# Oral toxicological studies of D-002 in mice

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**RESUMEN.** El D-002 es una mezcla de alcoholes alifáticos primarios de alto peso molecular aislada y purificada a partir de la cera de las abejas (*Apis mellifera*) a la que ha sido demostrada moderada actividad antiinflamatoria y efectiva actividad antiulcerosa. En este trabajo se investigó su posible toxicidad aguda y subcrónica (60 d de tratamiento) oral en ratones. En el ensayo agudo, se administraron dosis únicas de 500, 1 500, 2 500 y 5 000 mg/kg y en el subcrónico, dosis repetidas de 25, 125 y 625 mg/kg. En ninguno de los estudios se observó mortalidad, ni signos tóxicos atribuibles al tratamiento. Tampoco, fueron observados cambios significativos en ninguna de las variables analizadas (peso corporal, porcentaje del peso de los órganos y lesiones histopatológicas). En el estudio subcrónico fueron investigados los efectos de la droga en el proceso de espermatogénesis y no se observaron cambios significativos entre los grupos. Estos resultados permitieron afirmar que la administración oral del D-002 resulta muy segura en estas condiciones experimentales.

**ABSTRACT.** D-002 is a natural mixture of higher aliphatic primary alcohols isolated and purified from beeswax (*Apis mellifera*), wherein triacontanol is one of its main components. D-002 (5-100 mg/kg) orally administered shows moderate antiinflammatory and effective antiulcer activity in different experimental models. The present study shows the results of oral acute and subchronic (60 d) administration of D-002 to Swiss mice. In the acute study five groups were included: a control and four groups treated with D-002 at 500, 1 500, 2 500 and 5 000 mg/kg, meanwhile, in the subchronic study a control and three treated groups with 25, 125 and 625 mg/kg were included. Neither mortality nor toxic symptoms were observed. No significant changes on body weight, organ weight percent and histopathological findings were observed. The effects of the subchronic administration of D-002 on mouse spermatogenesis were, also, investigated and no significant differences between groups were observed. The acute and the subchronic studies did not show evidences of any drug-related toxicity. It is concluded that D-002 is a safe drug under these experimental conditions.

## INTRODUCTION

D-002 is a defined mixture of higher aliphatic primary alcohols isolated from beeswax (*Apis mellifera*) which contains triacontanol, followed by octacosanol, dotriacontrianol, hexacosanol and tetracosanol, meanwhile tetratriacontanol is a minor component. Preclinical pharmacology showed D-002 administered orally induces mild antiinflammatory and effective antiulcer activity in different experimental models.[1-3] Previous toxicological studies have shown that oral acute, subacute and subchronic toxicity of D-002 in rats is practically absent.[4,5]

It is accepted that mouse is the second rodent specie in preclinical toxicological evaluations. Then, the present work was aimed to investigate the $LD_{50}$ and the subchronic administration of D-002 to Swiss mice of both sexes.

In addition, the effects of the subchronic administration of D-002 on mouse spermatogenesis were also investigated.

## MATERIALS AND METHODS

### Animals

Young adults Swiss mice of both sexes, weighing 18-20 g were obtained from the Centro de Productos Veterinarios, Cuba and adapted to laboratory conditions for a week. Animals were kept in cages with free access to water and food *ad libitum*. The cages were kept in controlled experimental conditions, (25 ± 2)°C, humidity 55-60 % and 12 h light/dark cycles.

### Administration and dosage

Mice were distributed randomly in experimental groups of 8 and 10 animals by sex by dose for the acute and the subchronic assays respectively.

