Effects of D-002 on behavioural patterns in mice

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Received: March 27th, 2008 Accepted: August 14th, 2008

Palabras clave: D-002, conducta, actividad exploratoria, plato caliente, varilla rotatoria, evitación pasiva.
Key words: D-002, behaviour, exploratory behaviour, hot plate, rotating rod, passive avoidance.

ABSTRACT. D-002 is a mixture of six high molecular-weight aliphatic alcohols purified from beeswax with antiulcer and antioxidant effects proven in experimental and clinical studies and with anti-inflammatory effects demonstrated experimentally. Experimental toxicology has not shown evidence of potential neurotoxicity of D-002. Nevertheless, adverse side effects related with the Nervous System are among the most frequent treatment-related adverse effects. Then, despite the lack of potential neurotoxicity detected in experimental toxicology and that the chemical nature of D-002 did not suppose to expect neurotoxic effects of D-002, it was explored its effects on classical behavioral patterns in rodents, frequently used as part of the battery of tests used to determine the potential neurotoxicity of new substances, like D-002. The present study was aimed to investigate the effects of single and repeat (30 d) doses of D-002 on the following behavioural patterns in mice: exploratory activity in open field, response to hot plate, performance in the rotating rod (horizontal screening test) and passive avoidance single trial test. Animals were randomised into four groups (10 mice/group): a control group treated with the acacia gum H_2O vehicle and three groups treated with D-002 at 25, 125 and 250 mg/kg, respectively. D-002 orally administered as single or repeat doses (25 - 250 mg/kg) did not change significantly the exploratory activity in the open field, the responses to the painful stimulus in the hot plate, the holding and performance on the horizontal screening test and the learning retention in the passive avoidance single trial test.

RESUMEN. El D-002 es una mezcla de seis alcoholes alifáticos de alto peso molecular purificada de la cera de abejas con efectos antiulcerosos y antioxidantes demostrados en modelos experimentales y estudios clínicos, y efectos antinflamatorios demostrados experimentalmente. Los estudios de toxicología experimental no han mostrado evidencias de que el D-002 presente potencial neurotoxicidad. Sin embargo, los efectos adversos relacionados con el Sistema Nervioso se encuentran entre los más frecuentes asociados a tratamientos de diversas áreas terapéuticas. Por ello, a pesar de la ausencia de neurotoxicidad observada en los estudios de toxicología y a que la naturaleza química del D-002 no hacía probable esperar efectos neurotóxicos, se exploraron sus efectos sobre patrones conductuales clásicos en ratones, frecuentemente usados para determinar la posible neurotoxicidad de nuevas sustancias, como es el caso del D-002. El presente estudio tuvo como objetivo investigar los efectos del tratamiento oral con D-002 (dosis únicas y repetidas durante 30 d) sobre los ensayos de conducta siguientes: actividad exploratoria en campo abierto, respuesta al plato caliente, sujeción y ejecución en varilla rotatoria y evitación pasiva de una sola prueba de aprendizaje en ratones. Los animales se distribuyeron aleatoriamente en cuatro grupos experimentales (10 animales/grupo): uno control que recibió el vehículo goma acacia/H_20 y tres tratados con D-002 (25, 125 y 250 mg/kg). El tratamiento oral con dosis únicas y repetidas de D-002 (25-250 mg/kg) no modificó la conducta exploratoria en campo abierto, la respuesta al dolor en el plato caliente, la sujeción y ejecución motora en varilla rotatoria y la retención del aprendizaje en el ensayo de evitación pasiva de una sola prueba.

