

Anxiolytic-like effects of 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate in mice

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Abstract

The pharmacological effects of 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate (PTMB), a novel synthetic benzodiazepine, were examined in mice. In the elevated plus-maze test of anxiety, 0.3-1 mg/kg diazepam *ip* ($F(3,53) = 3.78$; $P < 0.05$) and 1-10 mg/kg PTMB *ip* increased ($F(5,98) = 3.26$; $P < 0.01$), whereas 2 mg/kg picrotoxin *ip* decreased ($F(3,59) = 8.32$; $P < 0.001$) the proportion of time spent in the open arms, consistent with an anxiolytic action of both benzodiazepines, and an anxiogenic role for picrotoxin. In the holeboard, 1.0 mg/kg diazepam *ip* increased ($F(3,54) = 2.78$; $P < 0.05$) and 2 mg/kg picrotoxin *ip* decreased ($F(3,59) = 4.69$; $P < 0.01$) locomotor activity. Rotarod assessment revealed that 1 mg/kg diazepam *ip* and 3, 10 and 30 mg/kg PTMB *ip* produced significant motor incoordination compared to vehicle control ($F(4,70) = 7.6$; $P < 0.001$). These data suggest that the recently synthesized PTMB compound possesses anxiolytic activity and produces motor incoordination similar to those observed with diazepam.

Key words

- Anxiolytic
- 1,5-Benzodiazepine
- Diazepam
- Elevated plus-maze
- Holeboard
- Picrotoxin

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Benzodiazepine compounds have been reported to be the most extensively consumed psychoactive drugs worldwide due to their anxiolytic and anticonvulsant activity. However, undesirable side effects like muscle relaxation, sedation, physical dependence, tolerance, ataxia and memory impairment have been associated with the use of benzodiazepines. A variety of novel agents capable of interacting with benzodiazepine receptors have been investigated in order to develop non-sedative anxiolytic agents (1-3). Two main hypotheses for the develop-

ment of such anxiolytic drugs have been proposed. The first suggests that different benzodiazepine receptor subtypes may be responsible for the behavioral effects of benzodiazepine compounds. According to this hypothesis, two receptor subtypes, benzodiazepine (ω_1) and benzodiazepine (ω_2) receptors, exist in different brain areas responsible for different physiological functions (2). The alternative hypothesis proposes the development of benzodiazepine receptor partial agonists which present lower intrinsic efficacy sufficient to maintain the

anxiolytic and anticonvulsant responses, but insufficient to induce the side effects seen with full agonists (2,4).

Most of the benzodiazepines used in clinical therapeutics are 1,4-benzodiazepines (5-7), since several products were generated by introducing substituents at different positions of the benzodiazepine ring of diazepam. Modifications in the structure of the ring have also been made, and the anxiolytic effect of 1,5-benzodiazepines (clobazam) has been described (8,9). However, considerably less is known about the effects of substituents on 1,5-benzodiazepines compared to the 1,4 group. In the present study we investigated the anxiolytic potential of the recently synthesized 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate (PTMB) (10). The effects of this new benzodiazepine on spontaneous coordinated motor movements were also evaluated.

Male albino mice (35-40 g) from our breeding stock were used in all experiments. All drugs were dissolved in 25% propylene glycol in distilled water.

The effect of the compounds on spontaneous locomotor activity and exploratory behavior was assessed by the holeboard test, as previously reported (11). The apparatus was a round arena (34 cm in diameter) with its floor divided into 21 equal areas. The arena had four holes 3 cm in diameter equally spaced on the floor. Twenty-five min after the intraperitoneal injection of vehicle (25% propylene glycol, v/v), diazepam (0.1-1 mg/kg), PTMB (0.3-30 mg/kg) or picrotoxin (0.2-2 mg/kg) (Sigma Chemical Co., St. Louis, MO, USA), the animals were observed for 5 min in the apparatus. The number of rearing responses, number of areas crossed with all paws, number of head dips and time spent head dipping were recorded and the animals were then immediately transferred to the elevated plus-maze apparatus.

