L) consisted of 10 male and 10 female rats each. Pups were provided the same

concentrations of NBD in drinking water as were provided to their respective dams during gestation. Dams were euthanized according to Ramazzini Institute protocols. Necropsy was not performed on F_0 dams. Feed and dosed water were available ad libitum. F_1 rats were housed two to three animals/sex/cage. Cages were periodically rotated. Details of the study design and animal maintenance are summarized in Table 3.

Swiss mice were 10 weeks old when the experiment started. They were quarantined for 7 days and were approximately 11 weeks old on the first day of exposure. Mice were provided NBD in drinking water for 4 weeks at the following concentrations: 0, 6.25, 12.5, 25, 50, or 100 mg/L; tap water served as the control. The control and high exposure groups (0 and 100 mg/L, respectively) consisted of 20 male and 20 female mice each. All other exposed groups (6.25, 12.5, 25, and 50 mg/L) consisted of 10 male and 10 female mice each. Feed and dosed water were available ad libitum. Mice were housed up to five/sex/cage. Cages were periodically rotated. Details of the study design and animal maintenance are summarized in Table 3.

During the 4-week studies, rats and mice were observed three times daily for signs of mortality or moribundity, except Sundays and nonworking days when they were observed twice daily. Clinical observations, body weights, and daily feed and water consumption were recorded initially, weekly thereafter, and at study termination. After the 4 weeks of exposure, 10 animals per sex in the control and high (25 mg/L for rats; 100 mg/L for mice) exposure groups were monitored without exposure for a 2-week recovery period.

At the end of the 4-week studies, animals were anesthetized with a carbon dioxide/oxygen mixture, blood was collected from the caudal vena cava of 10 rats/sex/group and 5 mice/sex/group for hematology and clinical chemistry, and the animals were then euthanized via exsanguination. Blood was collected into tubes containing tripotassium ethylenediaminetetraacetic acid (K_3EDTA) for hematology and into tubes containing lithium heparin for clinical chemistry. Hematological parameters were measured using a Procyte Dx hematology analyzer (IDEXX), and clinical chemistry parameters were measured using a Catalyst DX clinical chemistry analyzer (IDEXX). Urine samples were collected from 10 rats/sex/group for standard urinalysis following approximately 16 hours in individual metabolic cages. Urine specific gravity was measured using a Euromax hand refractometer RF312. All other urine endpoints were measured with Combur10 test M urine test strips (Roche) and a Urisys 1800 semi-automated urinalysis system (Roche). The clinical pathology parameters measured are listed in Table 3.

Necropsies were performed on all F_1 male and female rats and all mice. Organ weights were determined for the adrenal gland, bladder, brain (with cerebellum and medulla/pons), heart, kidneys, liver, mediastinal lymph nodes, ovaries, prostate, seminal vesicle (with coagulating gland), spleen, testes and epididymides, thymus, and uterus (with cervix) from all rats and mice. Tissues for microscopic examination were fixed and preserved in 70% alcohol, processed, trimmed, embedded in paraffin, sectioned to a thickness of 3–6 μm, and stained with hematoxylin and eosin (H&E). Complete histopathological examinations were performed by the study laboratory pathologist on 10 animals from the control male and female groups and 10 animals from the high exposure male and female groups. Table 3 lists the tissues and organs examined.

Three-month Studies

Study Design in Rats

F_0 female Sprague Dawley rats were 17 weeks old when the experiment started. They were mated and quarantined for 7 days and were approximately 20 weeks old on the first day of exposure. GD 0 was defined as the first day with evidence of mating. Siblings were allocated to separate groups, and groups were normalized to attain similar group average body weights before mating. Randomization was performed using a random number generator. Beginning on GD 6, groups of eight F_0 female rats were provided NBD in drinking water throughout gestation at the following concentrations: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L; tap water served as the control. Throughout lactation, all groups, apart from the control group, were provided NBD in drinking water at a concentration of 1.56 mg/L due to the unexpected pup mortality observed during lactation in the 4-week study at all higher exposure concentrations. Feed and dosed water were available ad libitum.

F_0 female rats were housed individually during gestation and with their respective litters during lactation. F_0 female rats were weighed on GDs 5, 7, 9, 12, 15, 18, and 21; LDs 1, 4, 7, 10, 14, 17, 21, and 24; and at weaning. Feed and water consumption were also measured on those days. The day of parturition was considered LD 0 for dams and PND 0 for pups. Pup body weights by sex and litter were recorded on PNDs 1, 4, 7, 10, 14, 17, 21, 24, and at weaning.

On the day the last litter reached PND 28, pups were weaned, and 10 animals/sex/group were randomly selected and housed 2 or 3/sex/cage. Weaning marked the beginning of the 3-month study. After weaning, groups of 10 male and 10 female F_1 rats were provided NBD in drinking water for 3 months at the following concentrations: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L; tap water served as the control. Pups were provided the same concentration of NBD in drinking water as their respective dams received during gestation. Dams were euthanized according to Ramazzini Institute protocols. Necropsy was not performed on dams. Feed and dosed water were available ad libitum. Cages were periodically rotated. Details of the study design and animal maintenance are summarized in Table 3.

Study Design in Mice

Swiss mice were 11 weeks old when the experiment started. They were quarantined for 7 days and were approximately 11 weeks old on the first day of exposure. Mice were provided NBD in drinking water for 3 months at the following concentrations: 0, 6.25, 12.5, 25, 50, or 100 mg/L; tap water served as the control. The control and high exposure groups (0 and 100 mg/L, respectively) consisted of 30 male and 30 female mice each. Satellite groups of 15 male and 15 female mice in the control and high exposure groups were included for in-life observations. All other exposed groups (6.25, 12.5, 25, and 50 mg/L) consisted of 15 male and 15 female mice each. Feed and dosed water were available ad libitum. Mice were housed up to five/sex/cage. Cages were periodically rotated. Details of the study design and animal maintenance are summarized in Table 3.

Clinical Examinations and Pathology

During the 3-month studies, rats and mice were observed three times daily for signs of mortality or moribundity, except Sundays and nonworking days when they were observed twice daily.

Clinical observations, body weights, and daily feed and water consumption were recorded initially, weekly thereafter, and at study termination.

At the end of the 3-month studies, animals were anesthetized with a carbon dioxide/oxygen mixture, blood was collected from the caudal vena cava of 10 rats/sex/group and 5 mice/sex/group for hematology and clinical chemistry, and the animals were then euthanized via exsanguination. Blood was collected into tubes containing K_3EDTA for hematology and into tubes containing lithium heparin for clinical chemistry. Hematology parameters were measured using a Procyte Dx hematology analyzer (IDEXX), and clinical chemistry parameters were measured using a Catalyst DX clinical chemistry analyzer (IDEXX). Urine samples were collected from 10 rats/sex/group for standard urinalysis. Urine specific gravity was measured using a Euromax hand refractometer RF312. All other urine endpoints were measured with Combur10 test M urine test strips (Roche) and a Urisys 1800 semi-automated urinalysis system (Roche). The clinical pathology parameters measured are listed in Table 3.

Necropsies were performed on all F_1 male and female rats and all mice (excluding satellite groups). Organ weights were determined for the adrenal gland, bladder, brain (with cerebellum and medulla/pons), heart, kidneys, liver, mediastinal lymph nodes, ovaries, prostate, seminal vesicle (with coagulating gland), spleen, testes and epididymides, thymus, and uterus (with cervix) from all rats and mice. Tissues for microscopic examination were fixed and preserved in 70% alcohol, processed, trimmed, embedded in paraffin, sectioned to a thickness of 3 to 6 µm, and stained with H&E. Complete histopathological examinations were performed by the study laboratory pathologist on all animals. Table 3 lists the tissues and organs examined. Peer review of the pathology observations, conducted by the Division of Translational Toxicology (DTT) pathologists, focused on outcomes from the 3-month studies with select review of observations from the 4-week studies to confirm temporal consistencies.

Internal Concentration Assessment

Following NBD exposure via drinking water for 3 months, plasma and urine samples were collected from rats and mice, stored at −70°C, and shipped to Battelle (Columbus, OH) for analysis of nicotine and cotinine concentrations. On PND 90, all animals (10 rats/sex/group, 30 mice per sex for the control and high exposure groups and 15 mice per sex for all other exposed groups) were placed individually in metabolic cages for approximately 16 hours. During this time, animals had free access to the drinking water formulations and feed. The following morning, urine and plasma samples were collected from 10 rats/sex/group and 5 randomly selected mice/sex/group. Animals were anesthetized by inhalation using a mixture of carbon dioxide and oxygen (70% and 30%, respectively), blood was collected from the cava vein into tubes containing K_3EDTA, and the animals were then euthanized via exsanguination. The samples were gently inverted for 30 seconds to mix the contents and centrifuged at 1,500 rpm for 10 minutes at 4°C. Plasma was harvested (two aliquots, approximately 150 µL each) and stored at −70°C until analysis.

Plasma and urine study samples from rats and mice, calibration standards, blanks, and quality control samples were processed by a solid-phase extraction, eluting with 5% ammonium hydroxide in methanol, and analyzed by LC-MS/MS (Table 3; Appendix C). The urine samples were analyzed for creatinine. Additionally, the samples were screened for nicotine metabolites, including trans-3′-hydroxy cotinine, (S)-cotinine N-oxide, cotinine N-β-D-glucuronide, (R,S)-

norcotinine, (R,S)-nornicotine, trans-3′-hydroxy cotinine O-β-D-glucuronide, (1′S,2′S)-nicotine 1′-oxide, and nicotine N-β-D-glucuronide.

Table 3. Experimental Design and Materials and Methods in the Four-week and Three-month Drinking Water Studies of Nicotine Bitartrate Dihydrate

Four-week Studies	Three-month Studies
Study Laboratory	
Cesare Maltoni Cancer Research Center, Ramazzini Institute (Bologna, Italy)	Same as in 4-week studies
Strain and Species	
Rats: Sprague Dawley	Same as in 4-week studies
Mice: Swiss	
Animal Source	
Cesare Maltoni Cancer Research Center (Bologna, Italy)	Same as in 4-week studies
Time Held Before Studies	
F_0 female rats: 7 days	Same as in 4-week studies
Mice: 7 days	
Average Age When Exposure Began	
F_0 female rats: 20 weeks	Same as in 4-week studies
Mice: 11 weeks	
Date of First Exposure	
F_0 female rats: October 31, 2016	F_0 female rats: April 25, 2017
Mice: October 31, 2016	Mice: February 21, 2017
Duration of Exposure	
F_0 female rats: GD 6 through LD 21 (halted ~4 days prior to weaning due to high pup mortality)	F_0 female rats: GD 6 through LD 28
F_1 rats: Perinatal plus 4 weeks	F_1 rats: Perinatal plus 13 weeks
Mice: 4 weeks	Mice: 13 weeks
Date of Last Exposure	
F_0 female rats: January 18, 2017	F_0 female rats: September 14, 2017
Mice: November 29, 2016	Mice: May 24, 2017
Necropsy Dates	
F_1 rats: January 16–19, 2017	F_1 rats: September 11–15, 2017
F_1 rats (recovery): January 30–31, 2017	
Mice: November 29–30, 2016	Mice: May 22–25, 2017
Mice (recovery): December 12, 2016	
Average Age at Necropsy	
F_1 rats: 8 weeks	F_1 rats: 17 weeks
Mice: 15 weeks	Mice: 24 weeks

Four-week Studies	Three-month Studies
Size of Study Groups	
F_0 female rats: 5	F_0 female rats: 8
F_1 rats: 10/sex for the 4-week exposure (0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L) plus 10/sex for the 4-week exposure with 2-week recovery period (0 and 25 mg/L)	F_1 rats: 10/sex
Mice: 10/sex for the 4-week exposure (0, 6.25, 12.5, 25, 50, or 100 mg/L) plus 10/sex for the 4-week exposure with 2-week recovery period (0 and 100 mg/L)	Mice: 30/sex (0 and 100 mg/L); 15/sex (6.25, 12.5, 25, 50 mg/L)
Animals per Cage	
F_1 rats: 2 to 3/sex	Same as in 4-week studies
Mice: Up to 5/sex	
Method of Animal Identification	
F_0 female rats: Cage card	Same as in 4-week studies
F_1 rats: Cage card and ear punch	
Mice: Cage card and ear punch	
Diet	
Pelleted feed (Laboratorio Dottori Piccioni Srl, Gessate, Milan, Italy), available ad libitum, changed every 2–3 days	Same as in 4-week studies
Water	
Tap water (Bologna municipal supply), either untreated or containing a formulation of nicotine bitartrate dihydrate via dark glass bottles, available ad libitum, changed daily	Same as in 4-week studies
Cages	
During exposure: polycarbonate cages (41 × 25 × 18 cm) with stainless-steel wire tops, rotated periodically	Same as in 4-week studies
After exposure: polycarbonate and polymethylpentene metabolic cages (Techniplast, Buguggiate, Italy)	
Bedding	
Shallow layer of white wood shavings, changed every 2–3 days	Same as in 4-week studies
Animal Room Environment	
Temperature: 22°C ± 3°C Relative humidity: 50% ± 20% Natural and artificial light: 12 hours/day Room air changes: 10/hour	Same as in 4-week studies

Toxicity Studies of Nicotine Bitartrate Dihydrate Administered in Drinking Water to Sprague Dawley Rats and Swiss Mice

Four-week Studies	Three-month Studies
Exposure Concentrations	
Rats: 0, 1.56, 3.12, 6.25, 12.5, or 25 mg/L in drinking water available ad libitum; from approximately 4 days before weaning until the start of the 4-week study, animals in all exposure groups were given untreated tap water	Rats: 0, 1.56, 3.12, 6.25, 12.5 or 25 mg/L in drinking water available ad libitum; dams in all exposed groups received only 1.56 mg/L during lactation
Mice: 0, 6.25, 12.5, 25, 50, or 100 mg/L in drinking water available ad libitum	Mice: Same as in 4-week studies
Type and Frequency of Observation	
F_0 female rats: Observed three times daily, except Sundays and nonworking days when they were observed twice daily; daily feed and water consumption and body weights were measured on GDs 5, 7–12, 15, 18, and 21; LDs 1, 4, 7, 14, and 21.	F_0 female rats: Observed three times daily, except Sundays and nonworking days when they were observed twice daily; daily feed and water consumption and body weight were measured on GDs 5, 7, 9, 12, 15, 18, and 21; LDs 1, 4, 7, 10, 14, 17, 21, 24, and at weaning.
F_1 rats: Observed three times daily, except Sundays and nonworking days when they were observed twice daily; clinical observations were recorded initially and at least weekly thereafter until study termination; body weights were measured by sex and litter on PNDs 4, 7, 14, and 21; individual body weights were measured the first day after weaning, then weekly thereafter until study termination; daily feed and water consumption and body weights were measured per cage the first day after weaning and then weekly thereafter until study termination; water consumption in metabolic cages was measured at study termination.	F_1 rats: Observed three times daily, except Sundays and nonworking days when they were observed twice daily; clinical observations were recorded initially and at least weekly thereafter until study termination; body weights were measured by sex and litter on PNDs 1, 4, 7, 10, 14, 17, 21, 24, and at weaning; individual body weights were measured the first day after weaning, then weekly thereafter until study termination; daily feed and water consumption and body weights were measured per cage the first day after weaning and then weekly thereafter until study termination; water consumption in metabolic cages was measured at study termination.
During the recovery period, rats in the control and 25 mg/L recovery groups were observed three times daily except Sundays and nonworking days when they were observed twice daily; clinical observations were recorded initially and weekly thereafter until study termination; individual daily feed and water consumption and body weights were measured on the first day of water administration and weekly thereafter until study termination.	

Four-week Studies	Three-month Studies
Mice: Observed three times daily, except Sundays and nonworking days when they were observed twice daily; clinical observations were recorded initially and at least weekly thereafter until study termination; daily feed and water consumption and body weights were measured on day 1, day 3, and then weekly until study termination.	Mice: Observed three times daily, except Sundays and nonworking days when they were observed twice daily; clinical observations were recorded initially and at least weekly thereafter until study termination; daily feed and water consumption and body weights were measured on day 0, day 1, and then weekly until study termination.
During the recovery period, mice in the control and 100 mg/L recovery groups were observed three times daily except Sundays and nonworking days when they were observed twice daily; clinical observations were recorded initially and weekly thereafter until study termination; individual daily feed and water consumption and body weights were measured on the first day of water administration and weekly thereafter until study termination.	
Method of Euthanasia	
Exsanguination while under CO_2/O_2 anesthesia	Same as in 4-week studies
Necropsy	
Necropsies were performed on all F_1 rats and all mice. Organs weighed at study termination were adrenal gland, bladder, brain (with cerebellum and medulla/pons), heart, kidneys, liver, mediastinal lymph nodes, ovaries, prostate, seminal vesicle (with coagulating gland), spleen, testes and epididymides, thymus, and uterus (with cervix).	Same as in 4-week studies
Clinical Pathology	
F_1 rats: At the end of the study, blood and urine were collected from 10 male and 10 female rats per group for clinical chemistry, hematology, and urinalysis. Following the 2-week recovery period, blood was collected from 10 male and 10 female rats from only the control and high exposure groups for clinical chemistry, hematology, and urinalysis.	F_1 rats: At the end of the study, blood and urine were collected from 10 male and 10 female rats per exposure group for clinical chemistry, hematology, and urinalysis.
Hematology: Erythrocyte count, mean corpuscular volume, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count and differential, reticulocyte count, platelet count, red cell distribution width, platelet distribution width, mean platelet volume, and plateletcrit.	*Hematology:* Same as in 4-week studies
Clinical chemistry: Sodium, potassium, chloride, glucose, inorganic phosphates, calcium, globulins, total cholesterol, triglycerides, blood urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, gamma glutamyl transferase, alkaline phosphatase, and total bilirubin.	*Clinical chemistry:* Same as in 4-week studies
Urinalysis: Appearance, volume, specific gravity, pH, total protein, glucose, ketone bodies, urobilinogen, bilirubin, and occult blood.	*Urinalysis:* Appearance, volume, specific gravity, pH, total protein, glucose, ketone bodies, urobilinogen, bilirubin, occult blood, and creatinine.

Four-week Studies	Three-month Studies
Mice: At the end of the 4-week exposure, blood was collected for clinical chemistry and hematology from 5 male and 5 female mice randomly selected from each exposure group. Following the 2-week recovery period, blood was collected for clinical chemistry and hematology from 5 male and 5 female mice randomly selected from the control and high exposure groups.	Mice: At the end of the study, blood was collected for clinical chemistry and hematology from 10 male and 10 female mice randomly selected from each exposure group.
Hematology: Erythrocyte count, mean corpuscular volume, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count and differential, reticulocyte count, platelet count, red cell distribution width, platelet distribution width, mean platelet volume, and plateletcrit.	*Hematology:* Same as in 4-week studies
Clinical chemistry: Sodium, potassium chloride, glucose, inorganic phosphates, calcium, globulins, total cholesterol, triglycerides, blood urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, gamma glutamyl transferase, alkaline phosphatase, and total bilirubin.	*Clinical chemistry:* Same as in 4-week studies
Urinalysis: None	*Urinalysis:* Creatinine
Histopathology	
Complete histopathology was performed on control and high exposure group animals. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bladder, gallbladder (mice only), brain (with cerebellum and medulla/pons), cranium, esophagus, Harderian gland, heart, kidneys, large intestine (with the Peyer's patches), liver (caudate and main), lungs, lymph nodes (mediastinal, mesenteric, subcutaneous), mammary gland (axillary and inguinal, right and left), ovaries, pancreas, parathyroid, pituitary gland, prostate, salivary gland, seminal vesicle (with coagulating gland), skeletal muscle of the leg (with sciatic nerve), skin and subcutaneous tissue, small intestine, spinal cord (cervical, thoracic, and lumbar), spleen, sternum (bone marrow), stomach (forestomach and glandular), testes and epididymides, thymus, thyroid gland, tongue, trachea, uterus (with cervix), and vagina.	Complete histopathology was performed on all animals. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bladder, brain (with cerebellum and medulla/pons), cranium, esophagus, Harderian gland, heart, kidneys, large intestine (with the Peyer's patches), liver (caudate and main), lungs, lymph nodes (mediastinal, mesenteric, subcutaneous), mammary gland (axillary and inguinal, right and left), ovaries, pancreas, parathyroid, pituitary gland, prostate, salivary gland, seminal vesicle (with coagulating gland), skeletal muscle of the leg (with sciatic nerve), skin and subcutaneous tissue, small intestine, spinal cord (cervical, thoracic, and lumbar), spleen, sternum (bone marrow), stomach (forestomach and glandular), testes and epididymides, thymus, thyroid gland, tongue, trachea, uterus (with cervix), and vagina.
Internal Concentration Assessment	
Not reported	F_1 rats: Plasma and urine concentrations of nicotine and cotinine were measured in all animals (10/sex/group) at study termination, following a 16-hour period in metabolic cages during which animals had free access to feed and the assigned group drinking water formulations.

Four-week Studies	Three-month Studies
	Mice: Plasma and urine concentrations of nicotine and cotinine were measured in five randomly selected animals/sex/group at study termination, following a 16-hour period in metabolic cages during which animals had free access to feed and the assigned group drinking water formulations.

GD = gestation day; LD = lactation day; PND = postnatal day.

Statistical Methods

Statistical methods were chosen based on distributional assumptions as well as on the need to incorporate within-litter correlation among animals (for the perinatal rat studies). For F_1 animal data during the lactation phase, data were summarized as a mean of litter means to appropriately account for within-litter correlation prior to performing statistical analysis. After weaning, data were summarized by individual animals. Unless specifically mentioned, all endpoints were tested for a trend across exposure groups, followed by pairwise tests for each exposed group against the control group. Significance of all trend and pairwise tests is determined by a p value of ≤ 0.05 and is reported at both 0.05 and 0.01 levels.

Calculation and Analysis of Nonneoplastic Lesion Incidences

The incidences of nonneoplastic lesions are presented as numbers of animals bearing such lesions at a specific anatomical site and the numbers of animals with that site examined microscopically. Fisher's exact test,[37] a procedure that uses the overall proportion of affected animals, was used to determine significance between exposed and control animals. In the perinatal and 3-month studies, the Cochran-Armitage linear trend test was used to test for significant trends for select lesion categories.[38]

Analysis of Continuous Variables

Body weight, body weight gain, water and feed consumption, and clinical pathology data, including organ weight, hematology, and clinical chemistry, were statistically investigated through one-way analysis of variance (ANOVA) followed by the Dunnett test (when applicable).[39]

For the perinatal and 3-month studies in rats and mice, urine creatinine and internal concentration assessment data were analyzed using the nonparametric multiple comparison methods of Shirley[40] (as modified by Williams[41]) and Dunn.[42] The Jonckheere test[43] was used to assess the significance of the exposure-related trends and to determine whether a trend-sensitive test (the Shirley test) would be more appropriate for pairwise comparisons than a test that does not assume a monotonic exposure-related trend (the Dunn test). Before statistical analysis, outliers identified using the Dixon and Massey test[44] were examined by NIEHS personnel, and biologically implausible values (likely due to experimental error) were eliminated from the analysis. When individual concentration values were provided as "below the limit of detection" (LOD), one-half the LOD was used as a substitute value. However, if 80% or more of the values in the control group were below the LOD, the mean was reported as "BD" to indicate the values were "below detection" and no statistical analysis was performed on the endpoint.

Quality Assurance Methods

An internal quality assurance unit at the Ramazzini Institute monitored scientific, operative, and structural requirements, including experimental conduct, data recording, analysis and storage of samples, personnel qualifications, and adequacy of all equipment used. A separate audit of the draft NIEHS Report was conducted for internal consistency and accuracy compared to supplemental data sources.

Results

Data Availability

All study data were evaluated. Data relevant for evaluating toxicological findings are presented here. All study data are available in the Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NIEHS-DATA-NIEHS-11.[45]

Two-week Palatability Study in Rats and Mice

All rats and mice completed the 2-week palatability study of nicotine bitartrate dihydrate (NBD) except for rats exposed to 100 mg nicotine/L (mg/L), which were euthanized after 24 hours in compliance with animal welfare guidelines because of severe reductions in water consumption (78% in males and 88% in females) and mean body weight (7% in males and 12% in females) (Appendix D). All NBD dose formulations refer to the freebase (-)-nicotine concentrations. No exposure-related gross or microscopic lesions occurred in exposed rats or mice. Final mean body weight was significantly decreased in male and female rats exposed to 50 mg/L, and final mean water consumption was significantly decreased in male and female rats exposed to 50 mg/L and female rats exposed to 12.5 and 25 mg/L. Water consumption was significantly decreased by mice at most exposure concentrations, although the study still complied with animal welfare guidelines. In conclusion, exposure to NBD in drinking water at concentrations up to 25 mg/L (rats) or 100 mg/L (mice) for 2 weeks was well tolerated, and the solutions were considered palatable.

Rats

Perinatal and Four-week Dose Range-finding Study (Perinatal Phase)

Exposure to NBD had no effects on the percentages of dams with litters, gestation length, or litter size (Table 4). On postnatal day (PND) 1, litter sizes for exposed groups were 74%–97% of the control group; however, these decreases were not exposure-related (Table 4).

Table 4. Summary of the Disposition of Rats during Perinatal Exposure in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
F_0 Reproductive Performance						
Time-mated Females (GD 6)[b]	5	5	5	5	5	5
Females Pregnant (%)[b]	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
Females Not Pregnant (%)[b]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dams Not Delivering with Evidence of Pregnancy (%)[b]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dams with Litters on LD 0 (%)[b]	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
Gestation Length (Days)[b,c]	22 ± 0	21.8 ± 0.45	22 ± 0	22 ± 0	22 ± 0	21.8 ± 0.45
Mean Litter Size and Survival						
Litter Size on PND 1[b,d,e,f]	17.2 (5)	12.8 (5)	16.6 (5)	14.2 (5)	15.4 (5)	15.6 (5)

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Mean Number of Male Pups per Litter on PND 4[b,d,e]	9 (5)	5.6 (5)	10 (5)	6.2 (5)	9.2 (5)	7.4 (5)
Mean Number of Female Pups per Litter on PND 4[b,d,e]	7.6 (5)	6.8 (5)	5.6 (5)	7.8 (5)	6 (5)	6.6 (5)
Survival per Litter[e,g]						
Total dead: PND 1–18	6 (4)	2 (2)	5 (3)	2 (2)	1 (1)	8 (3)
Total dead: PND 19–24[h]	0 (5)	0 (5)	22** (4)	2 (2)	13** (5)	5* (2)
Weaned Males/Females[b]	44/36	28/34	34/22	29/38	37/25	34/31

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day; LD = lactation day; PND = postnatal day.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]No statistical analyses were performed on these endpoints.
[c]Gestation length calculated for sperm-positive females that delivered a litter. Data are presented as a mean ± standard deviation.
[d]Data are presented as a mean (number of dams).
[e]Each exposed group was compared to the vehicle control group with Fisher's exact test.
[f]Pups were sexed on PND 4.
[g]Total dead in exposure group (number of litters).
[h]Due to high pup mortality during lactation, nicotine bitartrate dihydrate exposure was interrupted, and all animals were given tap water instead of dosed water from ~4 days before weaning until the start of the 4-week study.

All F_0 dams survived until study termination (Table 5), and no clinical observations recorded during the study were considered related to exposure. Mean body weights of all groups of F_0 dams were similar at the beginning of gestation (gestation day [GD] 5); however, mean body weight gain of the 25 mg/L group was lower than that of the control group (Table 5; Figure 2). By the end of gestation (GD 21), mean body weight of the 25 mg/L group was 90% of that of the control group. Throughout lactation, mean body weights of 25 mg/L dams remained ≤90% of that of the control group, reaching 86% by lactation day (LD) 21 (Figure 2; Appendix D).

Table 5. Summary of Survival, Mean Body Weights, and Body Weight Gains of F_0 Female Rats during Gestation and Lactation in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Parameter[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Gestation Body Weight						
Gestation Day						
5[d]	314.0 ± 15.6 (5)	316.0 ± 10.8 (5)	310.0 ± 21.8 (5)	305.0 ± 5.0 (5)	302.0 ± 9.1 (5)	320.0 ± 11.2 (5)
21	475.0 ± 23.5 (5)	479.0 ± 36.0 (5)	469.0 ± 32.7 (5)	446.0 ± 8.2 (5)	448.0 ± 5.7 (5)	427.0 ± 25.1* (5)
Gestation Weight Change						
Gestation Day Interval						
5–21	161.0 ± 14.3 (5)	163.0 ± 25.6 (5)	159.0 ± 15.2 (5)	141.0 ± 12.4 (5)	146.0 ± 6.5 (5)	107.0 ± 18.2** (5)

Parameter[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Lactation Body Weight						
Lactation Day						
1	327.0 ± 16.0 (5)	326.0 ± 15.6 (5)	322.0 ± 14.8 (5)	306.0 ± 11.9 (5)	302.0 ± 4.5 (5)	292.0 ± 22.2** (5)
21	359.0 ± 13.4 (5)	360.0 ± 15.4 (5)	346.0 ± 19.8 (5)	339.0 ± 17.8 (5)	324.0 ± 15.6* (5)	307.0 ± 18.9** (5)
Lactation Weight Change						
Lactation Day Interval						
1–21	32.0 ± 14.0 (5)	34.0 ± 18.2 (5)	24.0 ± 9.6 (5)	33.0 ± 11.5 (5)	22.0 ± 16.8 (5)	15.0 ± 3.5 (5)

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation (number of dams). Body weight data are presented in grams.
[d]Body weights were evaluated 1 day prior to study start and initiation of exposure on gestation day 6.

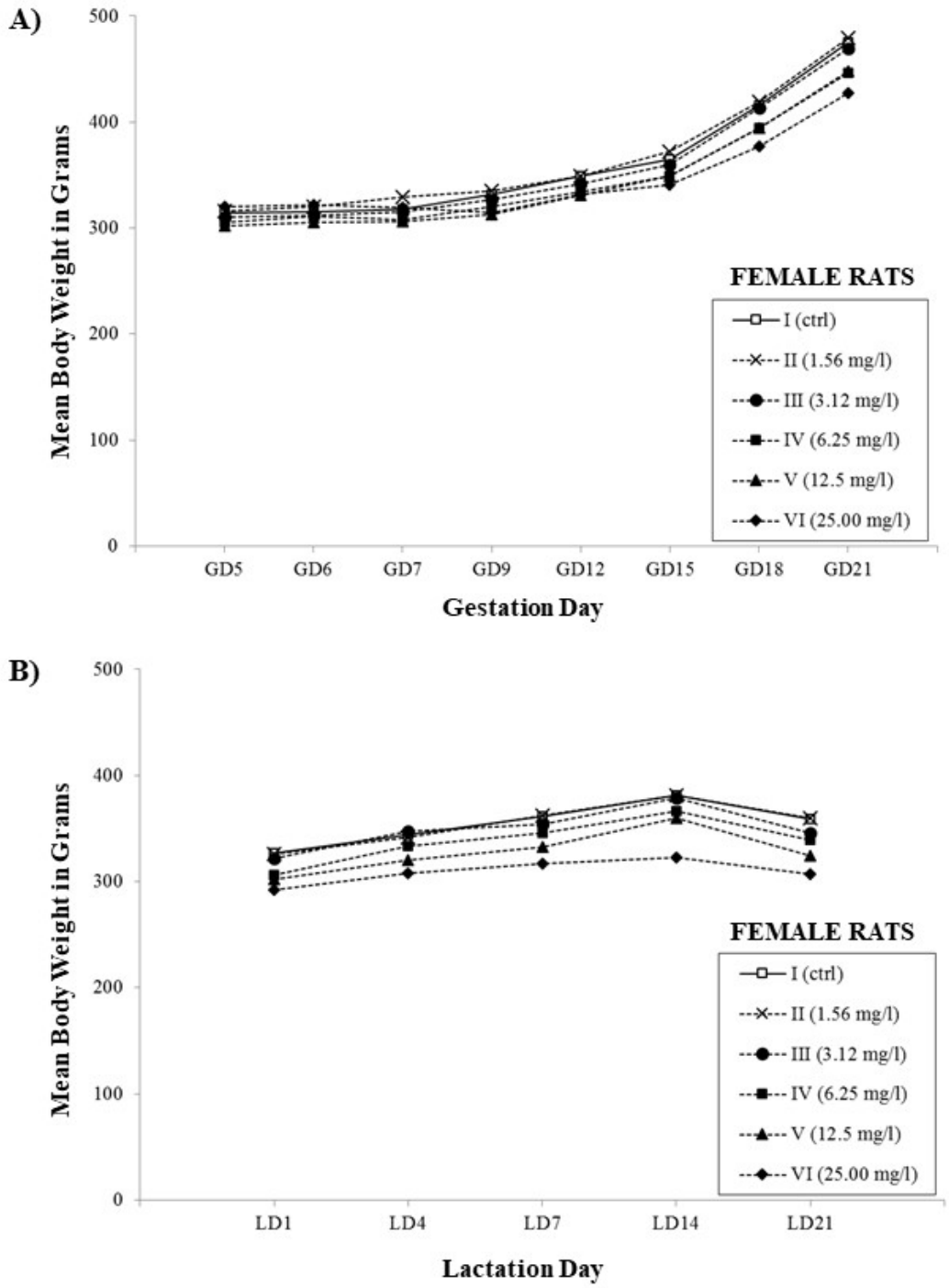

Figure 2. Gestation and Lactation Growth Curves for F_0 Female Rats in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) gestation and (B) lactation.

