

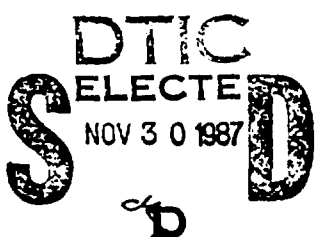
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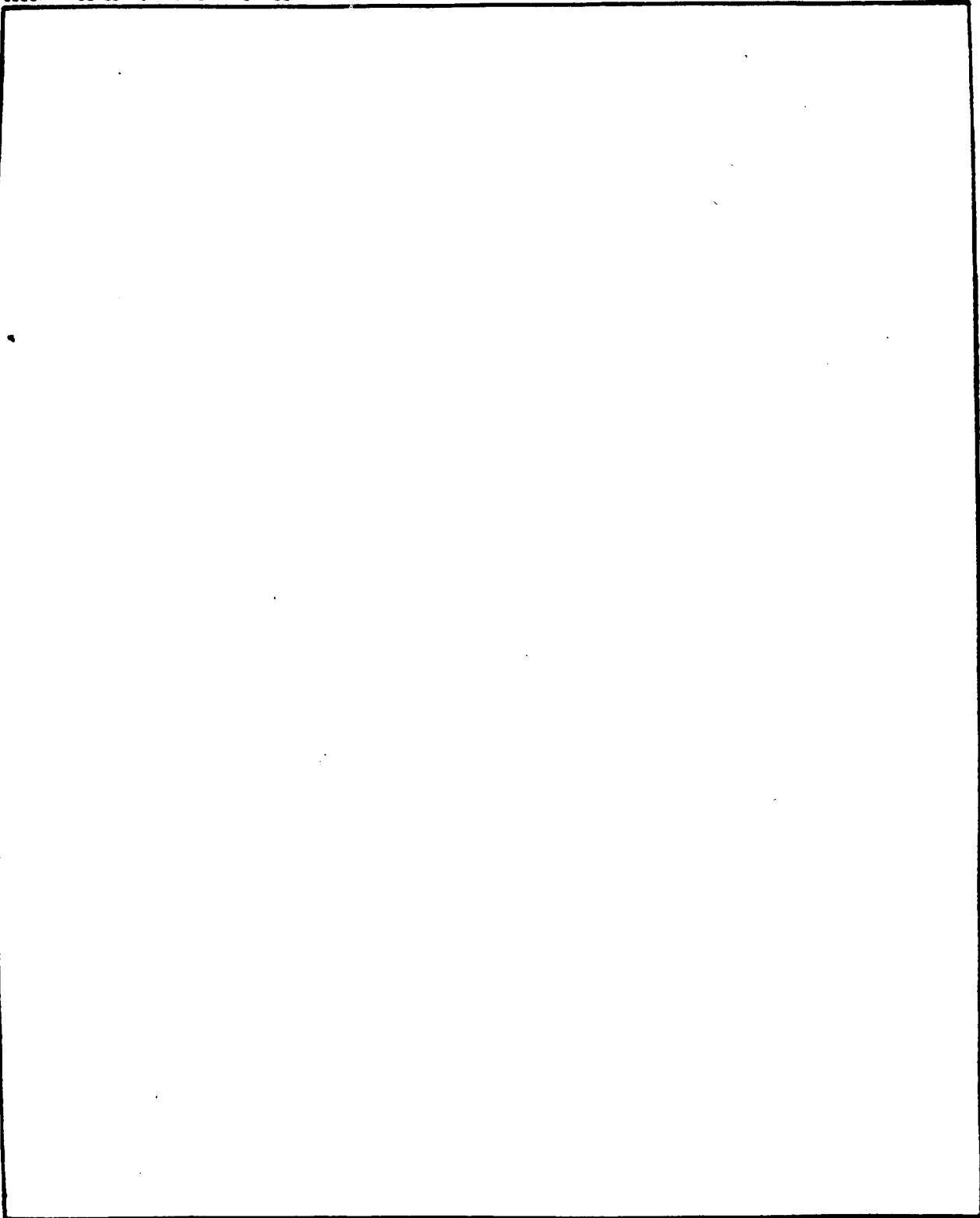
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MOTOR PERFORMANCE EFFECTS OF PROPYLENE GLYCOL DINITRATE IN THE RAT