The product was supplied by Dalmer Laboratories, Havana, Cuba. The used batch (021093) fulfilled the quality criteria specifications checked by gas chromatography.[6] D-002 dose was adjusted for weight gain. The suspensions were orally administered by gastric garage (10 mL/kg) between 9:30-11:30 a.m.[7] Control group received a similar volume of Acacia gum-water. This vehicle was used according to recommendations for non water soluble products.[8]

The following doses were selected for the acute study: 500, 1 500, 2 500 and 5 000 mg/kg. Taking into account the results of the acute study and the pharmacological screening, the doses selected for the subchronic study were 25, 125 and 625 mg/(kg · d). A control group only receiving the equivalent volume of vehicle was included in both stud-

ies. To achieve the desired dose, in the acute study the animals received two successive administration in a 60 min interval. In the subchronic study the treatment was administered 6 d a week for 60 d .

**Clinical and behaviour examinations**

All animals were daily observed to monitor clinical signs of toxicity during 14 and 60 d in acute and subchronic studies, respectively. Daily observations included scrutiny for changes in skin, eyes, mucus membranes, orifices and clinical signs of the respiratory, circulatory, autonomic and central nervous systems, somatomotor activities and behavioural changes. In both studies the body weight was recorded on the day the treatment started and on the day of the sacrifice. In addition, in the subchronic study the body weight was also recorded weekly and the food consumption was measured during all the treatment.

**Histophatological examinations**

At the end of the studies, all animals were sacrificed. At autopsy, abdominal, thoracic and cranial cavities were examined, the organ weight were measured and organ weight to body weight ratio X100 was calculated.

Samples from all organs and tissues were taken according to Chhrabra *et al.*[9] Tissue samples were fixed in 10 % buffered formalin solution for histophatological processing. Paraffin sections were stained by hematoxylin and eosin for examination by light microscopy.

**Sperm count and morphology assay**

The epididymis of male mice from the subchronic assay were taken for analysis of sperm count and morphology.[9,10] Samples were prepared and sperm count was performed in a Newbawer chamber. A proteolytic enzyme, 0.25 % trypsin was added to the sperm suspension in order to destroy connective tissue. Sperm heads were classified as normal, amorphous, banana type and without hook.

**Statistical analysis**

Statistical evaluations of body weight, organ weights percent, sperm count and morphology were made using Kruskal Wallis test (no parametric ANOVA).[11] Histophatological findings were compared using the Fisher's Exact Probability test ($p < 0.05$).

## RESULTS AND DISCUSSION

**Mortality and clinical signs**

No death occurred during the studies and no drug-related gross clinical signs were observed.

**Body weight analysis and food consumption**

Comparisons between treated and control groups demonstrated acute or subchronic administrations of D-002 by oral route did not induce changes on body weight gain in males and females (Table 1 and Figures 1-2). Neither the food consumption changed with the subchronic treatment in any grou.

**Sperm count and morphology**

The sperm count and morphology assays have been incorporated into the subchronic toxicity studies. This approach eliminates the need of additional studies on a number of chemicals, thus limiting the use of animals and reducing costs.[10] No significant differences were obtained in any comparison (Tables 2 and 3). These results demonstrate that D-002 does not induce morphological changes in sperm head, reflecting an absence of direct effect on sperm process.

**Histopathological study**

No significant changes on organ weight were found as result of the effects of acute and subchronic treatment (Tables 4 and 5) . Microscopic observations only showed lesions commonly described in this species (Table 6). No significant differences were obtained in the statistical comparisons.

**Table 1**. Effect of single oral dose of D-002 on body weight.

| Group | Males | | Females | |
|---|---|---|---|---|
| Dose (mg/kg) | $\bar{X}$ ± DS) (g) | | | |
| | Before | After | Before | After |
| Control | 23.2 ± 2.1 | 31.0 ± 2.2 | 20.5 ± 1.8 | 25.9 ± 3.0 |
| 500 | 23.1 ± 1.8 | 30.9 ± 1.6 | 20.1 ± 2.1 | 24.8 ± 4.3 |
| 1 500 | 23.1 ± 2.0 | 30.1 ± 3.1 | 20.2 ± 1.8 | 25.0 ± 2.2 |
| 2 500 | 23.1 ± 1.5 | 29.8 ± 2.0 | 20.5 ± 1.9 | 23.5 ± 2.7 |
| 5 000 | 23.2 ± 1.5 | 30.7 ± 2.5 | 20.4 ± 2.4 | 23.4 ± 2.6 |

Before: day of the starting of the treatment.
After: day of the sacrifice.

