

# High doses of D-003, an antiplatelet drug purified from sugar cane wax, on bleeding time and coagulation indexes in rats

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Palabras clave: D-003, hipocolesterolémico, antiagregante plaquetario, tiempo de sangrado, indicadores de la coagulación.  
Key words: D-003, cholesterol-lowering effects, antiplatelet effects, bleeding time, coagulation markers.

**RESUMEN.** El D-003 es una nueva droga en desarrollo, la cual, consiste en una mezcla de ácidos alifáticos superiores provenientes de la cera de la caña de azúcar (*Saccharum officinarum* L.). Estudios realizados en roedores han demostrado que la administración oral de D-003 inhibe la agregación plaquetaria al colágeno y al ADP e incrementa el tiempo de sangrado en ratas Sprague Dawley. El trabajo tuvo como objetivo demostrar si el incremento del tiempo de sangrado inducido por la administración de D-003 se debe a una acción sobre el proceso de coagulación. Las ratas fueron aleatoriamente distribuidas en cuatro grupos experimentales (8 animales/grupo): un grupo control que recibió el vehículo goma acacia-agua, dos grupos tratados con D-003 (1 000 y 2 000 mg/kg, respectivamente) y otro tratado con warfarina como control positivo. Finalizado el período de tratamiento, los animales fueron anestesiados y se cuantificó el tiempo de sangrado por incisión del extremo terminal de la cola, posteriormente, los animales fueron sacrificados y 3 mL de sangre fueron colectados para la posterior determinación del tiempo de protrombina y el tiempo parcial de tromboplastina activado con kaolín. El D-003 (1 000 y 2 000 mg/kg) incrementó significativamente el tiempo de sangrado, pero no modificó los indicadores de la coagulación estudiados. Los tiempos de protrombina y el parcial de tromboplastina activado con kaolín fueron similares en los grupos tratados con D-003 y el control de vehículo, a diferencia de lo observado en los animales que se les administró warfarina, donde el incremento de estos indicadores fue significativo. El D-003 no afecta los indicadores de la coagulación estudiados, por lo que el incremento del tiempo de sangrado inducido por el D-003 no está relacionado con su acción sobre el proceso de coagulación.

**ABSTRACT.** D-003 is a mixture of very long-chain aliphatic acids with cholesterol-lowering and concomitant antiplatelet effects. Studies conducted in rodents evidenced that D-003 orally administered inhibited collagen and ADP-induced platelet aggregation and increased bleeding time (BT) in Sprague Dawley rats. Elucidate whether the increase of BT induced with high doses of D-003 is related to coagulations indexes. Rats were randomly distributed into four groups (eight animal/group). A control group received only the acacia gum-water vehicle, two groups orally treated with D-003 (1 000 and 2 000 mg/kg, respectively) and a group treated with warfarin as positive control. At treatment completion, rats were anesthetized and BT was measured after making a standardized incision in the tail, after that, animals were exsanguinated through section of the inferior *vena cava*. Whole blood were collected and the coagulation markers here investigated were prothrombin time (PT) and kaolin-activate partial thromboplastin time (KPTT). D-003 (1 000 and 2 000 mg/kg) significantly increased BT values, but did not affect the coagulation indexes here studied. Thus, PT and KPTT values in D-003 treated groups were similar to those of the controls, while warfarin significantly

increased both indexes. D-003 did not affect coagulation indexes here assessed. Hence, increase on BT induced with high doses of D-003 is not related to impairment of coagulation process, but probably to the inhibition of platelet aggregation.

## INTRODUCTION

D-003 is a drug under experimental research consisting of a mixture of high molecular weight primary aliphatic acids isolated and purified from sugarcane (*Saccharum officinarum*, L.) wax.<sup>1</sup> The main component of D-003 is 1-octacosanoic acid, followed by 1-triacontanoic, 1-dotriacontanoic and 1-tetracontanoic acids. Also, 1-tetracosanoic, 1-pentanoic, 1-hexatriacontanoic, 1-hexacosanoic, 1-nonacosanoic, 1-tritriacontanoic, 1-hentriacontanoic and 1-pentatriacontanoic acids are present as minor components.<sup>1</sup>

D-003 presents antiplatelet,<sup>2-5</sup> antithrombotic<sup>5-7</sup> and cholesterol-lowering effects<sup>3,8-13</sup> proven in experimental models and healthy volunteers, which could be useful for manage atherosclerosis and its thromboembolic complications.

