Pharmacological and Toxicological Tests

University of Bologna

Institute of Pharmacology Pharmacodynamics and Toxicology

Director: Prof. Pietro Dessi

Bologna – Strada Maggiore, 45 – Tel. 276.557

Doctor Bruno Manica of Verona sent us a proprietary preparation, made up into sugar-coated tablets, named CERNILTON, in order that we should carry our pharmacological and toxicological tests.

The composition of the preparation is as follows:

One tablet contains:

Cernitin T60…….60 mg

Cernitin GBX_1…….....3 mg

Excipients…….q.s.

The clinical use proposed for the preparation is that of an anti-inflammatory agent, in a dose of 2-4 tablets/day.

The studies in our Institute were extended in two directions: first a group of experiments was carried out to estimate the activity of CERNILTON, by comparison with known drugs. Having established its activity we went on to study its toxicity in order to estimate the therapeutic coefficient.

EXPERIMENTS AND RESULTS

1. ACTIVITY
   a) carrageenin-induced oedema test in the normal and the adrenalectomized rat.
   b) cotton pellet granuloma test.
2. TOXICITY
   a) acute
   b) chronic
   c) foetal
   d) anaphylactic action

1. ACTIVITY

a) carrageenin-induced oedema in the normal and adrenalectomized rat.

In order to study carrageenin-induced oedema, the test preparation, administered by gastric intubation, was compared with phenylbutazone and indomethacin. The doses administered are reported in table 1, together with the results. Local oedema from carrageenin in the normal and adrenalectomized rat was produced, in the animal's paw, by means of the injection in the plantar region of 0.1 ml of a 0.5% solution of carrageenin in 0.9% NaCl.

The volume of the paw was measured in the individual rats with a gauge, three hours after the injection of the carrageenin.

The experiment was carried out on male rats of weight around 220 G, divided into three groups, two of which were used for the comparative assessment with indomethacin and phenylbutazone.

TABLE 1

Percentage inhibition of carrageenin-induced oedema in rat's paw.

Substance Tested	Dose	Number of Animals	Mode of Administration	% inhibition
Cernilton	½ tab/kg	10	Oral	33.7
	1 tab/kg	10	Oral	44.2
	1.5 tab/kg	10	Oral	56.8
	2 tab/kg	10	Oral	65.4
Indomethacin	0.5 mg/kg	10	i.p.	20.4
	1.0 mg/kg	10	i.p.	34.5
	2.0 mg/kg	10	i.p.	38.3
	3.0 mg/kg	10	i.p.	46.7
Phenylbutazone	25 mg/kg	10	i.p.	26.2
	50 mg/kg	10	i.p.	39.3
	100 mg/kg	10	i.p.	52.6
	200 mg/kg	10	i.p.	60.7

Examination of the data reported in table 1 shows that CERNILTON demonstrates inhibitory activity on carrageenin-induced oedema.

The test of the inhibition of carrageenin-induced oedema was repeated on the adrenalectomized rat, in order to avoid the liberation of corticosteroids. A single dose of 1.5 tablets/kg of CERNILTON was administered to a group of 10 rats by gastric intubation. From this experiment it was apparent that the oral administration of 1.5 tablets/kg of CERNILTON produces an inhibition of oedema amounting to 53.2%. This results is of the same order of magnitude as that found in the non-adrenalectomized rat (56.8%).

This finding enables stress to be excluded as the cause of anti-inflammatory activity by means of release of corticosteroids.

b) Cotton pellet granuloma test.

This test, consisting in the subcutaneous implantation of cotton pellets of initial weight 15 ± 0.2 mg, sterilized at 120° C for 2 hours and moistened with penicillin (50 µg/pellet), was carried out under light ether anesthesia in male rats. After 8 days the pellets were removed, dried for 24 hours at 60° C and then weighed again. The experiment was performed on 40 rats weighing around 200 G, divided into 4 groups, respectively: controls, treated with CERNILTON, treated with Indomethacin, and treated with Phenylbutazone.