Anxiolytic activity was measured using the elevated plus-maze paradigm (11). The plus-maze was made of wood and consisted

of two open arms measuring 30 x 5 cm and two closed arms measuring 30 x 5 x 14.5 cm. The arms extended from a central platform measuring 5 x 5 cm. The open arms, the central platform and the floor of the closed arms were covered with linoleum. The side walls were 14.5 cm high and were made of glass, in order to ensure that the light levels in the open and closed arms were approximately the same. The apparatus was elevated 38.5 cm from the floor by a support that could not be reached by the animals. The animals were placed in the central platform of the apparatus facing one of the open arms. The number of entries and the time spent in closed and open arms was recorded for 5 min.

The effect of the new compound on coordinated motor movements was assessed using the rotarod test (12). Mice were trained to stay in a rotarod apparatus (3.0 cm in diameter, 8 rpm) for more than 1 min. Twenty-four hours later the animals were injected with vehicle, diazepam (1 mg/kg) or PTMB (3-30 mg/kg), and placed in the apparatus 25 min later. The latency (in seconds) to drop off the rotarod was recorded up to a limit of 240 s.

All behavioral data were scored by two observers who were not aware of the animal's treatment, with >90% agreement between them.

Statistical analysis of behavioral data was carried out by one-way ANOVA. *Post hoc* tests were carried out by the Student-Newman-Keuls test. F-values are shown only if $P < 0.05$.

The effect of picrotoxin, diazepam and PTMB on the behavior of the animals in the holeboard is shown in Table 1. Picrotoxin (2 mg/kg) administration caused a reduction in the locomotor activity of the animals ($F(3,59) = 4.69$; $P < 0.01$), in the number of rearing responses ($F(3,59) = 7.73$; $P < 0.01$), in the number of head dips and in the time spent head dipping ($F(3,59) = 10.09$; $P < 0.001$ and $F(3,59) = 8.23$; $P < 0.001$, respectively). In

contrast, diazepam caused an increase in the number of head dipping responses at the dose of 0.1 mg/kg ($F(3,54) = 4.16$; $P < 0.01$) and an increase in locomotor activity at the dose of 1 mg/kg ($F(3,54) = 2.78$; $P < 0.05$). Other behavioral parameters (time spent head dipping and number of rearing responses) were not affected by diazepam administration (Table 1). PTMB administration caused a reduction in the number of rearing responses in the holeboard ($F(5,98) = 2.57$; $P < 0.05$), but had no effect on the number of head dips or on time spent head dipping and on locomotor activity.

Statistical analysis of plus-maze data revealed that picrotoxin decreased the proportion of time spent in the open arms ($F(3,59) = 8.32$; $P < 0.001$; Figure 1A), confirming previous literature data which suggest an anxiogenic role for this compound. Moreover, the classic anxiolytic benzodiazepine, diazepam, significantly increased the proportion of time spent in the open arms ($F(3,53) = 3.78$; $P < 0.05$; Figure 1B). These results confirm the suitability of the method used in the present study, and agree with previous literature data (1,2,11). The new benzodiazepine (PTMB), similarly to diazepam, increased the proportion of time spent in the open arms ($F(5,98) = 3.26$; $P < 0.01$; Figure 1C), suggesting an anxiolytic role for this compound. Diazepam, picrotoxin and PTMB had no effect on the number of open or closed arm entries (data not shown).

The effect of anxiolytic doses of PTMB or diazepam on the motor coordination of mice was evaluated by the rotarod test. Statistical analysis of the latency to fall from the rotarod revealed that diazepam (1 mg/kg) and PTMB (3, 10 and 30 mg/kg) produced significant motor incoordination compared to vehicle control ($F(4,70) = 7.6$; $P < 0.001$; Figure 2).