Experimental studies conducted in rabbits showed that D-003 orally administered (5 to 200 mg/kg) to rabbits for 30 d significantly reduced serum total cholesterol (TC) and low density lipoprotein-cholesterol

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(LDL-C) levels, whereas increased high density lipoprotein-cholesterol (HDL-C) values.<sup>9-12</sup> Also, comparative studies of the effects of D-003 and other cholesterol-lowering drugs, like policosanol,<sup>10</sup> lovastatin<sup>11</sup> and fluvastatin<sup>12</sup> in rabbits show that D-003 was more effective for lowering LDL-C than policosanol, but similar to both statins. Nevertheless, D-003 increased serum HDL-C levels more effectively than all comparison drugs. D-003 (5 to 50 mg/d) has also shown cholesterol-lowering effects in healthy volunteers and patients with Type II hypercholesterolemia, its effects being characterised by reduction of serum LDL-C and TC levels, and markedly raises of HDL-C.<sup>3,13</sup>

On the other hand, D-003 orally administered also induces anti-platelet effects in animal models and humans,<sup>2,6</sup> an effect that appears to be associated to the reduction of thromboxane A<sub>2</sub> (TxA<sub>2</sub>) and increasing prostacyclin (Pgl<sub>2</sub>) serum levels.<sup>6,7</sup>

Studies conducted in rodents evidenced that D-003 orally administered (25 to 200 mg/kg) inhibited collagen and ADP-induced platelet aggregation, while from 50 to 200 mg/kg significantly and dose dependently increased bleeding time (BT) in Sprague Dawley rats.<sup>2</sup>

The increase on BT induced with D-003 could be related to the inhibition of platelet aggregation, but haemostatic mechanism includes other physiological premises, like blood coagulation factors. It is generally accepted that prothrombin time (PT) and kaolin-activate partial thromboplastin time (KPTT) are the best available screening tests for evaluating any effect on the extrinsic and intrinsic coagulation system, respectively.<sup>14,15</sup>

For instance, blood coagulation disturbances can be induced with oral anticoagulant drugs, like warfarin, which is commonly used as positive control for assessing anti-thrombotic drugs acting by reducing vitamin K dependent blood coagulation factors.<sup>16</sup>

Previous studies of the oral toxicity of repeated doses of D-003 have shown that doses up to 1 000 or 1 500 mg/kg administered for 9 and 3 months, respectively, did not impair platelet count or induce signs of drug-related toxicity.<sup>17-19</sup>

Although the relevance of the BT increase observed in such studies was minimised, the possibility

of some adaptation process induced after long-term treatment was not discarded. Hence the aim of this work was to elucidate whether the increase of BT induced with high doses of D-003 administered for a time as short as prevent appearance of adaptation mechanisms, causes individual values above normal range and if such effect involves changes on major coagulation markers.

## MATERIALS AND METHODS

### Animals

Male Sprague Dawley rats weighing 210 to 230 g were purchased from the National Center for Laboratory Animal Production (CENPALAB, Havana City, Cuba). Animals were adapted to laboratory conditions for 7 d being randomly distributed among experimental groups, housed three per cage in a room with controlled conditions: temperature (25 ± 2) °C, relative humidity (RH) (65 ± 4) % and 12 h-light 12 h-dark cycles. Food (standard rodent chow manufactured in CENPALAB) and water were provided *ad libitum* during the whole study.

Animal handling was conducted according to the ethical principles for animal care and management recommended in Cuban Guidelines and Standard Operational Procedures.

### Drugs and treatments

D-003 was supplied by the Chemistry Department of the Center of Natural Products and its purity was checked by gas chromatography. Taking into account that D-003 is highly insoluble in water, it was suspended in acacia gum-water vehicle (10 mg/mL).

Warfarin was purchased from the Cuban Medical Pharmaceutical Industry (IMEFA) and dissolved in 0.9 % isotonic NaCl solution.

Rats were randomly distributed in four groups (eight animal/groups): a control group receiving only the acacia gum/water vehicle, two groups orally treated with D-003 (1 000 and 2 000 mg/kg, respectively) and a group treated with warfarin as positive control.