INTRODUCTION

D-002 is a mixture of high molecular weight aliphatic alcohols (tetracosanol, hexacosanol, octacosanol, triacontanol, dotriacontanol and tetratriacontanol) purified from beeswax, further referred to as beeswax alcohols (BWA). Triacontanol is the most abundant component of D-002.[1]

D-002 has shown antiulcer[2-8] and antioxidant effects[9-12] both in rats and humans as well as anti-inflammatory effects in experimental models.[13]

Experimental toxicology has shown that D-002 is safe. There are no evidences of potential neurotoxicity according to daily observations and post-mortem examinations.[14-18]

Nevertheless, adverse side effects related with the Nervous System are among the most frequent treatment-related adverse effects.[19,20] No neurotoxicity has been detected in D-002 in experimental toxicology and, due to its chemical nature, it is not supposed to produce neurotoxic effects. However, it is advisable to

explore its effects on classical behavioural patterns in rodents, frequently used as part of the battery of tests used to determine the potential neurotoxicity of new substances, like D-002.[19,20]

Historically, classical toxicological studies, including final anatomo-histological analyses have been used to assess gross evidence of neurotoxicity. Usually, those studies do not include formal assessment of the impact of the treatments on quantifiable behaviours. This explains the increasing interest at the scientific and regulatory level in the use of specific animal behavioural methods for evaluating neurotoxicity as the best approach for fully characterizing the range of effects of a given compound. This approach prove invaluable in predicting effects that might be expected to occur in human populations exposed to specific neurotoxicants.[21]

Therefore, this study investigated the effects of single and repeat doses of D-002 on behavioural patterns in mice.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (25 - 30 g) from the Cuban Veterinary Centre (CUBAVET, Havana City, Cuba) were adapted for 15 d to lab conditions [(25 ± 2) °C; (65 ± 5) % relative humidity, 12 h light/dark cycles]. Mice had free access to tap water and to standard rodent chow, acquired in the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba).

The study was performed according to Cuban recommendations for Using Laboratory Animals and the Cuban Code of Good Laboratory Practices.

Quality specifications of the batch of D-002 used (Chemistry Department, Centre of Natural Products, Havana City) were verified before preparation of solutions. For dosing, D-002 was suspended in acacia gum/H_2O vehicle (10 mg/ml). After corroborating stability, suspensions were prepared weekly.

Administration and dosage

Two experiments were conducted to assess the effects of single and repeated doses of D-002 on each behavioural pattern. Mice were randomised into four groups of 10 mice each: a control group treated with the vehicle and three other groups treated with D-002 at 25, 125 and 250 mg/kg, respectively. This is the dose range in which D-002 has demonstrated anti-inflammatory,[13] antiulcer,[2-6] and antioxidant[9,10] effects in experimental studies.

Treatments were given orally, using gastric gavage (1 mL/200 g). Single doses were given two hours prior to the tests, while repeated doses were administered for 30 d . In the repeated dose study, the last dose was given about 24 h prior to the test, in order to discard that any effect could be attributable to the last dose given in the day of the experiment.

Experimental procedures

Mice were deprived of food 12 h prior to the experiment, while the access to tap water remained *ad libitum*. Experiments were conducted always in the morning (from 8:30-12:30 a.m.) in a lab room dedicated only to perform behavioural tests.

Exploratory activity in open field

This test was conducted according to the modification of this test reported by Fernandez *et al*.[22] Mice were introduced in a plastic round device (15 cm x 30 cm) with a central circle (10 cm diameter). The number of mice rears (R) and the number of times they cross (C), through the central circle were counted. Observation was performed during six minutes counted from the moment of placing the mice into the device. The global exploratory activity was estimated as the sum of R + C.

Hot-plate test

This test was conducted according to Holzer *et al*.[23] Mice were placed in a hot-test device in which heat [a temperature controlled at (57 ± 2) °C] was the painful stimulus. Then, the elapsed times to the first responses (latencies) of the animals (paw licks or mice jumps) to the painful stimulus were recorded. The duration of the assay was 60 s counted from the moment of placing the mice on the device. If no response is observed in this time interval, response should be recorded as absent, and time as 60 s.

Modified rotating rod test (horizontal screen test)

The test was conducted as described by Coughenour *et al*.[24] Mice were placed in the top of six square-wire-screens (13 cm x 13 cm) mounted horizontally to a metal single rotating rod (130 cm x 1.5 cm) cm, through a fix perpendicular arm of 9 cm x 1,5 cm, so that six mice should be assessed simultaneously. A 180° turn (15 r/min) was performed. The numbers of mice fallen and of those that fail to reach the opposite side of the grid were quantified. All mice were tested two days before the beginning of the treatment (single or repeated doses) to discard any animal own failure to perform the test, as recommended.