Before initiation of exposure (GD 5), mean water consumption (mL/animal/day) by the 25 mg/L group was approximately 83% of that of the control group (Table 6). Beginning on GD 6, water consumption by the 25 mg/L group dropped dramatically and was <60% of that of the control group for most of the remaining exposure period, ending with a mean water consumption value approximately 37% of that of the control group on GD 21 (Appendix D). Starting on GD 8, mean water consumption by the 12.5 mg/L group was <70% of that of the control group for most of the remaining exposure period, whereas mean water consumption by the 1.56, 3.12, and 6.25 mg/L groups was <90% of that of the control group for most of the remaining time points.

On LD 1, mean water consumption by the 25 mg/L group was approximately 73% of that of the control group; at all subsequent measurements, it was 53%–61% of that of the control group (Table 6; Appendix D). From LD 4 to LD 21, mean water consumption by the 12.5 mg/L group was ≤80% of that of the control group. Water consumption by the other exposed groups was <90% of that of the control group during most of lactation but within 10% of the control group by LD 21.

Table 6. Summary of Water and Nicotine Bitartrate Dihydrate Consumption by F_0 Female Rats during Gestation and Lactation in the Perinatal and Four-week Dose Range-finding Drinking Water Study[a]

Parameter	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Gestation Day (mL/animal/day)[b,c]						
5[d]	46.0 ± 8.4 (5)	35.6 ± 6.1 (5)	43.2 ± 5.8 (5)	46.4 ± 6.1 (5)	40.0 ± 8.5 (5)	38.0 ± 4.2 (5)
21	51.2 ± 3.3 (5)	35.6 ± 6.7** (5)	36.4 ± 7.0** (5)	40.4 ± 7.0 (5)	31.6 ± 7.4** (5)	18.8 ± 6.1** (5)
Chemical Intake (mg/kg/day)[e,f]						
GD 5–21	0	0.2	0.4	0.8	1.4	2.0
Lactation Day (mL/animal/day)[b,c]						
1	40.0 ± 6.5 (5)	39.2 ± 2.7 (5)	41.2 ± 5.8 (5)	42.4 ± 8.5 (5)	33.6 ± 6.7 (5)	29.2 ± 5.9* (5)
21	153.2 ± 23.1 (5)	148.0 ± 22.5 (5)	146.8 ± 22.1 (5)	140.4 ± 22.0 (5)	123.2 ± 21.7 (5)	84.4 ± 15.8** (5)

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day.
[a]Concentrations in drinking water refer to freebase (-)-nicotine only.
[b]Data are presented as mean ± standard deviation (number of dams).
[c]Each exposed group was compared to the vehicle control group with the Dunnett test.
[d]Water consumption was evaluated 1 day prior to study start and initiation of exposure on gestation day 6.
[e]Chemical intake calculated as: ([exposure concentration × water consumption]/[average body weight of day range]).
[f]No statistical analysis performed on the chemical intake data.

Mean feed consumption by the 25 mg/L group was <90% of that of the control group from GD 6 to GD 12, after which consumption decreased significantly, ending at approximately 51% of that of the control group on GD 21 (Table 7; Appendix D). Mean feed consumption by the 12.5 mg/L group was consistently <90% of that of the control group, beginning at GD 8, and <90% that of the other exposed groups at some time points.

Similar to the pattern observed in gestation, mean feed consumption throughout lactation was lower for all exposed groups relative to the control group, with only a couple of exceptions early

in lactation (Table 7). By the end of lactation (LD 21), mean feed consumption was 70% of that of the control group for the 25 mg/L group and 78% for the 12.5 mg/L group. The remaining exposed groups had mean feed consumption values within 10% of the control group at LD 21.

Table 7. Summary of Feed Consumption by F_0 Female Rats during Gestation and Lactation in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Parameter[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Gestation Day (g/animal/day)						
5[d]	24.0 ± 2.2 (5)	25.0 ± 3.5 (5)	26.0 ± 4.2 (5)	26.0 ± 2.2 (5)	25.0 ± 6.1 (5)	27.0 ± 5.7 (5)
21	37.0 ± 2.7 (5)	26.0 ± 8.2* (5)	27.0 ± 4.5 (5)	27.0 ± 4.5 (5)	27.0 ± 4.5 (5)	19.0 ± 8.9** (5)
Lactation Day (g/animal/day)						
1	25.0 ± 0.0 (5)	30.0 ± 7.1 (5)	30.0 ± 0.0 (5)	24.0 ± 4.2 (5)	23.0 ± 4.5 (5)	22.0 ± 2.7 (5)
21	100.0 ± 10.0 (5)	95.0 ± 7.9 (5)	90.0 ± 10.0 (5)	92.0 ± 7.6 (5)	78.0 ± 15.2** (5)	70.0 ± 10.0** (5)

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Data are presented as mean ± standard deviation (number of dams).
[c]Each exposed group was compared to the vehicle control group with the Dunnett test.
[d]Feed consumption was evaluated 1 day prior to study start and initiation of exposure on gestation day 6.

There were no significant changes in preweaning pup mean body weights; however, both male and female pups exposed to 25 mg/L had lower body weights at all time points and were <90% of the control group by PND 14 for females and PND 21 for males. Mean body weights of male and female pups in the 25 mg/L groups were approximately 88%–93% and 86%–92%, respectively, of that of the control groups (Table 8).

Table 8. Summary of Preweaning F_1 Male and Female Rat Pup Mean Body Weights Following Perinatal Exposure to Nicotine Bitartrate Dihydrate[a]

Postnatal Day[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Male						
4	9.2 ± 0.8 (45/5)	10.4 ± 1.1 (28/5)	9.0 ± 0.9 (50/5)	9.5 ± 1.2 (31/5)	8.6 ± 0.4 (46/5)	8.5 ± 0.9 (37/5)
7	12.4 ± 1.8 (44/5)	14.8 ± 1.1 (28/5)	12.3 ± 1.3 (50/5)	12.9 ± 2.0 (31/5)	11.5 ± 0.7 (46/5)	11.5 ± 1.2 (37/5)
14	22.1 ± 2.7 (44/5)	26.4 ± 2.7 (28/5)	22.0 ± 1.9 (50/5)	22.9 ± 4.6 (31/5)	21.0 ± 1.3 (46/5)	20.2 ± 2.4 (37/5)
21	31.6 ± 4.9 (44/5)	39.8 ± 5.9 (28/5)	29.8 ± 3.6 (40/5)	33.1 ± 7.6 (30/5)	29.4 ± 2.6 (44/5)	27.7 ± 4.2 (37/5)
Female						
4	8.8 ± 0.8 (38/5)	10.1 ± 0.7 (34/5)	8.5 ± 1.1 (28/5)	9.0 ± 1.1 (39/5)	8.3 ± 0.6 (30/5)	8.1 ± 1.2 (33/5)

Postnatal Day[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
7	11.9 ± 1.5 (37/5)	13.9 ± 1.3 (34/5)	11.4 ± 1.8 (28/5)	12.1 ± 2.0 (39/5)	11.0 ± 0.6 (29/5)	10.8 ± 1.6 (33/5)
14	21.5 ± 2.6 (36/5)	25.4 ± 2.6 (34/5)	20.8 ± 2.9 (28/5)	21.8 ± 4.1 (38/5)	20.0 ± 1.7 (29/5)	19.1 ± 3.1 (33/5)
21	30.5 ± 5.0 (36/5)	38.1 ± 5.5 (34/5)	29.5 ± 3.9 (24/5)	31.1 ± 6.8 (38/5)	28.2 ± 3.4 (28/5)	26.3 ± 5.2 (33/5)

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant differences were noted at $p \leq 0.05$.
[c]Data are presented as the mean of litter means ± standard deviation (number of pups/number of litters). Body weight data are presented in grams.

During lactation (PND 19–24), pup mortality in the 3.12, 12.5, and 25 mg/L groups was significantly increased compared to the control group. In total, 42 pups died across all exposed groups, whereas no mortality was observed in the control group during the same period. Because of the observed mortality, exposure was suspended in all groups at approximately 4 days before weaning; thereafter, no additional pup mortality was observed, suggesting that the higher incidence of mortality was exposure related. At necropsy, pups from the exposed groups who died between PNDs 19 and 24 presented gross lesions in the intestine, which correlated histopathologically with moderately diffuse intestinal inflammation (Appendix D).

Perinatal and Four-week Dose Range-finding Study (Postweaning Phase)

After exposure was suspended (LD 22–25, depending on parturition date of the litter), pups were weaned on LD 26–29 and were not exposed again until 4 days after weaning. After resumption of exposure, no additional mortalities occurred until study termination (Table 9).

Mean body weights of the 1.56 mg/L male and female groups were significantly higher than the control group at study day 21, with significantly higher weight gain from study day 1 to 21 in males and females. At higher concentrations, the mean body weights of the 12.5 and 25 mg/L male and female groups were <90% of those of the control groups on study day 1 (Table 9; Figure 3). The 12.5 mg/L groups were within 10% of the control groups by study day 14 for female rats and study day 21 for male rats. Mean body weights of the 25 mg/L male and female groups remained <90% of those of their respective control groups for the entire exposure period; however, mean body weights of the 25 mg/L male and female recovery groups were within 10% of those of the control groups by the end of the recovery period (Table 9). No clinical observations observed during the study were considered exposure related (Appendix D).

Table 9. Summary of Survival, Mean Body Weights, and Body Weight Gains of Male and Female Rats in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate, with Two-week Recovery Period[a]

Study Day[b,c,d]	0 mg/L		1.56 mg/L		3.12 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L	
	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n
Male												
Exposure Period												
1	89.4 ± 9.2	20	97.2 ± 12.2	10	82.5 ± 11.1	10	89.1 ± 19.5	10	76.1 ± 10.2*	10	74.7 ± 10.3**	20
7[e]	152.9 ± 11.6	20	168.3 ± 18.1	10	144.3 ± 14.2	10	151.4 ± 24.0	10	131.3 ± 16.4	10	126.7 ± 14.9	20
14[e]	202.2 ± 13.8	20	221.7 ± 19.3	10	194.9 ± 16.1	10	198.2 ± 25.9	10	177.5 ± 20.7	10	169.6 ± 18.6	20
21	251.9 ± 15.2	20	276.6 ± 17.8*	10	246.0 ± 15.8	10	254.2 ± 30.9	10	227.2 ± 22.6*	10	215.4 ± 21.5**	20
28[e,f]	297.1 ± 18.4	20	333.5 ± 23.0	10	293.8 ± 20.2	10	289.3 ± 30.5	10	278.6 ± 27.8	10	248.8 ± 23.2	20
Recovery Period[g]												
35	331.8 ± 13.0	10	–	0	–	0	–	0	–	0	301.8 ± 27.0**	10
42[e,f]	364.4 ± 14.4	10	–	0	–	0	–	0	–	0	337.6 ± 27.6	10
Weight Change												
1–21	162.5 ± 11.7	20	179.4 ± 6.7**	10	163.5 ± 8.4	10	165.1 ± 18.8	10	151.1 ± 13.2	10	140.7 ± 12.3**	20
Female												
Exposure Period												
1	81.8 ± 7.7	20	87.6 ± 9.2	10	74.8 ± 12.1	10	81.3 ± 15.6	10	68.4 ± 9.5**	10	67.8 ± 10.1**	20
7[e]	128.5 ± 9.6	20	139.9 ± 8.3	10	123.4 ± 12.7	10	127.9 ± 17.3	10	112.3 ± 10.1	10	107.9 ± 12.3	20
14[e]	151.6 ± 10.8	20	168.2 ± 9.3	10	150.2 ± 13.0	10	154.4 ± 17.4	10	138.8 ± 10.3	10	132.2 ± 11.0	20
21	177.1 ± 13.3	20	197.2 ± 11.0**	10	175.8 ± 14.2	10	176.5 ± 17.9	10	160.1 ± 9.3**	10	153.7 ± 13.3**	20
28[e,f]	194.2 ± 16.3	20	222.3 ± 16.6	10	202.5 ± 12.0	10	202.2 ± 18.1	10	178.2 ± 12.6	10	173.7 ± 13.7	20
Recovery Period[g]												
35	219.7 ± 15.2	10	–	0	–	0	–	0	–	0	200.6 ± 14.8*	10
42[e,f]	237.9 ± 14.8	10	–	0	–	0	–	0	–	0	218.1 ± 16.8	10
Weight Change												
1–21	95.3 ± 7.6	20	109.6 ± 3.4**	10	101.0 ± 6.0	10	95.2 ± 7.1	10	91.7 ± 4.7	10	86.0 ± 6.2**	20

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation. Body weight data are presented in grams.
[d]Study day 1 is the day animals were placed on study after weaning. Study day 1 corresponds to postnatal day 30 of the last litter born.
[e]No statistical analysis performed on this endpoint.
[f]On study day 28, measurements were taken after 10 rats from each exposure group were moved to metabolic cages for 16 hours. On study day 42 (recovery period), measurements were taken after 10 rats each from the control group and 25 mg/L group were moved to metabolic cages for 16 hours.
[g]Data for the recovery period were collected for only 10 rats each from the control group and 25 mg/L group.

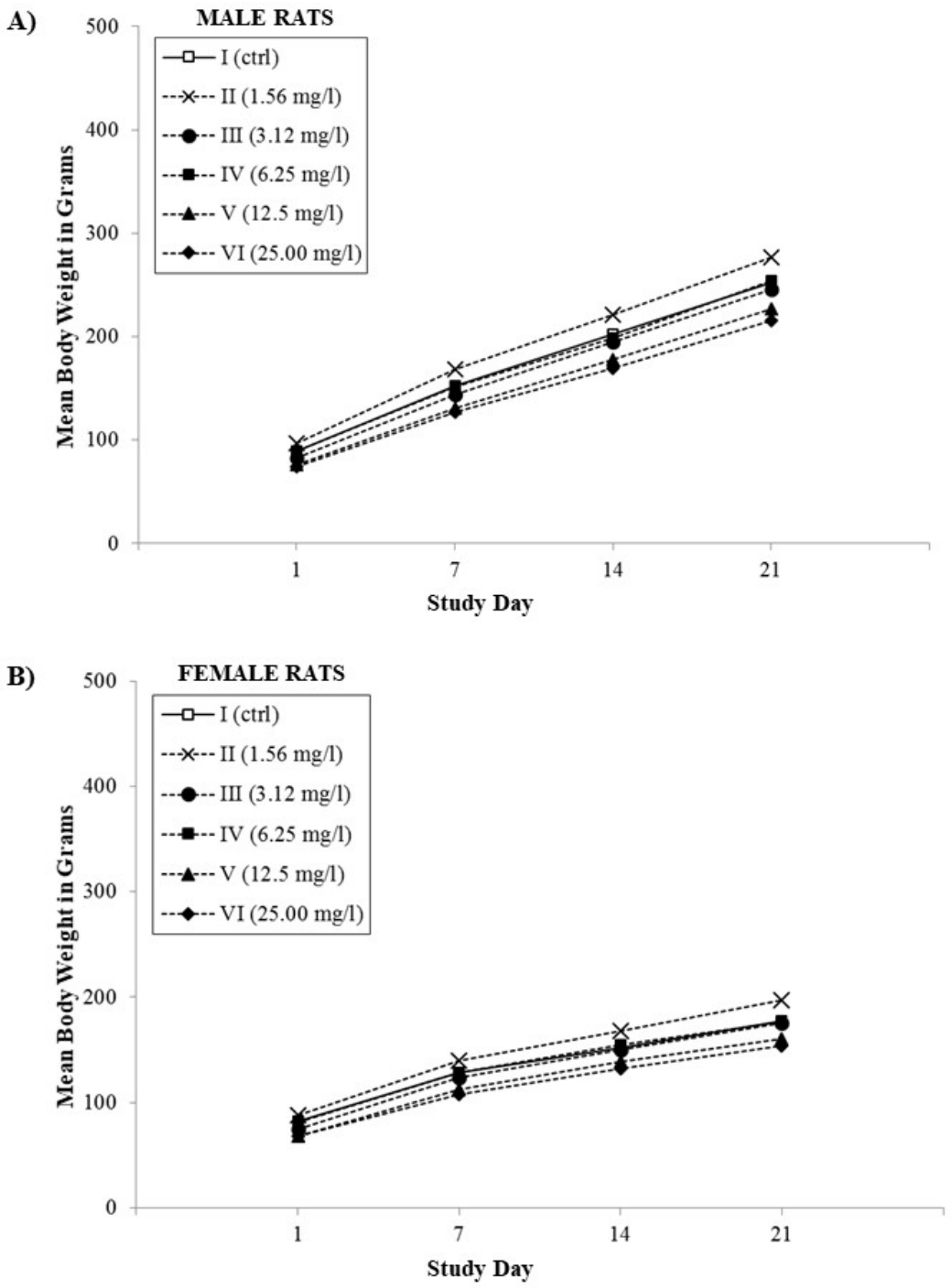

Figure 3. Growth Curves for Male and Female Rats in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) males and (B) females.

In male rats, mean water consumption (mL/animal/day) by the ≥6.25 mg/L groups was <90% of that of the control group for the entire 4 weeks of exposure, with consumption by the 25 mg/L group <70% of that of the control group (Table 10). During the 2-week recovery period (with no exposure), mean water consumption by the 25 mg/L group was similar to that of the control group (Table 10).

In female rats, mean water consumption by the 25 mg/L group was ≤61% of that of the control group throughout exposure (Table 10) and did not return to control group values during recovery, reaching 84% at the end of recovery week 2 (study day 42) (Table 10). Mean water consumption by the 12.5 mg/L group was approximately 70% of that of the control group throughout exposure (Table 10). For the other exposed groups, mean water consumption was generally within 10% to 15% of that of the control group.

Table 10. Summary of Water and Nicotine Bitartrate Dihydrate Consumption by Male and Female Rats in the Perinatal and Four-week Dose Range-finding Drinking Water Study, with Two-week Recovery Period[a]

Study Day	0 mg/L		1.56 mg/L		3.12 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
Water (mL/animal/day)[b,c]												
Exposure Period												
1	20.8 ± 2.1	20	21.7 ± 2.8	10	22.3 ± 3.9	10	18.4 ± 2.5	10	14.0 ± 0.8**	10	12.3 ± 1.7**	20
21	39.0 ± 4.6	20	38.9 ± 2.3	10	37.5 ± 2.9	10	32.3 ± 1.6**	10	31.7 ± 3.2**	10	26.5 ± 2.1**	20
28[d]	42.6 ± 1.2	10	–	0	–	0	–	0	–	0	45.2 ± 10.1	10
Recovery Period[e]												
35	43.2 ± 5.2	10	–	0	–	0	–	0	–	0	39.9 ± 2.5	10
42[d,f]	37.8 ± 16.8	10	–	0	–	0	–	0	–	0	38.4 ± 17.5	10
Chemical Intake (mg/kg/day)[f,g,h]												
1–21	0	20	0.3	10	0.6	10	1.0	10	2.0	10	3.5	20
Female												
Water (mL/animal/day)[b,c]												
Exposure Period												
1	19.9 ± 1.4	20	21.6 ± 2.3	10	22.7 ± 5.2	10	19.2 ± 1.6	10	13.8 ± 1.4**	10	11.3 ± 1.8**	20
21	28.7 ± 3.7	20	31.2 ± 1.3	10	28.6 ± 3.0	10	26.3 ± 1.1	10	20.7 ± 2.8**	10	15.6 ± 3.1**	20
28[d]	35.3 ± 0.6	10	–	0	–	0	–	0	–	0	31.1 ± 1.7**	10
Recovery Period[e]												
35	34.9 ± 2.2	10	–	0	–	0	–	0	–	0	27.3 ± 1.1**	10
42[d,f]	38.8 ± 16.8	10	–	0	–	0	–	0	–	0	32.4 ± 14.1	10
Chemical Intake (mg/kg/day)[f,g,h]												
1–21	0	20	0.3	10	0.6	10	1.1	10	2.0	10	3.2	20

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
**Statistically significant at $p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation.
[d]On study day 28, measurements were not taken for the 10 rats from each exposure group moved to metabolic cages. On study day 42 (recovery period), measurements were taken after 10 rats each from the control and high exposure groups were moved to metabolic cages for 16 hours.
[e]Data for the recovery period were collected for only 10 rats each from the control group and 25 mg/L group.
[f]No statistical analysis performed on the chemical intake data.
[g]Chemical intake calculated as: ([exposure concentration × water consumption]/[average body weight of day range]).
[h]Milligrams of freebase (-)-nicotine consumed/kg body weight/day.

In male rats, mean feed consumption by the 25 mg/L group was <90% of that of the control group for the entire 4 weeks of exposure; during the 2-week recovery period (with no exposure), mean feed consumption was similar to that of the control group (Table 11). Mean feed consumption by the 3.12, 6.25, and 12.5 mg/L groups was <90% of that of the control group on study days 1 (12.5 mg/L group only), 7, and 21 (Appendix D). In female rats, mean feed consumption by the 25 mg/L group was <90% of that of the control group throughout most of the exposure period and returned to within 10% of the control group during the recovery period (Table 11). Mean feed consumption by the 12.5 mg/L group was approximately 76% of that of the control group on study day 1 but was within 10% of the control group for the remainder of the study.

Table 11. Summary of Feed Consumption by Male and Female Rats in the Perinatal and Four-week Drinking Dose Range-finding Water Study of Nicotine Bitartrate Dihydrate, with Two-week Recovery Period[a]

Study Day[b,c]	0 mg/L		1.56 mg/L		3.12 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
Feed (g/animal/day)												
Exposure Period												
1	13.8 ± 1.8	20	14.5 ± 1.7	10	12.4 ± 0.6	10	12.8 ± 2.4	10	10.3 ± 1.6**	10	9.6 ± 1.4**	20
21	26.1 ± 3.8	20	27.9 ± 1.9	10	23.3 ± 2.0	10	22.6 ± 0.8	10	23.2 ± 2.5	10	21.7 ± 2.2*	20
28[d]	25.2 ± 2.4	10	–	0	–	0	–	0	–	0	25.6 ± 0.8	10
Recovery Period[e]												
35	26.3 ± 0.6	10	–	0	–	0	–	0	–	0	25.1 ± 2.5	10
Female												
Feed (g/animal/day)												
Exposure Period												
1	12.2 ± 0.8	20	12.9 ± 0.5	10	11.8 ± 1.5	10	12.0 ± 0.7	10	9.3 ± 1.2**	10	9.1 ± 1.4**	20
21	16.1 ± 2.0	20	18.7 ± 2.5	10	16.6 ± 2.1	10	17.3 ± 1.0	10	15.1 ± 1.3	10	12.5 ± 2.9*	20
28[d]	17.3 ± 0.3	10	–	0	–	0	–	0	–	0	18.5 ± 0.8*	10
Recovery Period[e]												
35	18.2 ± 2.5	10	–	0	–	0	–	0	–	0	16.8 ± 0.9	10

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Data are presented as mean ± standard deviation.
[c]Each exposed group was compared to the vehicle control group with the Dunnett test.
[d]On study day 28, measurements were not taken for the 10 rats from each exposure group moved to metabolic cages.
[e]Data for the recovery period were collected for only 10 rats each from the control group and 25 mg/L group.

At the end of the 4-week study, there were no exposure-related changes in the clinical chemistry parameters for either sex. There were no hematological findings in male rats, but the white blood cell counts and lymphocyte counts were significantly increased at all exposure concentrations with the exception of the 25 mg/L female exposed group (Appendix D). Causes of lymphocyte count increases in rats include excitation (physiological leukocytosis) or chronic inflammation. There was increased incidence of minimal intestinal inflammation in histopathologically examined female rats in the 25 mg/L group (Appendix D); animals used for clinical chemistry analysis, which exhibited significantly increased lymphocyte counts, were not evaluated histopathologically. While the significant increase in the lymphocyte counts may have been due to mild inflammation or antigenic stimulation, biological variability could not be ruled out. No

changes in the white blood cell count or lymphocyte count were observed in the 2-week recovery animals, which included only control rats and rats in the 25 mg/L group. There were no marked changes in urinalysis measures that were considered biologically significant (Appendix D).

In male rats, mean absolute liver weight for the 25 mg/L group was 72% of that of the control group, and relative liver weight was also significantly decreased (Table 12). Mean absolute urinary bladder and prostate weights (combined) were significantly decreased in the 25 mg/L group compared to those of the control group. There were no histopathological correlations with these organ weight changes (Table 13; Appendix D). Male rats in the 25 mg/L group had several other organs with mean absolute weights <90% of those of the control group, including the thymus and mediastinal lymph nodes (combined), heart, spleen, kidneys, and adrenal glands; however, relative organ weights were similar to those of the control group. The lower absolute weights probably reflect differences in terminal body weights. In male rats, all mean organ weights of the 25 mg/L group were within 10% of the control group after postexposure recovery, except for the absolute spleen, thymus, and mediastinal lymph node weights (Appendix D).

In female rats, mean absolute and relative adrenal gland weights were significantly decreased in the 25 mg/L group compared to the control group (Table 12). One animal was recorded as having agenesis of the adrenal gland (Appendix D). Mean absolute weights of the heart and uterus of the 25 mg/L group were <90% of those of the control group; however, the relative organ weights indicate this change was probably due to differences in terminal body weights. Several groups had absolute organ weights that were ≥110% of those of the control group; however, relative organ weights were similar to those of the control group, indicating that the differences were likely due to body weight differences. These organs included the spleen, liver, and kidney of the 1.56 mg/L group; the liver of the 3.12 mg/L group; and the liver and ovaries of the 6.25 mg/L group. In female rats, mean absolute spleen, liver, and uterus weights of the 25 mg/L group were ≤90% of those of the control group after postexposure recovery; however, given the relative weights, these changes were most likely due to body weight differences (Appendix D).

Table 12. Summary of Select Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male and Female Rats in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a,b,c]

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
n	10	10	10	10	10	10
Male						
Terminal Body Wt. (g)	303.40 ± 20.35	333.50 ± 22.99*	293.80 ± 20.18	289.30 ± 30.51	278.60 ± 27.78	246.90 ± 22.61**
Liver						
Absolute (g)	11.896 ± 1.251	13.265 ± 1.187*	11.912 ± 0.810	11.662 ± 1.436	10.787 ± 0.816	8.565 ± 1.286**
Relative (mg/g)[d]	39.256 ± 3.816	39.750 ± 1.610	40.571 ± 1.653	40.791 ± 6.955	38.840 ± 1.999	34.570 ± 2.740*
Urinary Bladder and Prostate (Combined)						
Absolute (g)	0.392 ± 0.052	0.429 ± 0.077	0.430 ± 0.153	0.396 ± 0.076	0.316 ± 0.055	0.261 ± 0.057**
Relative (mg/g)	1.289 ± 0.133	1.288 ± 0.218	1.449 ± 0.437	1.389 ± 0.318	1.134 ± 0.175	1.051 ± 0.191
Thymus and Mediastinal Lymph Nodes (Combined)						
Absolute (g)	0.888 ± 0.147	0.906 ± 0.125	0.871 ± 0.104	0.760 ± 0.127	0.822± 0.074	0.653 ± 0.065**
Relative (mg/g)	2.943 ± 0.552	2.731 ± 0.439	2.991 ± 0.513	2.651 ± 0.506	2.969 ± 0.326	2.666 ± 0.398
Heart						
Absolute (g)	1.147 ± 0.064	1.259 ± 0.159	1.122 ± 0.083	1.074 ± 0.110	1.044 ± 0.075	0.935 ± 0.072**
Relative (mg/g)	3.785 ± 0.159	3.769 ± 0.324	3.820 ± 0.145	3.744 ± 0.512	3.762 ± 0.213	3.798 ± 0.203
Spleen						
Absolute (g)	0.632 ± 0.067	0.689 ± 0.077	0.627 ± 0.082	0.575 ± 0.054	0.634 ± 0.088	0.548 ± 0.133
Relative (mg/g)	2.091 ± 0.267	2.072 ± 0.241	2.131 ± 0.209	2.001 ± 0.241	2.291 ± 0.366	2.215 ± 0.477
Kidneys						
Absolute (g)	2.267 ± 0.121	2.551 ± 0.236**	2.219 ± 0.165	2.320 ± 0.179	2.220 ± 0.206	1.961 ± 0.227**
Relative (mg/g)	7.498 ± 0.568	7.649 ± 0.449	7.558 ± 0.336	8.097 ± 1.033	7.983 ± 0.394	7.937 ± 0.510
Adrenal Glands						
Absolute (g)	0.062 ± 0.019	0.061 ± 0.014	0.051 ± 0.010	0.058 ± 0.006	0.057 ± 0.013	0.045 ± 0.009**
Relative (mg/g)	0.207 ± 0.075	0.181 ± 0.036	0.174 ± 0.030	0.202 ± 0.026	0.204 ± 0.045	0.182 ± 0.033
Female						
Terminal Body Wt. (g)	190.50 ± 18.67	222.30 ± 16.56**	202.50 ± 12.30	202.20 ± 18.08	178.20 ± 12.62	177.00 ± 12.16
Liver						
Absolute (g)	7.329 ± 1.655	9.104 ± 0.945**	8.459 ± 0.551	8.086 ± 0.638	6.927 ± 0.905	7.032 ± 0.994
Relative (mg/g)	38.152 ± 5.646	41.121 ± 4.829	41.804 ± 2.045	40.029 ± 0.836	38.818 ± 3.814	39.578 ± 3.382
Heart						
Absolute (g)	0.786 ± 0.055	0.887 ± 0.063**	0.821 ± 0.078	0.799 ± 0.073	0.755 ± 0.089	0.702 ± 0.059**
Relative (mg/g)	4.141 ± 0.194	4.001 ± 0.303	4.051 ± 0.272	3.963 ± 0.350	4.228 ± 0.329	3.963 ± 0.179
Spleen						
Absolute (g)	0.434 ± 0.038	0.546 ± 0.177*	0.448 ± 0.046	0.504 ± 0.130	0.449 ± 0.076	0.429 ± 0.074
Relative (mg/g)	2.287 ± 0.217	2.453 ± 0.522	2.208 ± 0.131	2.483 ± 0.545	2.517 ± 0.377	2.435 ± 0.444
Kidney						
Absolute (g)	1.489 ± 0.115	1.711 ± 0.109**	1.583 ± 0.081	1.593 ± 0.129	1.498 ± 0.115	1.454 ± 0.115
Relative (mg/g)	7.836 ± 0.401	7.715 ± 0.486	7.826 ± 0.300	7.894 ± 0.436	8.407 ± 0.372**	8.214 ± 0.279
Adrenal Glands						
Absolute (g)[e]	0.061 ± 0.009	0.065 ± 0.015	0.060 ± 0.007	0.061 ± 0.007	0.054 ± 0.004	0.046 ± 0.010**
Relative (mg/g)[e]	0.320 ± 0.028	0.292 ± 0.066	0.294 ± 0.026	0.301 ± 0.029	0.304 ± 0.030	0.258 ± 0.054**

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Uterus						
Absolute (g)	0.480 ± 0.129	0.472 ± 0.105	0.528 ± 0.167	0.440 ± 0.185	0.461 ± 0.172	0.387 ± 0.150
Relative (mg/g)	2.548 ± 0.802	2.130 ± 0.467	2.606 ± 0.801	2.192 ± 0.913	2.603 ± 1.043	2.181 ± 0.810
Ovaries						
Absolute (g)	0.122 ± 0.022	0.142 ± 0.023	0.135 ± 0.014	0.145 ± 0.019	0.137 ± 0.026	0.125 ± 0.023
Relative (mg/g)	0.642 ± 0.088	0.637 ± 0.087	0.666 ± 0.053	0.717 ± 0.086	0.775 ± 0.166*	0.706 ± 0.128

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard deviation.
[b]Statistical analysis performed with the Dunnett (pairwise) test.
[c]Concentrations in drinking water refer to freebase (-)-nicotine.
[d]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight per g body weight.
[e]One animal weighed in the 25 mg/L group had agenesis of the adrenal gland.

Histopathology

This section describes the incidences of select nonneoplastic lesions in the intestines (all sites), kidney, and testes. The incidences were not statistically significant. The control and 25 mg/L groups were the only groups examined microscopically.