**Table 2**. Sperm count after D-002 subchronic treatment.

| Dose (mg/kg) | Concentration* ($\bar{X}$ ± DS) |
|---|---|
| Control | 25.88 ± 10.40 |
| 25 | 15.75 ± 9.78 |
| 125 | 25.65 ± 10.99 |
| 625 | 20.07 ± 11.21 |

* Sperm $10^6$ by epididymis.

**Table 3**. Morphology of sperm head after D-002 subchronic treatment.

| Dose | Amorphous | Bananas | Whithout hook | Anormals |
|---|---|---|---|---|
| (mg/kg) | $\bar{X}$ ± DS) | | | |
| Control | 1.74 ± 0.55 | 0.42 ± 0.16 | 1.07 ± 1.20 | 8.24 ± 1.38 |
| 25 | 1.17 ± 0.47 | 0.32 ± 0.18 | 0.67 ± 0.53 | 2.17 ± 0.82 |
| 125 | 1.61 ± 1.14 | 0.35 ± 0.21 | 1.09 ± 1.18 | 3.05 ± 2.21 |
| 625 | 1.59 ± 0.66 | 0.36 ± 0.22 | 1.00 ± 0.75 | 2.95 ± 1.01 |

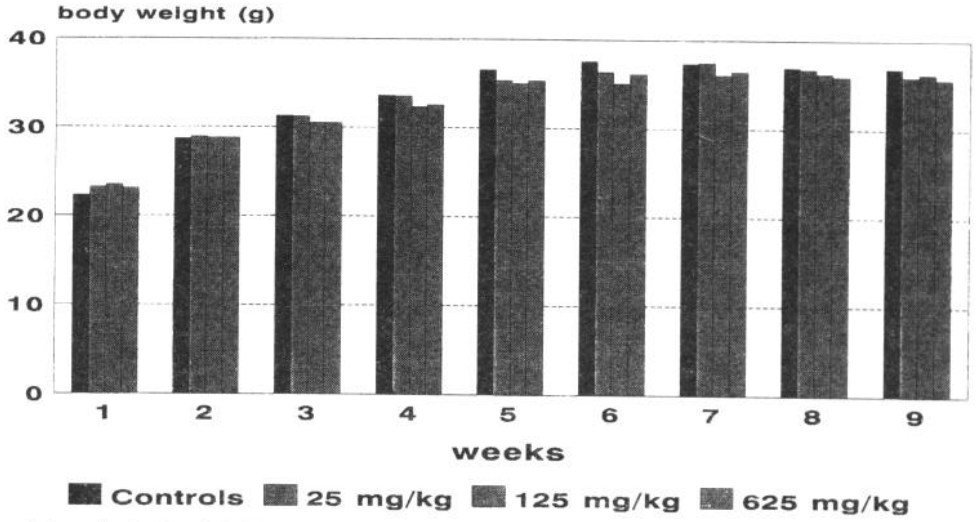


*Fig. 1. Body weight gain during D-002 60 d treatment in male mice.*

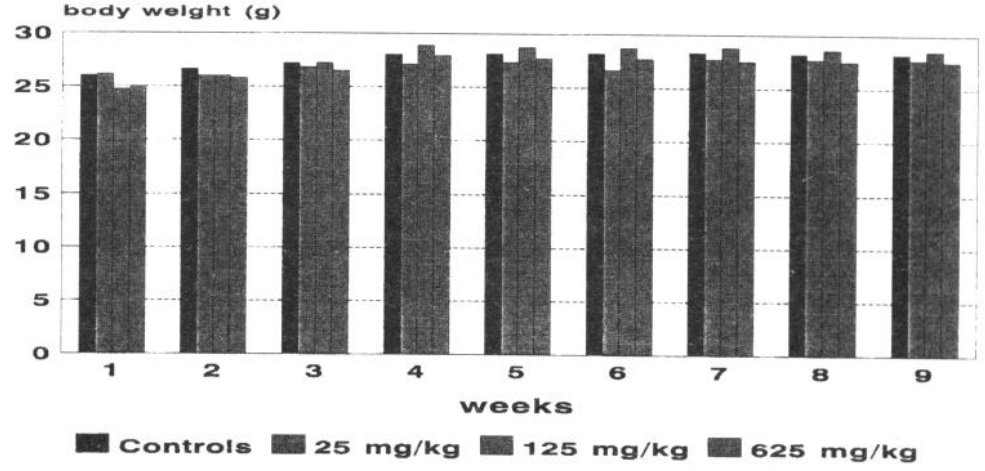


*Fig. 2. Body weight gain during D-002 60 d treatment in female mice.*

**Table 4.** Effect of single oral dose of D-002 on organ weight percent.

| Dose | Liver | Kidneys | | Heart | Spleen | Lungs | Thymus |
|---|---|---|---|---|---|---|---|
| | | Right | Left | | | | |
| (mg/kg) | | | | ($\bar{X}$ ± DS) (%) | | | |
| | | | | Males | | | |