Passive avoidance single trial test

This test consist in training the rodent to avoid an aversion stimulus presented as a punishment (electric shock) and afterwards the retention of such learning is assessed through the avoidance of such stimulus. In the single dose experiment treatments were given two hours prior to the first session of this assay, while in the repeated dose experiment the first session was held at the day 30th on treatment and the second session 24 hours after (so that mice were not treated in this second day of the test).

Mice were introduced in the front chamber of a two chamber device. The first chamber is a wide and illuminated compartment, which communicates to a second small and dark compartment. The small chamber has an electrified grid at the bottom and a guillotine-like door (Bartus 1982)[25]

The animal normally tends to enter into the dark compartment within one minute after being placed into the illuminated chamber. Once it enters into this dark and small compartment, the guillotine door is closed and a mild electric shock (0.5 mA for three seconds) is applied immediately, then concluding the first session. Twenty-four (24) hours later, the same experience is repeated. The number of animals that remain in the first illuminated compartments is recorded, since it represents the number of animals that retain what they learned (learning retention)

Statistical analysis

Statistical comparisons of continuous variables (exploratory activity and hot plate test) were done with the Mann Whitney U test, while comparisons of categorical variables (rotating rod and single trial passive avoidance test) were done with the Fishers

Exact Probability test, statistical significance taken at $p < 0.05$. Data were processed using the statistical software Statistics for Windows.

RESULTS

Table 1 shows the effect of oral treatment with D-002 on mice exploratory activity. Single or repeated doses (25-250 mg/kg) of D-002 administered orally did not significantly change the number of R, C or the global exploratory activity compared to the controls.

Similarly, orally administered D-002 at the doses and schemes tested did not modify the latency of the mice responses to the painful stimulus in the hot plate, since values of treated and control groups were statistically similar (Table 2).

Table 3 summarizes results of mice performance on the horizontal screening rotating rod test. No mice fall from the grids. On the other hand, the number of mice able to pass to the opposite side of their respective grids was similar in all groups. Obviously, no differences between control and treated groups were found.

Table 4 presents data of the passive avoidance single trial test. No significant differences were observed after treating the mice with single or repeated doses. Learning retention was similar in all groups.

DISCUSSION

This study demonstrates that oral treatment with D-002 (25-250 mg/kg) using single or repeat doses does not affect mice behavioural patterns assessed in this study.

Toxicological studies of D-002 has failed to demonstrate any D-002-related toxicity, even when it was administered at doses up to 625 mg/kg sub-chronically to rats and mice and for1 000 mg/kg to rats for 1 year.[14-18] Therefore, the present results were to be expected.

Classical toxicological studies can detect changes in nervous system structures at post-mortem examinations, and also usually include a battery of daily observations

Table 1. Effects of single and repeated doses of D-002 on mice exploratory behaviour.

Treatment	Dose (mg/kg)	Rears (R)	Crosses (C)	Total activity (R+C)
		Single dosing		
Control	0	32 ± 13	13 ± 6	46 ± 15
D-002	25	36 ± 10	16 ± 7	53 ± 14
D-002	125	36 ± 8	14 ± 4	50 ± 8
D-002	250	34 ± 18	17 ± 4	50 ± 10
		Repeated dosing		
Control	0	29 ± 12	14 ± 8	43 ± 13
D-002	25	35 ± 10	17 ± 6	51 ± 13
D-002	125	29 ± 15	17 ± 9	45 ± 23
D-002	250	29 ± 11	16 ± 8	45 ± 13

Data expressed as mean ± SD. R number of rears, C number of times mice crossed the central circle of the experimental device. All comparisons were not significant (Mann Whitney U test).