Intestines (all sites): Minimal inflammation of the intestine (all sites combined) was present in the 25 mg/L and control male and female rats (Table 13).

Kidney: Minimal focal or peritubular inflammation was present in the kidneys of 25 mg/L male and female rats and minimal focal inflammation was observed in the control groups (Table 13).

Testes: Degenerative lesions in the testes were recorded in three male rats in the 25 mg/L group; none were recorded in the control group (Table 13). These lesions included minimal tubule degeneration and mild tubule degeneration of multinucleated cells with necrosis, which are considered rare for Sprague Dawley rats of this age.

Table 13. Incidences of Select Nonneoplastic Lesions in Male and Female Rats in the Perinatal and Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

	0 mg/L	25 mg/L
Male		
Intestines (All Sites)[b]	10	10
Inflammation, minimal[c,d]	2	2
Kidney	10	10
Inflammation, peritubular, minimal	0	2
Inflammation, focal, minimal	2	3
Testes	10	10
Degeneration, tubules, minimal	0	1
Degeneration, tubules, multinucleated cells with necrosis, mild	0	2
Female		
Intestines (All Sites)	10	10
Inflammation, minimal	2	5
Kidney	10	9

	0 mg/L	25 mg/L
Inflammation, peritubular, minimal	0	1
Inflammation, focal, minimal	1	3

[a]Concentration in drinking water refers to freebase (-)-nicotine.
[b]Number of animals examined microscopically.
[c]Number of animals with lesion.
[d]The exposed group was compared to the vehicle control group using Fisher's exact (pairwise) test. No statistically significant findings were noted at $p \leq 0.05$.

Exposure Concentration Selection Rationale for the Perinatal and Three-month Study

The perinatal and 4-week study provided information for the selection of exposure concentrations for the perinatal and 3-month study in rats. In the perinatal and 4-week study, pup mortality was observed in all exposed groups during lactation, except at the lowest exposure concentration of 1.56 mg/L. Therefore, the exposure concentrations selected for the perinatal and 3-month study in rats were 0, 1.56, 3.12, 6.25, 12.5, and 25 mg/L (concentration of freebase (-)-nicotine from NBD) in drinking water, except during lactation, when all exposed groups, dams and pups, received 1.56 mg/L.

Perinatal and Three-month Study (Perinatal Phase)

All F_0 dams survived until study termination (Appendix D), and no clinical observations recorded during the study were considered related to exposure. All animals were pregnant, and there was no effect of NBD exposure on dams with litters, gestation length, or sex ratio (Table 14).

Table 14. Summary of the Disposition of Rats during Perinatal Exposure in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
F_0 Reproductive Performance[b]						
Time-mated Females (GD 6)	8	8	8	8	8	8
Females Pregnant (%)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)
Females Not Pregnant (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dams Not Delivering with Evidence of Pregnancy (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dams with Litters on LD 0 (%)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)
Gestation Length (Days)[c]	22 ± 0	22 ± 0	22 ± 0	22 ± 0	22 ± 0	22 ± 0
Mean Litter Size and Survival[b]						
Mean Number of Male Pups per Litter on PND 1[d,e,f]	6.6 ± 2.0 (8)	7.8 ± 1.8 (8)	8.5 ± 2.4 (8)	8.5 ± 2.4 (8)	8.1 ± 2.7 (8)	7.3 ± 1.8 (8)
Mean Number of Female Pups per Litter on PND 1[d,e,f]	6.6 ± 2.5 (8)	7.8 ± 1.7 (8)	7.1 ± 1.6 (8)	7.6 ± 2.3 (8)	7.9 ± 1.6 (8)	6.9 ± 1.6 (8)
Weaned Males/Females[f]	51/53	62/62	67/56	66/58	59/60	58/53

GD = gestation day; LD = lactation day; PND = postnatal day.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]No statistical analyses were performed on these endpoints.
[c]Gestation length calculated for sperm-positive females that delivered a litter. Data are presented as a mean ± standard deviation.
[d]Litters were not standardized in this study.
[e]Data are presented as mean ± standard deviation (number of dams).
[f]During lactation, all exposed animals received the same dose of 1.56 mg/L because of the high pup mortality during lactation in the previous 4-week study.

Throughout gestation and lactation, mean body weights of exposed groups of F_0 dams were within 10% of that of the control group (Table 15; Figure 4; Appendix D). The largest difference was observed at the end of gestation, when the mean body weight of the 25 mg/L dams was approximately 8% lower than that of the control group.

Table 15. Summary of Survival, Mean Body Weights, and Body Weight Gains of F_0 Female Rats during Gestation and Lactation in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Parameter[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L[d]	6.25 mg/L[d]	12.5 mg/L[d]	25 mg/L[d]
Gestation Body Weight						
Gestation Day						
5[e]	316.9 ± 16.7 (8)	323.1 ± 12.2 (8)	324.4 ± 20.3 (8)	320.0 ± 16.9 (8)	320.0 ± 16.5 (8)	325.6 ± 9.4 (8)
21	458.8 ± 11.3 (8)	475.6 ± 12.4 (8)	479.4 ± 33.5 (8)	476.9 ± 23.7 (8)	449.4 ± 17.2 (8)	422.5 ± 13.9** (8)
Gestation Weight Change						
Gestation Day Interval						
5–21	141.9 ± 11.3 (8)	152.5 ± 14.1 (8)	155.0 ± 16.5 (8)	156.9 ± 8.8 (8)	129.4 ± 11.2 (8)	96.9 ± 13.6** (8)
Lactation Body Weight						
Lactation Day						
1	327.5 ± 17.1 (8)	335.0 ± 14.6 (8)	333.8 ± 21.3 (8)	326.3 ± 20.0 (8)	305.6 ± 13.7* (8)	302.5 ± 9.3* (8)
28	314.4 ± 21.1 (8)	314.4 ± 12.4 (8)	316.3 ± 19.8 (8)	310.0 ± 15.8 (8)	306.9 ± 13.9 (8)	308.8 ± 17.5 (8)
Lactation Weight Change						
Lactation Day Interval						
1–28	−13.1 ± 13.3 (8)	−20.6 ± 9.8 (8)	−17.5 ± 17.3 (8)	−16.3 ± 13.8 (8)	1.3 ± 3.5 (8)	6.3 ± 13.3* (8)

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation (number of dams). Body weight data are presented in grams.
[d]During lactation, all exposed animals received the same drinking water concentration of 1.56 mg/L because of the high pup mortality during lactation in the previous 4-week study.
[e]Body weights were evaluated 1 day prior to study start and initiation of exposure on gestation day 6.

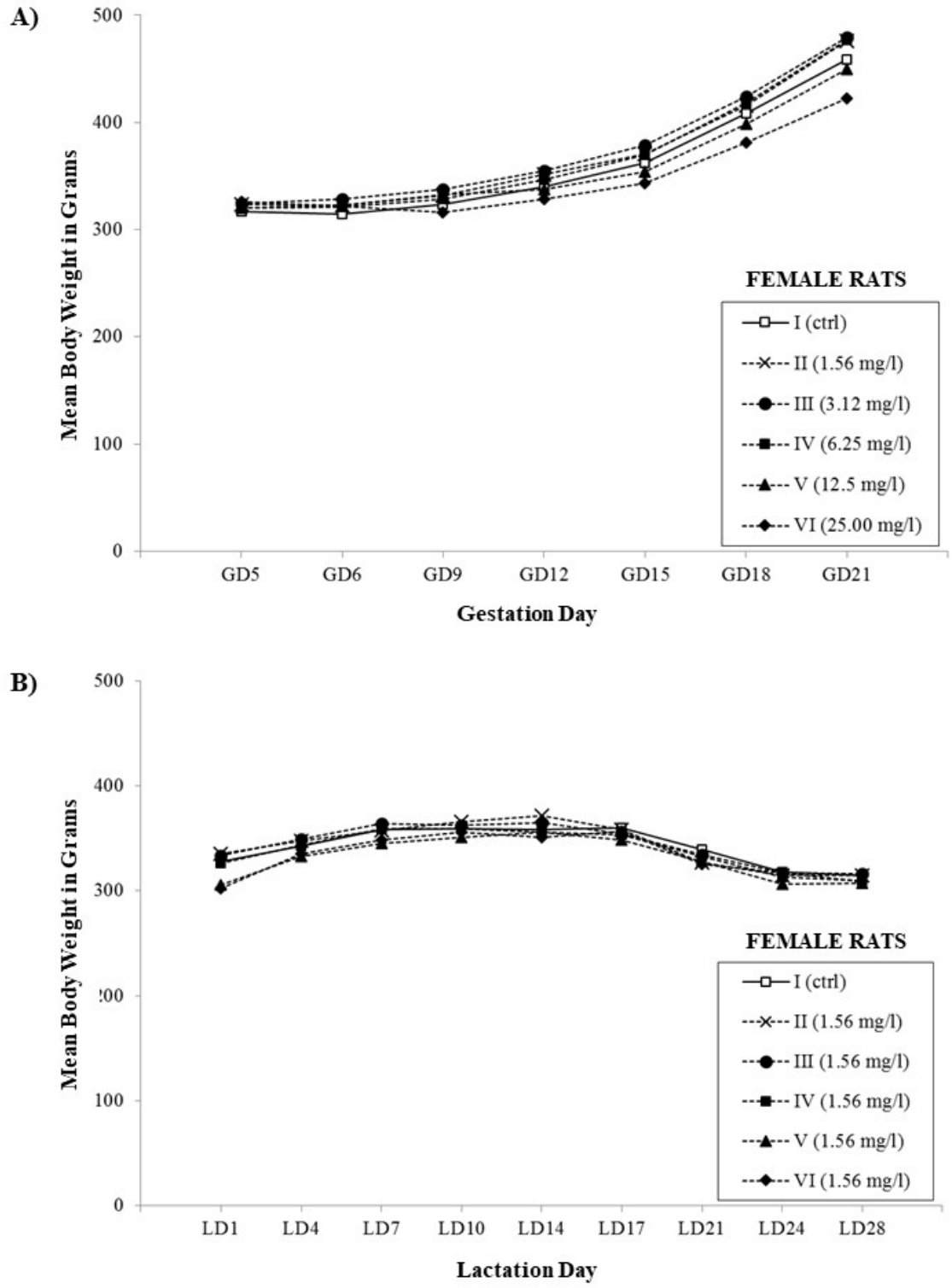

Figure 4. Gestation and Lactation Growth Curves for F_0 Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) gestation and (B) lactation.

Before exposure (GD 5), mean water consumption (mL/animal/day) by dams in the 25 mg/L group was approximately 118% of that of the control group (Table 16). On GD 6, however, it fell to 47% of that of the control group (Appendix D). Throughout gestation, mean water consumption by the 25 mg/L group remained less than that of the control group and was approximately 58% of that of the control group on GD 21 (Table 16). The 6.25 and 12.5 mg/L groups also consumed less water on average than the control group (significantly reduced at 12.5 mg/L on GD 21). During lactation, when exposure was limited to 1.56 mg/L, mean water consumption by all exposed groups was similar to that of the control group (Table 16; Appendix D).

Table 16. Summary of Water and Nicotine Bitartrate Dihydrate Consumption by F_0 Female Rats during Gestation and Lactation in the Perinatal and Three-month Drinking Water Study[a]

Parameter	0 mg/L	1.56 mg/L	3.12 mg/L[b]	6.25 mg/L[b]	12.5 mg/L[b]	25 mg/L[b]
Gestation Day (mL/animal/day)[c,d]						
5[e]	37.1 ± 5.1 (8)	42.5 ± 7.1 (8)	45.3 ± 7.6 (8)	40.6 ± 6.2 (8)	40.1 ± 4.5 (8)	43.8 ± 7.2 (8)
21	42.0 ± 8.3 (8)	41.8 ± 7.4 (8)	39.8 ± 7.7 (8)	38.8 ± 10.3 (8)	24.8 ± 5.2** (8)	24.5 ± 5.6** (8)
Chemical Intake (mg/kg/day)[f,g]						
GD 5–21	0	0.2	0.4	0.7	1	1.4
Lactation Day(mL/animal/day)[c,d]						
1	45.5 ± 5.6 (8)	42.3 ± 13.4 (8)	49.8 ± 7.4 (8)	42.5 ± 8.6 (8)	54.0 ± 6.7 (8)	55.8 ± 8.2 (8)
28	282.5 ± 33.1 (8)	303.5 ± 50.5 (8)	309.8 ± 44.6 (8)	290.5 ± 25.6 (8)	297.3 ± 51.8 (8)	282.8 ± 63.6 (8)

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
**Statistically significant at $p \leq 0.01$.
GD = gestation day.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]During lactation, all exposed animals received the same exposure concentration of 1.56 mg/L because of the high pup mortality during lactation in the previous 4-week study.
[c]Data are presented as mean ± standard deviation (number of dams).
[d]Each exposed group was compared to the vehicle control group with the Dunnett test.
[e]Water consumption was evaluated 1 day prior to study start and initiation of exposure on gestation day 6.
[f]Chemical intake calculated as: ([exposure concentration × water consumption]/[average body weight of day range]).
[g]No statistical analysis performed on the chemical intake data.

Mean feed consumption by the 25 mg/L group was slightly higher (approximately 117%) than that of the control group prior to exposure on GD 5 (Table 17). On GD 6, however, it fell to 82% of that of the control group and remained <90% throughout gestation (Appendix D). Mean feed consumption by the 12.5 mg/L group was also less than that of the control group throughout gestation and was approximately 80% of that of the control group on GD 21, though not significantly different. During lactation, when exposure was limited to 1.56 mg/L, mean feed consumption by all exposed groups was similar to that of the control group (Table 17).

Table 17. Summary of Feed Consumption by F_0 Female Rats during Gestation and Lactation in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Parameter[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L[d]	6.25 mg/L[d]	12.5 mg/L[d]	25 mg/L[d]
Gestation Day (g/animal/day)						
5[e]	21.9 ± 3.7 (8)	23.1 ± 5.3 (8)	24.4 ± 4.2 (8)	20.6 ± 5.0 (8)	22.5 ± 2.7 (8)	25.6 ± 3.2 (8)
21	28.1 ± 4.6 (8)	31.3 ± 5.8 (8)	28.8 ± 5.8 (8)	26.9 ± 3.7 (8)	22.5 ± 2.7 (8)	23.8 ± 4.4 (8)
Lactation Day (g/animal/day)						
1	27.5 ± 4.6 (8)	27.5 ± 9.3 (8)	28.1 ± 7.5 (8)	25.6 ± 7.3 (8)	30.6 ± 5.0 (8)	33.8 ± 5.2 (8)
28	148.1 ± 10.3 (8)	166.3 ± 16.9 (8)	161.3 ± 10.3 (8)	160.0 ± 14.9 (8)	164.4 ± 14.0 (8)	155.0 ± 16.5 (8)

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Data are presented as mean ± standard deviation (number of dams).
[c]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant findings were noted at $p \leq 0.05$.
[d]During lactation, all exposed animals received the same exposure concentration of 1.56 mg/L because of the high pup mortality during lactation in the previous 4-week study.
[e]Feed consumption was evaluated 1 day prior to study start and initiation of exposure on gestation day 6.

For F_1 male and female rats, pup viability, number of live pups per litter, live litter size, and sex ratio were similar to the control groups during lactation (Table 14, Table 18).

On PND 1, mean body weights of male and female pups in the 12.5 and 25 mg/L groups were 93% and 91% (males) and 93% and 89% (females) of control animals, respectively. During lactation, male and female pups in the ≤12.5 mg/L groups had mean body weights that were 85%–92% of the control groups from PND 4 through PND 28 (Table 18; Figure 5; Appendix D). Mean body weights of male and female pups in the 25 mg/L group were similar to those of the control groups on PND 28.

Table 18. Summary of Preweaning F_1 Male and Female Rat Pup Mean Body Weights Following Perinatal Exposure to Nicotine Bitartrate Dihydrate[a]

Postnatal Day[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L[d]	6.25 mg/L[d]	12.5 mg/L[d]	25 mg/L[d]
Male						
1	7.5 ± 0.5 (53/8)	7.1 ± 0.4 (62/8)	7.3 ± 0.5 (68/8)	7.4 ± 0.4 (68/8)	7.0 ± 0.3 (65/8)	6.9 ± 0.5 (58/8)
4	10.4 ± 1.0 (52/8)	9.3 ± 0.7 (62/8)	9.6 ± 1.0 (67/8)	9.5 ± 1.0 (68/8)	9.2 ± 0.8 (63/8)	9.6 ± 1.0 (58/8)
7	14.1 ± 1.9 (51/8)	12.7 ± 1.1 (62/8)	12.9 ± 1.5 (67/8)	12.5 ± 1.8 (67/8)	12.6 ± 1.2 (62/8)	13.4 ± 1.5 (58/8)
14	25.1 ± 3.2 (51/8)	22.1 ± 1.9 (62/8)	22.5 ± 2.6 (67/8)	22.2 ± 3.7 (66/8)	23.9 ± 5.2 (60/8)	23.4 ± 2.7 (58/8)
21	38.9 ± 6.4 (51/8)	33.1 ± 3.5 (62/8)	33.9 ± 4.7 (67/8)	33.3 ± 6.3 (66/8)	34.2 ± 3.8 (59/8)	35.9 ± 5.0 (58/8)

Postnatal Day[b,c]	0 mg/L	1.56 mg/L	3.12 mg/L[d]	6.25 mg/L[d]	12.5 mg/L[d]	25 mg/L[d]
28	67.1 ± 9.7 (51/8)	59.2 ± 5.4 (62/8)	59.9 ± 7.7 (67/8)	58.6 ± 8.6 (66/8)	60.3 ± 4.9 (59/8)	63.2 ± 7.7 (58/8)
Female						
1	7.1 ± 0.5 (53/8)	6.7 ± 0.3 (62/8)	6.8 ± 0.4 (57/8)	6.8 ± 0.5 (61/8)	6.6 ± 0.3 (63/8)	6.3 ± 0.5 (55/8)
4	9.8 ± 1.1 (53/8)	8.9 ± 0.6 (62/8)	9.1 ± 0.9 (57/8)	8.9 ± 1.2 (59/8)	8.7 ± 0.7 (62/8)	9.1 ± 0.9 (54/8)
7	13.5 ± 1.9 (53/8)	12.2 ± 1.3 (62/8)	12.3 ± 1.5 (57/8)	12.4 ± 1.8 (58/8)	12.1 ± 1.0 (61/8)	12.7 ± 1.5 (54/8)
14	23.6 ± 3.1 (53/8)	21.4 ± 2.3 (62/8)	21.6 ± 2.6 (56/8)	21.5 ± 3.7 (58/8)	21.6 ± 2.3 (60/8)	22.5 ± 2.9 (53/8)
21	37.6 ± 6.6 (53/8)	31.6 ± 4.0 (62/8)	32.8 ± 5.4 (56/8)	33.1 ± 6.9 (58/8)	33.4 ± 3.4 (60/8)	35.5 ± 5.2 (53/8)
28	63.7 ± 9.5 (53/8)	55.8 ± 6.1 (62/8)	57.4 ± 8.0 (56/8)	56.9 ± 9.0 (58/8)	57.9 ± 4.4 (60/8)	61.1 ± 7.8 (53/8)

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant findings were noted at $p \leq 0.05$.
[c]Data are presented as the mean of litter means ± standard deviation (number of pups/number of litters). Body weight data are presented in grams.
[d]During lactation, all exposed dams received the same exposure concentration of 1.56 mg/L because of the high pup mortality during lactation in the previous 4-week study.

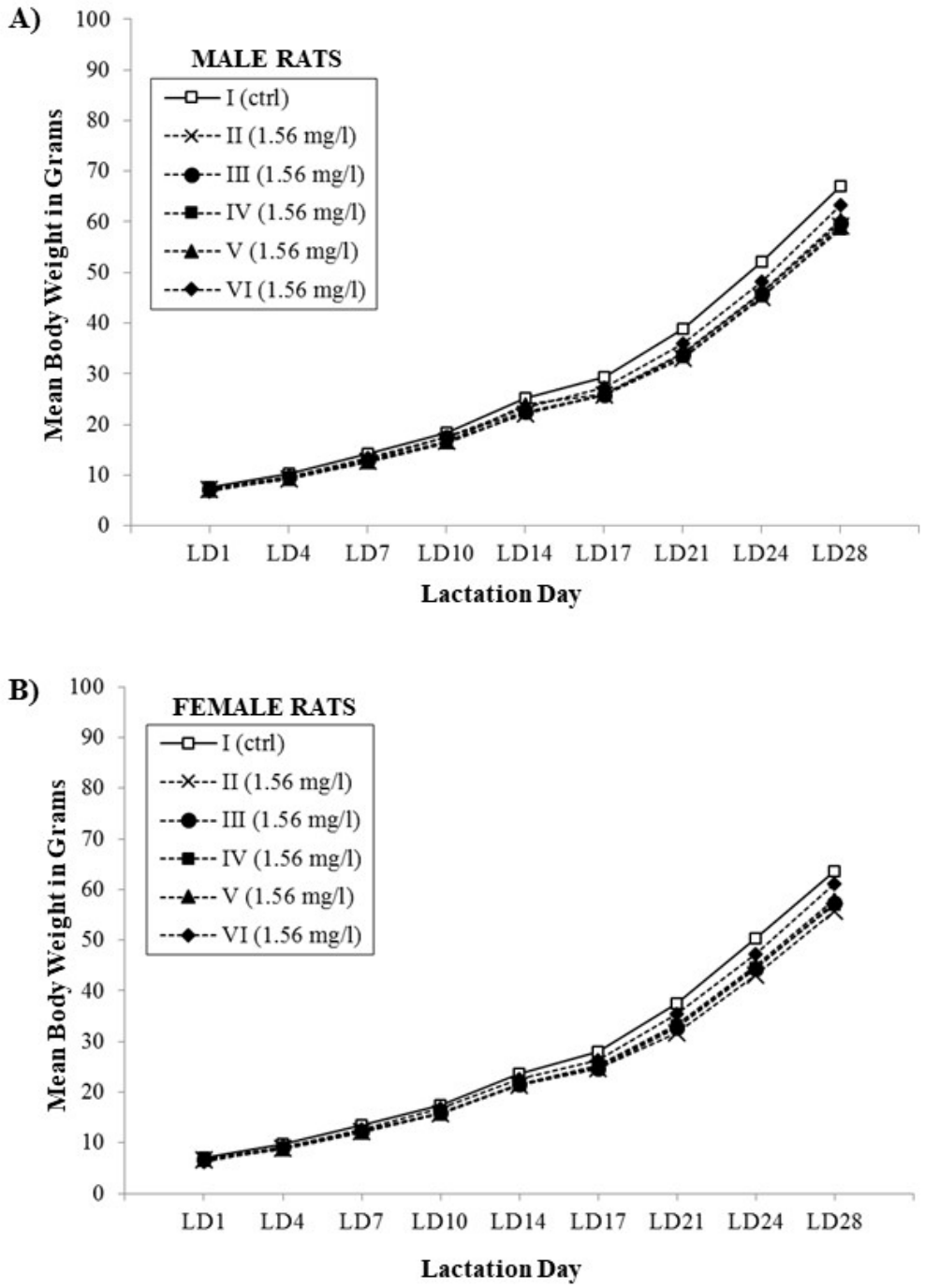

Figure 5. Preweaning Growth Curves for Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) males and (B) females.

Perinatal and Three-month Study (Postweaning Phase)

All male and female rats survived to study termination, and no clinical observations recorded during the study were considered exposure related (Appendix D).

Mean body weights of all exposed groups of male and female rats were 83%–87% of those of the control groups in week 1 (Table 19; Figure 6). By week 4, nearly all mean body weights of all exposed groups of both sexes were within 10% of the control groups (Appendix D). At week 12, mean body weights of male rats in the 25 mg/L group remained significantly decreased at 90% of the control group, and all other exposed groups were within 5% of the control group. Mean body weights of exposed female rats were also lower at 12 weeks but within 7% of that of the control group.

Table 19. Summary of Survival, Mean Body Weights, and Body Weight Gains of Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Week[b,c]	0 mg/L		1.56 mg/L		3.12 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L	
	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n
Male												
Body Weight												
1[d]	92.1 ± 6.6	10	77.3 ± 5.6**	10	76.1 ± 9.0**	10	79.5 ± 6.6**	10	79.0 ± 6.8**	10	79.6 ± 5.9**	10
12[e]	480.7 ± 28.5	10	484.3 ± 26.3	10	475.2 ± 35.5	10	469.0 ± 28.2	10	455.3 ± 27.4	10	431.3 ± 32.0**	10
Body Weight Change												
1–12	388.6 ± 22.7	10	407.0 ± 27.3	10	399.1 ± 36.4	10	389.5 ± 25.7	10	376.3 ± 29.6	10	351.7 ± 32.2*	10
Female												
Body Weight												
1[d]	85.2 ± 6.1	10	70.4 ± 3.5**	10	70.5 ± 9.2**	10	74.3 ± 8.7**	10	74.3 ± 3.9**	10	74.5 ± 5.4**	10
12[e]	292.6 ± 16.7	10	289.6 ± 18.4	10	279.8 ± 19.8	10	276.3 ± 18.0	10	280.7 ± 18.6	10	271.7 ± 18.3	10
Body Weight Change												
1–12	207.4 ± 18.6	10	219.2 ± 19.6	10	209.3 ± 18.2	10	202.0 ± 17.4	10	206.4 ± 18.5	10	197.2 ± 20.7	10

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation. Body weight data are presented in grams.
[d]Week 1 mean body weights represent body weights taken on the day of weaning.
[e]Week 12 mean body weights represent body weights taken before rats in all exposure groups were moved to metabolic cages for 16 hours during week 13.

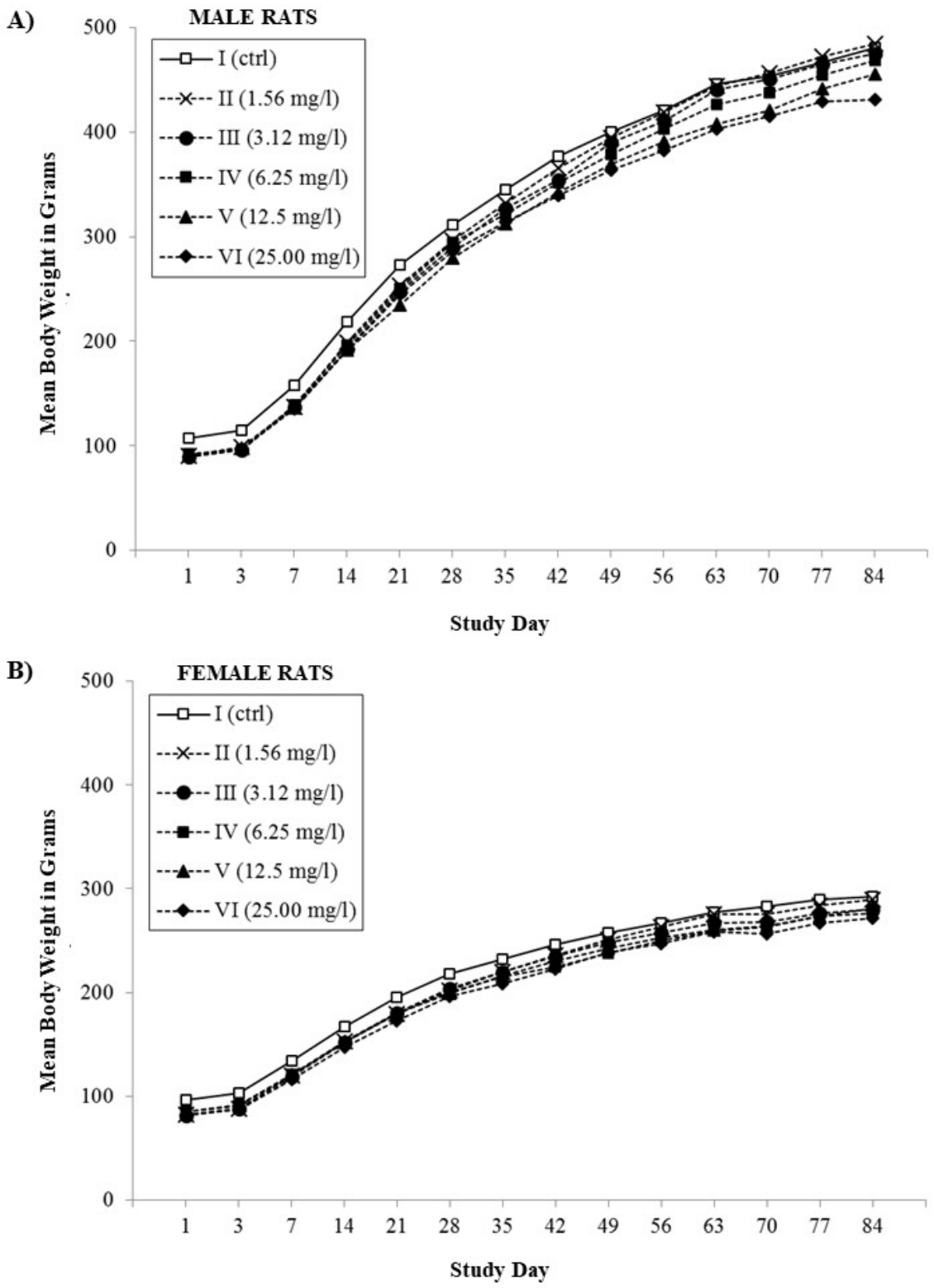

Figure 6. Growth Curves for Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) males and (B) females.

In male rats, mean water consumption (mL/animal/day) by the 25 mg/L group was less than that of the control group throughout the entire postweaning period (Table 20; Appendix D). During week 12, mean water consumption by the 25 mg/L group was 62% of that of the control group. The 6.25 and 12.5 mg/L groups had lower mean water consumption compared to the control group for most of the postweaning period, but consumption was similar to the control group in the last week of the study. In female rats, mean water consumption was less than that of the control group throughout the entire postweaning period for groups exposed to ≥6.25 mg/L (Table 20). During week 12, mean water consumption by the 6.25 and 12.5 mg/L groups was 91% and 82%, respectively, of that of the control group (not significantly different). During week 12, mean water consumption by the 25 mg/L was 58% of that of the control group.

Table 20. Summary of Water and Nicotine Bitartrate Dihydrate Consumption by Male and Female Rats in the Perinatal and Three-month Drinking Water Study[a]

Week	0 mg/L		1.56 mg/L		3.12 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
Water (mL/animal/day)[b,c]												
1	28.3 ± 1.9	10	24.3 ± 1.0	10	28.0 ± 4.1	10	23.8 ± 1.5*	10	23.5 ± 0.6*	10	19.5 ± 2.5**	10
12[d]	39.0 ± 3.6	10	40.8 ± 4.6	10	41.6 ± 4.5	10	39.3 ± 3.2	10	35.8 ± 4.0	10	24.1 ± 2.1**	10
Chemical Intake (mg/kg/day)[e,f,g]												
1–12	0	10	0.2	10	0.4	10	0.8	10	1.5	10	2.3	10
Female												
Water (mL/animal/day)[b,c]												
1	26.3 ± 2.1	10	24.9 ± 1.4	10	24.1 ± 3.7	10	20.7 ± 2.8*	10	20.8 ± 1.9*	10	15.6 ± 1.3**	10
12[d]	29.6 ± 1.8	10	32.8 ± 3.0	10	30.8 ± 3.8	10	26.8 ± 4.8	10	24.3 ± 2.3	10	17.2 ± 1.8**	10
Chemical Intake (mg/kg/day)[e,f,g]												
1–12	0	10	0.2	10	0.5	10	0.8	10	1.5	10	2.2	10

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation.
[d]Week 12 mean water consumption represents water consumption taken before rats in all exposure groups were moved to metabolic cages for 16 hours during week 13.
[e]Chemical intake calculated as: ([exposure concentration × water consumption]/[average body weight of day range]).
[f]No statistical analysis performed on the chemical intake data.
[g]Milligrams of freebase (-)-nicotine consumed/kg body weight/day.

In male rats, mean feed consumption on study day 1 was lower for all exposed groups compared to the control group (Appendix D), with consumption by the 25 mg/L group at 62% of that of the control group. By week 3, however, mean feed consumption by the 25 mg/L group was 90% of that of the control group and was similar to the control group for the remainder of the study (Table 21; Appendix D). Mean feed consumption by other exposed groups was sporadically less than that of the control group, but the differences were slight and not consistent throughout the study. Therefore, these differences were not considered biologically or toxicologically relevant.

In female rats, mean feed consumption by the 25 mg/L group was less than that of the control group, although the largest differences were found early in the study; by week 5, mean feed consumption reached 94% of that of the control group (Table 21; Appendix D). Mean feed consumption by the 12.5 mg/L group also was less than that of the control group throughout most of the study, but the differences were less than those of the 25 mg/L group. Other exposed

groups had sporadic differences in mean feed consumption, but these were not considered biologically or toxicologically relevant.