| Control | 6.63 ± 0.5 | 0.76 ± 0.11 | 0.76 ± 0.11 | 0.53 ± 0.08 | 0.75 ± 0.20 | 0.85 ± 0.14 | 0.27± 0.06 |
| 500 | 6.00 ± 0.3 | 0.76 ± 0.06 | 0.72 ± 0.04 | 0.54 ± 0.06 | 0.71 ± 0.16 | 0.88 ± 0.23 | 0.23± 0.06 |
| 1 500 | 6.41 ± 1.1 | 0.79 ± 0.06 | 0.80 ± 0.09 | 0.59 ± 0.08 | 0.75 ± 0.14 | 0.93 ± 0.23 | 0.26 ± 0.09 |
| 2 500 | 6.54 ± 0.4 | 0.81 ± 0.13 | 0.80 ± 0.11 | 0.58 ± 0.06 | 0.68 ± 0.21 | 0.83 ± 0.09 | 0.22 ± 0.06 |
| 5 000 | 6.48 ± 0.6 | 0.80 ± 0.09 | 0.80 ± 0.09 | 0.53 ± 0.05 | 0.74 ± 0.10 | 0.83 ± 0.06 | 0.26 ± 0.09 |
| | | | | Females | | | |
| Control | 6.17 ± 0.6 | 0.72 ± 0.05 | 0.68 ± 0.06 | 0.56 ± 0.06 | 0.78 ± 0.13 | 0.90 ± 0.19 | 0.38 ± 0.04 |
| 500 | 6.03 ± 0.3 | 0.76 ± 0.07 | 0.72 ± 0.06 | 0.60 ± 0.11 | 0.71 ± 0.11 | 0.90 ± 0.09 | 0.33 ± 0.11 |
| 1 500 | 6.40 ± 0.7 | 0.74 ± 0.06 | 0.73 ± 0.06 | 0.63 ± 0.11 | 0.71 ±0.11 | 0.94 ± 0.09 | 0.39 ± 0.10 |
| 2 500 | 6.28 ± 0.7 | 0.75 ± 0.08 | 0.74 ± 0.06 | 0.65 ± 0.15 | 0.91 ± 0.42 | 0.97 ± 0.06 | 0.34 ± 0.06 |
| 5 000 | 6.62 ± 1.2 | 0.73 ± 0.08 | 0.68 ± 0.07 | 0.51 ± 0.07 | 0.84 ± 0.43 | 0.91 ± 0.03 | 0.35 ± 0.08 |

Data are mean values for each group ± SD of organ weight to body weight ratio X100.

