Regulatory Toxicology and Pharmacology 70 (2014) S35–S42

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Health assessment of gasoline and fuel oxygenate vapors: Neurotoxicity evaluation

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ARTICLE INFO

Article history:
Available online 28 May 2014

Keywords:
Neurotoxicity
Glial fibrillary acidic protein (GFAP)
Astrogliosis
Gasoline vapor condensates
Methyl tertiary butyl ether
Ethyl t-butyl ether
t-Amyl methyl ether
Diisopropyl ether
Ethanol
t-Butyl alcohol

ABSTRACT

Sprague–Dawley rats were exposed via inhalation to vapor condensates of either gasoline or gasoline combined with various fuel oxygenates to assess potential neurotoxicity of evaporative emissions. Test articles included vapor condensates prepared from "baseline gasoline" (BGVC), or gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA). Target concentrations were 0, 2000, 10,000 or 20,000 mg/mg^3 and exposures were for 6 h/day, 5 days/week for 13 weeks. The functional observation battery (FOB) with the addition of motor activity (MA) testing, hematoxylin and eosin staining of brain tissue sections, and brain regional analysis of glial fibrillary acidic protein (GFAP) were used to assess behavioral changes, traditional neuropathology and astrogliosis, respectively. FOB and MA data for all agents, except G/TBA, were negative. G/TBA behavioral effects resolved during recovery. Neuropathology was negative for all groups. Analyses of GFAP revealed increases in multiplebrain regions largely limited to males of the G/EtOH group, findings indicative of minor gliosis, most significantly in the cerebellum. Small changes (both increases and decreases) in GFAP were observed for other test agents but effects were not consistent across sex, brain region or exposure concentration.

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1. Introduction

The 1990 amendments to the Clean Air Act (CAA) mandated the use of oxygenates in motor gasoline. In 1994, the U.S. Environmental Protection Agency (EPA) issued a final rule under the Act which added new health effects information and testing requirements to the Agency's existing registration requirements. As described in more detail in a companion paper (Henley et al., 2014), requirements include inhalation exposures to evaporative emissions of the gasoline or additive in question. The health endpoints include assessments for standard subchronic toxicity, neurotoxicity, genotoxicity, immunotoxicity, developmental and reproductive toxicity, and chronic toxicity/carcinogenicity. The results of chronic toxicity testing of gasoline and gasoline combined with MTBE have already been reported (Benson et al., 2011). This paper describes the results of neurotoxicity testing submitted to EPA.

Test materials evaluated in the 13 week toxicity studies included vapor condensates prepared from an EPA described "baseline gasoline" (BGVC), as well as gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA). The goal of the studies was to provide information on the extent to which the use of oxygenates in gasoline might alter the hazard of evaporative emissions that are encountered during refueling of vehicles, compared to those from gasoline alone. The animals evaluated for neurotoxicity were exposed concurrently with animals involved in a subchronic inhalation toxicity study of the materials described above (Clark et al., 2014). Additional groups of animals were exposed to the test materials concurrently to evaluate the effects of exposure on immunotoxicity and genotoxicity, the results of which are described elsewhere (White et al., 2014; Schreiner et al., 2014).

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http://dx.doi.org/10.1016/j.yrtph.2014.05.002
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Astrogliosis is the activated state of astrocytes, a characteristic feature of all types of CNS damage. A hallmark of astrogliosis, often termed "reactive gliosis," is the intracellular accumulation of astroglial filaments, the major protein component of which is glial fibrillary acidic protein (GFAP). Thus, an increase in the brain concentration of GFAP serves as a biochemical indicator of astrogliosis and, as such, enhanced expression of GFAP is a biomarker of neurotoxicity. To validate the use of GFAP as a biomarker of neurotoxicity, prototype neurotoxicants were administered to experimental animals and the effects of these agents on the tissue content of GFAP was determined by immunoassay (O'Callaghan, 1991a, 2002; Norton et al., 1992; O'Callaghan and Sriram, 2005; O'Callaghan et al., 2014). Assays of GFAP were found to reveal dose-, time- and region-dependent patterns of neurotoxicity at toxicant dosages below those that cause light microscopic evidence of cell loss or damage (O'Callaghan, 1988; Norton et al., 1992; O'Callaghan and Sriram, 2005; O'Callaghan et al., 2014). Moreover, the temporal and regional increments in GFAP correspond to the temporal and regional patterns of neuronal damage, as revealed by sensitive silver stains (Balaban et al., 1988; Balaban, 1992). These findings indicate that assaying brain regional levels of GFAP represents a sensitive, simple and quantitative approach for evaluation of nervous system damage (O'Callaghan, 1991a, 2000; Norton et al., 1992; O'Callaghan and Sriram, 2005).