Table 21. Summary of Feed Consumption by Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Week[b,c]	0 mg/L		1.56 mg/L		3.12 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
Feed (g/animal/day)												
1	20.6 ± 1.1	10	19.1 ± 1.4	10	18.7 ± 0.7	10	17.7 ± 1.1	10	17.2 ± 1.1	10	17.7 ± 1.7	10
12[d]	25.3 ± 0.5	10	27.1 ± 4.8	10	27.7 ± 3.1	10	27.2 ± 1.6	10	26.2 ± 0.8	10	23.8 ± 2.0	10
Female												
Feed (g/animal/day)												
1	17.1 ± 1.3	10	15.4 ± 1.2	10	15.3 ± 2.6	10	14.8 ± 1.5	10	14.2 ± 1.1	10	14.2 ± 0.6	10
12[d]	17.4 ± 0.1	10	18.8 ± 2.3	10	16.6 ± 3.6	10	18.8 ± 3.3	10	17.9 ± 2.8	10	16.3 ± 2.4	10

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Data are presented as mean ± standard deviation.
[c]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant findings were noted at $p \leq 0.05$.
[d]Week 12 mean feed consumption represents feed consumption taken before rats in all exposure groups were moved to metabolic cages for 16 hours during week 13.

At the end of the 3-month study, there were several significant changes observed in both the clinical chemistry and hematology measurements in males and females. These changes were mild, not exposure related, not consistent between species or sex, or lacked biological significance. Thus, none of the observed significant changes were considered related to NBD exposure. There were no marked changes in urinalysis measures that were considered biologically significant (Appendix D).

In male rats, some sporadic and non-significant reductions in mean absolute organ weights (<90% of that of the control group) were accompanied by lower mean relative organ weights; these included the liver of the 25 mg/L group; the adrenal glands of the 3.12, 6.25, and 12.5 mg/L groups; and the urinary bladder and prostate (combined) of the 1.56, 3.12, and 25 mg/L groups (Table 22). In female rats, the mean absolute liver weight of the 25 mg/L group was 86% of that of the control group. However, the mean relative liver weight was unchanged from that of the control male and female rats in the presence of significant body weight decreases. In addition, several organs had non-significantly higher absolute weights ≥110% of that of the control group; these included the spleen of the 1.56 mg/L group; the adrenal glands and uterus of all exposed groups; and the ovaries of the 1.56, 12.5, and 25 mg/L groups (Table 22).

Table 22. Summary of Select Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a,b,c]

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
n	10	10	10	10	10	10
Male						
Terminal Body Wt. (g)	494.40 ± 31.05	493.00 ± 35.46	491.50 ± 36.57	480.30 ± 27.19	463.80 ± 28.58	447.40 ± 27.53**
Liver						
Absolute (g)	15.423 ± 1.581	14.859 ± 1.902	15.749 ± 1.640	14.830 ± 1.341	14.224 ± 1.750	13.806 ± 1.451
Relative (mg/g)[d]	31.141 ± 1.667	30.068 ± 2.242	32.006 ± 1.715	30.867 ± 1.957	30.586 ± 2.056	30.839 ± 2.389
Adrenal Glands						
Absolute (g)	0.078 ± 0.023	0.081 ± 0.021	0.070 ± 0.010	0.065 ± 0.017	0.065 ± 0.013	0.077 ± 0.021
Relative (mg/g)	0.159 ± 0.045	0.164 ± 0.041	0.143 ± 0.027	0.136 ± 0.034	0.140 ± 0.029	0.174 ± 0.052
Urinary Bladder and Prostate (Combined)						
Absolute (g)	0.901 ± 0.242	0.789 ± 0.226	0.750 ± 0.139	0.854 ± 0.093	0.861 ± 0.206	0.727 ± 0.148
Relative (mg/g)	1.821 ± 0.477	1.606 ± 0.446	1.537 ± 0.325	1.784 ± 0.229	1.858 ± 0.444	1.629 ± 0.352
Heart						
Absolute (g)	1.477 ± 0.059	1.520 ± 0.102	1.470 ± 0.082	1.461 ± 0.094	1.371 ± 0.086	1.367 ± 0.119*
Relative (mg/g)	2.997 ± 0.203	3.094 ± 0.243	2.998 ± 0.157	3.049 ± 0.226	2.959 ± 0.161	3.059 ± 0.234
Female						
Terminal Body Wt. (g)	302.40 ± 19.41	297.40 ± 21.57	284.60 ± 15.32	283.80 ± 19.01	287.30 ± 20.45	273.20 ± 11.50**
Liver						
Absolute (g)	10.595 ± 0.917	10.204 ± 1.195	9.934 ± 0.983	9.808 ± 0.930	9.920 ± 0.876	9.148 ± 0.911**
Relative (mg/g)	35.018 ± 1.689	34.264 ± 2.580	34.860 ± 2.213	34.548 ± 2.275	34.543 ± 1.975	33.461 ± 2.744
Adrenal Glands						
Absolute (g)	0.072 ± 0.011	0.084 ± 0.017	0.084 ± 0.018	0.083 ± 0.026	0.096 ± 0.022	0.089 ± 0.016
Relative (mg/g)	0.239 ± 0.037	0.285 ± 0.058	0.294 ± 0.063	0.292 ± 0.086	0.336 ± 0.081**	0.324 ± 0.058*

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Spleen						
Absolute (g)	0.546 ± 0.055	0.624 ± 0.064	0.582 ± 0.112	0.579 ± 0.065	0.599 ± 0.053	0.554 ± 0.062
Relative (mg/g)	1.808 ± 0.184	2.100 ± 0.198	2.044 ± 0.361	2.040 ± 0.185	2.088 ± 0.150	2.033 ± 0.262
Uterus						
Absolute (g)	0.527 ± 0.069	0.595 ± 0.105	0.601 ± 0.169	0.610 ± 0.147	0.706 ± 0.185	0.595 ± 0.169
Relative (mg/g)	1.756 ± 0.293	2.014 ± 0.380	2.111 ± 0.556	2.153 ± 0.524	2.456 ± 0.612*	2.183 ± 0.638
Ovaries						
Absolute (g)	0.150 ± 0.030	0.174 ± 0.041	0.159 ± 0.024	0.160 ± 0.037	0.168 ± 0.033	0.169 ± 0.026
Relative (mg/g)	0.496 ± 0.104	0.586 ± 0.140	0.560 ± 0.087	0.561 ± 0.116	0.589 ± 0.140	0.619 ± 0.093

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard deviation.
[b]Statistical analysis performed with the Dunnett (pairwise) test.
[c]Concentrations in drinking water refer to freebase (-)-nicotine.
[d]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.

Histopathology

This section describes incidences of select nonneoplastic lesions in the kidney, larynx, mandibular lymph node, stomach, large and small intestines, pancreas, Harderian gland, testes, and salivary glands. Multiple organ systems exhibited minimal to mild focal inflammation or changes in lymphoid tissues that did not follow any consistent dose response, making associations with exposures uncertain.

Kidney: Minimal focal or mild diffuse inflammation in the kidney was present in exposed male and female rats and control rats (Table 23). One male rat in the 25 mg/L group and one female rat in the 12.5 mg/L group also had hyperplasia of the transitional epithelium of the pelvis (Appendix D).

Larynx: Minimal inflammation of the larynx was present in all exposed male rat groups (Table 23). Similarly, in exposed female rat groups, minimal laryngeal inflammation was present at exposure concentrations of ≥6.25 mg/L. These lesions were not considered biologically significant.

Mandibular lymph node: The incidence of minimal chronic reactive lymphadenitis was higher in most of the exposed male groups than in the control group and was significantly increased in the 25 mg/L group (Table 23). A similar pattern was apparent in female rats, with minimal chronic reactive lymphadenitis present in seven rats in the 25 mg/L group compared to three rats in the control group.

Stomach: Minimal hyperkeratosis of the forestomach was observed at 6.25 and 12.5 mg/L in males and at 6.25 mg/L in females (Table 23).

Large and small intestines: Higher incidences of minimal inflammation of the rectum were present in males from the 3.12, 12.5, and 25 mg/L groups, and females in the 1.56, 6.25, 12.5, and 25 mg/L groups, compared to respective control groups (Table 23). Minimal inflammation of the duodenum was observed in females in the 3.12, 12.5, and 25 mg/L groups but not in control females (Table 23). This lesion was also observed in exposed males but at incidences that did not exceed that of the control group.

Pancreas: Minimal focal inflammation of the pancreas was observed in female rats in the 1.56, 3.12, and 25 mg/L groups but not in control females (Table 23). This lesion was observed in male rats at each exposure concentration, except 6.25 mg/L, and was observed in one rat in the control group.

Harderian gland: Minimal focal inflammation was increased in all but the 3.12 mg/L exposed group of females compared to control females (Table 23). This lesion was not increased in male rats.

Testes: Degenerative lesions of the testes were observed in two rats in the two highest exposed groups (Table 23). There was a positive trend for the incidence of rats bearing lesions.

Salivary glands: Minimal focal inflammation was observed in all but the 12.5 mg/L group of males, including in one rat in the control group (Table 23). Single instances were observed in the 3.12 and 25 mg/L female groups.

Table 23. Incidences of Select Nonneoplastic Lesions in Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
n[b]	10	10	10	10	10	10
Male						
Kidney						
Inflammation, focal, minimal[c]	3	5	3	5	9**	3
Inflammation, diffuse, mild	0	1	0	0	0	0
Larynx						
Inflammation, minimal	0	2	1	2	2	3
Lymph Node, Mandibular						
Lymphadenitis, chronic reactive, minimal	2	5	4	4	2	8*
Stomach, Forestomach						
Hyperkeratosis, minimal	0	0	0	2	3	0
Intestine, Large, Rectum						
Inflammation, minimal	1	1	5	1	2	2
Intestine Small, Duodenum						
Inflammation, minimal	2	2	2	1	0	1
Pancreas						
Inflammation, focal, minimal	1	2	1	0	2	1
Harderian Gland						
Inflammation, focal, minimal	1	1	1	0	0	1
Testes						
Degeneration, fibrosis tubules	0	0	0	0	1	0
Degeneration, seminiferous tubules, minimal	0	0	0	0	1	1
Degeneration, germinal epithelium, mild	0	0	0	0	0	1
Mineralization, tubules, minimal	0	0	0	0	1	1
Hyperplasia, interstitial cells	0	0	0	0	0	1
Salivary Glands						
Inflammation, focal, minimal	1	2	1	3	0	1
Female						
Kidney						
Inflammation, focal, minimal	2	1	4	5	5	2
Inflammation, diffuse, mild	0	0	0	0	1	1

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Larynx						
Inflammation, minimal	0	0	0	1	4	1
Lymph Node, Mandibular						
Lymphadenitis, chronic, reactive, minimal	3	4	3	3	2	7
Stomach, Forestomach						
Hyperkeratosis, minimal	0	0	0	1	0	0
Intestine, Large, Rectum						
Inflammation, minimal	0	3	0	4	2	2
Intestine Small, Duodenum						
Inflammation, minimal	0	0	1	0	3	1
Pancreas						
Inflammation, focal, minimal	0	2	2	0	0	4
Harderian Gland						
Inflammation, focal, minimal	1	4	1	4	3	2
Salivary Glands						
Inflammation, focal, minimal	0	0	1	0	0	1

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control.
*Statistically significant at $p \leq 0.05$ by Fisher's exact test; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Number of animals examined microscopically.
[c]Number of animals with lesion.

Mice

Four-week Dose Range-finding Study

All mice survived until study termination, and no clinical observations recorded during the study were considered exposure related (Table 24; Appendix D).

Mean body weights of all exposed groups of male and female mice were within 10% of those of the control groups throughout the study (Table 24; Figure 7). At the end of exposure, mean body weights of the 100 mg/L male and female groups were within 5% of the respective control groups.

Table 24. Summary of Survival, Mean Body Weights, and Body Weight Gains of Male and Female Mice in the Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate, with Two-week Recovery Period[a]

Study Day[b,c]	0 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L		50 mg/L		100 mg/L	
	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n
Male												
Exposure Period												
0	41.3 ± 2.7	20	41.1 ± 3.7	10	42.8 ± 2.8	10	40.6 ± 4.4	10	41.8 ± 3.7	10	40.5 ± 3.5	20
1[d,e]	41.8 ± 2.2	20	41.8 ± 3.6	10	43.3 ± 2.6	10	41.8 ± 4.1	10	43.2 ± 3.7	10	41.8 ± 3.5	20
3[e]	42.4 ± 2.5	20	42.5 ± 4.0	10	44.0 ± 2.6	10	42.4 ± 4.0	10	43.8 ± 3.7	10	41.7 ± 3.8	20
7[e]	43.0 ± 2.4	20	42.2 ± 4.2	10	44.7 ± 2.9	10	42.6 ± 4.2	10	43.4 ± 4.0	10	42.2 ± 3.9	20
14[e]	43.0 ± 2.4	20	43.0 ± 3.8	10	44.2 ± 3.1	10	42.5 ± 4.0	10	43.6 ± 3.6	10	42.5 ± 4.0	20
21	44.9 ± 3.0	20	43.7 ± 4.1	10	45.2 ± 2.9	10	44.0 ± 4.9	10	44.4 ± 3.7	10	43.3 ± 4.5	20
28[f]	44.5 ± 3.2	20	41.7 ± 4.6	10	42.6 ± 3.3	10	41.9 ± 6.3	10	42.1 ± 4.1	10	43.4 ± 3.9	20
Recovery Period[g]												
35	44.7 ± 2.8	10	–	0	–	0	–	0	–	0	43.4 ± 3.6	10
42[e,f]	45.6 ± 2.8	10	–	0	–	0	–	0	–	0	43.6 ± 4.9	10
Weight Change												
0–21	3.6 ± 1.0	20	2.6 ± 1.2	10	2.4 ± 1.5	10	3.4 ± 1.3	10	2.6 ± 1.4	10	2.8 ± 2.1	20
Female												
Exposure Period												
0	33.8 ± 3.1	20	33.4 ± 2.2	10	33.4 ± 2.9	10	31.4 ± 2.8	10	33.2 ± 3.7	10	33.4 ± 3.3	20
1[d,e]	33.8 ± 3.3	20	34.4 ± 2.3	10	33.2 ± 2.6	10	31.1 ± 2.9	10	33.5 ± 3.2	10	34.4 ± 3.8	20
3[e]	33.6 ± 3.0	20	34.9 ± 2.1	10	33.9 ± 2.6	10	32.0 ± 2.8	10	33.3 ± 3.0	10	33.8 ± 3.5	20
7[e]	34.7 ± 3.0	20	35.1 ± 3.2	10	34.7 ± 2.7	10	32.4 ± 3.6	10	33.1 ± 3.1	10	34.6 ± 3.5	20
14[e]	34.8 ± 3.6	20	35.0 ± 3.5	10	34.9 ± 3.3	10	32.1 ± 2.9	10	34.5 ± 3.6	10	35.1 ± 3.9	20
21	35.1 ± 2.9	20	36.8 ± 3.1	10	35.6 ± 2.6	10	33.3 ± 2.7	10	34.5 ± 3.7	10	35.8 ± 4.0	20
28[f]	34.8 ± 3.5	20	34.5 ± 3.7	10	33.8 ± 2.1	10	32.3 ± 3.8	10	33.2 ± 3.2	10	34.1 ± 3.5	20

Toxicity Studies of Nicotine Bitartrate Dihydrate Administered in Drinking Water to Sprague Dawley Rats and Swiss Mice

Study Day[b,c]	0 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L		50 mg/L		100 mg/L	
	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n	Av. Wt. (g)	n
Recovery Period[g]												
35	36.2 ± 3.7	10	–	0	–	0	–	0	–	0	35.6 ± 2.7	10
42[e,f]	37.4 ± 3.5	10	–	0	–	0	–	0	–	0	36.5 ± 3.6	10
Weight Change												
0–21	1.4 ± 1.5	20	3.1 ± 2.2	10	2.2 ± 1.8	10	1.9 ± 2.2	10	1.3 ± 1.1	10	2.4 ± 1.8	20

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant findings were noted at $p \leq 0.05$.
[c]Data are presented as mean ± standard deviation. Body weight data are presented in grams.
[d]Study day 1 is the day animals were placed on study.
[e]No statistical analysis performed on this endpoint.
[f]On study day 28, measurements were taken after 10 mice from each exposure group were moved to metabolic cages for 16 hours. On study day 42 (recovery period), measurements were taken after 10 mice each from the control and 100 mg/L groups were moved to metabolic cages for 16 hours.
[g]Data for the recovery period were collected for only 10 mice in the control group and 100 mg/L group.

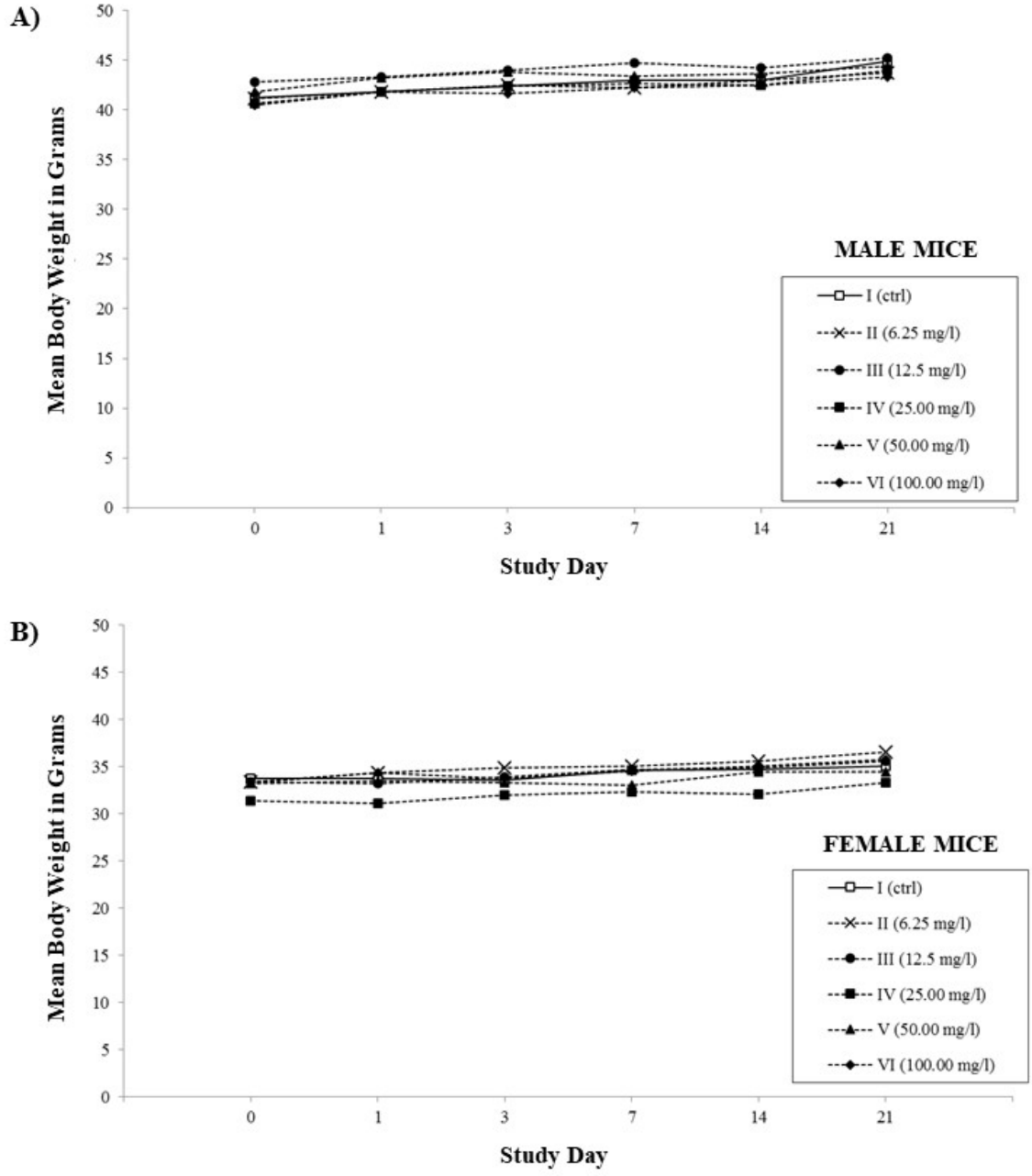

Figure 7. Growth Curves for Male and Female Mice in the Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) males and (B) females.

In male mice, mean water consumption (mL/animal/day) by all exposed groups fluctuated throughout the study compared to the control group, with consumption as low as 79% on study day 21 for the 100 mg/L group (Table 25). A similar pattern was observed in female mice, wherein mean water consumption by the 100 mg/L group was 79% of that of the control group on study day 21. None of the reductions in water consumption were significant. Mean water consumption by the 100 mg/L male and female recovery groups was approximately 95% and 112%, respectively, of that of the control groups.

Table 25. Summary of Water and Nicotine Bitartrate Dihydrate Consumption by Male and Female Mice in the Four-week Dose Range-finding Drinking Water Study, with Two-week Recovery Period[a]

Study Day	0 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L		50 mg/L		100 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
Water (mL/animal/day)[b,c]												
Exposure Period												
0	7.0 ± 0.5	20	6.2 ± 1.4	10	6.0 ± 0.6	10	6.6 ± 0.3	10	6.4 ± 1.1	10	6.8 ± 0.7	20
1[d]	7.3 ± 0.8	20	6.2 ± 0.8	10	6.8 ± 0.6	10	7.0 ± 0.8	10	6.8 ± 0.6	10	6.8 ± 0.6	20
21	7.1 ± 0.8	20	6.0 ± 1.1	10	6.4 ± 1.1	10	6.0 ± 1.1	10	6.2 ± 0.3	10	5.6 ± 0.6	20
28[e]	7.2 ± 0.6	10	–	0	–	0	–	0	–	0	7.8 ± 0.3	10
Recovery Period[f]												
35	7.8 ± 0.3	10	–	0	–	0	–	0	–	0	7.4 ± 1.4	10
Chemical Intake (mg/kg/day)[g]												
0–21	0	20	1	10	2	10	3.8	10	7.6	10	14	20
Female												
Water (mL/animal/day)[b,c]												
Exposure Period												
0	6.5 ± 0.5	20	6.2 ± 0.3	10	5.4 ± 0.3	10	5.8 ± 1.4	10	6.2 ± 0.8	10	6.1 ± 0.4	20
1[d]	6.9 ± 0.8	20	6.8 ± 1.1	10	6.6 ± 0.3	10	7.2 ± 1.7	10	6.2 ± 0.8	10	6.4 ± 0.5	20
21	6.3 ± 0.9	20	5.2 ± 0.0	10	6.2 ± 0.3	10	6.0 ± 0.0	10	5.4 ± 0.3	10	5.0 ± 0.8	20
28[e]	8.0 ± 2.8	10	–	0	–	0	–	0	–	0	8.0 ± 1.7	10
Recovery Period[f]												
35	6.6 ± 0.8	10	–	0	–	0	–	0	–	0	7.4 ± 1.4	10
Chemical Intake (mg/kg/day)[g]												
0–21	0	20	1.2	10	2.4	10	5.0	10	9.1	10	15.3	20

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant differences were identified at $p \leq 0.05$.
[c]Data are presented as mean ± standard deviation.
[d]Study day 1 is the day animals were placed on study.
[e]For study day 28, measurements are reported for only 10 mice each in the control and 100 mg/L recovery groups.
[f]Data for the recovery period were collected for only 10 mice each in the control group and 100 mg/L group.
[g]Milligrams of nicotine bitartrate dihydrate consumed/kg body weight/day.

Throughout the study, mean feed consumption by exposed groups was similar to that of the control groups for both male and female mice. While sporadic differences from the control group were found, they were not consistent and did not indicate an exposure-related effect (Appendix D).

No clinical chemistry or hematological changes were observed in exposed male or female mice compared to control mice (Appendix D).

In female mice, mean absolute weights of the thymus and mediastinal lymph nodes (combined), adrenal glands, uterus, and ovaries were lower in all exposed groups compared to those of the control group (Table 26). However, there were no significant exposure concentration-related decreases in organ weights. The mean absolute weight of the adrenal glands in the exposed groups was 67%–80% of that of the control group, but the mean weight in the control group was higher than that recorded in the female recovery control group (Appendix D). In fact, the mean absolute weight of the adrenal glands in the 100 mg/L recovery group was 145% of that of the recovery control group. These observations, along with other sporadic differences in mean organ weights, were not considered toxicologically relevant because they lacked consistency, exposure concentration-related response, and histopathological correlations.

Likewise, in male mice, there were sporadic occurrences of organ weights being <90% or >110% of those of the control group. None of the occurrences were consistent, exhibited a pattern of exposure-concentration relatedness, or had histopathological correlations; thus, they were not considered toxicologically relevant. These observations were true for both the 4-week study groups and the recovery groups (Appendix D).

Table 26. Summary of Select Organ Weights and Organ-Weight-to-Body-Weight Ratios for Female Mice in the Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a,b,c]

	0 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L	100 mg/L
n	10	10	10	10	10	10
Terminal Body Wt. (g)	33.50 ± 3.06	34.50 ± 3.75	33.80 ± 2.10	32.30 ± 3.80	33.20 ± 3.22	34.40 ± 4.17
Thymus and Mediastinal Lymph Nodes (Combined)						
Absolute (g)	0.052 ± 0.022	0.041 ± 0.014	0.042 ± 0.009	0.042 ± 0.013	0.049 ± 0.010	0.049 ± 0.009
Relative (mg/g)[d]	1.531 ± 0.512	1.201 ± 0.431	1.253 ± 0.277	1.306 ± 0.374	1.476 ± 0.305	1.422 ± 0.186
Adrenal Glands						
Absolute (g)	0.015 ± 0.008	0.010 ± 0.007	0.012 ± 0.005	0.012 ± 0.007	0.011 ± 0.002	0.012 ± 0.005
Relative (mg/g)	0.431 ± 0.206	0.293 ± 0.164	0.344 ± 0.140	0.377 ± 0.255	0.325 ± 0.052	0.363 ± 0.134
Uterus						
Absolute (g)	0.230 ± 0.075	0.189 ± 0.059	0.171 ± 0.080	0.161 ± 0.064	0.175 ± 0.080	0.212 ± 0.091
Relative (mg/g)	6.898 ± 2.312	5.558 ± 1.864	5.060 ± 2.431	4.888 ± 1.382	5.258 ± 2.256	6.300 ± 2.913
Ovaries						
Absolute (g)	0.063 ± 0.019	0.055 ± 0.016	0.059 ± 0.024	0.054 ± 0.029	0.050 ± 0.016	0.058 ± 0.021
Relative (mg/g)	1.861 ± 0.493	1.633 ± 0.523	1.746 ± 0.654	1.648 ± 0.788	1.518 ± 0.470	1.695 ± 0.591

[a]Data are presented as mean ± standard deviation.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test. No statistically significant findings were noted at $p \leq 0.05$.
[c]Concentrations in drinking water refer to freebase (-)-nicotine.
[d]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight per g body weight.

Histopathology

This section describes the incidences of select nonneoplastic lesions in the kidney, intestines (all sites), liver, and Harderian gland. The incidences were not statistically significant. The control and 100 mg/L groups were the only groups examined microscopically.

Kidney: Minimal focal inflammation of the kidney was present in exposed and control male and female mice, and moderate diffuse inflammation was diagnosed in one exposed male (Table 27).

Stomach and intestines (all sites): In exposed and control male and female mice, inflammation was present (Table 27).

Liver: Minimal focal necrosis with inflammation was present in three female mice in the 100 mg/L group but was considered a background lesion (Table 27). The lesion consisted of one or several small areas of necrosis with an associated mixed inflammatory cell response.

Harderian gland: Minimal focal inflammation was present in exposed male and female mice (Table 27). This lesion was not observed in control mice.

Table 27. Incidences of Select Nonneoplastic Lesions in Male and Female Mice in the Four-week Dose Range-finding Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

	0 mg/L	100 mg/L
n[b]	10	10
Male[c]		
Kidney		
Inflammation, focal, minimal[d]	1	4
Inflammation, diffuse, moderate	0	1
Intestines (All Sites)		
Inflammation, minimal	4	6
Harderian Gland		
Inflammation, focal, minimal	0	1
Female		
Kidney		
Inflammation, focal, minimal	1	1
Stomach, Glandular		
Inflammation, focal	0	1
Intestines (All Sites)		
Inflammation, minimal	3	2
Liver		
Necrosis, focal, minimal, with inflammation	0	3
Harderian Gland		
Inflammation, focal, minimal	0	3

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Number of animals examined microscopically.
[c]The exposed group was compared to the vehicle control group with Fisher's exact (pairwise) test. No statistically significant differences were identified at $p \leq 0.05$.
[d]Number of animals with lesion.

Exposure Concentration Selection Rationale for the Three-month Study

The 4-week study informed selection of exposure concentrations for the 3-month study in mice. Exposure to drinking water solutions with NBD ≤100 mg/L for 4 weeks was well tolerated, and therefore the exposure concentrations selected for the 3-month study in mice were the same as the 4-week study (0, 6.25, 12.5, 25, 50, and 100 mg/L).

Three-month Study

All mice survived until study termination, and no clinical observations recorded during the study were considered exposure related (Appendix D).

Mean body weights of all exposed groups of male and female mice were within 10% of those of the control groups throughout the study (Table 28; Figure 8). At study termination, mean body weights of the 100 mg/L male and female groups were approximately 93% and 95% of those of their respective control groups.

Table 28. Summary of Survival, Mean Body Weights, and Body Weight Gains of Male and Female Mice in the Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

Week[b,c]	0 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L		50 mg/L		100 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
1[d]	40.4 ± 2.8	30	40.4 ± 3.2	15	40.6 ± 2.4	15	39.7 ± 3.4	15	42.3 ± 3.6	15	40.2 ± 3.1	30
12[e]	46.0 ± 3.9	30	47.1 ± 5.2	15	44.5 ± 3.7	15	45.1 ± 5.2	15	45.5 ± 3.2	15	42.9 ± 4.1*	30
Weight Change												
1–12	5.7 ± 2.6	30	6.7 ± 2.6	15	3.9 ± 2.2	15	5.5 ± 3.4	15	3.1 ± 2.1**	15	2.7 ± 2.1**	30
Female												
1[d]	31.6 ± 3.5	30	31.8 ± 3.2	15	31.4 ± 3.4	15	29.9 ± 2.7	15	32.1 ± 2.5	15	31.0 ± 3.5	30
12[e]	38.6 ± 5.7	30	37.9 ± 4.8	15	37.7 ± 5.2	15	37.6 ± 4.6	15	37.3 ± 3.3	15	36.8 ± 4.8	30
Weight Change												
1–12	7.0 ± 3.5	30	6.1 ± 3.1	15	6.3 ± 2.7	15	7.7 ± 2.7	15	5.2 ± 2.0	15	5.8 ± 2.2	30

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation. Body weight data are presented in grams.
[d]Week 1 mean body weights represent body weights taken the day prior to study start.
[e]Week 12 mean body weights represent body weights taken before mice in all exposure groups were moved to metabolic cages for 16 hours during week 13.