The rationale for selecting doses was to choose low doses with pharmacological activity and highest doses near to those ranges considered as upper cut-off in studies of oral toxicity after repeated doses.<sup>17-20</sup> Treatment duration was selected as 14 d considering that half-life of platelets is between 7 to 10 d.

Vehicle and D-003 treatments were administered through gastric gavage (1 mL/kg) for 14 d. Warfarin was administered by intraperitoneal route (i.p.) (0.25 mg/kg) for the last three days of this period.

### Observations and determinations

Each animal was observed daily in the morning between 8:30 to 12:30 for detection of behavioral changes or evidence of subtle blood loss. Body weight gain was recorded weekly. Furthermore, during the autopsies, cavities and organs were observed carefully. Animals received no food for 12 h prior to the experiments.

### Bleeding time (BT) assessment

At treatment completion, rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.). BT was measured after making a standardized incision in the tail according to the method of Dejana *et al.* (1982).<sup>21</sup> In brief, animal tail was immersed in an isotonic (0.9 %) saline solution (37 °C) for 5 min and immediately cut off at 4 mm from the distal end. BT was recorded in seconds.

### Assessment of coagulation markers

**Prothrombin time and kaolin-activate partial thromboplastin time.** For blood sampling and after assessing BT, animals were anaesthetized and exsanguinated through section of the inferior *vena cava*. Three (3) mL of whole blood were collected into plastic tubes containing 0.3 mL of sodium citrate solution (3.8 %) and centrifuged at 1 000 X g for 10 min at 25 °C to prepare platelet-poor plasma (PPP).

The coagulation markers here researched were PT and KPTT, which were measured by routine methods.<sup>22,23</sup> Briefly, acetone extracted rabbit brain thromboplastin, obtained from IMEFA was used as thromboplastin and to prepare partial thromboplastin. A glass tube containing 0.1 mL of thromboplastin and 0.1 mL of 0.25 mol/L Cl<sub>2</sub>Ca (BDH) solution was warmed to 37 °C in a water bath for 2 min followed by the addition of 0.1 mL of PPP, the time taken for a fibrin clot to develop was viewed as PT. In a similar way, 0.1 mL of PPP added to 0.1 mL mixture imidazol-kaolin (Fluka, Chemie) suspension in a glass tube was maintained at 37 °C for 4 min and 0.1 mL of a 0.025 mol/L Cl<sub>2</sub>Ca solution were added. The time taken for a clot to form was considered KPTT. Both determinations PT

and KPTT were recorded in minutes.

### Statistical analyses

Data are expressed as mean  $\pm$  standard deviation. Two tailed Mann Whitney U test was used for comparisons between groups. Statistical significance was *a priori* established for  $\alpha = 0.05$ . Statistical analyses were performed with the Statistics software for Windows.

## RESULTS AND DISCUSSION

No deaths and no clinical symptoms of toxicity occurred during the study. Also, body weight gain was similar in D-003-treated and control groups, indicating that D-003 was well tolerated by the animals (Table 1). It must be noted that no bleeding experience was observed during daily observations.

Autopsy revealed a normal appearance of organs and cavities of D-003 treated animals and vehicle control. Otherwise, all warfarin-treated rats showed hemorrhagic zones in the stomach, intestine and liver.

D-003 (1 000 and 2 000 mg/kg) significantly increased BT values, but the effect induced with both doses was the same (Table 2). The increase on BT here shown is consistent with the results reported by Molina *et al.*,<sup>2</sup> who found maximal effects induced with D-003 orally administered on BT were achieved with 25 mg/kg.

The increase on BT could be potentially toxic, since it can lead to hemorrhage manifestations in organs and cavities. Thus, hemorrhage episodes are clinical signs occasionally seen in toxicity studies. Hemostasis process results of a complex and dynamic interplay of two important events: platelet activity and coagulation process.<sup>15</sup> Therefore, the increase of BT can reflect an imbalance of at least one of these events.

Nevertheless, the results allow to classify the increase on BT induced with D-003 as moderate, since doses above 25 mg/kg orally administered as repeated doses do not induce higher increases of such index. In addition, BT individual values of D-003-treated animals remained within normal range of such indicator in the rat.<sup>21</sup> By contrast, the group treated with warfarin showed the expected increase in BT, values resulting significantly higher than control and D-003 groups.