**Table 5.** Effect of subchronic treatment of D-002 on organ weight percent.

| Dose (mg/kg) | Liver | Kidneys Right | Kidneys Left | Heart | Spleen | Lungs | Thymus |
|---|---|---|---|---|---|---|---|
| | | | | (X̄ ± DS) (%) | | | |
| | | | | Males | | | |
| Control | 4.68 ± 0.47 | 0.80 ± 0.08 | 0.81 ± 0.07 | 0.50 ± 0.07 | 0.69 ± 0.07 | 0.45 ± 0.11 | 0.10 ± 0.03 |
| 25 | 4.91 ± 0.59 | 0.83 ± 0.11 | 0.81 ± 0.06 | 0.48 ± 0.11 | 0.69 ± 0.10 | 0.41 ± 0.12 | 0.11 ± 0.05 |
| 125 | 5.02 ± 0.66 | 0.77 ± 0.09 | 0.78 ± 0.09 | 0.45 ± 0.03 | 0.62 ± 0.08 | 0.39 ± 0.12 | 0.10 ± 0.03 |
| 625 | 4.95 ± 0.79 | 0.81 ± 0.13 | 0.80 ± 0.11 | 0.58 ± 0.06 | 0.72 ± 0.12 | 0.51 ± 0.19 | 0.09 ± 0.03 |
| | | | | Females | | | |
| Control | 5.30 ± 1.00 | 0.66 ± 0.09 | 0.64 ± 0.08 | 0.48 ± 0.06 | 0.81 ± 0.07 | 0.56 ± 0.14 | 0.23 ± 0.05 |
| 25 | 4.81 ± 0.85 | 0.64 ± 0.10 | 0.63 ± 0.07 | 0.49 ± 0.05 | 0.83 ± 0.11 | 0.60 ± 0.18 | 0.21 ± 0.07 |
| 125 | 5.01 ± 0.70 | 0.62 ± 0.07 | 0.59 ± 0.05 | 0.50 ± 0.05 | 0.78 ± 0.08 | 0.54 ± 0.12 | 0.21 ± 0.05 |
| 625 | 5.09 ± 0.53 | 0.60 ± 0.04 | 0.58 ± 0.05 | 0.50 ± 0.05 | 0.86 ± 0.08 | 0.64 ± 0.15 | 0.27 ± 0.05 |

**Table 6.** Histopathological findings in D-002 oral acute and subchronic assays.

| Study | Sex | Dose (mg/kg) | Observation | N |
|---|---|---|---|---|
| Acute | Male | 5 000 | Liver: vacuolar degeneration. | 2/8 |
| | Female | Control | Liver: inflammatory infiltrate | 1/8 |
| | Female | 5 000 | Intestine: haemorragic necrosis of mucosa. | 1/8 |
| | Female | 5 000 | Liver: focus of necrosis. | 1/8 |
| Subchronic | Male | Control | Kidney: inflammatory infiltrate. | 3/10 |
| | Male | 625 | Liver: inflammatory infiltrate | 1/10 |
| | Female | 625 | Liver: inflammatory infiltrate. | 1/10 |

Since these lesions are commonly described in mice,[12] no significant differences with the control were observed and no tendency with the dose increase was appreciated, it was conclude that such lesions are not D-002 treatment related.

These results agree with those of previous oral toxicological assays of this product conducted in rats (acute, subacute, subchronic and chronic studies).[4,5] In these studies was demonstrated D-002 has a broad margin of safety in these experimental conditions. The results observed in these acute and subchronic studies realized in mice, a second rodent specie, confirm it.

## CONCLUSIONS

The results showed that $LD_{50}$ of D-002 in mice was higher than 5 g/kg . On the other hand, D-002 orally administered for 60 d up to 625 mg/kg did not induce any drug-related toxicity.

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