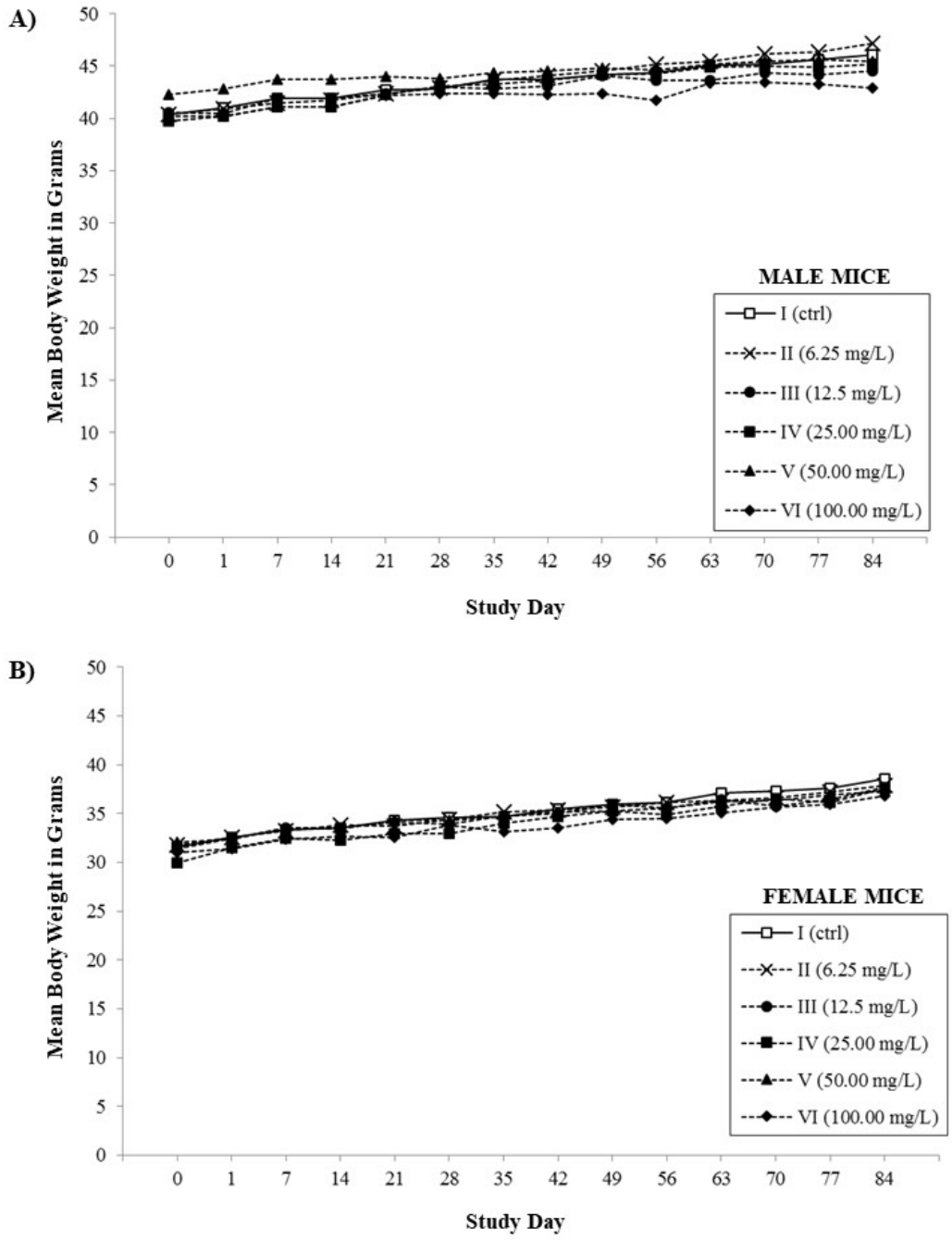

Figure 8. Growth Curves for Male and Female Mice in the Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate

Growth curves are shown for (A) males and (B) females.

In male mice, mean water consumption (mL/animal/day) by the 100 mg/L group was <90% of that of the control group for most of the first half of the study, as well as for the last 3 weeks (Table 29). Other exposed groups had sporadic weeks with water consumption less than that of the control group, but they were not consistent or considered biologically or toxicologically significant. In female mice, beginning on study day 1, mean water consumption was <90% of that of the control group by all exposed groups for most of the study. The largest differences were in the 100 mg/L group, which had mean water consumption <80% of that of the control group during most weeks.

Table 29. Summary of Water and Nicotine Bitartrate Dihydrate Consumption by Male and Female Mice in the Three-month Drinking Water Study[a]

Week	0 mg/L		6.25 mg/L		12.5 mg/L		25 mg/L		50 mg/L		100 mg/L	
	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n	Av.	n
Male												
Water (mL/animal/day)[b,c]												
1[d]	7.7 ± 0.7	30	7.6 ± 0.8	15	7.3 ± 0.5	15	8.1 ± 0.9	15	8.0 ± 1.1	15	7.3 ± 0.9	30
12[e]	7.4 ± 0.9	30	7.3 ± 0.9	15	7.3 ± 0.6	15	8.1 ± 1.6	15	7.6 ± 1.1	15	6.4 ± 0.5	30
Chemical Intake (mg/kg/day)[f]												
1–12	0	30	1	15	2	15	4.4	15	8.1	15	15	30
Female												
Water (mL/animal/day)[b,c]												
1[d]	7.9 ± 1.4	30	7.3 ± 1.2	15	7.7 ± 0.8	15	7.1 ± 1.2	15	8.1 ± 2.0	15	8.1 ± 1.3	30
12[e]	8.1 ± 0.9	30	7.3 ± 0.8	15	7.7 ± 0.8	15	7.5 ± 1.3	15	7.2 ± 0.8	15	5.9 ± 0.3**	30
Chemical Intake (mg/kg/day)[f]												
1–12	0	30	1.4	15	2.4	15	5.1	15	9.6	15	18.6	30

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group.
**Statistically significant at $p \leq 0.01$.
[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group with the Dunnett test.
[c]Data are presented as mean ± standard deviation.
[d]Week 1 mean water consumption represents water consumption taken the day prior to study start.
[e]Week 12 mean water consumption represents water consumption taken before mice in all exposure groups were moved to metabolic cages for 16 hours during week 13.
[f]Milligrams of nicotine bitartrate dihydrate consumed/kg body weight/day.

Throughout the study, mean feed consumption by the exposed groups was similar to that of the control groups for both male and female mice. Sporadic differences from the control groups were neither consistent nor related to exposure concentrations and did not indicate an exposure-related effect (Appendix D).

At the end of the 3-month study, there were some significant changes observed in both the clinical chemistry and hematology measures from male and female mice. These changes were mild, not exposure related, not consistent between species or sex, or lacked biological significance. Thus, none of the observed significant changes were considered related to NBD exposure. In exposed groups of mice, mean absolute and relative weights of the adrenal glands in

males and of the spleen and thymus in females were <90% of that of the control group; however, changes were not significant (Table 30; Appendix D).

Table 30. Summary of Select Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male and Female Mice in the Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a,b,c]

	0 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L	100 mg/L
n	15	15	15	15	15	15
Male						
Terminal Body Wt. (g)	40.07 ± 3.01	41.33 ± 4.95	39.07 ± 3.26	39.00 ± 4.64	39.13 ± 2.53	38.07 ± 3.01
Adrenal Glands						
Absolute (g)	0.013 ± 0.014	0.008 ± 0.007	0.007 ± 0.002	0.009 ± 0.009	0.007 ± 0.003	0.010 ± 0.007
Relative (mg/g)[d]	0.311 ± 0.343	0.197 ± 0.165	0.179 ± 0.064	0.233 ± 0.189	0.170 ± 0.073	0.265 ± 0.198
Seminal Vesicles and Coagulating Gland						
Absolute (g)	0.456 ± 0.106	0.435 ± 0.125	0.472 ± 0.165	0.459 ± 0.103	0.461 ± 0.114	0.407 ± 0.056
Relative (mg/g)	11.362 ± 2.306	10.652 ± 3.408	12.258 ± 4.965	11.822 ± 2.567	11.675 ± 2.283	10.685 ± 1.287
Female						
Terminal Body Wt. (g)	33.67 ± 4.51	34.07 ± 4.64	33.13 ± 4.76	33.27 ± 4.15	32.33 ± 3.13	32.60 ± 4.27
Spleen						
Absolute (g)	0.171 ± 0.174	0.117 ± 0.027	0.114 ± 0.022	0.117 ± 0.031	0.125 ± 0.061	0.121 ± 0.029
Relative (mg/g)	4.994 ± 4.914	3.497 ± 0.952	3.463 ± 0.512	3.540 ± 0.985	3.848 ± 1.717	3.698 ± 0.734
Thymus						
Absolute (g)	0.048 ± 0.042	0.038 ± 0.016	0.037 ± 0.008	0.040 ± 0.008	0.036 ± 0.016	0.038 ± 0.011
Relative (mg/g)	1.393 ± 1.140	1.129 ± 0.437	1.109 ± 0.221	1.215 ± 0.346	1.125 ± 0.448	1.174 ± 0.311

[a]Data are presented as mean ± standard deviation.
[b]Each exposed group was compared to the vehicle control group with the Dunnett (pairwise) test. No statistically significant findings were noted at $p \leq 0.05$.
[c]Concentrations in drinking water refer to freebase (-)-nicotine.
[d]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight per g body weight.

Histopathology

This section describes the incidences of select nonneoplastic lesions in the kidney, urinary bladder, stomach, large and small intestines, and Harderian gland. Multiple organ systems exhibited minimal to mild focal inflammation that did not follow any consistent exposure concentration response, making associations with exposures uncertain.

Kidney: A positive trend of mild diffuse inflammation was observed in female treated mice (Table 31).

Urinary bladder: Minimal focal inflammation of the urinary bladder was present in two male mice in the 6.25 mg/L group, one male mouse in the 12.5 mg/L group, and two female mice in each of the 25 and 100 mg/L groups (Table 31).

Stomach, glandular: Minimal inflammation of the glandular stomach was present in all exposed groups, but not in the control group, of female mice (Table 31).

Large and small intestines: Minimal inflammation of the colon (males only), rectum (males and females), and duodenum (males only) was observed in only exposed animals, and no cases of inflammation of these tissues were observed in control mice (Table 31). A nonsignificant trend of higher incidence of minimal inflammation of the jejunum was observed in exposed female mice (Table 31). No cases of minimal inflammation of the jejunum were observed in control mice (Table 31).

Harderian gland: Minimal focal inflammation occurred in exposed and control female mice (Table 31).

Salivary glands: Mild focal inflammation occurred to a higher extent in exposed male mice compared to control mice (Table 31).

Table 31. Incidences of Select Nonneoplastic Lesions in Male and Female Mice in the Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a]

	0 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L	100 mg/L
Male[b]						
Kidney[c]	15	15	15	15	15	15
Inflammation, focal, minimal[d]	4	2	2	3	4	5
Inflammation, diffuse, mild	4	1	1	1	1	4
Urinary Bladder	13	15	15	14	15	15
Inflammation, focal, minimal	0	2	1	0	0	0
Stomach, Glandular	13	14	15	15	15	14
Inflammation, minimal	1	0	2	1	1	0
Intestine, Large, Rectum	15	15	15	15	15	15
Inflammation, minimal	0	0	1	1	1	2
Intestine, Large, Colon	15	15	15	15	15	15
Inflammation, minimal	0	0	2	1	3	1
Intestine, Small, Jejunum	15	15	15	15	15	15
Inflammation, minimal	0	1	0	0	1	0
Intestine, Small, Duodenum	15	15	15	15	15	15
Inflammation, minimal	0	0	0	0	2	0
Pancreas	15	15	15	15	15	15
Inflammation, focal, minimal	0	1	0	0	1	0
Harderian Gland	15	15	15	15	15	15
Inflammation, focal, minimal	2	1	0	0	1	1
Salivary Glands	15	15	15	15	15	15
Inflammation, focal, mild	2	6	1	6	5	4

	0 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L	100 mg/L
Female						
Kidney	15	15	15	15	15	15
Inflammation, focal, minimal	1	4	0	2	4	2
Inflammation, diffuse, mild	0	1	0	2	2	3
Urinary Bladder	15	15	15	15	15	15
Inflammation, focal, minimal	0	0	0	2	0	2
Stomach, Glandular	15	15	15	15	15	15
Inflammation, minimal	0	1	2	4	2	2
Intestine, Large, Rectum	15	15	15	15	15	15
Inflammation, minimal	0	1	0	1	3	0
Intestine, Large, Colon	15	15	15	15	15	15
Inflammation, minimal	2	4	4	1	2	0
Intestine, Small, Jejunum	15	15	15	15	15	15
Inflammation, minimal	0	1	1	0	0	3
Intestine, Small, Duodenum	15	15	15	15	15	15
Inflammation, minimal	1	2	2	0	0	0
Pancreas	15	15	15	15	15	15
Inflammation, focal, minimal	0	0	1	2	2	1
Harderian Gland	15	15	15	15	15	15
Inflammation, focal, minimal	1	0	3	2	2	2
Salivary Glands	15	15	15	15	15	15
Inflammation, focal, mild	5	7	8	9	5	5

[a]Concentrations in drinking water refer to freebase (-)-nicotine.
[b]Each exposed group was compared to the vehicle control group using Fisher's exact (pairwise) test. No statistically significant findings were noted at $p \leq 0.05$.
[c]Number of animals examined microscopically.
[d]Number of animals with lesion.

Internal Concentration Assessment

Plasma and urine samples from male and female Sprague Dawley rats and Swiss mice were collected after the 3-month studies and were analyzed for nicotine and cotinine and screened for eight metabolites: trans-3'-hydroxy cotinine, (S)-cotinine N-oxide, cotinine N-β-D-glucuronide, (R,S)-norcotinine, (R,S)-nornicotine, trans-3'-hydroxy cotinine O-β-D-glucuronide, (1'S,2'S)-nicotine 1'-oxide, and nicotine N-β-D-glucuronide. For both rats and mice, cotinine concentrations were similar to or higher than nicotine concentrations, and concentrations in urine were higher than in plasma (Table 32, Table 33). In general, the concentrations for both nicotine and cotinine increased with increasing exposure. All control group samples for both species, matrices, and analytes were below the limit of quantitation (LOQ), except for one mouse urine nicotine sample, which was slightly above the LOQ. Creatinine was present in all urine samples and showed some elevation in the higher exposure groups.

In addition to nicotine and cotinine concentrations in plasma and urine samples, a qualitative assessment was performed to determine the presence of the metabolites and concentration estimates. Metabolites were present in varying concentrations in both plasma and urine. The summaries of these results are presented in Table C-3. Biological sample analysis graphs of nicotine and cotinine are also presented in Appendix C.

Table 32. Summary of Internal Concentration Data for Male and Female Rats in the Perinatal and Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a,b,c]

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
n	5	5	5	5	5	5
Nicotine Concentration[d]						
Male						
Plasma (ng/mL)	1.66 ± 0.784**	2.14 ± 0.378	12.6 ± 5.83**	9.57 ± 4.56**	6.14 ± 1.65*	23.6 ± 10.9**
Urine (ng/mL)	2.38 ± 0.812**	299 ± 88.2**	600 ± 96.8**	1,070 ± 138**	1,940 ± 578**	3,800 ± 658**
Creatinine-adjusted urine (ng/mg)	3.3 ± 1.1**	397.0 ± 95.7**	926.3 ± 222.8**	1,044.6 ± 121.9**	1,999.3 ± 466.7**	3,028.6 ± 501.2**
Female						
Plasma (ng/mL)	0.551 ± 0.101**	3.22 ± 1.67**	7.22 ± 2.41**	5.18 ± 1.31**	7.68 ± 3.38**	37.4 ± 12.5**
Urine (ng/mL)	2.10 ± 0.510**	247 ± 25.9**	588 ± 104**	606 ± 152**	1,340 ± 375**	2,920 ± 358**
Creatinine-adjusted urine (ng/mg)	4.0 ± 0.9**	500.3 ± 46.3**	1,303.1 ± 189.5**	923.6 ± 238.9**	2,237.4 ± 476.3**	2,977.5 ± 335.9**
Cotinine Concentration[d]						
Male						
Plasma (ng/mL)	BD[e]	19.0 ± 4.02	41.8 ± 11.3	41.7 ± 6.81	81.2 ± 13.7	210 ± 33.4
Urine (ng/mL)	1.57 ± 0.130**	337 ± 88.4**	624 ± 84.6**	1,030 ± 123**	2,040 ± 394**	5,290 ± 763**
Creatinine-adjusted urine (ng/mg)	2.2 ± 0.2**	456.7 ± 102.1**	976.4 ± 238.8**	1,006.9 ± 98.0**	2,169.0 ± 304.0**	4,237.0 ± 605.5**
Female						
Plasma (ng/mL)	BD[e]	24.5 ± 2.66	69.2 ± 8.09	59.3 ± 12.3	120 ± 12.1	259 ± 34.6
Urine (ng/mL)	2.45 ± 0.168**	345 ± 36.9**	737 ± 64.9**	976 ± 198**	2,050 ± 299**	5,840 ± 455**
Creatinine-adjusted urine (ng/mg)	4.7 ± 0.4**	705.0 ± 78.2**	1,679.7 ± 196.5**	1,485.5 ± 314.1**	3,528.2 ± 404.1**	6,014.6 ± 602.7**

	0 mg/L	1.56 mg/L	3.12 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L
Creatinine Concentration[d]						
Male						
Urine (mg/dL)	71.8 ± 5.8**	73.0 ± 9.0	73.0 ± 13.2	101.4 ± 4.8	91.6 ± 7.8	127.0 ± 10.4**
Female						
Urine (mg/dL)	53.4 ± 5.1**	49.4 ± 2.5	47.2 ± 7.8	66.4 ± 1.9	57.8 ± 3.8	100.6 ± 11.4**

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
BD = below detection; group did not have over 20% of its values above the limit of detection (LOD).
[a]Statistical analysis performed by the Jonckheere (trend) and the Shirley or Dunn (pairwise) tests.
[b]Data are presented as mean ± standard error.
[c]Concentrations in drinking water refer to freebase (-)-nicotine.
[d]If over 20% of the values for nicotine or cotinine in the plasma or urine of animals in a group were above the LOD, one-half the LOD value was substituted for values below the LOD. All values for creatinine in urine below the LOD were substituted with one-half the LOD.
[e]If 80% or more of the values in the vehicle control group were below the LOD, no mean and standard error were calculated and no statistical analysis was performed.

Table 33. Summary of Internal Concentration Data for Male and Female Mice in the Three-month Drinking Water Study of Nicotine Bitartrate Dihydrate[a,b,c]

	0 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L	100 mg/L
n	5	5	5	5	5	5
Nicotine Concentration[d]						
Male						
Plasma (ng/mL)	1.44 ± 0.772**	7.35 ± 2.99	18.7 ± 9.40*	27.1 ± 7.07**	56.7 ± 6.04**	183 ± 38.7**
Urine (ng/mL)	5.05 ± 0.747**	42.9 ± 9.68*[e]	129 ± 15.4**[e]	193 ± 69.9**	656 ± 153**	1,360 ± 681**
Creatinine-adjusted urine (ng/mg)	37.1 ± 8.1**	286.3 ± 80.8*	1,360.0 ± 399.6**	1,593.6 ± 659.0**	2,784.5 ± 785.4**	4,448.7 ± 1,296.2**
Female						
Plasma (ng/mL)	BD[f]	3.87 ± 1.25	5.33 ± 1.10	18.6 ± 5.48	33.7 ± 12.1	142 ± 51.8
Urine (ng/mL)	6.43 ± 1.48**	40.3 ± 7.85*	55.5 ± 16.3**	120 ± 29.6**	237 ± 47.9**	616 ± 141**
Creatinine-adjusted urine (ng/mg)	66.0 ± 18.9**	498.6 ± 204.7*	435.0 ± 256.8*	956.7 ± 355.2**	1,430.2 ± 336.3**	3,530.5 ± 1,519.0**

	0 mg/L	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L	100 mg/L
Cotinine Concentration[d]						
Male						
Plasma (ng/mL)	1.14 ± 0.352**	22.7 ± 4.25**	178 ± 90.3**	185 ± 23.2**	343 ± 64.8**	857 ± 111**
Urine (ng/mL)	0.663 ± 0.058**	105 ± 22.0**	718 ± 166**	546 ± 196**	1,490 ± 147**	4,940 ± 2,290**
Creatinine-adjusted urine (ng/mg)	4.9 ± 1.0**	818.9 ± 384.2**	9,323.2 ± 3,258.6**	5,238.9 ± 2,372.8**	6,625.6 ± 1,548.4**	16,486.8 ± 4,457.9**
Female						
Plasma (ng/mL)	BD[f]	21.2 ± 6.09	41.0 ± 11.3	112 ± 25.6	198 ± 44.6	1,030 ± 146
Urine (ng/mL)	0.701 ± 0.116**[e]	146 ± 63.1*	337 ± 90.4**	529 ± 88.3**	1,250 ± 261**	2,850 ± 285**
Creatinine-adjusted urine (ng/mg)	6.6 ± 1.2**	3,108.3 ± 2,469.3*	2,532.1 ± 1,330.8**	3,558.4 ± 839.6**	6,821.6 ± 864.0**	14,785.9 ± 5,888.5**
Creatinine Concentration[d]						
Male						
Urine (mg/dL)	15.6 ± 2.8	18.4 ± 3.5	13.2 ± 5.7	14.2 ± 3.6	27.8 ± 6.6	27.4 ± 6.8
Female						
Urine (mg/dL)	11.2 ± 2.1**	11.6 ± 2.2	20.8 ± 4.4	16.8 ± 4.0	18.2 ± 3.7	27.4 ± 6.4*

Statistical significance for an exposed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
BD = below detection; group did not have over 20% of its values above the limit of detection (LOD).
[a]Statistical analysis performed by the Jonckheere (trend) and the Shirley or Dunn (pairwise) tests.
[b]Data are presented as mean ± standard error.
[c]Concentrations in drinking water refer to freebase (-)-nicotine.
[d]If over 20% of the values for nicotine or cotinine in the plasma or urine of animals in a group were above the LOD, one-half the LOD value was substituted for values below the LOD. All values for creatinine in urine below the LOD were substituted with one-half the LOD.
[e]Two urine nicotine observations from 6.25 and 12.5 mg/L males and one urine cotinine observation from a control female were excluded from analysis as outliers.
[f]If 80% or more of the values in the vehicle control group were below the LOD, no mean and standard error were calculated and no statistical analysis was performed.

Discussion

Evidence of the devastating health effects of smoking tobacco was summarized in the first report of the Surgeon General Advisory Committee on Smoking and Health in 1964, which reviewed the more than 7,000 articles already available at that time linking smoking to disease.[46] In the nearly 60 years since the issuance of that report, many thousands of research papers have been published on the toxicity of tobacco smoke constituents, including formaldehyde, nitrosamines, carbon monoxide, and particulates. Less attention was given to understanding the role of nicotine, which accounts for more than 95% of tobacco alkaloids.[3] Although recognized to possess potent pharmacological and addictive properties, nicotine is delivered in electronic cigarettes and is now a common ingredient in products designed to reduce or stop smoking, including gum and dermal patches. All these smoking-reduction aids are widely available over the counter.

In 2003, the modern electronic cigarette was developed, and this nicotine delivery system was introduced into the U.S. market in the mid-2000s.[47] Electronic cigarette usage among women shortly before, during, or after pregnancy was estimated to be 7% in 2015.[48] Among children, usage has increased rapidly during the last decade, as suggested in a survey indicating 4.9% of middle school and 20.7% of high school age students used electronic cigarettes in 2018.[49]

This pattern of increasing use of products containing nicotine, without the concurrent presence of other tobacco constituents, either combusted or chewed, prompted interest in further characterization of the toxicity and carcinogenicity of nicotine in rodent models.

Drinking water was chosen as the route of administration for these studies to mimic the episodic use of smoking replacement gums and electronic cigarettes and because absorption of nicotine across the oral mucosa is a primary route of delivery during use of these products.[35] To select the test article, brief toxicokinetic studies of freebase (-)-nicotine and nicotine bitartrate dihydrate (NBD), a dihydrate salt complex, were performed first using a single gavage administration to male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats. Toxicokinetic parameters, including the maximum plasma concentration (C_{max}) and area under the plasma-concentration-versus-time curve (AUC), and conversion to the first metabolite cotinine were similar for both compounds. Because the NBD salt was more stable in aqueous solution, it was selected for these studies (Appendix B).

The palatability of NBD in dosing solutions was tested in Sprague Dawley rats and Swiss mice. In rats, the highest exposure concentration (100 mg/L) was discontinued after 24 hours because of a severe reduction in water consumption and reductions in body weight. Exposure to 50 mg/L induced significant reductions in body weights and water consumption in both sexes of rats by the end of the 2-week palatability study. Both the 50 and 100 mg/L exposure concentrations were considered unpalatable for rats. Therefore, the concentrations selected for the perinatal and 4-week study in rats were 0, 1.56, 3.12, 6.25, 12.5, and 25 mg/L. In mice, although some decreases in water consumption were observed, all exposure concentrations ≤100 mg/L were considered palatable and were selected for the 4-week study.

Rat dams receiving the two highest NBD concentrations (12.5 and 25 mg/L) in the perinatal and 4-week study consumed less feed and water, and the 25 mg/L dams gained significantly less weight during gestation and lactation than control dams. The mean number of pups per litter

varied somewhat across the exposed groups but with no discernible exposure concentration-related pattern. Likewise, early pup mortality, which is common in Sprague Dawley rats that produce large litters, occurred in the control group as well as in exposed groups, again without a discernible pattern. Although body weight gains of pups were not markedly different from control pups, at the end of the lactation phase, an unusual wave of pup mortality was observed across exposed groups at concentrations ≥3.12 mg/L. In total, 42 pups died in the exposed groups, whereas no mortality was observed in the control group during the same period (postnatal day [PND] 19–24). At necropsy, some pups presented with gross lesions in the intestine, which correlated histopathologically with moderate diffuse intestinal inflammation. For this reason, NBD exposure was stopped for all exposed groups approximately 4 days before weaning. Thereafter, no additional pup mortality occurred, suggesting the deaths were exposure related. After weaning, pups' NBD exposure was resumed at the concentrations given previously to their respective dams, resulting in no further mortality.

The possible reasons for this unexpected pup mortality remain speculative, but Slotkin and colleagues have extensively studied prenatal nicotine exposure and consequent postnatal deaths in rat pups exposed to periods of atmospheric hypoxia as a model to explore the strong statistical link between fetal exposure to cigarette smoke and sudden infant death syndrome.[28; 50] Noting that a peak of natural cholinergic stimulation during the second postnatal week is critical for the proper establishment of aspects of cerebro-cortical cytoarchitecture in Sprague Dawley rat pups, Slotkin's group established that premature stimulation and resulting up-regulation and acquisition of cholinergic receptors[51] by prenatal nicotine exposure leads to neurobehavioral abnormalities that appear immediately following birth and continue to express through adolescence.[28; 50] Slotkin and colleagues concluded that prenatal nicotine exposure results in an intolerance to postnatal hypoxia by interfering with heart rate and respiration control, causing a marked fall in pup heart and respiration rates leading to death instead of the normal increase in heart rate and respiration during hypoxic episodes. At the time of writing, this phenomenon has not been sufficiently studied to determine an expectation concerning dose response, and it presumably would also be sensitive to potential differences in maternal behaviors between litters that may limit oxygen availability to pups. Dams can nurse pups in a blanket posture, wherein the dam lays over the pups, or in an arched back or a passive posture, wherein the dam lays on her back or side, respectively.[52] These different nursing positions may limit the oxygen available to pups. Therefore, an expectation of a traditional dose response in pup mortality, which was not seen in this study, may not be justified. Although the phenomenon described by Slotkin's group may account in large part for the unexpected pattern of pup mortalities in this study, the observation that from PND 19 to PND 24, mortality did not occur at the lowest NBD exposure concentration group in the 4-week study—and that administration of this low exposure concentration during lactation (discussed below) resulted in all rat pups surviving to study termination in the 3-month study—suggests an additional role for nicotine during a vulnerable phase of postnatal maturation at the higher exposure concentrations.

At the end of the perinatal and 4-week study in rats, there were minor decreases in relative liver weights in males and relative adrenal weights in females at the top NBD exposure concentrations of 25 mg/L. Hematological findings of a significant increase in lymphocyte counts in females in all exposure groups except the highest (25 mg/L) were observed. Histopathological examination of the control and 25 mg/L groups revealed few significant lesions. Microscopic examination of the 25 mg/L and control groups (the only exposure groups evaluated) noted minimal

inflammation of the intestine was increased in females but was not significant. Inflammation in the kidney was also noted in both sexes, and testicular lesions were noted in a few animals, but could not clearly be related to NBD exposure.

For the perinatal and 3-month study in rats, the design of the study and NBD drinking water concentrations were the same as in the perinatal and 4-week study, with the exception that all NBD-exposed groups received the lowest concentration of NBD during lactation. This approach was an attempt to prevent the late pup mortality seen in the lactational phase of the perinatal and 4-week study, and no pups died during the corresponding phase of the 3-month study. Patterns of reduced feed and water consumption by dams receiving the higher concentrations of NBD during gestation were similar to those in the earlier study and resulted again in reduced body weight at the top concentration (25 mg/L). There were no effects on litter size, sex ratio, pup survival, or body weight gains during lactation. Other than differences in body weights, there were no remarkable in-life effects of the NBD exposures on pups during the 3-month, postweaning study.

The pathology peer review of the perinatal and 3-month rat study did not identify any clear target organs associated with exposure to NBD. Various minimal to mild degenerative lesions in the testes were observed in a few animals in the 12.5 and 25 mg/L groups in the 3-month males and in the 25 mg/L group in the perinatal and 4-week study, and although rare at this young age in this strain of rat, they were not considered exposure related. Minimal inflammation was observed in a number of organs of rats from the perinatal and 4-week and 3-month studies, and while the incidence of this lesion was often greater in the NBD-exposed groups than in control rats, it rarely exhibited a clear exposure concentration response.

There is extensive and complex literature on nicotine and inflammation, recently reviewed by Zhang et al.[53] The clearest evidence for proinflammatory effects of nicotine comes from studies of oral mucosal inflammation and periodontitis, a disease leading to frequent tooth loss in tobacco users. Dussor et al.[54] in studies of buccal tissues isolated from Sprague Dawley rats, provided evidence that nicotine enhances the release of the proinflammatory factor calcitonin gene-related peptide through a neurogenic mechanism. Studies have shown an enhanced intestinal inflammatory response from nicotine in mouse models of Chron's disease, but nicotine appears to be primarily anti-inflammatory in mouse models of ulcerative colitis.[55] Nicotine has been extensively studied as a potential therapeutic agent to reduce inflammation in a wide variety of animal and human tissue models of immune or inflammation-related diseases, including inflammatory bowel disease, arthritis, multiple sclerosis, sepsis and endotoxemia, pancreatitis, and myocarditis. The interest in nicotine as an anti-inflammatory agent is because it activates cholinergic neural pathways that suppress inflammatory responses and because it acts directly on immune cells that can enhance apoptosis, inhibit inflammatory cytokine release, or modulate other inflammation processes.[53]

It is not clear whether the age dependencies and/or pharmacodynamics of these conflicting actions of nicotine on inflammatory processes could account for the observed patterns of minimal to mild inflammation in this study. Some of the more convincing evidence for proinflammatory responses was in the larynx, mandibular lymph nodes, large intestine, and kidney, the effect on which might be expected from direct oral exposures to nicotine. However, the generally minimal severity of these changes would suggest that they are presumably of nominal toxicological significance in animals once the cholinergic neural anti-inflammatory circuits become fully developed.

In the 4-week and 3-month studies in mice, no effects on survival, or biologically significant changes in body weight, feed consumption, or water consumption, were observed. There were no significant differences in organ weights. Histopathological evaluation of mice exposed to the highest concentration (100 mg/L) in the 4-week study noted a few instances of minimal or moderate inflammation in several organs, including the kidney in males and the Harderian gland in females; however, none were significant. In the 3-month study, there were incidences of increased minimal to mild inflammation in the kidney in male and female mice. Minimal inflammation in the jejunum, urinary bladder, and glandular stomach was also noted in female mice, with less evidence in these organs in exposed males. There were indications of increases in minimal focal inflammation in the Harderian gland and rectum in both sexes. Again, significance was lacking for all these measures, but when taken together with the sporadic occurrences of inflammatory lesions in the perinatal and 4-week and 3-month rat studies, they suggest a generalized minimal proinflammatory state associated with the NBD exposures. And, as with the findings from the rat studies, the toxicological significance of these minor inflammatory responses is uncertain.

When comparing mice to rat dams, mice were able to tolerate higher NBD drinking water concentrations. This observation was previously described by Matta et al.[19] and may be due to differences in perception of taste and/or species differences in absorption and circulating half-life of nicotine (6–7 minutes in mice, approximately 1 hour in rats, and 2 hours in humans and nonhuman primates).[3; 56-58] In the current toxicokinetic studies, following oral administration of freebase (-)-nicotine or NBD, the time at which C_{max} occurred was typically between 0.5 and 1 hour; for cotinine (a major metabolite of nicotine), it was 4–4.67 hours for freebase (-)-nicotine and 4–5.33 hours for NBD, which suggests a slow conversion of nicotine to cotinine. No apparent sex-related effects on the toxicokinetic parameters or systemic exposure of cotinine were observed. Compared to nicotine, cotinine had a longer half-life, smaller apparent clearance of the central compartment and apparent volume of distribution for the central compartment, and greater systemic exposure (Appendix B).