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Propylene glycol dinitrate (PGDN), a major constituent of a liquid torpedo propellant, produces incoordination and impairment of balance in humans. This study was conducted to evaluate the rat as a model for PGDN-induced motor performance decrement, and to determine if direct application of PGDN onto neural tissue is a useful alternative to other routes of exposure. PGDN was injected onto the cisterna magna (ic) of adult Sprague-Dawley rats trained on the accelerod, a test of motor performance. Three groups of 13-14 male rats each received a single dose of either 5 or 10 μ l PGDN or 25 μ l sterile saline (control) while anesthetized with halothane. Accelerod performance was measured 12 min after ic injection, then hourly for 6 h, and at 24 h. Injections were evaluated using a five-stage screening criterion to eliminate grossly traumatized subjects, to verify the accuracy of the injection, and to determine the extent of mechanical damage. Eighteen out of 41 subjects passed the five-stage screen. A significant decrease in performance occurred during the first 2 h following injection of 10 μ l PGDN compared to the control and the 5- μ l groups. No significant differences were seen between the 5- μ l and control groups. These data confirm previous findings of PGDN-induced changes in human motor performance, suggesting that the rat may be a useful model for further PGDN neurobehavioral assessment. The data also indicate that ic injection may be an effective alternative to other routes of exposure for materials with appropriate chemical and biological properties if an evaluation screen is used.

INTRODUCTION

Propylene glycol dinitrate (PGDN) is a major constituent of a volatile liquid torpedo propellant, Otto Fuel II, used by the U.S. Navy.

The authors gratefully acknowledge the assistance of C. Boward and G. G. Kessell in training and testing the experimental subjects and B. Jackson in statistical analyses.

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Based on animal research, the major toxic effects of PGDN are hemosiderin deposits in the liver and kidneys, hypotension, and methemoglobin formation (Anderson et al., 1976; Clark and Litchfield, 1969; Jones et al., 1972). Human studies have shown that PGDN also produces headaches, nasal congestion, dizziness, impairment of motor coordination and balance, eye irritation, disruption in the organization of the visual evoked response, and changes in oculomotor function (Stewart et al., 1974; Horvath et al., 1981). In the work place, inhalation is the main route of exposure for personnel handling the propellant, although considerable potential also exists for percutaneous absorption. An occupational exposure survey by Carmichael and Lieben (1963) of explosive workers suggests serious PGDN-induced physiologic consequences, i.e., headaches, nausea, vomiting, lowered blood pressure, and increased pulse rate. No animal research has been done to follow up these clinical observations. Developing alternative animal models to test substances such as PGDN might be important due to restrictive regulations regarding human research. The primary objective of this study was to determine the neuromotor effects of PGDN using the rat/accelerod model, which has been shown to be a good test of motor performance following neurotoxic insult (Bogo et al., 1981; 1984).

Human inhalation studies conducted to date have been exposures to Otto Fuel II, which includes stabilizers, dyes, and desensitizers, as well as PGDN (Horvath et al., 1981; Stewart et al., 1974), or mixtures of PGDN and unidentified inert diluents (Anderson and Mehl, 1973; Jones et al., 1972; Mattsson et al., 1981), but none have used the nitrate ester alone. This is primarily because PGDN has a significant vapor pressure at room temperature and is highly flammable and explosive. Considering these characteristics of PGDN, intracisternal (ic) administration might be preferred over inhalation exposure for procedural reasons because it is a direct route of exposure, which makes it easier to control the accuracy and precision of the dose, and because the very small quantities of material necessary to conduct the experiment contribute to a safer work environment for the investigator. Further experimental advantages accrue because systemic degradation to the mononitrate by interaction with hemoglobin or enzymes is reduced and the blood-brain barrier is circumvented (Clark and Litchfield, 1969; Needleman and Hunter, 1965; Chasseaud et al., 1978). Therefore, the second objective of this study was to determine the feasibility of the ic route of exposure in the rat for modeling human inhalation response to PGDN.