**Table 1.** Mean body weight during the study.

Group	Doses (mg/kg)	Body weight $\pm$ S.D. (g)		
		Initial	7 d	14 d
Control	0	216.7 $\pm$ 12.4	243.7 $\pm$ 12.8	321.7 $\pm$ 7.9
D-003	1 000	216.2 $\pm$ 12.1	241.5 $\pm$ 13.3	323.0 $\pm$ 27.7
	2 000	217.0 $\pm$ 10.6	243.6 $\pm$ 7.6	329.5 $\pm$ 27.0
Warfarin	0.25	216.0 $\pm$ 9.4	242.6 $\pm$ 10.5	327.2 $\pm$ 16.7

**Table 2.** Effect of D-003 on bleeding time, prothrombin time and kaolin-activate thromboplastin time.

Group	Doses (mg/kg)	Bleeding time	PT	KPTT
		(s)		
Control	0	225 $\pm$ 26 <sup>b</sup>	27 $\pm$ 3	32 $\pm$ 5
D-003	1 000	453 $\pm$ 128 <sup>ab</sup>	28 $\pm$ 5	35 $\pm$ 4
	2 000	480 $\pm$ 111 <sup>ab</sup>	27 $\pm$ 3	32 $\pm$ 4
Warfarin	0.25	812 $\pm$ 141 <sup>a</sup>	63 $\pm$ 7 <sup>a</sup>	128 $\pm$ 37 <sup>a</sup>

a)  $p < 0.001$  - control group. b)  $p < 0.001$  - warfarin group. Mann Witney U Test.

As per artificial convention, the coagulation cascade is classified in intrinsic, extrinsic and common pathways. PT is an indicator commonly used for assessing extrinsic pathway factors, while KPTT is used for the factors of the intrinsic coagulation pathway.<sup>24,25</sup>

D-003 did not affect the coagulation indicators here studied. Thus, PT and KPTT values in D-003 treated groups were similar to those of the controls, while warfarin significantly increased both parameters. Hence, differently from warfarin, which is considered as a multifactorial coagulation inhibitor,<sup>26</sup> the effects of D-003 on BT here reported are not associated with major changes on coagulation process, being probable that depends only of the antiplatelet efficacy of D-003.

Up to date, antiplatelet effects of D-003 have been demonstrated in rodents,<sup>2,4,18</sup> dogs<sup>19</sup> and human healthy volunteers.<sup>3,4</sup> Antiplatelet effects of D-003 are related to a reduction of TxA<sub>2</sub> and concomitant increase of PGI<sub>2</sub> serum levels.<sup>6,7</sup> Moreover, a protective effect on lipid peroxidation induced by D-003 in animals and humans could contribute with its antiplatelet effect.<sup>27,28</sup>

D-003-induced increases on BT have been researched in rodents,<sup>2,4,18</sup> dogs<sup>19</sup> and humans.<sup>3</sup> In particular, a phase I clinical study carried out in healthy volunteers investigated whether D-003 administered from 5 to 50 mg as single or repeated doses for 30 d could increase BT values. Results showed that D-003 increased BT values only at the high-

est dose researched (50 mg), that individual values remained within normal range and that the increase on BT was reversible after treatment stopping.<sup>3</sup> Another study carried out in healthy volunteers treated with 20 mg/d demonstrated that antiplatelet effects of D-003 were related to a decrease on TxA<sub>2</sub> and concomitant increase on PGI<sub>2</sub> serum levels.<sup>6,7</sup> This fact, together with no changes observed on coagulation parameters investigated in such studies reinforce the idea that the increase on BT caused with D-003 is an effect accompanied by a low potential risk.

## CONCLUSIONS

The present study demonstrates that oral treatment with high repeated doses of D-003 to Sprague Dawley increase BT values in a moderate extent, being not greater than increases induced with low doses. Also, D-003 did not induce drug-related toxicity; including haemorrhage manifestations. D-003 did not affect coagulation indicators here assessed. Hence, increase on BT induced with high doses of D-003 is not related to impairment of coagulation process, but probably with the inhibition of platelet aggregation.

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