In rats and mice, nicotine and cotinine concentrations in plasma and urine from the 3-month studies were largely dose proportional to the exposure concentrations. Nicotine intake was also estimated by normalizing nicotine and cotinine concentrations using water consumption data (data not shown). Overall, observations did not change when data were normalized to nicotine intake and did not shed light on the observed lack of dose-response for many of the minor pathology findings discussed above. The nicotine plasma concentrations from rats (Figure C-1) and mice (Figure C-3) ranged from below to somewhat above reported human plasma concentrations (15–80 ng/mL) following smoking one cigarette (approximately 2 mg nicotine/cigarette).[35; 59-63] Cotinine concentrations in rat (Figure C-2) and mouse (Figure C-4) plasma also ranged below and above reported human plasma concentrations. Blood cotinine concentrations were reported to range between 250 and 350 ng/mL for regular smokers and up to 900 ng/mL for heavy smokers.[36; 64-66]

Nicotine metabolism in humans and other species is well studied, and various metabolites are reported.[3] In this study, in addition to nicotine and cotinine concentrations in plasma and urine samples, a qualitative assessment was performed to determine the presence and concentration estimates of some of the main nicotine and cotinine metabolites (trans-3'-hydroxycotinine, cotinine-N-oxide, cotinine N-β-D-glucuronide, norcotinine, nornicotine, trans-3'-hydroxycotinine-O-B-D-glucuronide, nicotine-1'-oxide, and nicotine N-β-D-glucuronide) that

are reported in the literature. Metabolites were present in varying concentrations in both plasma and urine of rats and mice (Table C-3). Metabolite concentrations in mouse plasma were higher than in rat plasma, which may be due to mice having significantly higher in vivo CYP2A activity relative to rats.[67] Cotinine N-β-D-glucuronide concentrations were the highest in rat and mouse plasma within all the metabolites analyzed. Although trans-3'-hydroxy cotinine was not detected in rat plasma, it was present at relatively low concentrations in mouse plasma across all exposure groups. Trans-3'-hydroxycotinine and its glucuronide conjugate are reported as the main urinary nicotine metabolites detected in smokers.[3; 68-72] These metabolites were reported as minor in rats and with no measurable glucuronidation activity in rat or mouse liver microsomes.[3] Trans-3'-hydroxy cotinine O-β-D-glucuronide, nicotine N-β-D-glucuronide, and cotinine N-β-D-glucuronide in urine were not reportable in the current analyses, largely due to interfering peaks observed in the chromatography in rat and mouse samples.

In conclusion, studies were performed to examine the palatability, metabolism, toxicokinetic, and toxic responses to NBD administered in drinking water to rats beginning on gestation day (GD) 6 and extending through 4 weeks or 3 months postweaning and to adult mice similarly exposed to NBD in drinking water for 4 weeks or 3 months. Mice tolerated higher NBD drinking water concentrations relative to rats. Rates of nicotine clearance were higher in both rodent species than in humans. Other than reductions in feed and water consumption and resulting lower body weight gains at the higher exposure levels, there were no clinical findings considered to be of clear toxicological significance for the NBD exposures in rats or mice. However, there was a high rate of mortality of rat pups receiving NBD at concentrations ≥3.12 mg/L during the late-lactation phase of the perinatal and 4-week study that was unexpected and may be related to inhibition of normal cholinergic neural development by prenatal nicotine exposures. No mortality was observed in the control group and in the exposed group at 1.56 mg/L during the same period. This finding prompted a change in the design of the perinatal and 3-month study in rats to provide all groups of exposed lactating dams the same lowest NBD exposure concentration (1.56 mg/L), which effectively prevented pup mortalities during this apparently vulnerable developmental period. The only other findings of note were observations of generally minimal inflammation across many organ systems in rats and mice. These were often of low incidence, not related to exposure concentration, and were not considered to be of clear toxicological significance.

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Appendix A. Chemical Characterization and Dose Formulation Studies

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A.1. Procurement and Characterization of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate

Nicotine as freebase (-)-nicotine and nicotine bitartrate dihydrate (NBD) were obtained from Siegfried (Zofingen, Switzerland) via Interchem Corporation (Paramus, NJ) in single lots (lots 1515L008 and 1531H012, respectively). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Battelle (Columbus, OH). Reports on analyses performed in support of the nicotine studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

Lot 1515L008 of freebase (-)-nicotine was a colorless to yellow or brownish liquid prior to handling. It arrived in two bottles, and the bottles were not combined because of the hazardous nature of the material. The bottles were homogenized by inverting them 10 times to mix. Bulk freebase (-)-nicotine was stored at room temperature (13°C–25°C), protected from light, and under inert gas with desiccant.

Lot 1531H012 of NBD was a white or almost-white powder. It was homogenized by first removing the test article bag from the outer container and then sealing the inner plastic bag of the test article in a second plastic bag. The contents were kneaded for approximately 1 minute, and then the bag was rotated 180° and the contents were kneaded again for approximately 1 minute. This process of rotating and kneading was repeated six times to homogenize the material. The homogenized test article was then repackaged into 10 1-L amber high-density polyethylene (HDPE) bottles. The containers were capped and sealed with tape and then stored at room temperature (13°C–25°C) with desiccant.

The lot identities were confirmed using infrared (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopies, and high-performance liquid chromatography (HPLC) with mass spectrometry (MS). The IR spectra (Figure A-1, Figure A-4) were consistent with the structure of NBD for lot 1531H012 (no reference spectrum available) and with the freebase (-)-nicotine reference spectrum (No. DLX 1359, Bio-Rad “Know It All” v.14.1.209.0) for lot 1515L008. The ^{1}H and ^{13}C spectra (Figure A-2, Figure A-3, Figure A-5, Figure A-6) were consistent with the structures predicted by the Advanced Chemistry Development spectral prediction program (Version 14.00).[73]

HPLC/MS supported the identity of nicotine in both lots, as the mass spectra and fragmentation were consistent with nicotine (Table A-1, System A). Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN). The relative amounts of carbon, hydrogen, nitrogen, and oxygen for lot 1531H012 (43.43%, 5.93%, 45.37%, and 5.55%, respectively) were within 3% of the theoretical values for NBD. The relative amounts of carbon, hydrogen, and nitrogen for lot 1515L008 (73.69%, 8.32%, and 17.10%, respectively) were within 4% of the theoretical values for freebase (-)-nicotine.

The moisture content of lots 1515L008 and 1531H012 was determined by Karl Fischer titration and performed at Galbraith Laboratories (Knoxville, TN). The specific rotation and water analyses were performed at Exova Inc. (Santa Fe Springs, CA). Differential scanning calorimetry (DSC) was attempted for both lots, but the analysis of NBD resulted in broad duplicate overlapping peaks due to the dihydrate salt complex nature of the compound, and accurate purity and melting points could not be determined. The volatile content was determined by

thermogravimetric analysis (TGA). The purity profiles were determined using HPLC with ultraviolet (UV) detection. Additionally, the purity of freebase (-)-nicotine lot 1515L008 was supported by gas chromatography with flame ionization detection (GC/FID).

For freebase (-)-nicotine lot 1515L008, Karl Fischer titration yielded an average water content of 0.18 ± 0.01($\bar{d}$)% ($\bar{d}$ is the deviation between two samples). The specific rotation analysis indicated an average of −133.2°C, which was 4°C different from the value reported on the certificate of analysis (COA) from the manufacturer (−137.1°C) but consistent with the specification of freebase (-)-nicotine (−143°C to −130°C).[74] The water analysis indicated 0.23% water was present in the test article, congruent with Galbraith's Karl Fischer titration results but slightly higher than the 0.02% reported on the COA. DSC measured the boiling point as 244.9°C, which was consistent with freebase (-)-nicotine's known boiling point.[4] TGA did not detect volatile or nonvolatile impurities. The purity of the two containers was determined separately by HPLC/UV at 254 nm and was 99.6% for both containers (Table A-1, System B). Two impurity peaks representing 0.4% of the total area were present in the chromatograms. Furthermore, ^{1}H NMR confirmed identical structures and purity between the two containers. Impurity signals detected near 3.71 ppm and 1.23 ppm of the ^{1}H NMR spectra were indicative of an ethyl group and assigned to the probable presence of ethanol. GC/FID (Table A-2, System E) also detected one impurity present at 0.27%, resulting in an overall purity of 99.7%. The purity values measured by HPLC/UV and GC/FID were consistent with the 99.3% purity reported on the COA. Given the ^{1}H NMR results, the impurities measured by HPLC/UV and GC/FID were likely ethanol.

For NBD lot 1531H012, Karl Fischer titration yielded an average water content of 8.97 ± 0.49($\bar{d}$)%, which was within 2% of the theoretical value (7.23%), based on the molecular formula of NBD, and slightly higher than the 7.41% reported on the COA. The specific rotation analysis indicated an average degree of +23.0°C, which was consistent with the 22.8°C reported on the COA. The water analysis indicated 7.2% water, which was consistent with the 7.41% reported on the COA. TGA results corresponded with literature reports for NBD and indicated no detectable volatile or nonvolatile impurities. The purity determined by HPLC/UV at 254 nm was 100%, which was consistent with the 100% purity reported on the COA (Table A-1, System B). A second HPLC system using UV detection at 210 nm was used to estimate purity—no impurity peaks representing >0.1% of the total peak area were detected (Table A-1, System C).

Accelerated stability studies of freebase (-)-nicotine and NBD were conducted on lots 1515L008 and 1531H012, respectively. Samples of each test article were stored in four individual 4-mL amber glass vials for 15 days at approximately 60°C, room temperature, approximately 5°C, and approximately −20°C. The appearance of each sample was noted before and after storage, and purity was analyzed with HPLC/UV at 210 nm (Table A-1, System C). Freebase (-)-nicotine samples were stable for at least 15 days at approximately −20°C, 5°C, and room temperature. Storing freebase (-)-nicotine at approximately 60°C reduced the purity to 96.7%. NBD samples were stable when stored in sealed glass containers for at least 15 days at temperatures of −20°C to 60°C.

A.2. Preparation and Analysis of Dose Formulations

The dose formulations for the toxicokinetic studies were prepared once at Battelle (Columbus, OH) by mixing freebase (-)-nicotine or NBD in ASTM Type I water at a concentration of 0.1 mg/mL. Test articles were weighed into a mixing container, diluted to the final volume with vehicle, and stirred with a magnetic stir plate and stir bar until dissolved. While stirring, 10-mL aliquots of each formulation were dispensed for analysis and retention (archive) samples. Final formulations were dispensed into appropriately labeled individual amber glass bottles for dosing and then sealed and placed in a secondary container. The freebase (-)-nicotine formulations were stored at 2°C–8°C, and the NBD formulations were stored at room temperature (Table A-3). Formulations were analyzed prior to dosing by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Table A-1, System D), using a calibration curve from 0.01 to 0.4 µg/mL of nicotine in diluent (10:90 acetonitrile:20 mM ammonium bicarbonate in water [v/v]) and nicotine-d3 (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) as an internal standard (IS). The preadministration nicotine concentrations in all formulations used for the study were within 10% of the target concentrations (Table A-5).

Dose preparation and analysis for the 4-week studies are not presented in this report. The dose formulations for the 3-month studies were prepared weekly at the Ramazzini Institute (Bologna, Italy) by mixing NBD with tap water to obtain the desired concentrations for each species (Table A-4).

For the rat study, formulations were prepared at concentrations of 0, 1.56, 3.12, 6.25, 12.5, and 25 mg nicotine/L. For the mouse study, formulations were prepared at concentrations of 0, 6.25, 12.5, 25, 50, and 100 mg/L. The typical formulation volume prepared was 50 L. Dose calculations used to prepare the formulations were based on the molecular weight of nicotine, and weights were normalized to salt molecular weight. Dose formulations were stored at room temperature in HDPE plastic bottles, covered with black plastic foil to avoid light exposure, and dispensed to the animals daily in dark glass bottles. Aliquots (from three different time points) of each preadministration formulation dose were sent frozen in an insulated container to Battelle (Columbus, OH) for analysis, where they were stored at −30°C to −15°C until analyzed.

Preadministration formulations for the 3-month studies were analyzed by LC-MS/MS (Table A-1, System D) using a calibration curve from 0.01 mg/L to 0.4 mg/L of nicotine in diluent (ASTM Type I water) and nicotine-d3 as an IS. Each formulation dose was diluted with ASTM Type I water into the quantitation range of the analytical method. Blanks of ASTM Type I water were used to assess system suitability. The results of the preadministration formulation analysis of the 3-month studies are shown in Table A-6 (rats) and Table A-7 (mice). Multiple formulations had results outside the typical NIEHS acceptance criteria for accuracy and precision (average concentration within 10% of the target concentration and relative standard deviation [RSD] values within 5%). However, these deviations were not considered to significantly affect the studies. All mouse formulations were within 10% of the target concentration, and all rat formulations were within 10%, except for the 1.56 mg/L formulations from the first and second shipment, which were 59.0% and 15.7% above target, respectively.

Prior to study start, the degradation of nicotine in drinking water formulations was investigated. At the lowest exposure concentration, some loss of nicotine was observed when formulations were prepared in tap water (Columbus, OH) but not in deionized water, and this was attributed to

the presence of chlorine in tap water.[75] Because in Europe, where the study laboratory was located, water disinfection is done mainly via ozonation with much lower levels of chlorine, tap water was used to prepare formulations.

The RSD values of all formulations were within the NIEHS acceptance limit of 5%, with the exception of the 50 mg/L formulation from the 3-month mouse study analyzed on March 24, 2017; the 12.5, 25, and 50 mg/L formulations from the 3-month mouse study analyzed on April 14, 2017; the 6.25 mg/L formulation from the perinatal and 3-month rat study analyzed from April 28, 2017, to May 1, 2017; and the 6.25, 12.5, and 25 mg/L formulations from the 3-month mouse study analyzed on May 26, 2017. After removing two outliers by the Q-test, these formulations had RSD values from 6.2% to 9.2% (values not shown). The control (0 mg/mL) formulations were below the limit of quantitation (BLOQ).

Table A-1. Liquid Chromatography Systems Used in the Toxicokinetic, Four-week, and Three-month Studies of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate

Chromatography	Detection System	Column	Mobile Phase
System A			
High-performance liquid chromatography	Mass spectrometry, positive ion	Phenomenex Gemini C18 (150 × 4.6 mm ID, 5 µm particle size)	A: 10 mM ammonium bicarbonate, pH 10.5 (aq) B: Acetonitrile Gradient program: A:B 95:5 for 3 minutes; 95:5 to 75:25 in 25 minutes; 75:25 to 60:40 in 4 minutes; 60:40 to 95:5 in 1 minute; hold at 95:5 for 3 minutes 1 mL/min flow rate
System B			
High-performance liquid chromatography	Ultraviolet (254 nm)	Phenomenex Gemini C18 (150 × 4.6 mm ID, 5 µm particle size)	A: 10 mM ammonium bicarbonate, pH 10.5 (aq) B: Acetonitrile Gradient program: A:B 95:5 for 3 minutes; 95:5 to 75:25 in 25 minutes; 75:25 to 60:40 in 4 minutes; 60:40 to 95:5 in 1 minute; hold at 95:5 for 3 minutes 1 mL/min flow rate
System C			
High-performance liquid chromatography	Ultraviolet (210 nm)	Thermo Scientific Acclaim Trinity P1 (50 × 3 mm ID, 3 µm particle size)	A: 4 mmol dibasic sodium phosphate, 20 mmol monobasic sodium phosphate, 81 µmol tetrasodium pyrophosphate decahydrate, pH 6.3 (aq) B: Acetonitrile Gradient program: A:B Isocratic 75:25 0.6 mL/min flow rate

Chromatography	Detection System	Column	Mobile Phase
System D			
High-performance liquid chromatography	Tandem mass spectrometry, positive ion	Phenomenex Kinetex, XB-C18, (150 × 4.6 mm ID, 5 µm particle size) Phenomenex Security Guard, C18, 4 × 2 mm	A: 10 mM ammonium bicarbonate (aq) B: Methanol Gradient program: A:B 95:5 for 0.1 minute; 95:5 to 20:80 in 5 minutes; hold at 20:80 for 2 minutes; 20:80 to 95:5 in 0.5 minutes; hold at 95:5 for 4.5 minutes 400 µL/min flow rate

ID = internal diameter.

Table A-2. Gas Chromatography Systems Used in the Toxicokinetic, Four-week, and Three-month Studies of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate

Detection System	Column	Carrier Gas	Oven Temperature Program
System E			
Flame ionization	Restek Stabilwax (30 m × 0.32 mm ID, 1-µm film thickness)	Helium, ~1 mL/min	50°C, no hold, then 10°C/min to 250°C, held for 5 minutes

ID = internal diameter.

Table A-3. Preparation and Storage of Dose Formulations in the Toxicokinetic Study of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate

Freebase (-)-Nicotine	Nicotine Bitartrate Dihydrate
Preparation	
Formulations for gavage administration were prepared in ASTM Type I water at a concentration of 0.1 mg/mL by first weighing the appropriate amount of test article into a mixing container and then diluting to the final volume with vehicle. Formulations were stirred with a magnetic stir plate and stir bar until the test article dissolved. The mixed formulations were dispensed into appropriately labeled individual amber glass bottles for dosing and then sealed and placed in a secondary container.	Same as freebase (-)-nicotine
Chemical Lot Number	
1515L008 (Siegfried, Zofingen, Switzerland)	1531H012 (Siegfried, Zofingen, Switzerland)
Storage Conditions	
Individual sealed amber glass bottles at 2°C–8°C	Individual sealed amber glass bottles at room temperature
Study Laboratory	
Battelle (Columbus, OH)	Same as freebase (-)-nicotine

Table A-4. Preparation and Storage of Dose Formulations in the Three-month Studies of Nicotine Bitartrate Dihydrate

Preparation
The dose formulations were prepared by mixing nicotine bitartrate dihydrate with tap water and then diluting to the five required final concentrations.
Chemical Lot Number
1531H012 (Siegfried, Zofingen, Switzerland)
Storage Conditions
During the study, dose formulations were stored at room temperature in high-density polyethylene plastic bottles, covered with black plastic foil. Samples were shipped frozen from the Ramazzini Institute to Battelle in insulated containers and stored frozen (−30°C to −15°C) at Battelle prior to analysis.
Study Laboratory
Cesare Maltoni Cancer Research Center, Ramazzini Institute (Bologna, Italy)
Analytical Laboratory
Battelle (Columbus, OH)

Table A-5. Results of Preadministration Analyses of Dose Formulations for Male and Female Rats in the Toxicokinetic Study of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate

	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)[a]	Difference from Target (%)
Freebase (-)-Nicotine	June 21, 2016	0.1	0.103 ± 0.001	2.6
Nicotine Bitartrate Dihydrate[b]	June 21, 2016	0.1	0.101 ± 0.002	0.6

[a]Results of triplicate analyses.
[b]Nicotine bitartrate dihydrate target concentration is corrected for salt and water with a correction factor of 0.3255 (162.2/498.2 g/mol).

Table A-6. Results of Preadministration Analyses of Dose Formulations for Male and Female Rats in the Perinatal and Three-month Study of Nicotine Bitartrate Dihydrate

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)[a]	Difference from Target (%)
April 22, 2017	April 28, 2017–May 1, 2017	0	BLOQ	NA
		1.56	2.48 ± 0.13	59.0
		3.12	3.00 ± 0.16	−3.8
		6.25	6.27 ± 0.42	0.4
		12.5	12.0 ± 0.5	−3.9
		25.0	24.8 ± 1.2	−0.9
May 15, 2017	May 26, 2017	0	BLOQ	NA
		1.56	1.80 ± 0.03	15.7
		3.12	NA	NA
		6.25	NA	NA

Date Prepared	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)[a]	Difference from Target (%)
		12.5	NA	NA
		25.0	NA	NA
July 24, 2017	August 2, 2017	0	BLOQ	NA
		1.56	1.53 ± 0.05	−1.7
		3.12	3.28 ± 0.05	5.3
		6.25	6.19 ± 0.28	−1.0
		12.5	12.8 ± 0.2	2.7
		25.0	25.3 ± 0.8	1.2

BLOQ = below the limit of quantitation; NA = not applicable.
[a]Results of triplicate analyses.

Table A-7. Results of Preadministration Analyses of Dose Formulations for Male and Female Mice in the Three-month Study of Nicotine Bitartrate Dihydrate

Date Formulated	Date Analyzed	Target Concentration (mg/L)	Determined Concentration (mg/L)[a]	Difference from Target (%)
February 21, 2017	March 10, 2017	0	BLOQ	NA
		6.25	6.30 ± 0.13	0.8
		12.5	12.7 ± 0.2	4.7
		25.0	25.5 ± 0.1	1.9
		50.0	50.3 ± 1.0	0.5
		100.0	100 ± 2	0.3
March 20, 2017	March 24, 2017	0	BLOQ	NA
		6.25	6.54 ± 0.33	4.7
		12.5	12.3 ± 0.2	−1.6
		25.0	25.0 ± 0.6	0.1
		50.0	51.5 ± 0.2[b]	3.0
		100.0	101 ± 2	0.6
April 7, 2017	April 14, 2017	0	BLOQ	NA
		6.25	6.05 ± 0.29	−3.1
		12.5	11.5 ± 0.8	−7.9
		25.0	24.5 ± 2.1	−1.9
		50.0	49.5 ± 3.6	−0.9
		100.0	99.0 ± 3.6	−1.0
May 19, 2017	May 26, 2017	0	BLOQ	NA
		6.25	6.41 ± 0.59	2.6
		12.5	12.4 ± 0.8	−0.5
		25.0	26.2 ± 0.4[b]	4.7
		50.0	52.5 ± 1.5	5.0
		100.0	104 ± 1	4.5

BLOQ = below the limit of quantitation; NA = not applicable.
[a]Results of triplicate analyses.
[b]Calculation performed with one outlier excluded.

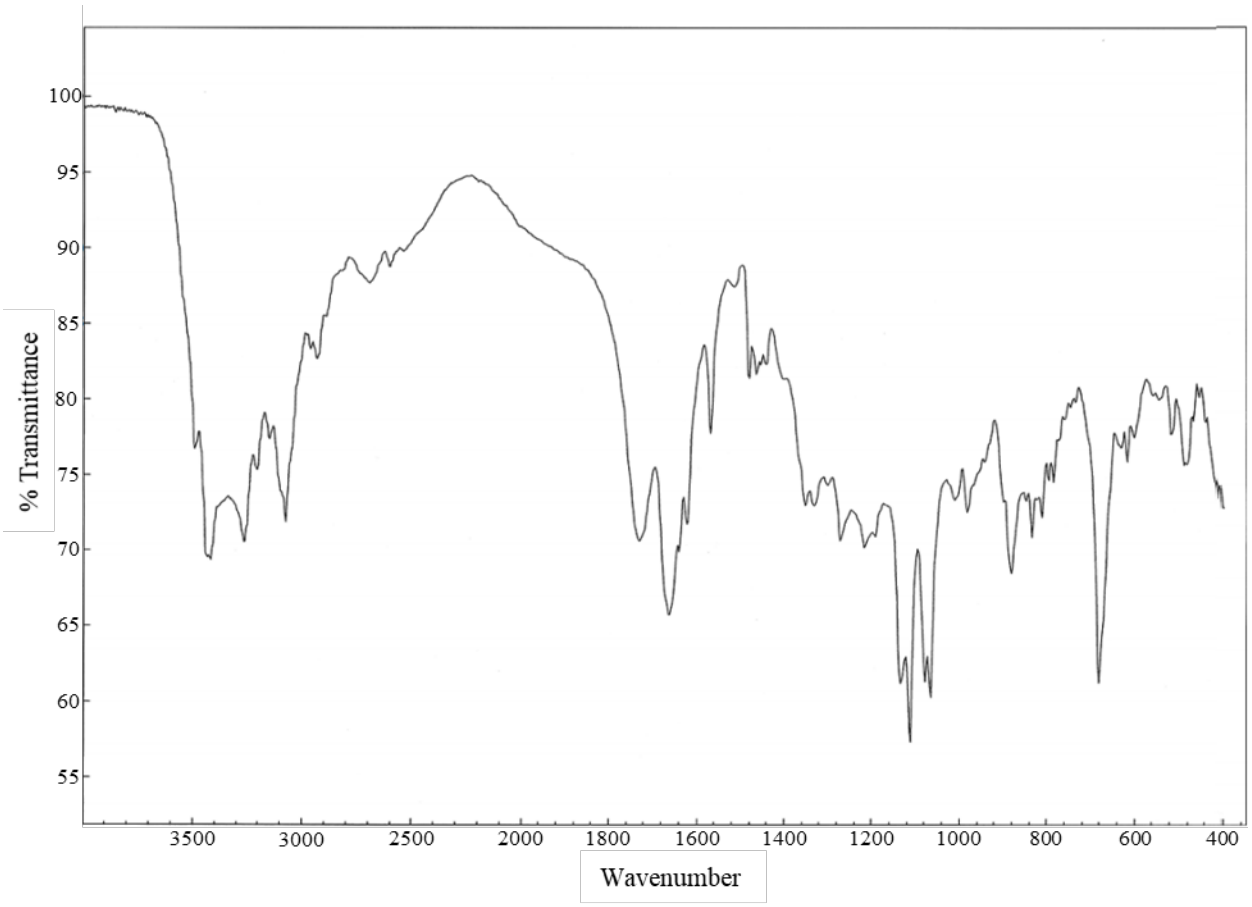

Figure A-1. Infrared Absorption Spectrum of Nicotine Bitartrate Dihydrate

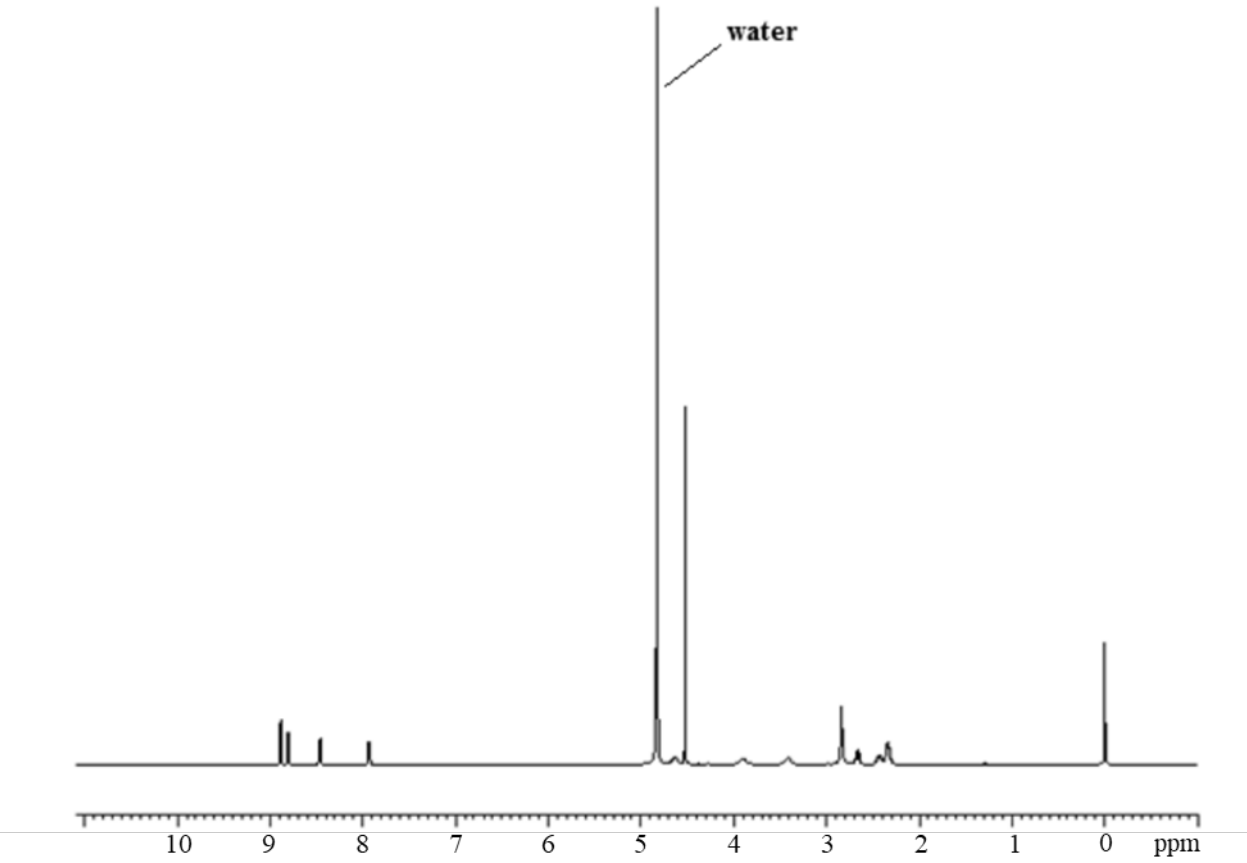

Figure A-2. ^{1}H Nuclear Magnetic Resonance Spectrum of Nicotine Bitartrate Dihydrate

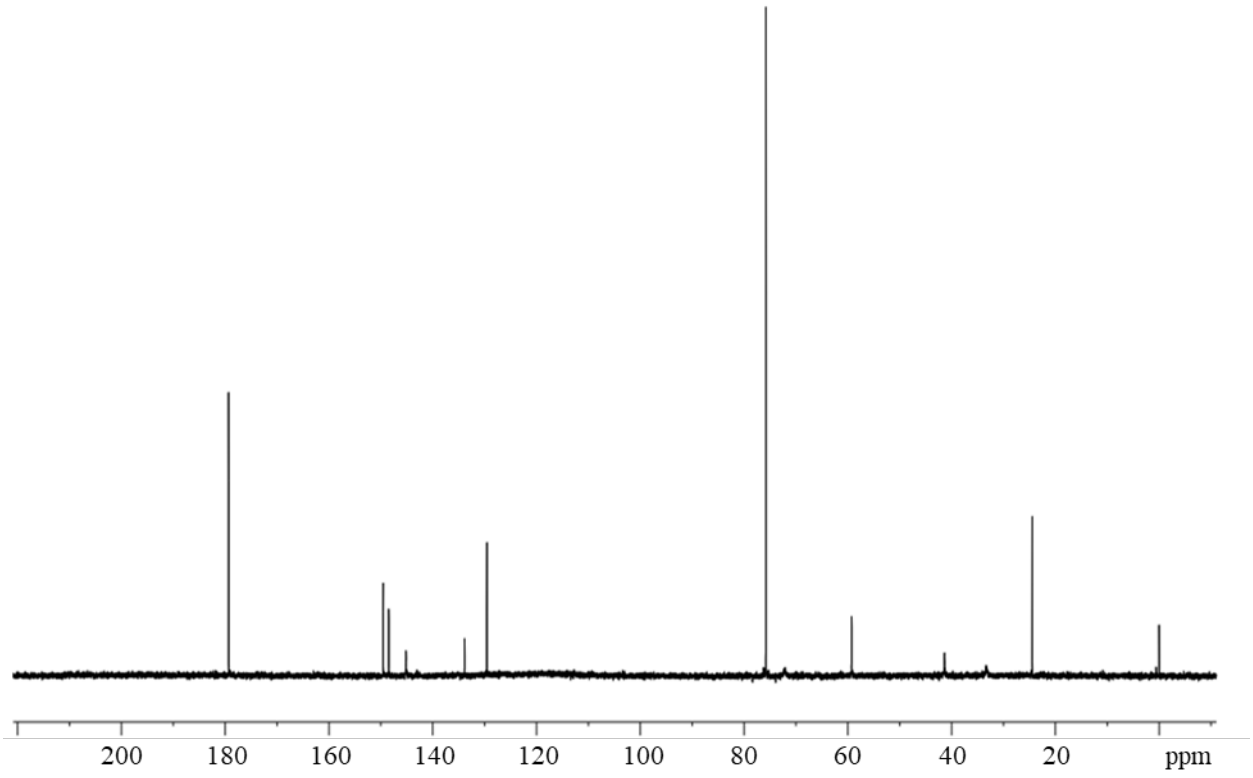

Figure A-3. ^{13}C Nuclear Magnetic Resonance Spectrum of Nicotine Bitartrate Dihydrate