METHODS

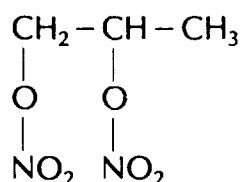
Subjects

Subjects were 41 male Sprague-Dawley rats that weighed 425 ± 9 g. Subjects were individually housed in polycarbonate cages and main-

tained in keeping with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council. Food and water were available ad libitum, and room temperature was kept at $22 \pm 1^\circ\text{C}$.

Test Material

Propylene glycol dinitrate (PGDN) in ethanol solution was obtained from the Naval Propellant Plant, Indian Head, Md., and refrigerated until ready for use. PGDN or 1,2-propanediol has the following chemical structure:



Portions of 100 μl were precipitated out of alcohol stock solution using ice-cold sterile saline. The PGDN droplets were washed three times with saline to remove the remaining alcohol. Purity of the neat PGDN was verified by gas-liquid chromatography and electron-capture detection as $>99.995\%$.

Dosing Procedures

Anesthesia was induced by a halothane-saturated atmosphere in a desiccating jar. The semiconscious subject was placed in the anesthetic-filled cylinder at the top of the resting block (Fig. 1). A rubber band looped behind the upper incisors and secured at the front of the resting block to hold the subject snugly in the cylinder. Once the tail-pinch reflex ceased, the following occurred. The tip of the guide needle was angled freehand (as shown in the left of Fig. 2), and it was inserted through the shaved skin into the previously marked ligaments between the occipital bone and the first vertebra. As the guide-needle holder (1-cc tuberculin syringe) was rotated down 90° , the tip of the needle was used to locate the upper edge of the foramen magnum. When the needle tip pierced the dura, the 33-gauge internal cannula was used as a probe to gently slide in and out of the guide needle to determine when the needle tip was no longer occluded by the dura. The guide needle was then rotated further down to lift the cerebellum and allow the cannula to be inserted 1.0–1.5 mm into the cisternal space (as shown in the right of Fig. 2). The cannula position relative to the guide needle and thus the depth of the cannula insertion were determined by using the graduations on the 1-cc glass tuberculin syringe referenced to a mark on the cannula. In preliminary tests of the procedure, injection of methylene blue demonstrated penetration and depo-

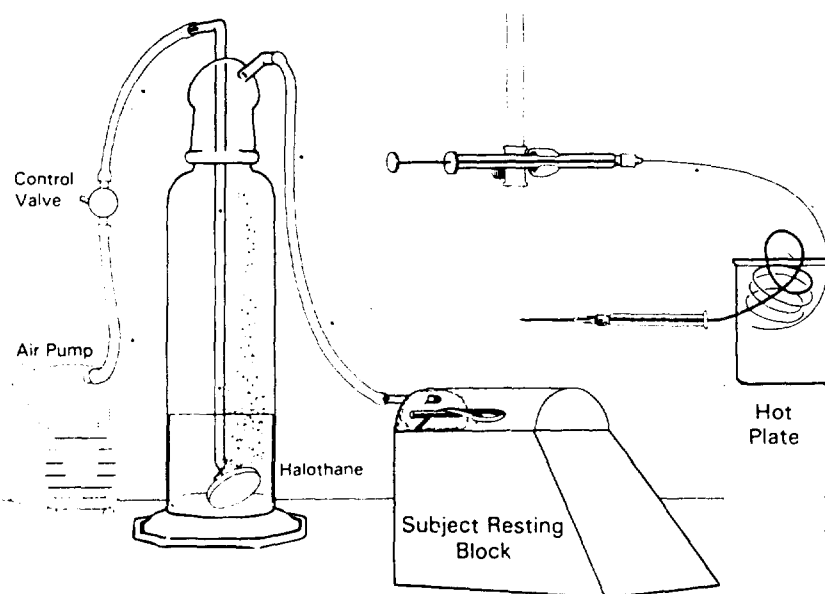


FIGURE 1. Anesthesia generation and ic injection apparatus. The anesthetic was generated by an air pump and halothane-filled glass container shown on the left. Halothane was pumped to the gauze-filled end on top of the rat resting block. The 1-cc glass tuberculin syringe was mounted on a ring stand. The polyethylene tube (0.015 in. inside diameter and 20 in. long) passed through the hot-plate-heated water maintained at 37°C to the cannula (33 gauge, 4 in. long) and the rat on the resting block.

sition within the cisterna magnum, as opposed to the ventricles and solid tissue.