The holeboard test has been claimed to be suitable to assess directed exploratory behavior, since it allows the discrimination between exploratory activity and nonspe-

cific motor activity in rodents (5,11,13). In the holeboard, 2 mg/kg picrotoxin significantly decreased the number of head dips, the time spent head dipping, the number of rearing responses and the number of squares crossed with all paws. These results suggest that picrotoxin caused a general reduction of the spontaneous activity of the animals and, therefore, little can be said about the specific effects of this compound on directed exploratory behavior (11). Diazepam (0.1 mg/kg), on the other hand, significantly increased the number of head dips, without altering the time spent head dipping. This result contrasts with the previously reported lack of effect of other anxiolytic compounds such as chlordiazepoxide, pentobarbital and ethanol on head dipping performance of mice subjected to the holeboard (11). PTMB (30 mg/kg) significantly reduced the number of rearing responses, but had no effect on the other behavioral parameters evaluated in the holeboard test. This effect seems to be causally related to the evident ataxia that these animals developed, which was confirmed by the rotarod test (see Figure 2).

Table 1 - Effect of picrotoxin, diazepam and 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate (PTMB) on the behavior of mice in the holeboard test.

Data are reported as means \pm SEM; N = 14-18 mice per group. * $P < 0.05$ compared to vehicle (Student-Newman-Keuls test).

Drug (mg/kg)	Number of rearings	Number of head dips	Time spent head dipping	Motor activity
Vehicle	20.2 \pm 3.1	34.2 \pm 2.6	54.0 \pm 5.1	116.7 \pm 10.5
Picrotoxin (0.2)	21.8 \pm 3.0	36.2 \pm 2.3	51.8 \pm 6.3	102.9 \pm 8.1
Picrotoxin (0.6)	16.8 \pm 2.1	41.8 \pm 3.1	52.3 \pm 5.0	116.7 \pm 8.5
Picrotoxin (2.0)	6.0 \pm 1.7*	20.4 \pm 2.9*	21.2 \pm 5.5*	72.0 \pm 11.1*
Vehicle	15.4 \pm 2.2	34.5 \pm 3.0	46.5 \pm 4.4	108.1 \pm 12.9
Diazepam (0.1)	15.0 \pm 1.8	51.8 \pm 4.1*	60.8 \pm 5.9	126.5 \pm 14.0
Diazepam (0.3)	15.4 \pm 1.8	42.6 \pm 4.2	60.4 \pm 7.7	125.6 \pm 10.2
Diazepam (1.0)	10.1 \pm 2.5	36.8 \pm 3.6	42.6 \pm 6.5	158.7 \pm 16.8*
Vehicle	16.4 \pm 2.2	40.9 \pm 2.9	45.6 \pm 2.5	107.9 \pm 11.6
PTMB (0.3)	17.5 \pm 3.0	38.7 \pm 2.3	42.0 \pm 3.5	106.1 \pm 9.6
PTMB (1.0)	18.4 \pm 2.3	43.0 \pm 2.8	51.1 \pm 3.6	105.4 \pm 10.5
PTMB (3.0)	13.7 \pm 1.6	42.9 \pm 3.6	54.7 \pm 6.6	107.2 \pm 7.9
PTMB (10)	11.3 \pm 1.8	37.3 \pm 2.8	43.8 \pm 3.6	103.7 \pm 8.9
PTMB (30)	9.7 \pm 1.8*	39.0 \pm 3.4	44.4 \pm 5.3	116.6 \pm 11.0

The elevated plus-maze is currently one of the most widely used models of animal anxiety, having been employed by many re-

Figure 1 - Effects of picrotoxin, diazepam and 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate (PTMB) on percent time spent in the open arms by mice submitted to a 5-min test in the elevated plus-maze. Data are reported as means \pm SEM; N = 13-18 mice per group. *P<0.05 compared to vehicle (Student-Newman-Keuls test).