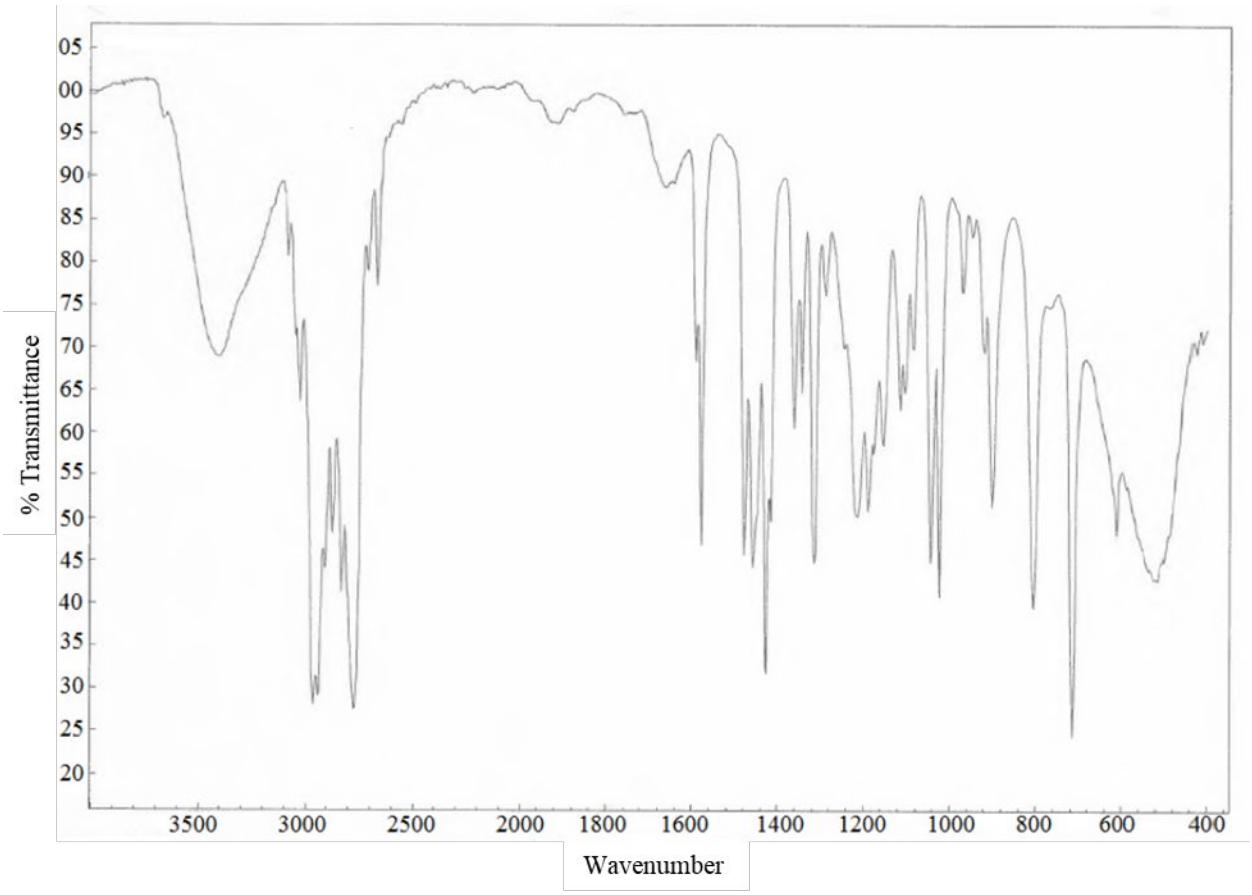

Figure A-4. Infrared Absorption Spectrum of Freebase (-)-Nicotine

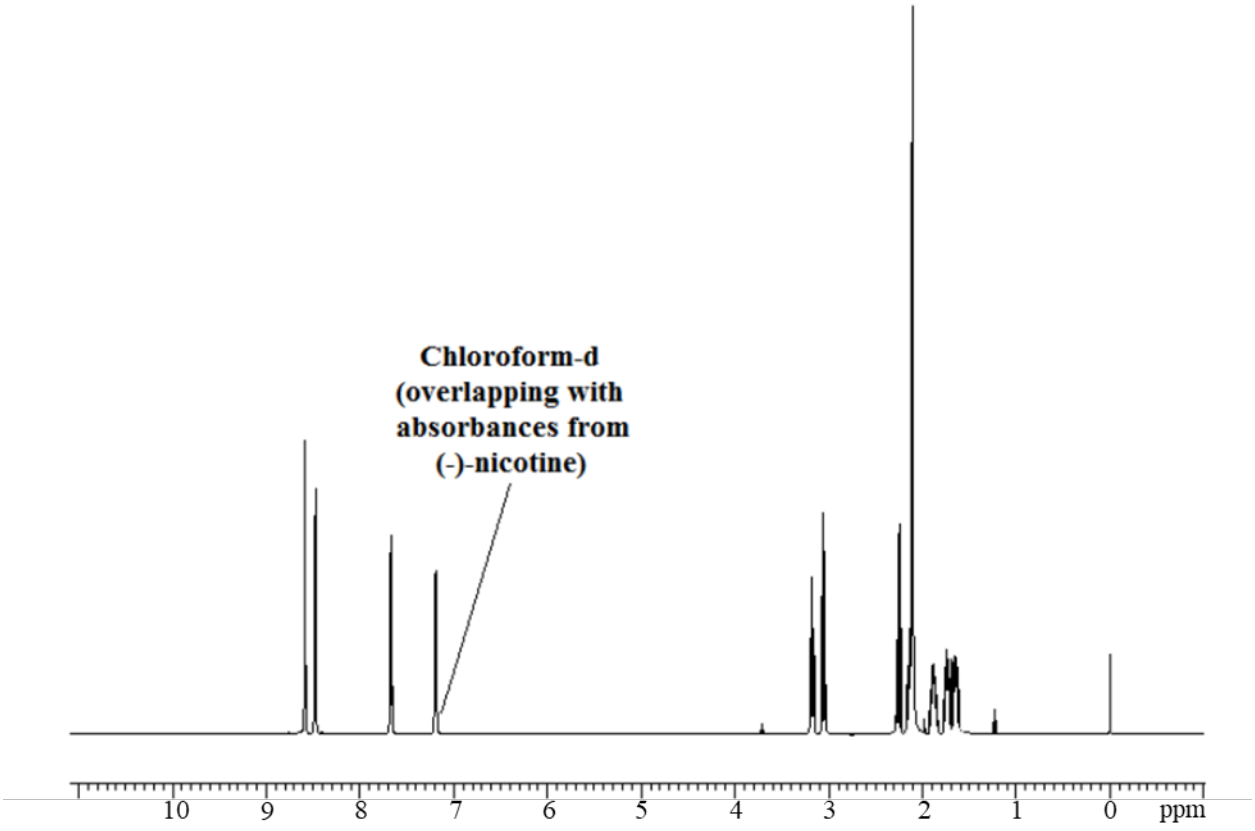

Figure A-5. ^{1}H Nuclear Magnetic Resonance Spectrum of Freebase (-)-Nicotine

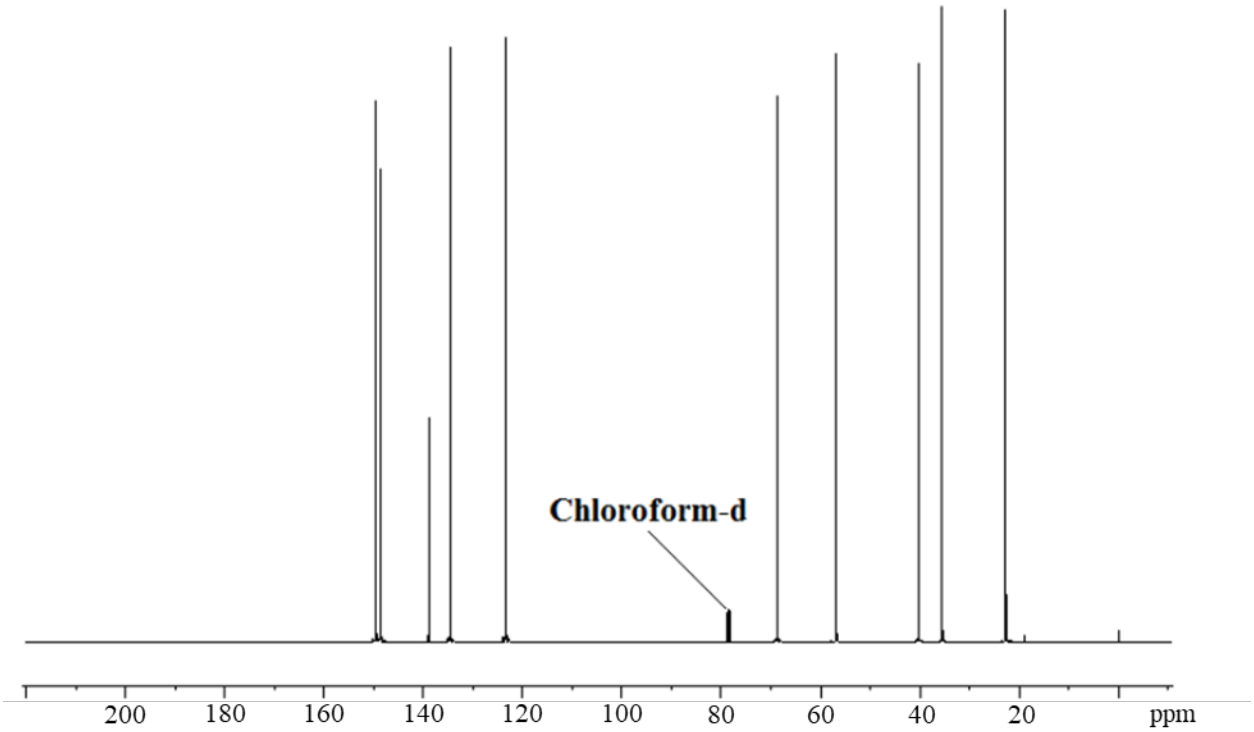

Figure A-6. ^{13}C Nuclear Magnetic Resonance Spectrum of Freebase (-)-Nicotine

Appendix B. Toxicokinetic Studies in Rats

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B.1. Formulation Preparation and Analysis

Dose formulations for the toxicokinetic studies were prepared once at Battelle (Columbus, OH) by mixing freebase (-)-nicotine or nicotine bitartrate dihydrate (NBD) in ASTM Type I water at a concentration of 0.1 mg/mL. The freebase (-)-nicotine formulations were stored at 2°C–8°C, and the NBD formulations were stored at room temperature (Table A-3). Formulations were analyzed prior to dosing by liquid chromatography with tandem mass spectrometry (LC-MS/MS) under the conditions presented in Appendix A. The preadministration nicotine concentrations in all formulations used for the study were within 10% of the target concentrations; sample analysis results are reported in Appendix A.

B.2. Animal Source and Welfare

Male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats (approximately 10 weeks old) were obtained from Envigo (Indianapolis, IN). Studies were approved by the Battelle (Columbus, OH) Animal Care and Use Committee. Animals were housed in a facility that is fully accredited by AAALAC International. Animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals.[76] Animals were quarantined immediately upon receipt. During the approximately 4-day quarantine period, all animals were observed twice daily for morbidity and mortality. Randomization was stratified by body weight to produce similar group mean weights using Provantis software (Instem, Version 8.6.1.2). Each study animal was assigned a unique identification number by the randomization program and identified by a cage card and an indelible ink tail marking.

B.3. Study Design and Dose Administration

For each group, three animals per sex were administered a single gavage dose, either freebase (-)-nicotine or NBD in ASTM Type I water at a dose of 0.5 mg/kg. Gavage dosing was administered using a 15-gauge gavage needle with a 3 mL syringe. Dosing volumes were 5 mL/kg body weight. Animals were weighed on the day before dosing during randomization to calculate dosing volume. Because the tartrate salt crystallizes as the dihydrate, a monoisotopic mass of 498.2 g/mol was used for calculating freebase (-)-nicotine concentrations. Freebase (-)-nicotine represents 32.56% of the monoisotopic mass of the NBD compound. Morbidity/mortality checks were performed on all animals twice daily (prior to 10 a.m. and at or after 2 p.m., separated by at least 6 hours) during the study, except for the day of receipt and the day of study termination. Clinical observations for signs of toxicity were recorded prior to dose administration. Details of the study design and animal maintenance are summarized in Table B-1.

Table B-1. Experimental Design and Materials and Methods in the Toxicokinetic Study of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate

Freebase (-)-Nicotine	Nicotine Bitartrate Dihydrate
Study Laboratory	
Battelle (West Jefferson, OH)	Same as freebase (-)-nicotine
Strain and Species	
Sprague Dawley (Hsd:Sprague Dawley® SD®)	Same as freebase (-)-nicotine
Animal Source	
Envigo (Indianapolis, IN)	Same as freebase (-)-nicotine
Time Held Before Studies	
4 days	Same as freebase (-)-nicotine
Average Age When Studies Began	
10 weeks	Same as freebase (-)-nicotine
Date of Dosing	
June 28, 2016 (male rats and female rats)	Same as freebase (-)-nicotine
Duration of Dosing	
Single dose administered via gavage over 5 minutes	Single dose administered via gavage over 7 minutes
Size of Study Groups	
Three male rats and three female rats	Same as freebase (-)-nicotine
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as freebase (-)-nicotine
Animals per Cage	
1	Same as freebase (-)-nicotine
Method of Animal Identification	
Cage card and tail mark	Same as freebase (-)-nicotine
Diet	
Irradiated NTP-2000 wafer feed (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed weekly	Same as freebase (-)-nicotine
Water	
Tap water (West Jefferson, OH municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available ad libitum	Same as freebase (-)-nicotine
Cages	
Upon receipt: solid polycarbonate cage (Lab Products, Inc., Seaford, DE), changed at least twice weekly	Same as freebase (-)-nicotine
During study period: Raturn Housing System of the Culex (BASi, West Lafayette, IN), changed at least twice weekly	

Freebase (-)-Nicotine	Nicotine Bitartrate Dihydrate
Bedding	
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed with cage changes	Same as freebase (-)-nicotine
Animal Room Environment	
Temperature: 72°F ± 3°F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Same as freebase (-)-nicotine
Dose	
0.5 mg/kg administered via gavage (prepared in ASTM Type I water at concentration of 0.1 mg/mL); 5 mL/kg dosing volume. Dosing volumes were calculated based on body weights obtained on the day prior to dose administration.	Same as freebase (-)-nicotine
Type and Frequency of Observation	
Observed twice daily except for day of receipt and day of study termination. Weighed on the day prior to dose administration (during randomization). Clinical observations for signs of toxicity were recorded prior to dose administration.	Same as freebase (-)-nicotine
Internal Concentration Assessment	
Blood samples were collected via catheter from three animals at each of 15 time points (predose and at 0.08, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hours postdose). Plasma was harvested from blood samples via centrifugation within 1 hour of blood collection. Plasma was evaluated for nicotine and cotinine (major metabolite) concentrations. Plasma was also qualitatively monitored for the following metabolites: trans-3'-hydroxycotinine, cotinine-N-oxide, cotinine N-β-D-glucuronide, norcotinine, nornicotine, trans-3'-hydroxycotinine-O-β-D-glucuronide, nicotine-1'-oxide, and nicotine N-β-D-glucuronide.	Blood samples were collected via catheter from three animals at 15 time points (predose and at 0.08, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hours postdose), with one deviation: Animal 253 (female rat dosed with NBD) did not undergo blood sample collection at the 72-hour postdose time point. Plasma was harvested from blood samples via centrifugation within 1 hour of blood collection. Plasma was evaluated for nicotine and cotinine (major metabolite) concentrations. Plasma was also qualitatively monitored for the following metabolites: trans-3'-hydroxycotinine, cotinine-N-oxide, cotinine N-β-D-glucuronide, norcotinine, nornicotine, trans-3'-hydroxycotinine-O-β-D-glucuronide, nicotine-1'-oxide, and nicotine N-β-D-glucuronide.
Toxicokinetic Analysis	
Plasma concentration-versus-time data were evaluated for aberrant concentration and time point values, as well as for any evidence of misdosing. Arithmetic and semilog plots of the plasma concentration-versus-time data sets were prepared for nicotine and cotinine. The concentration-versus-time profiles were analyzed using noncompartmental analysis with extravascular input (WinNonlin, Version 8.0, Certara, Princeton, NJ). Plasma toxicokinetic parameters were estimated for nicotine and cotinine in male and female animals.	Same as freebase (-)-nicotine

NBD = nicotine bitartrate dihydrate.

Blood samples (approximately 250 μL) were collected prior to dosing (predose) and at 14 time points after dose administration (0.08, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hours postdose) from each animal that had a blood collection catheter placed in the carotid artery. Animals were conscious and freely moving during the blood collection periods while samples were automatically collected with the Culex instrument (BASi, West Lafayette, IN). Blood samples were collected from three animals at each of the 15 time points, except for the 72-hour time point when blood samples from only two female rats dosed with NBD were collected. Each blood sample was transferred into a tripotassium ethylenediaminetetraacetic acid-containing tube by the Culex and maintained at a refrigerated temperature (approximately 5°C) until it was separated into plasma by centrifugation (within 1 hour of blood collection). Plasma was placed into cryovials, stored on dry ice, and then transferred to an approximately −70°C freezer until analysis.

Concentrations of nicotine and cotinine, a major metabolite of nicotine, were measured by LC-MS/MS (Appendix A). Samples below the limit of detection (LOD) were designated as below the LOD (BLOD). With one exception due to a missing sample at 72 hours (due to patency issues), blood samples were collected from at least three animals at 15 time points, except for the 72-hour time point at which samples were collected from only two female rats dosed with NBD. For each time point, the mean was calculated.

Additionally, the following metabolites were qualitatively monitored in plasma samples: trans-3'-hydroxycotinine, cotinine-N-oxide, cotinine N-β-D-glucuronide, norcotinine, nornicotine, trans-3'-hydroxycotinine-O-B-D-glucuronide, nicotine-1'-oxide, and nicotine N-β-D-glucuronide. The standards used for this analysis [S-(-)-cotinine, nicotine-d3, cotinine-d3, trans-3'-hydroxy cotinine, cotinine-N-oxide, cotinine N-β-D-glucuronide, (R,S)-norcotinine, (R,S)-nornicotine, trans-3'-hydroxy cotinine O-β-D-glucuronide, (1'S,2'S)-nicotine 1'-oxide, and nicotine N-β-D-glucuronide] were purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada).

B.4. Toxicokinetic Analysis

Plasma concentration-versus-time data were evaluated for aberrant concentration and time point values as well as for any evidence of misdosing. Individual animal data were examined for acceptable agreement between the target and actual collection times (i.e., within 5% for time points ≤4 hours and within 15 minutes for time points >4 hours). If substantial evidence indicated an explanation for potential aberrant concentrations, the value was reported but flagged with an explanation for its omission from the data set. Concentration-versus-time data sets for both nicotine and cotinine were evaluated using noncompartmental analysis (NCA) (WinNonlin, Version 8.0, Certara, Princeton, NJ) with extravascular input. No weighting factors were used to obtain the initial estimates for NCA. The software algorithm was allowed to select the points used to calculate slope of the terminal linear phase by determining an optimal determination coefficient (R^2). A glossary of kinetic symbols and definitions is provided in Table B-2.

Table B-2. Analytical Glossary of Kinetic Symbols and Definitions Used in Toxicokinetic Studies

Parameter	Noncompartmental Analysis	Definition
$C_{max\_observed}$	C_{max}	Observed maximum plasma concentration
$T_{max\ observed}$	T_{max}	Time at which observed C_{max} occurs
T_{last}	T_{last}	Time at which last plasma concentration occurs
Lambda_z	Lambda_z	Noncompartmental analysis (NCA) terminal elimination rate constant, NCA k_e or k_{elim}
Half-life	HL_Lambda_z	Lambda z half-life, $t_{1/2}$, the terminal elimination half-life based on noncompartmental analysis
Cl	Cl_pred	Clearance of central compartment
Cl1_F	Cl_F_pred	Apparent clearance of the central compartment, also Cl_F for gavage groups in noncompartmental model
V1	Vz_pred	Volume of distribution of the central compartment, includes Vd and V volume of distribution
V1_F	Vz_F_pred	Apparent volume of distribution for the central compartment, includes Vd_F, V_F for oral groups, and Vc_F
MRT	$MRT_{INF\ pred}$	Mean residence time
AUC_0-T	AUC_{last}	Area under the plasma-concentration-versus-time curve, AUC, from time t_i (initial) to t_f (final), AUC_{last}
$AUC_{\infty\_predicted}$	AUC_{INF_pred}	Area under the plasma-concentration-versus-time curve, AUC, extrapolated

B.5. Toxicokinetic Results

Following a single oral administration of freebase (-)-nicotine and NBD at 0.5 mg/kg in rats, the absorption of nicotine was rapid, occurring within the first hour for all but one animal. Comparisons of male and female rats revealed no apparent sex-related differences in toxicokinetic parameters, including systemic exposure parameters such as the maximum plasma concentration (C_{max}) and area under the plasma-concentration-versus-time curve (AUC), of

nicotine following either freebase (-)-nicotine or NBD administration (Table B-3). In addition, mean toxicokinetic parameters, including systemic exposure, were similar when comparing freebase (-)-nicotine and NBD. The only difference greater than twofold was the half-life for female rats, which was only 2.1-fold longer following NBD administration (3.82 versus 1.83 hours) and likely not biologically relevant.

Following oral administration of freebase (-)-nicotine and NBD in rats, concentrations of the metabolite cotinine rose with a $T_{max\_observed}$ of 4–5.33 hours for all animals (Table B-4). When compared to nicotine $T_{max\_observed}$, which was typically between 0.5 and 1 hour, this difference suggested a slow conversion of nicotine to cotinine. Comparisons of male and female rats revealed no apparent sex-related effects on the toxicokinetic parameters or systemic exposure of cotinine following freebase (-)-nicotine or NBD administration. Cotinine, following either freebase (-)-nicotine or NBD administration, for both male and female rats, had a longer half-life (1.4- to 3.4-fold) and greater systemic exposure ($C_{max\_observed}$ 2.6- to 4.0-fold; $AUC_{last\ \infty\_predicted}$ 8.8- to 11.6-fold) than nicotine; systemic exposure was similar when comparing freebase (-)-nicotine and NBD.

In conclusion, toxicokinetic parameters, including systemic exposure, were similar in male and female rats following a single oral administration of freebase (-)-nicotine and NBD at 0.5 mg/kg. Because freebase (-)-nicotine is less stable than NBD in solutions and the salt is easy to handle, NBD was selected as the test article.

Table B-3. Summary of Plasma Toxicokinetic Parameters of Nicotine in Male and Female Rats Following Gavage Administration of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate[a]

Parameter	Freebase (-)-Nicotine (0.5 mg/kg)		NBD (0.5 mg/kg)	
	Male	Female	Male	Female
$C_{max\_observed}$ (ng/mL)	50.5 ± 5.3	61.7 ± 12.0	49.5 ± 7.9	36.7 ± 3.0
$T_{max\_observed}$ (hr)	0.917 ± 0.083	0.583 ± 0.083	0.667 ± 0.083	0.917 ± 0.546
Half-life (hr)	2.70 ± NA	1.83 ± 0.41	3.25 ± 1.09	3.82 ± NA
Cl1_F (mL/hr/kg)	12,100 ± NA	10,800 ± 600	10,800 ± 900	10,400 ± NA
V1_F (mL/kg)	43,900 ± NA	28,800 ± 6,600	48,000 ± 12,900	50,900 ± NA
AUC_{last} (hr*ng/mL)	129 ± 6	153 ± 16	128 ± 9	126 ± 15
$AUC_{\infty\_predicted}$ (hr*ng/mL)	169 ± NA	186 ± 10	188 ± 15	211 ± NA

NBD = nicotine bitartrate dihydrate; $C_{max\_observed}$ = observed maximum plasma concentration; $T_{max\_observed}$ = time at which observed C_{max} occurs; NA = not applicable; Cl1_F = apparent clearance of the central compartment; V1_F = apparent volume of distribution for the central compartment; AUC_{last} = area under the plasma-concentration-versus-time curve from initial to final; $AUC_{\infty\_predicted}$ = area under the plasma-concentration-versus-time curve extrapolated to time equals infinity.
[a]Data are presented as mean of individual animal toxicokinetic parameters ± standard error.

Table B-4. Summary of Plasma Toxicokinetic Parameters of Cotinine in Male and Female Rats Following Gavage Administration of Freebase (-)-Nicotine and Nicotine Bitartrate Dihydrate[a]

Parameter	Freebase (-)-Nicotine (0.5 mg/kg)		NBD (0.5 mg/kg)	
	Male	Female	Male	Female
$C_{max\_observed}$ (ng/mL)	150 ± 21	160 ± 10	136 ± 21	147 ± 6
$T_{max\_observed}$ (hr)	4.00 ± 0.00	4.67 ± 0.67	4.00 ± 0.00	5.33 ± 0.67
Half-life (hr)	3.94 ± 0.44	6.17 ± 0.73	4.53 ± 0.49	5.52 ± 0.69
Cl1_F (mL/hr/kg)	1,250 ± 40	933 ± 60	1,210 ± 80	962 ± 58
V1_F (mL/kg)	7,110 ± 760	8,210 ± 670	7,960 ± 1140	7,690 ± 1190
AUC_{last} (hr*ng/mL)	1,280 ± 80	1,750 ± 130	1,520 ± 100	1,940 ± 120
$AUC_{\infty\_predicted}$ (hr*ng/mL)	1,600 ± 50	2,160 ± 150	1,660 ± 100	2,090 ± 130

NBD = nicotine bitartrate dihydrate; $C_{max\_observed}$ = observed maximum plasma concentration; $T_{max\_observed}$ = time at which observed C_{max} occurs; Cl1_F = apparent clearance of the central compartment; V1_F = apparent volume of distribution for the central compartment; AUC_{last} = area under the plasma-concentration-versus-time curve from initial to final; $AUC_{\infty\_predicted}$ = area under the plasma-concentration-versus-time curve extrapolated to time equals infinity.
[a]Data are presented as mean of individual animal toxicokinetic parameters ± standard error.

Appendix C. Nicotine Bitartrate Dihydrate Internal Concentration Assessment

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C.1. Sample Collection

C.1.1. Urine

After nicotine bitartrate dihydrate (NBD) administration via drinking water for 3 months, urine samples were collected from rats and mice, stored at –70°C, and shipped to Battelle (Columbus, OH) for biological sample analysis and evaluation of nicotine, cotinine, and creatinine concentrations. At week 12 of the 3-month studies, rats and mice were removed from study cages and placed in individual metabolism cages for 16 hours. Drinking water formulations and feed were available ad libitum. Urine samples were collected the following morning from all rats (10/sex/group) and randomly selected mice (5/sex/group) for internal concentration assessment.

C.1.2. Blood

At study termination, rats and mice were anesthetized with a carbon dioxide and oxygen mixture (70% and 30%, respectively), and blood was collected from all rats (10/sex/group) and randomly selected mice (5/sex/group) for internal concentration assessment. Blood was drawn from the cava vein into tubes containing potassium ethylenediaminetetraacetic acid (K_3EDTA). The tubes were gently inverted for 30 seconds to mix the contents and centrifuged at 1,500 rpm for 10 minutes at 4°C. Plasma was harvested (two aliquots, approximately 150 µL each) and stored at −70°C until analysis.

C.2. Sample Analysis

Nicotine and cotinine levels in samples from the 3-month studies were quantified using validated analytical methods, and method validation data are given in Table C-1 and Table C-2. Creatine was also quantified in rat and mouse urine. Qualitative measurements of metabolites trans-3'-hydroxy cotinine, (S)-cotinine N-oxide, cotinine N-β-D-glucuronide, (R,S)-norcotinine, (R,S)-nornicotine, trans-3'-hydroxy cotinine O-β-D-glucuronide, (1'S,2'S)-nicotine 1'-oxide, and nicotine N-β-D-glucuronide were also conducted on the rat and mouse plasma and urine (Table C-3).

Plasma and urine samples collected from male and female Sprague Dawley rats and male and female Swiss mice were received frozen on dry ice from the Ramazzini Institute (Bologna, Italy) and were stored at approximately –70°C when not being analyzed. The following matrix blanks used to prepare the calibration and quality control (QC) standards were received from BioIVT (Westbury, NY): male Sprague Dawley rat plasma (lot RAT329738) and male Swiss Webster mouse plasma (lot MSE263476) containing K_3EDTA as an anticoagulant, male Sprague Dawley rat urine (lot RAT329735), and male Swiss Webster mouse urine (lot MSE263474). The following matrix blanks used to prepare the QC stability standards were received from the Ramazzini Institute (Bologna, Italy): male and female Sprague Dawley rat plasma and male and female Swiss mouse plasma containing K_3EDTA as an anticoagulant, male and female Sprague Dawley rat plasma and urine, and male and female Swiss mouse urine.

Nicotine, cotinine, and the eight metabolites were measured using a calibration curve of seven matrix calibration standards ranging from 10 ng/mL to 200 ng/mL. Stock solutions for nicotine, cotinine, and each metabolite were combined and appropriately diluted to create a set of solvent standard spiking solutions in ASTM Type I water. On each day of extraction, the solvent

standard spiking solutions were diluted 1/10 in the appropriate blank matrix from BioIVT (rat or mouse, plasma or urine) to create the final matrix calibration standards. Racemic nicotine-d3 and cotinine-d3 (Toronto Research Chemicals, Inc [Toronto, Ontario, Canada]) were used as internal standards (IS). The working internal standard (WIS) solution at 150 ng/mL was made by dissolving the respective source chemicals in methanol and then diluting in an aqueous solution of 10% trichloroacetic acid (TCA). Standard solutions were stored refrigerated (at 2°C–8°C) when not in use.

Matrix QC standards were prepared in both rat and mouse plasma and urine matrices provided by BioIVT. Two standard concentrations were prepared at 150 ng/mL (high) and 15 ng/mL (low) and stored in individual aliquots at −70°C until use with each extraction set of the corresponding matrix. Similarly, matrix QC stability standards were prepared in the blank matrices provided by the Ramazzini Institute (Bologna, Italy) and stored at −70°C in the same freezer as the study samples. The matrix QC stability standards' results indicated there was no adverse effect on the samples during storage for nicotine and cotinine. The percentage difference between interrun-determined concentrations and expected concentrations are reported in Table C-2.

One hundred (100) µL of each matrix calibration standard, QC standard, blank, and study sample were transferred into individual microcentrifuge tubes. A 100 µL aliquot of the WIS was added to each tube, except for the blanks without IS. To the blanks without IS, 100 µL of 10% TCA in water was added. Each tube was mixed by vortex and centrifuged at maximum speed for approximately 5 minutes. Each well of a 96-well Oasis HLB solid-phase extraction plate (Waters, Milford, MA) was conditioned with 1 mL of methanol followed by 1 mL of 10% TCA in water. The wells were loaded with the supernatant of the pretreated sample. The samples were then eluted with 1 mL of 5% ammonium hydroxide in methanol into collection plates containing 100 µL of 1% hydrochloric acid. The eluent was evaporated to dryness under nitrogen at 40°C and then reconstituted with 100 µL of 10:90 methanol:10 mM ammonium bicarbonate in water. The samples were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Creatinine concentrations in urine were measured directly with a Roche (Basel, Switzerland) cobas® 6000 chemistry analyzer with the c501 module. Nicotine, cotinine, and creatinine concentrations in the study samples are listed in Table 32 and Table 33.

C.3. Instrumentation and Quantitation

Samples from the 3-month studies were analyzed using LC-MS/MS (Table C-1, System F) in positive ionization mode. The system was considered suitable for a given run if at least three consecutive injections of a 30 ng/mL standard in the appropriate matrix had a relative standard deviation (RSD) of ≤10% using area ratios. Drift was considered acceptable if the average area ratio of duplicate injections of the standard was within 15% of the prerun system suitability average area ratio. The performance of the calibration curve was evaluated before the analysis of each sample set. A successful calibration was indicated by the following: correlation coefficient (r) ≥0.98; RSD ≤±10.0% (except at the lower limit of quantitation [LLOQ], where RSD was ≤±20%); relative error (RE) ≤±15.0% (except at LLOQ, where RE was ≤±20%) (Table C-2).

Calibration curves relating response ratio of analyte to internal standard (after correction for the background levels for the 3-month studies only) and concentration of nicotine and cotinine were

constructed using a 1/X weighted linear regression. The analyte concentration in samples was calculated using response ratio, the regression equation, initial sample weight or volume, digestion volume, and dilution, when applicable. The concentration was reported as ng nicotine or cotinine/mL for plasma and urine and mg creatinine/dL for urine.

Data from study samples were considered valid if the system was deemed suitable and if QC standards passed two criteria: at least 67% of QC standards' concentrations were within 15% of its nominal value and at least 50% of the QC standards' RE was ≤15% at each target concentration level (low and high). All QC sets for each study sample set met these criteria.

Table C-1. Liquid Chromatography Systems Used for Internal Concentration Assessment Analysis in the Three-month Studies of Nicotine Bitartrate Dihydrate

Chromatography	Detection System	Column	Mobile Phase
System F			
High-performance liquid chromatography	Tandem mass spectrometry, positive ion	Phenomenex Gemini NX-C18, (50 × 2 mm ID, 3 µm particle size)	A: 20 mM ammonium bicarbonate (aq) B: Acetonitrile Gradient program: A:B 90:10 to 25:75 in 3 minutes; 25:75 to 90:10 in 0.1 minute; hold at 90:10 for 1.9 minutes 500 µL/min flow rate

ID = internal diameter.