The dose groups were 5 and 10 μ l PGDN and 25 μ l sterile saline (control). Sufficient saline was added to the PGDN injections to produce a total volume of 25 μ l. The test solutions were flushed from the preloaded polyethylene tube shown in Fig. 1 into the cisterna magna over a 30-s interval.

Task

The accelerod apparatus and procedure have been described in detail elsewhere (Bogo et al., 1981). Rats were trained to maintain balance for as long as possible on a gradually accelerating 2-in.-diameter rod elevated 6 in. above a grid-shock floor. The rotational velocity of the rod was increased at an average rate of 0.9 rev/min/s. A trial began by placing a subject on the stationary rod, and it lasted until the subject fell to the grid floor. Each training session lasted from 5 to 15 min (4–20 trials). It took an average of 9 d for a subject to learn the task. In the final stages of training, shock was given only for performance that lasted less than 30 s. The task was scored as performance duration, in seconds. The average performance time before receiving PGDN was 51 ± 3 (standard error) s, with performance averaged over three trials/ses-

sion/subject. PGDN testing began after stable daily performance of 30 s or above had been established.

Initial testing began 12 min after ic injection. Selection of this test time was based on a preliminary study with sham injections. In the pilot study, animals were rendered unconscious in a halothane-saturated atmosphere and were maintained on 3% halothane for about 2 min in order to shave, mark, and sham-inject them. For sham injections, the guide needle pierced the dura but the cannula was not extended (Fig. 2). As shown in Fig. 3, rats could perform at baseline levels within 6 min after removal from anesthesia. However, the performance trend did not return to the mean baseline performance until about 12 min after injection. Therefore, this latter time was used in the actual experiments. Beyond the 12-min interval, testing occurred hourly 6 times and at 24 h.

Analysis

The performance measure was time spent on the accelerod. Multiple one-way analyses of variance (ANOVA) were done to assess the effects of time after PGDN administration (Winer, 1962). The analyses were based on the difference between pre- and post-PGDN performance. A Bonferroni allocation for each ANOVA was in effect to compensate for potential multiple analyses errors (Miller, 1981).

Injection Evaluation Procedure

Prior experience with manual ic injection techniques and preliminary experiments with the current method indicated that trauma from

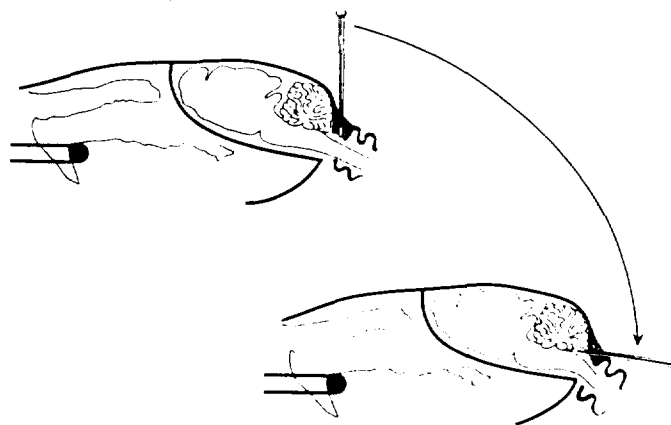


FIGURE 2. Detail of the ic injection procedure showing a side cutaway view of a rat head located on the resting block in Fig. 1. The left panel shows the incisor attached to a rubber band that maintained the head in the halothane saturated cylinder, with the needle and retracted cannula oriented perpendicular to the skull. In the right panel, the needle has been rotated 90° with the cannula inserted into the cisternal magnum.