Table 2. Effects of single and repeated doses of D-002 on mice response in the hot plate test

Treatment	Dose (mg/kg)	Latency of the response (seconds)[1]
	Single dosing	
Control	0	6 ± 3
D-002	25	4 ± 1
D-002	125	4 ± 1
D-002	250	5 ± 3
	Repeated dosing	
Control	0	5 ± 2
D-002	25	5 ± 3
D-002	125	5 ± 2
D-002	250	5 ± 2

Data expressed as mean ± SD. [1]Time (in seconds) at which mice had the first response (paw lick or mice jump) to the painful stimulus. All comparisons were not significant (Mann Whitney U test).

Table 3. Effects of single and repeated doses of D-002 on the horizontal screening rotating rod test.

Treatment	Dose (mg/kg)	Fallen mice	Mice unable to pass[1]
		Single dosing	
Control	0	0/10	1/10
D-002	25	0/10	0/10
D-002	125	0/10	1/10
D-002	250	0/10	1/10
		Repeated dosing	
Control	0	0/10	1/10
D-002	25	0/10	0/10
D-002	125	0/10	1/10
D-002	250	0/10	1/10

Data expressed as number of mice/groups. [1]To the other side of the grids. No mice fall from the grid. All comparisons were not significant (Fisher Exact Probability test).

Table 4. Effects of single and repeated doses of D-002 on passive avoidance single trial test.

Treatment	Dose (mg/kg)	Learning retention (n, %)
	Single dosing	
Control	0	9/10 (90 %)
D-002	25	10/10 (100 %)
D-002	125	8/10 (80 %)
D-002	250	8/10 (80 %)
	Repeated dosing	
Control	0	8/10 (80 %)
D-002	25	8/10 (80 %)
D-002	125	8/10 (80 %)
D-002	250	8/10 (80 %)

Data expressed as number of mice/groups. All comparisons were not significant (Fisher Exact Probability test).

that cover the overall animal behaviour that can be assessed qualitatively or semi-quantitatively. Therefore, subtle treatment-induced changes on these parameters could be ignored in the formal toxicity tests, being recommended as a part of the special safety pharmacology.[26]

The tests included in this study cover an acceptable range of behavioural patterns that, in case that the treatment can affect the Central Nervous System (CNS), the results of such tests should reflect it some how.

Open field exploratory activity test is widely used as screening method to assess the effects of substances on the CNS. It reflects the spontaneous activity of the animal in a strange environment, and provides information of disturbances on both exploratory and motor activity of the animals.[19,20,26,27] The number of rears is more explicitly linked to the exploratory activity while the number of crosses is a more direct expression of the motor activity. The total activity (R + C) involves the behavior as a whole, then, the fact that single or repeated oral dosage with D-002 did not affect any component of this behavior means that the product has no meaningful neurotoxic effect.

On the other hand, the response to the hot plate involves sensorial and motor components, as well the overall integrity of both components, so that the absence of effect of D-002 on this parameter indicates that it does not affect the sensitive, motor and central events involved in this response. This highlight that it is devoid of analgesic and neurotoxic effects, as well.[19,20,23,27]

The absence of effects of D-002 on the horizontal screening rotating rod test supports that the treatment did not affect all the events involved in the motor coordination and performance involved in this complex behavior, easily affected by treatments acting on the CNS with anxiolytic, hypnotic-sedatives and/or muscle-relaxant effects.[19,20,24,26,27]

Finally, the passive avoidance single trial test assesses whether a treatment can impair the short-term memory,

and if the changes are related to learning (training day) or to learning retention or memory (second day), which can be also impaired with some treatments.[19,20,25,27]

Therefore, the fact that D-002 given at doses from 25 to 250 mg/kg, a range in which it has produced anti-inflammatory, antiulcer and antioxidant effects in rodents,[2-6,9,10,13] did not affect the assessed behavioral patterns in mice, discards the negative results are due to inadequate substance exposure and well supports that it has not potential neurotoxicity.

CONCLUSIONS

This study demonstrates that single or repeated doses (25-250 mg/kg) of D-002 orally given for 30 d do not affect behavioural patterns (exploratory activity in open field, response in the hot plate test, performance on the horizontal screening rotating rod and learning retention in the passive avoidance single trial tests) in mice.

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