In table 2 below the data are expressed in terms of percentage inhibition of the weight of the granuloma compared with the untreated controls.

The anti-inflammatory action of CERNILTON is apparent, in this test, at a dose of 1 tablet/kg.

TABLE 2

Percentage inhibition of the “cotton pellet” granuloma.

Substance Tested	Dose	Number of Animals	% inhibition
Cernilton	1 tab/kg	10	36
Indomethacin	1.6 mg/kg	10	45.2
Phenylbutazone	100 mg/kg	10	42.7

2. TOXICITY

a) Acute Toxicity

The LD_{50} was estimated in the rat and the mouse after oral administration of the test preparation. The experiment was carried out using the logarithmic method of C.S. Weil (Biometrics, Sect. 1952) based on the administration of doses increasing in geometrical progression.

The experiment was carried out in 4 groups of 10 animals each, who were observed for a period of 48 hours after drug administration. The results obtained are reported in the following tables.

TABLE 3

Acute toxicity of CERNILTON in male Sprague-Dawley rats of weight 200-220 G.

Doses: tablets/kg	Mode of Administration	Number of Animals per group	Mortality	LD_{50}
1	Oral	10	0/10	> 8 tab/kg
2	Oral	10	0/10	
4	Oral	10	0/10	
8	Oral	10	0/10	

TABLE 4

Acute toxicity of CERNILTON in the male albino mouse weighing 22-25 G.

Doses: tablets/kg	Mode of Administration	Number of Animals per group	Mortality	LD_{50}
1	Oral	10	0/10	> 8 tab/kg
2	Oral	10	0/10	
4	Oral	10	0/10	
8	Oral	10	0/10	

b) *Chronic toxicity*

The test was performed on the Sprague-Dawley rat and the New Zea and rabbit. Treatment by the oral route was continued for a period of 180 consecutive days, the test preparation being mixed with the food ground up and made into a paste with water. Suitable containers prevented the dispersion of the food and ensured the total consumption of the substance given to the animals. The condition of the animals was determined periodically, and checks carried out on their weight gain, blood picture and renal function. At the end of the treatment the above observations were completed with determinations of the white cell formula and liver function, and macroscopical and microscopical examination of the principal organs.

A. Test on the RAT

20 Sprague-Dawley rats were used, divided into 2 groups of 10 each in the following way:

Group 1: controls
Group 2: treated with CERNILTON in a dose of 1 tablet/kg. The results obtained are as follows:

TABLE 5

Increase in body weight in the control rats and those treated with CERNILTON. Mean values ± S.E.

Days of Treatment	Controls	Treated
0	102 ± 1.4	101 ± 1.8
30	162 ± 2.7	160 ± 2.6
60	261 ± 3.8	262 ± 4.1
90	320 ± 4.9	315 ± 5.3
120	360 ± 5.7	365 ± 5.8
180	395 ± 6.1	393 ± 6.7

Examination of table 5 shows no significant variation in body weight of the animals treated with CERNILTON with the control animals.

BLOOD PICTURE

Erythrocyte and leukocyte counts were carried out periodically during the treatment of the rats with the test preparation and at the end of this treatment. The count, performed by means of a Thoma-Zeiss chamber on samples of blood taken from the animals' tails, gave the following results.

Adrenal: normal cortex and medulla.
Stomach and intestine: no lesions seen in the mucosa, submucosa or muscle layers.

CONCLUSIONS:

It is clear that the treatment with the preparation CERNILTON, continued over a period of 180 days, has no inhibitory effect on the body growth of the rat, nor on the blood picture, nor on hepatic or renal function. There are no resulting signs of damage to the principal organs. For this reason it is concluded that the preparation CERNILTON is not toxic to the Sprague-Dawley rat, even when treatment is for prolonged periods.