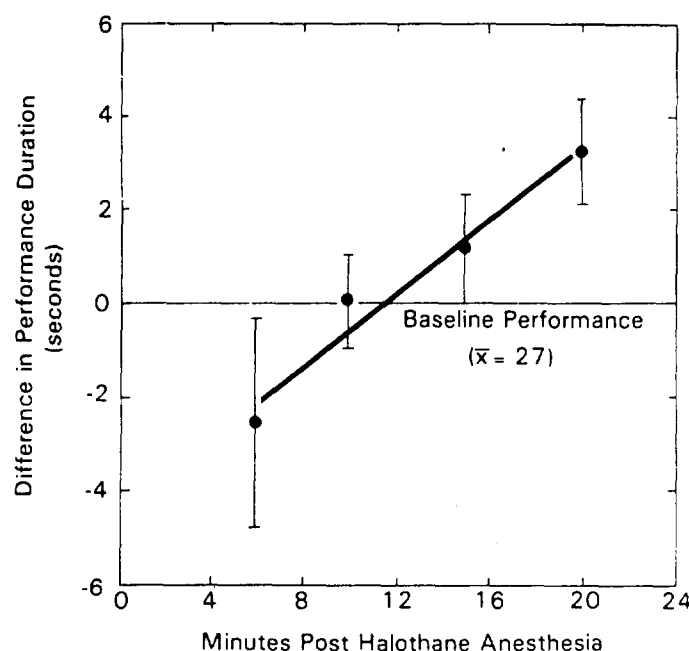


FIGURE 3. Mean $\pm$ SE of the difference in performance duration compared to baseline ($N = 6$). None of the performance trial periods were significantly different from baseline.

the injection could produce major aberrations in the performance data. Therefore, each injected animal was evaluated in a multistage, single-blind process to eliminate grossly traumatized subjects, verify the accuracy of the injection microscopically, and estimate the extent of any mechanical damage. A five-stage screen was used to reduce the likelihood of any aspect of the injection procedure contributing to the performance effects. The criteria were:

1. Subjective evaluation of the injection was judged satisfactory.
2. Subject recovered to baseline performance at 24 h.
3. Gross pathology of the brain at necropsy was normal. Any visible lesion, especially in the rhombencephalon and rostral section of the spinal cord, was cause for rejection.
4. Phase 1 histopathology examination. All surviving subjects ($N = 39$) were sacrificed 4–5 d after injection to allow sufficient time for a tissue response to chemical or mechanical insult. Serial 6- μ m coronal sections of the rhombencephalon and rostral portion of the spinal cord were sampled at 30- or 90- μ m intervals and stained with hematoxylin and eosin. Sections were evaluated for the appearance of needle or cannula tracks and/or related mechanical/hydraulic trauma without knowledge of the exposure conditions by a board-certified veterinary pathologist (J. Nold). Lesions were classified in descending order of severity as follows: (a) probably clinically sig-

- nificant (subject rejected); (b) possibly clinically significant (subject rejected); (c) probably not clinically significant (subject accepted)
5. Phase 2 histopathology examination. Tissues were classified as in stage 4, based on the degree and location of hemorrhage, necrosis, or inflammatory response. Even though some lesions may not have affected performance, they served as useful indicators of the locus of injection in the absence of significant mechanical trauma. Some lesions classified as "possibly clinically significant" were considered acceptable if the mechanical trauma that led to the lesion was considered "probably not clinically significant," i.e., the lesion was chemically rather than mechanically induced and was appropriately located. Although the five criteria become progressively more definitive, they are not weighted, i.e., rejection occurred at any stage.

Dose Groups

Subjects were assigned to the three groups over the course of the study so that baseline accelerated performance was equal. Subjects were added to the three groups until six trained subjects per group existed that were acceptable based on the injection evaluation procedure.

RESULTS

Of the 41 animals injected in 7 groups, 23 were excluded from the final analysis based on the five-stage screen as follows: (1) 2 subjects died during injection; (2) 7 subjects failed to recover behaviorally by 24 h; (3) even though behaviorally normal at 24 h, 14 subjects manifested cannula-placement errors or mechanical trauma sufficiently severe to be classified as "possibly clinically significant," and they were rejected.