Table C-2. Analytical Method Qualification and Stability Data for Nicotine Bitartrate Dihydrate and Cotinine in Plasma and Urine for the Three-month Studies

Validation Parameter	Rat Plasma	Rat Urine	Mouse Plasma	Mouse Urine
Matrix Concentration Range (ng/mL)				
LOQ (ng/mL)				
Nicotine	10.0	10.0	10.0	10.0
Cotinine	9.96	9.96	9.96	9.96
LOD (ng/mL)				
Nicotine	0.360	2.54	2.93	3.21
Cotinine	1.17	1.02	1.11	0.597
Correlation Coefficient (R^2)				
Nicotine	≥0.9973	≥0.9974	≥0.9992	≥0.9903
Cotinine	≥0.9892	≥0.9994	≥0.9987	≥0.9967
Precision and Accuracy				
Nicotine intraday % RSD	≤8.1	≤37.3	≤28.6	≤8.9
Nicotine intraday % RE	≤±5.3	≤39.3	≤22.7	≤±5.3
Nicotine interday % RSD	NA[a]	≤26.3	≤24.1	≤7.0
Nicotine interday % RE	NA[a]	≤20.0	≤10.7	≤2.0
Cotinine intraday % RSD	≤13.7	≤6.2	≤61.0	≤7.4
Cotinine intraday % RE	≤±16.8	≤4.0	≤61.7	≤±2.7

Validation Parameter	Rat Plasma	Rat Urine	Mouse Plasma	Mouse Urine
Cotinine interday % RSD	≤12.9	≤4.0	≤56.7	≤4.7
Cotinine interday % RE	≤±8.1	≤2.7	≤30.2	≤±1.3
QC Stability % RE[b]				
Nicotine	≤7.3	≤4.0	≤6.0	≤16.7
Cotinine	≤8.1	≤0.7	≤4.1	≤1.3

LOQ = limit of quantitation; LOD = limit of detection; RSD = relative standard deviation; RE = relative error; NA = not applicable; QC = quality control.
[a]QC samples were run on 1 day so there are no data to generate an interday value.
[b]Estimated by comparing response of matrix standards to solvent standards.

C.4. Results

Table C-3. Summary of Qualitative Assessment of Additional Metabolites in Rat and Mouse Plasma and Urine for the Perinatal and Three-month Study

Metabolite	Presence in Plasma[a]		Presence in Urine[a]	
	Rat	Mouse	Rat	Mouse
trans-3'-Hydroxy cotinine	NP	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups
(S)-Cotinine N-oxide	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups
Cotinine N-β-D-glucuronide	Present across all exposed groups	Present across all exposed groups	NR because a matrix peak interfered with the analyte, prohibiting quantitation	NR because a matrix peak interfered with the analyte, prohibiting quantitation
(R,S)-Norcotinine	Present in trace amounts in 1.56–12.5 mg/L groups, present in 25 mg/L group	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups
(R,S)-Nornicotine	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups
trans-3'-Hydroxy cotinine O-β-D-glucuronide	NP in 1.56 mg/L group, present in only a few samples in the 3.12–25 mg/L groups	Present in one sample in the 6.25 mg/L group, present in the remaining exposed groups	NP	Poor chromatography, unable to evaluate
(1'S,2'S)-Nicotine 1'-oxide	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups	Present across all exposed groups
Nicotine N-β-D-glucuronide	NP except trace amounts in the 25 mg/L group	NP or present in trace amounts in 6.25–25 mg/L groups, present in 50 and 100 mg/L groups	NR because a matrix peak interfered with the analyte, prohibiting quantitation	NR because a matrix peak interfered with the analyte, prohibiting quantitation

NP = not present; NR = not reportable.
[a]Results from the control groups are not reported.

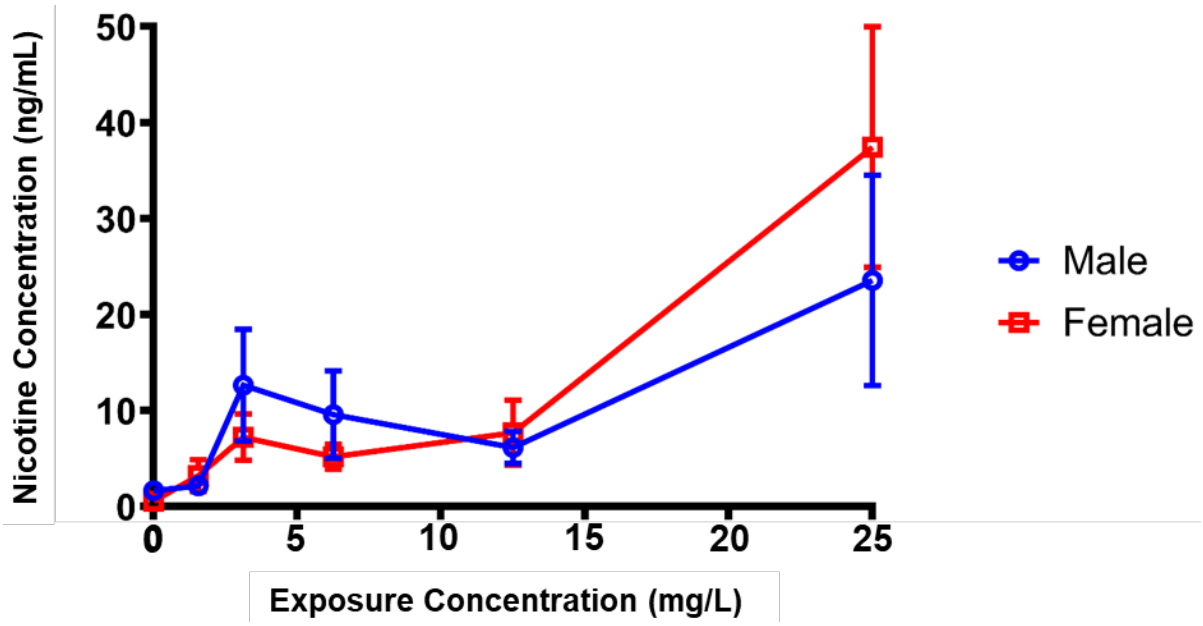

Figure C-1. Nicotine Plasma Concentrations in Male and Female Rats in the Perinatal and Three-month Study of Nicotine Bitartrate Dihydrate

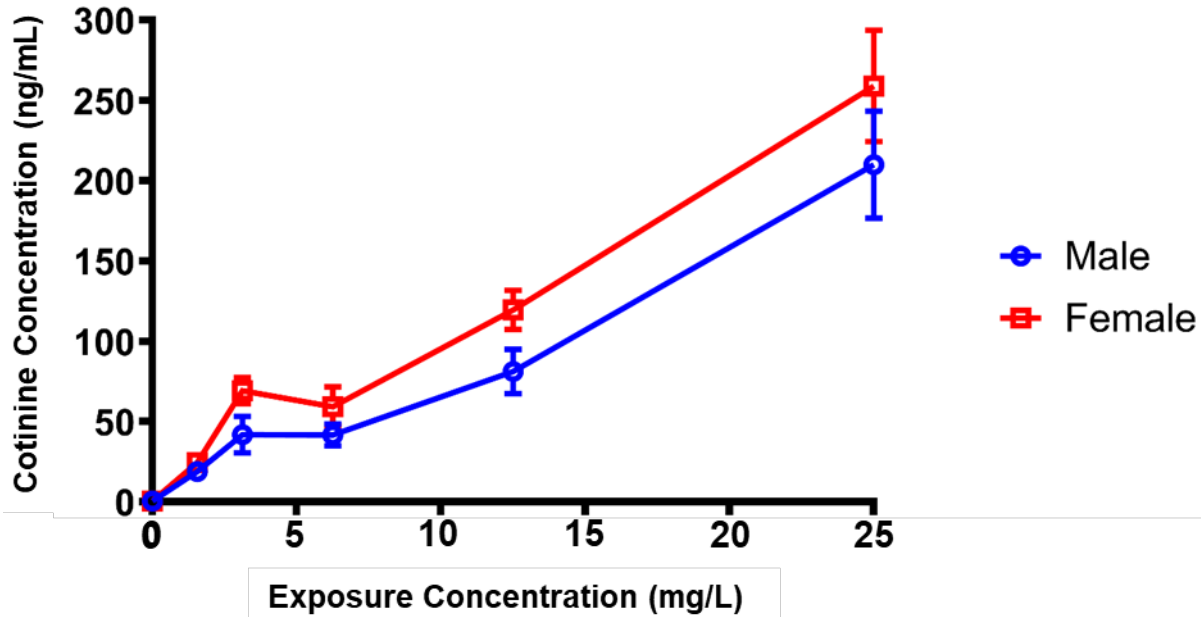

Figure C-2. Cotinine Plasma Concentrations in Male and Female Rats in the Perinatal and Three-month Study of Nicotine Bitartrate Dihydrate

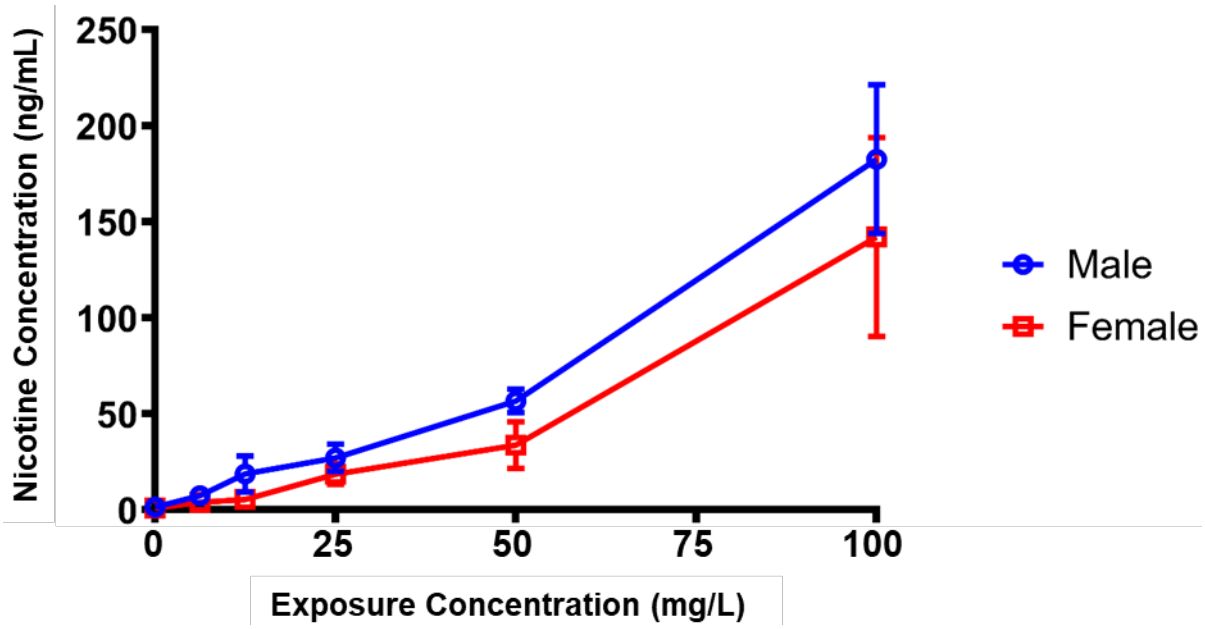

Figure C-3. Nicotine Plasma Concentrations in Male and Female Mice in the Three-month Study of Nicotine Bitartrate Dihydrate

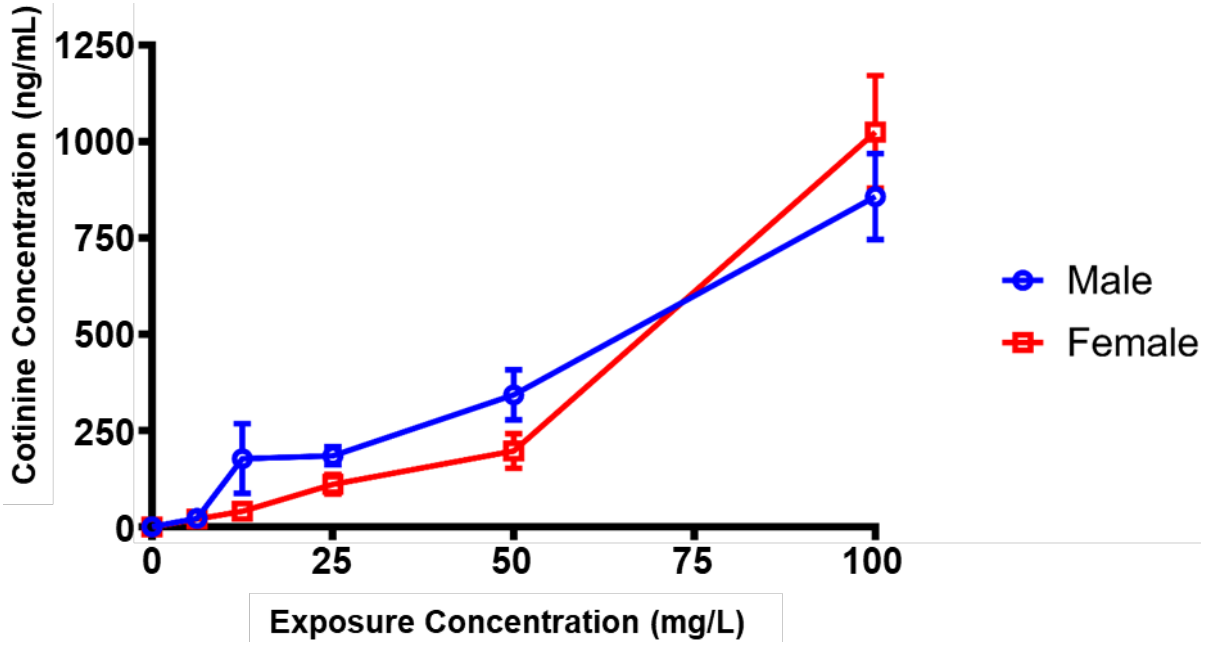

Figure C-4. Cotinine Plasma Concentrations in Male and Female Mice in the Three-month Study of Nicotine Bitartrate Dihydrate

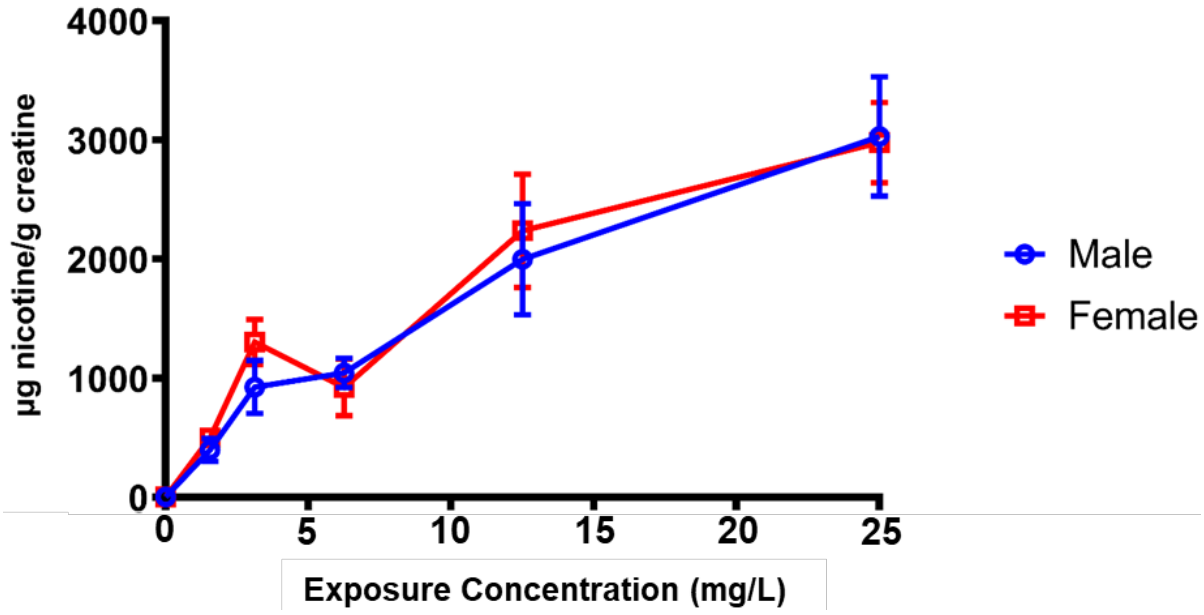

Figure C-5. Nicotine Urine Concentrations (Creatinine-adjusted) in Male and Female Rats in the Perinatal and Three-month Study of Nicotine Bitartrate Dihydrate

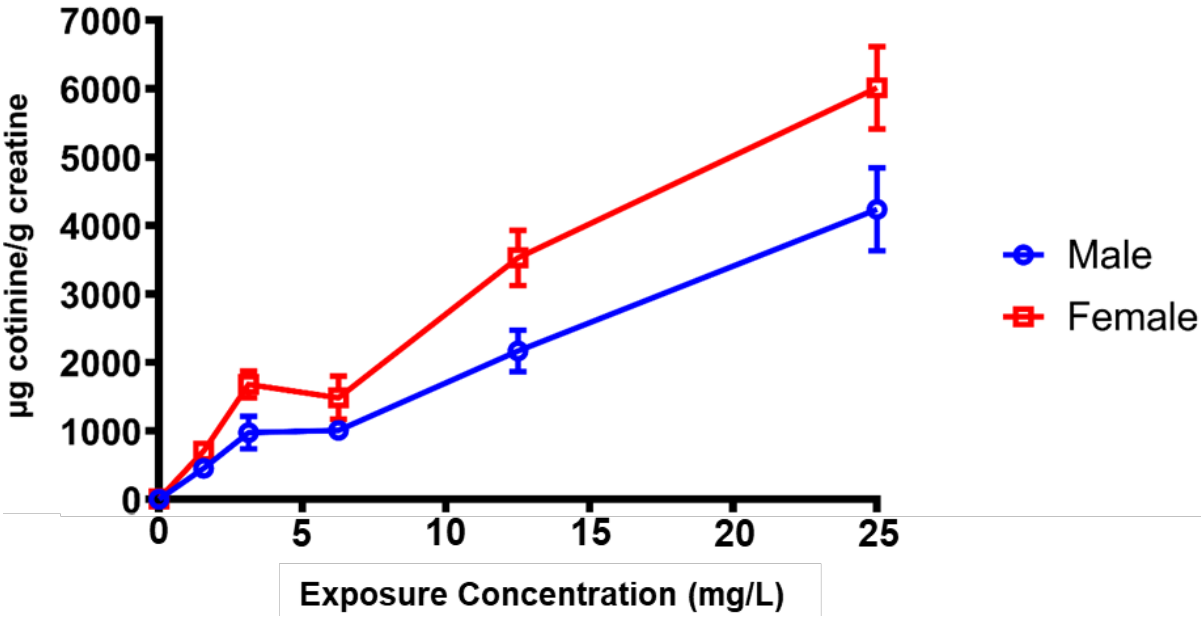

Figure C-6. Cotinine Urine Concentrations (Creatinine-adjusted) in Male and Female Rats in the Perinatal and Three-month Study of Nicotine Bitartrate Dihydrate

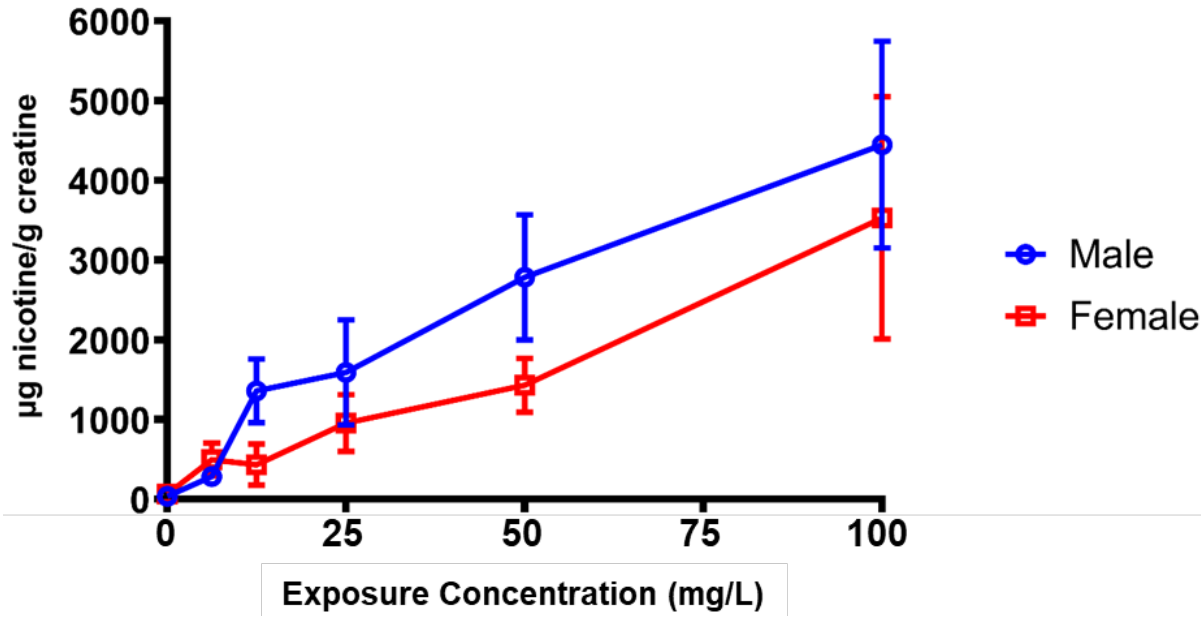

Figure C-7. Nicotine Urine Concentrations (Creatinine-adjusted) in Male and Female Mice in the Three-month Study of Nicotine Bitartrate Dihydrate

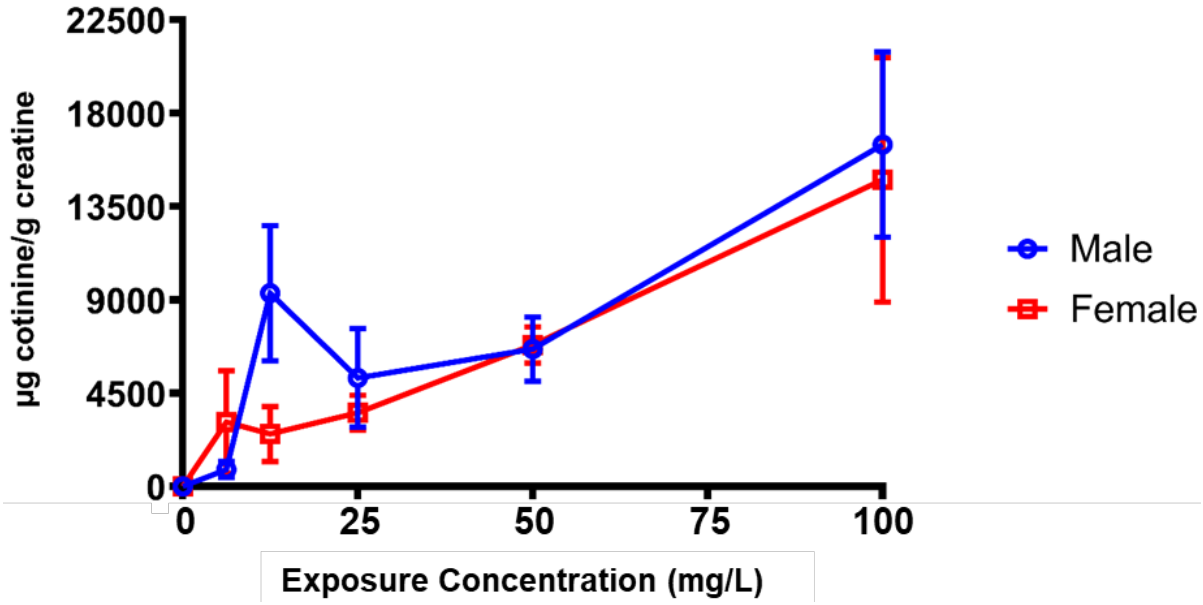

Figure C-8. Cotinine Urine Concentrations (Creatinine-adjusted) in Male and Female Mice in the Three-month Study of Nicotine Bitartrate Dihydrate

Appendix D. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NIEHS-DATA-NIEHS-11.[45]

D.1. Individual Animal Data - Rats

D.1.1. Two-week Palatability Study in Rats

Nicotine_2WeekPalatability_Rats_FEMALE_Body_Weight.xlsx

Nicotine_2WeekPalatability_Rats_FEMALE_Feed_Consumption.xlsx

Nicotine_2WeekPalatability_Rats_FEMALE_Water_Consumption.xlsx

Nicotine_2WeekPalatability_Rats_FEMALE_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_2WeekPalatability_Rats_MALE_Body_Weight.xlsx

Nicotine_2WeekPalatability_Rats_MALE_Feed_Consumption.xlsx

Nicotine_2WeekPalatability_Rats_MALE_Water_Consumption.xlsx

D.1.2. Perinatal and Four-week Dose Range-finding Study in Rats

Nicotine_4Week_Rats_DAM+PUP_Lactation_Feed_Consumption.xlsx

Nicotine_4Week_Rats_DAM+PUP_Lactation_Water_Consumption.xlsx

Nicotine_4Week_Rats_DAM_Gestation_Body_Weight.xlsx

Nicotine_4Week_Rats_DAM_Gestation_Feed_Consumption.xlsx

Nicotine_4Week_Rats_DAM_Gestation_Water_Consumption.xlsx

Nicotine_4Week_Rats_DAM_Lactation_Body_Weight.xlsx

Nicotine_4Week_Rats_FEMALE_Adult_Body_Weight.xlsx

Nicotine_4Week_Rats_FEMALE_Adult_Feed_Consumption.xlsx

Nicotine_4Week_Rats_FEMALE_Adult_Water_Consumption.xlsx

Nicotine_4Week_Rats_FEMALE_Adult_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_4Week_Rats_FEMALE_Clinical_Chemistry.xlsx

Nicotine_4Week_Rats_FEMALE_Clinical_Chemistry_Recovery.xlsx

Nicotine_4Week_Rats_FEMALE_Hematology.xlsx

Nicotine_4Week_Rats_FEMALE_Hematology_Recovery.xlsx

Nicotine_4Week_Rats_FEMALE_Nonneoplastic_Pathology.xlsx

Nicotine_4Week_Rats_FEMALE_Organ_Weight.xlsx

Nicotine_4Week_Rats_FEMALE_Organ_Weight_Recovery.xlsx

Nicotine_4Week_Rats_Litter_Mortality.xlsx

Nicotine_4Week_Rats_MALE_Adult_Body_Weight.xlsx

Nicotine_4Week_Rats_MALE_Adult_Feed_Consumption.xlsx

Nicotine_4Week_Rats_MALE_Adult_Water_Consumption.xlsx

Nicotine_4Week_Rats_MALE_Adult_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_4Week_Rats_MALE_Clinical_Chemistry.xlsx

Nicotine_4Week_Rats_MALE_Clinical_Chemistry_Recovery.xlsx

Nicotine_4Week_Rats_MALE_Hematology.xlsx

Nicotine_4Week_Rats_MALE_Hematology_Recovery.xlsx

Nicotine_4Week_Rats_MALE_Nonneoplastic_Pathology.xlsx

Nicotine_4Week_Rats_MALE_Organ_Weight.xlsx

Nicotine_4Week_Rats_MALE_Organ_Weight_Recovery.xlsx

Nicotine_4Week_Rats_PUP_Lactation_Body_Weight.xlsx

Nicotine_4Week_Rats_PUP_Postmortem_Pathology.xlsx

D.1.3. Perinatal and Three-month Study in Rats

PA44_-_3Month_Rats_Urinalysis_Summary.pdf

PA48_-_3Month_Rats_Tissue_Concentration_Summary.pdf

Nicotine_3Month_Rats_DAM+PUP_Lactation_Feed_Consumption.xlsx

Nicotine_3Month_Rats_DAM+PUP_Lactation_Water_Consumption.xlsx

Nicotine_3Month_Rats_DAM_Gestation_Body_Weight.xlsx

Nicotine_3Month_Rats_DAM_Gestation_Feed_Consumption.xlsx

Nicotine_3Month_Rats_DAM_Gestation_Water_Consumption.xlsx

Nicotine_3Month_Rats_DAM_Lactation_Body_Weight.xlsx

Nicotine_3Month_Rats_FEMALE_Adult_Body_Weight.xlsx

Nicotine_3Month_Rats_FEMALE_Adult_Feed_Consumption.xlsx

Nicotine_3Month_Rats_FEMALE_Adult_Water_Consumption.xlsx

Nicotine_3Month_Rats_FEMALE_Adult_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_3Month_Rats_FEMALE_Clinical_Chemistry.xlsx

Nicotine_3Month_Rats_FEMALE_Hematology.xlsx

Nicotine_3Month_Rats_FEMALE_Neoplastic_Pathology.xlsx

Nicotine_3Month_Rats_FEMALE_Nonneoplastic_Pathology.xlsx

Nicotine_3Month_Rats_FEMALE_Organ_Weight.xlsx

Nicotine_3Month_Rats_MALE_Adult_Body_Weight.xlsx

Nicotine_3Month_Rats_MALE_Adult_Feed_Consumption.xlsx

Nicotine_3Month_Rats_MALE_Adult_Water_Consumption.xlsx

Nicotine_3Month_Rats_MALE_Adult_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_3Month_Rats_MALE_Clinical_Chemistry.xlsx

Nicotine_3Month_Rats_MALE_Hematology.xlsx

Nicotine_3Month_Rats_MALE_Nonneoplastic_Pathology.xlsx

Nicotine_3Month_Rats_MALE_Organ_Weight.xlsx

Nicotine_3Month_Rats_PUP_Lactation_Body_Weight.xlsx

Nicotine_3Month_Rats_Removal_Reasons.xlsx

D.2. Individual Animal Data - Mice

D.2.1. Two-week Palatability Study in Mice

Nicotine_2WeekPalatability_Mice_FEMALE_Body_Weight.xlsx

Nicotine_2WeekPalatability_Mice_FEMALE_Feed_Consumption.xlsx

Nicotine_2WeekPalatability_Mice_FEMALE_Water_Consumption.xlsx

Nicotine_2WeekPalatability_Mice_MALE_Body_Weight.xlsx

Nicotine_2WeekPalatability_Mice_MALE_Feed_Consumption.xlsx

Nicotine_2WeekPalatability_Mice_MALE_Water_Consumption.xlsx

D.2.2. Four-week Dose Range-finding Study in Mice

Nicotine_4Week_Mice_FEMALE_Body_Weight.xlsx

Nicotine_4Week_Mice_FEMALE_Clinical_Chemistry.xlsx

Nicotine_4Week_Mice_FEMALE_Clinical_Chemistry_Recovery.xlsx

Nicotine_4Week_Mice_FEMALE_Feed_Consumption.xlsx

Nicotine_4Week_Mice_FEMALE_Hematology.xlsx

Nicotine_4Week_Mice_FEMALE_Hematology_Recovery.xlsx

Nicotine_4Week_Mice_FEMALE_Nonneoplastic_Pathology.xlsx

Nicotine_4Week_Mice_FEMALE_Organ_Weight.xlsx

Nicotine_4Week_Mice_FEMALE_Organ_Weight_Recovery.xlsx

Nicotine_4Week_Mice_FEMALE_Water_Consumption.xlsx

Nicotine_4Week_Mice_FEMALE_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_4Week_Mice_MALE_Body_Weight.xlsx

Nicotine_4Week_Mice_MALE_Clinical_Chemistry.xlsx

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Nicotine_4Week_Mice_MALE_Water_Consumption.xlsx

Nicotine_4Week_Mice_MALE_Water_Consumption_Metabolic_Cage.xlsx

D.2.3. Three-month Study in Mice

PA44_-_3Month_Mice_Urinalysis_Summary.pdf

PA48_-_3Month_Mice_Tissue_Concentration_Summary.pdf

Nicotine_3Month_Mice_FEMALE_Body_Weight.xlsx

Nicotine_3Month_Mice_FEMALE_Clinical_Chemistry.xlsx

Nicotine_3Month_Mice_FEMALE_Feed_Consumption.xlsx

Nicotine_3Month_Mice_FEMALE_Hematology.xlsx

Nicotine_3Month_Mice_FEMALE_Neoplastic_Pathology.xlsx

Nicotine_3Month_Mice_FEMALE_Nonneoplastic_Pathology.xlsx

Nicotine_3Month_Mice_FEMALE_Organ_Weight.xlsx

Nicotine_3Month_Mice_FEMALE_Water_Consumption.xlsx

Nicotine_3Month_Mice_FEMALE_Water_Consumption_Metabolic_Cage.xlsx

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Nicotine_3Month_Mice_MALE_Feed_Consumption.xlsx

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Nicotine_3Month_Mice_MALE_Nonneoplastic_Pathology.xlsx

Nicotine_3Month_Mice_MALE_Organ_Weight.xlsx

Nicotine_3Month_Mice_MALE_Water_Consumption.xlsx

Nicotine_3Month_Mice_MALE_Water_Consumption_Metabolic_Cage.xlsx

Nicotine_3Month_Mice_Removal_Reasons.xlsx

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