In this study, both standard neurobehavioral and motor toxicity assessments as well as the GFAP assay were used for assessing the potential neurotoxic effects of the fuel vapors described above. Although the EPA Guidelines for GFAP determination (US EPA, 1994a) specifies six regions to be analyzed, the analysis used in these studies was expanded to include an additional three areas of the brain to maximize the potential for detecting enhanced expression of GFAP due to exposure to the test substance.

2. Methods and materials

2.1. Test material exposures

Seven separate inhalation studies involving exposures to vapor condensates were conducted. Test materials included baseline gasoline (BGVC) and BGVC combined with methyl t-butyl ether (G/MTBE), ethanol (G/EtOH), t-amyl ethyl ether (G/TAME), ethyl t-butyl ether (G/ETBE), diisopropyl ether (G/DIPE), or t-butyl alcohol (G/TBA) at concentrations of 0, 2000, 10,000, 20,000 mg/m^3/day, 5 days a for a total of 65 exposures over 13 weeks, performed at Huntingdon Life Sciences (East Millstone, NJ). Ten adult Sprague–Dawley rats (5 males and 5 females) were used in each of the investigations to examine neuropathology and to determine changes in glial fibrillary acidic protein (GFAP) levels in areas of the brain. Motor activity and performance in the functional observational battery (FOB) was evaluated in all 20 animals. The animals were satellite groups of a larger subchronic toxicity study, and the generation and composition of the vapor concentrations as well as additional details on the exposure methodology are reported in companion articles (Henley et al., 2014; Clark et al., 2014).

2.2. Neurobehavioral studies

FOB and motor activity evaluations were staggered over several sessions and conducted on non-exposure days at least 16 h post-exposure. With the exception of the pretest, evaluations were performed "blind", i.e., the observer did not know the identity of the animal's exposure group. Evaluations were conducted once before initiation of exposures and again during the 4th, 8th and 13th week of exposures. Time of testing was balanced across treatment groups. Noise level was maintained within a level of 55–65 decibels by a white noise generator. Temperature, humidity and illumination were measured and recorded to ensure that variations in environmental conditions are minimal during all evaluations. The functional observational battery (Moser, 1989) was performed for all animals before evaluation of motor activity and included:

1. *Home Cage Evaluations*: posture, vocalization and palpebral closure.
2. *Handling Evaluations*: reactivity to general stimuli (handling); assessment of signs of autonomic function: lacrimation, salivation, altered fur appearance, or red crusty deposits around eyes.
3. *Open Field Evaluations*: arousal level and gait; count of urination and defecation; convulsions, tremors, abnormal movements or behaviors, excessive or repetitive actions; piloerection and exophthalmos.
4. *Reflex Assessments*: response to visual (approach response) and auditory (finger snap) stimuli; response to a tail pinch; pupillary function.
5. *Grip Strength* (Meyer et al., 1979): grip strength was measured using a grip strength meter (Columbus Instruments International Corporation, Columbus, Ohio).
6. *Landing Foot Splay*: each animal was dropped into a pan of sand from a height of one foot. The distance between the marks left by the hind paws was measured in centimeters.
7. *Hind limb Extensor Strength*: animals were held in a vertical position facing the observer with a firm grasp around the thorax. The observer placed one finger against the bottom of each hind paw and pressed toward the animal. Muscular resistance and pressure exerted by the animals were scored.
8. *Air Righting Ability*: animals were held upside down and dropped from a height of one foot into a container of bedding. The landing position of each animal was recorded.
9. *Body Weight*: animals were removed from their cages and weighed using a Mettler Balance, Model PE4000 (Mettler Instrument Corporation, Hightstown, New Jersey).
10. *Motor Activity*: using a modified version of Schulze's procedures (Schulze, 1990), the locomotor activity of all animals was monitored using an automated Photobeam Activity System (San Diego Instruments, Inc., San Diego, California). Sessions were 60 min in length; each session was divided into 12 intervals of 5 min.

2.3. Neuropathology

Following 13 weeks of exposures, designated animals (5/sex/group) were anesthetized with an IP injection of sodium pentobarbital and transcardially perfused with phosphate buffered saline followed by 1% glutaraldehyde and 4% paraformaldehyde in the same buffer. After perfusion, the tissues listed in Table 1 were obtained. Measurement of the size (length and width) and weight of the whole brain (cerebrum, cerebellum and pons-medulla) were made. All tissues were then placed into a fresh solution of the same fixative prior to processing.

Peripheral nerves were post-fixed in 1% osmium tetroxide, processed and embedded in epoxy resin, sectioned at approximately 2 microns and stained with toluidine blue. All other tissues, including the brain, eye with optic nerve, spinal cord, trigeminal ganglia, dorsal root ganglia, dorsal and ventral root fibers, lungs and trachea were processed by standard techniques, embedded in paraffin and sectioned at approximately 6 microns.

The tissues listed in Table 1 were examined microscopically for all animals as indicated. Tissues with macroscopic lesions were examined in all animals. Any abnormalities not noted during macroscopic postmortem examinations which were seen during histological processing were recorded.

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