Figure 4 depicts the performance profiles of the control and PGDN-dosed subjects ($N = 18$, 6 subjects/group) that recovered satisfactorily from the injections and were judged acceptable by the five-stage screening process. The performance profiles are the mean $\pm$ SE of the difference between the pre- and posttreatment performance duration. The 10- μ l subjects show a significant decrement in performance on the 12-, 60-, and 120-min trials. Recovery occurred at about h 4 or 5. No significant differences in motor performance were seen in the 5- μ l or saline-control groups.

DISCUSSION

The physiological manifestations of hypotension, vasodilation, and debilitating headaches produced by nitrate esters like PGDN have been recognized for some time based on clinical reports on explosive-industry workers (Carmichael and Lieben, 1963). Comparable vascular effects in laboratory animals have been demonstrated only in rats (Clark

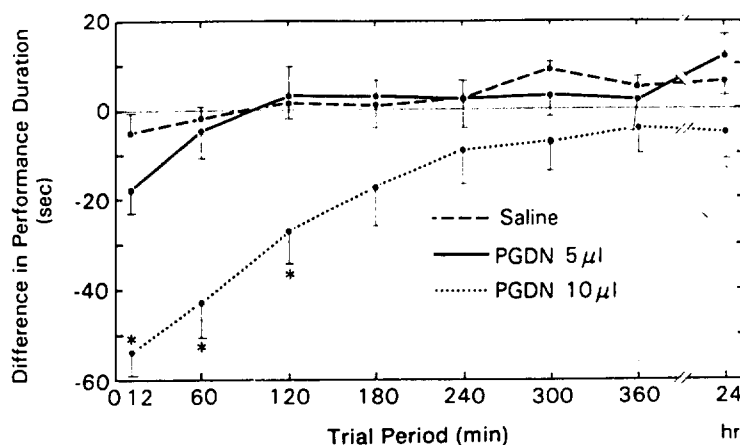


FIGURE 4. Performance profiles of 18 subjects in the 3 treatment groups ($N = 6/\text{group}$). Performance profiles are the mean $\pm$ SE of the difference between pre- and posttreatment performance duration. Significant effects are shown with asterisks ($p < 0.01$).

and Litchfield, 1969). More recently, Stewart et al. (1974) reported that exposure of people to PGDN vapor from Otto Fuel II at concentrations too low to induce hypotension produced motor performance and neurophysiological effects. Specifically, a 3-h exposure to 1.5 ppm PGDN induced significant changes in coordination and balance as observed in a modified Romberg test of balance and a coordination test of heel-to-toe walking. Horvath et al. (1981) conducted an occupational evaluation in humans exposed to PGDN by assessing motor performance and oculomotor function. In the latter study, both peak exposure concentrations of less than 0.25 ppm and short exposure periods (30–60 min) probably prevented the observation of significant decrements in motor performance or meaningful changes in oculomotor function.

The present study confirms in a rat model the human motor performance changes of Stewart et al. (1974). PGDN injected ic induced severe motor-performance decrement in the rat within 12 min of dosing at the 10- μ l dose level, but not at 5 μ l. Recovery from the high dose (approximately 33 mg/kg) was essentially linear with a gain in performance time of about 13 s/h over the 4 hourly posttreatment test periods. The effect of the 5- μ l injection was not significantly different from the saline treatment, but the performance curve during the first hour parallels the high-dose recovery curve. Since severe limitations exist in the use of human volunteers to establish toxicity threshold limits, the rat/accelerod findings suggest that it may be a useful alternative model in which to conduct future PGDN neurotoxicity research.