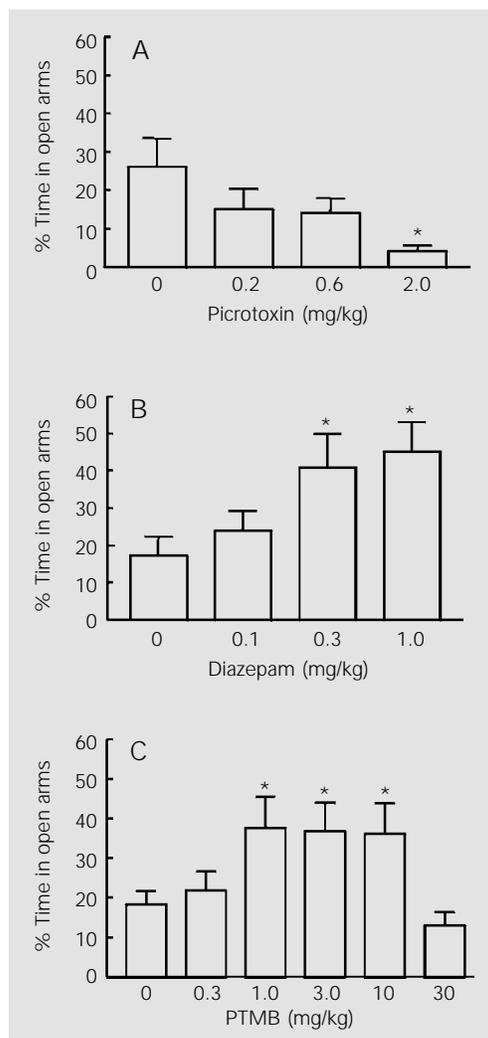
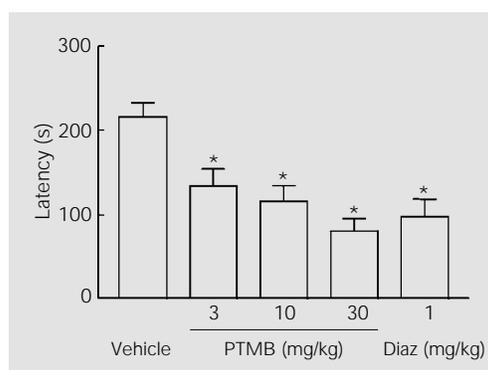


Figure 2 - Rotarod ataxia after 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate (PTMB) or diazepam (Diaz) administration to mice. Results are reported as seconds (means \pm SEM) to fall from the rotarod in a 4-min trial; N = 15 mice per group. *P<0.001 compared to vehicle (Student-Newman-Keuls test).



search laboratories in the past 6 years (14-16) and has been extensively validated for use with both rats (2,15) and mice (1,11,13,17). The conventional indices of anxiety in this test, percent of open arm entries and percent time spent in the open arm, are exquisitely sensitive to agents thought to act via the GABA_A receptor complex (i.e., benzodiazepines, barbiturates, ethanol, and neurosteroids) (14,17). For this reason, we chose this paradigm to investigate the anxiolytic potential of PTMB. Its validity in our study was supported by the observation that picrotoxin, a typical anxiogenic drug, and diazepam, a classic anxiolytic, significantly decreased and increased the proportion of time spent in the open arms, respectively (Figure 1A and B). The new benzodiazepine compound, similarly to diazepam, increased the proportion of time spent in the open arms (Figure 1C). These results are suggestive that PTMB has an anxiolytic-like effect in the plus-maze test.

The behavioral profile induced by PTMB is similar to that induced by diazepam, with both drugs having anxiolytic properties at intermediate doses and inducing ataxia at high doses. Nevertheless, PTMB, in contrast to diazepam, failed to increase the locomotor activity. These results suggest that PTMB has anxiolytic activity, but since it causes ataxia to the same extent as diazepam, its usefulness in clinical practice may be similar to that of diazepam. Further studies are needed to characterize the anxiolytic potential of PTMB in other behavioral paradigms and other biological activities, such as anti-convulsant activity and to determine whether the anxiolytic-like effect is prevented or blocked by a benzodiazepine receptor antagonist.

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