Although we observed motor deficits in the rat suggestive of those seen in humans, the absolute dose to the target neural tissue in our study was probably greater than in the human inhalation exposures because we used a direct application of the neat chemical unaffected

by systemic distribution and metabolism (Anderson and Smith, 1973; Stewart et al., 1974). In addition, the disparity in dose-response between the two species could be a function of the difference in the physical nature of the respective motor tests. In the human study, the modified Romberg test is performed on one leg, while no comparable restriction exists in the rat. Alternatively, if only the PGDN in solution is effective, saturation of the 200- μ l volume of cerebrospinal fluid in the rat would yield an effective dose level of approximately 1 mg/kg. Under this circumstance, the dose difference between the human and rat study may be reduced as much as 30-fold. Finally, the rapid onset of effect and the rapid recovery of the rats imply that the bulk of the PGDN is removed fairly quickly and that the recovery process involves a small residual amount of material locally absorbed in the tissue. The human rate of recovery from ataxia was not reported by Stewart et al. (1974).

We conducted related research with the rat/accelerod model using a 230-mg/kg dose of PGDN given subcutaneously (sc). The sc route was used so that the release of PGDN into the systemic circulation would occur over a longer duration than by oral administration, for instance, and immediate metabolism by the liver could be avoided. PGDN had a profound effect on performance over 6 h in the sc study. However, unlike the ic study, the sc effect may have been secondary to hypotension and methemoglobinemia-induced hypoxia. At ic-equivalent doses (i.e., 16 and 32 mg/kg, respectively, for the two dose levels in the present study), methemoglobin levels would not be expected to rise above control levels, and blood pressure would be likely to decrease by approximately 50 mm Hg for only a short period of time (Clark and Litchfield, 1969; Litchfield, 1971; Wyman et al., 1985). We chose the ic administration route to reduce the potential for secondary effects and to eliminate the rapid degradation that occurs in the liver following any route of administration that permits immediate access of major portions of the dose to systemic circulation (Kylin et al., 1964). Elimination of immediate systemic metabolism would also reduce the probability that degradation products would be a significant factor in the acute phase of neurotoxicity.

For acute dosing studies with volatile or flammable materials like PGDN, ic injection may offer several advantages over inhalation exposure, the route generally used to model human exposure. For example, the ic route is more direct and thus permits more quantitatively accurate dosing; the demanding technology and expense of running an inhalation system is eliminated; and research is possible with much smaller, safer quantities of the material. However, the ic technique has its own special problems. We found during preliminary work that simply discarding subjects with asymmetric locomotor behavior after injection did not exclude all subjects that had sustained significant injec-

tion trauma (Schanberg et al., 1967). In fact, three subjects that recovered fully within 24 h after injection were found at autopsy to have gross needle-puncture wounds in the dorsal spinal cord.

Any surgical rejection is undesirable, and this is more true for trained and conditioned animals, which are more valuable than naive subjects. The ic injection screen rejected 56% (23/41 subjects), suggesting that the screen is conservative; however, a closer look indicates that this may not be the total picture. Our 41 subjects were injected in 7 groups over 3 wk. In the first 4 groups, the rejection rate was 70% (16/23); however, in the last 3 groups, the rejection rate was almost halved to 39% (7/18). Further, 43% of the first 23 subjects were rejected based on stages 1–3 of the screen, which required little special expertise—i.e., death, failure to recover behaviorally, and gross pathology evaluation. However, of the last 18 subjects, only 2 were rejected based on stages 1–3. Thus, as the study progressed, the more sophisticated stages of the screen of histopathology evaluation for mechanical damage and necrosis were necessary to reject unsuitable animals, suggesting that technique refinement and improved accuracy is possible with practice.

It is interesting that 82% (32/39) of the surviving subjects performed normally, while 44% (14/32) of the subjects sustained lesions severe enough for rejection. A survey of recent literature on ic technique applications located 25 journal articles that used a manual injection methodology. When given, the descriptions of the injection technique were minimal, and only one article set any criteria for successful injections. If we had used a criterion for successful injection of "return to pretest performance within 24 h" (the criterion frequently reported), less than 25% of our rats would have been excluded. For these reasons, an evaluation screen is an important, if not crucial, adjunct in studies using an ic injection technique.

CONCLUSIONS

The present study confirms previously reported research of decrements in human motor performance produced by PGDN. The rat appears to be a useful animal model for assessing motor performance changes produced by PGDN. A selection criteria may be necessary to confirm the integrity and verify the accuracy of ic injections. In terms of directness, ic injections may be a reasonable alternative to inhalation administration.

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