B. Test on the rabbit

The test was carried out on 20 New Zealand rabbits divided into two experimental groups of 10 animals each in the following way:

Group 1: Control
Group 2: Treated with CERNILTON in a dose of 1 tablet/kg.

The test preparation was mixed with the diet, ground up and made into a paste with water. The control animals received only their food ground up and made into a paste.

The treatment was started in young animals, 50 days old, and continued for 180 days.

The results obtained are set out in the following tables.

TABLE 10

Body growth of the rabbits in the two experimental groups. Mean values per group ± S.E. expressed in Kg.

Days of Treatment	Controls	Treated
0	1.12 ± 0.011	1.18 ± 0.015
30	1.40 ± 0.024	1.43 ± 0.018
60	1.96 ± 0.032	1.95 ± 0.029
90	2.15 ± 0.047	2.18 ± 0.051
120	2.68 ± 0.059	2.73 ± 0.071
180	3.10 ± 0.064	3.17 ± 0.063

The body growth of the rabbits treated with CERNILTON does not differ significantly from that of the control animals.

TABLE 11

Blood picture. Erythrocytes (10^4 per mm^3) and leucocytes (10 per mm^3) in the blood of the rabbits. Mean values ± S.E.

Days of Treatment	Erythrocytes	
	Controls	Treated
0	974 ± 12.8	972 ± 14.1
60	979 ± 13.2	939 ± 17.1
120	942 ± 13.9	1012 ± 14.1
180	1004 ± 15.1	998 ± 15.9

Days of Treatment	Erythrocytes	
	Controls	Treated
0	648 ± 7.1	628 ± 6.7
60	610 ± 5.2	607 ± 4.3
120	624 ± 6.7	618 ± 7.2
180	643 ± 7.5	639 ± 7.8

The counts were carried out with a Thoma-Zeiss cell-counting chamber.

The values obtained in the animals treated with the test preparation do not differ from the normal.

The search for albumin and occult blood in the urines of the treated animals, carried out periodically during the treatment, gave consistently negative results.

Examination of peripheral blood films, prepared at the end of the treatment with the test preparation, enabled the mean values of the white cell formula to be worked out for each group, as shown in the following table.

TABLE 12

White cell formula of the rabbits in the two experimental groups. Mean values per group ± S.E.

	Lymphocytes	Monocytes	Granulocytes		
Group	%	%	Neutrophils	Eosinophils	Basophils
Controls	48.7 ± 1.2	4.9 ± 0.27	43.5 ± 1.62	1.9 ± 0.05	1.0 ± 0.01
Treated	47.8 ± 1.7	4.7 ± 0.30	44.5 ± 1.72	1.8 ± 0.06	1.2 ± 0.02

The determination of the serum GOT and GPT activities of the animals in the experimental groups was performed at the end of the period of chronic treatment by taking samples of blood from the marginal vein of the ear of each animal.

The test was performed with the colorimetric test of the Boehringer Company of Milan. The results obtained are set out in the following table.

TABLE 13

Serum GOT and GPT activities of the rabbits in the two experimental groups at the end of the chronic treatment. Mean values ± S.E.

Enzymatic Activity	Controls	Treated
SGOT: mU/ml	32.1 ± 2.27	31.5 ± 2.18
SGPT: mU/ml	17.8 ± 1.30	16.9 ± 1.32

Analysis of the above results shows no significant differences in the values obtained in the treated animals compared with those of the controls.

Macroscopical examination of the rabbits, sacrificed by carotid section at the end of the treatment, did not demonstrate any signs of damage to the organs of the animals subjected to chronic treatment with the preparation CERNILTON. Entirely normal appearances were found in the liver, kidney, spleen, heart, lungs, stomach, intestine and genital system.

The fresh weight of the principal organs in reported in the following table.

TABLE 14

Fresh weight of the organs of the rabbits in the two experimental groups. Mean values ± S.E.

Group	Liver G	Spleen G	Kidney G	Heart G	Adrenal mg
Controls	154 ± 1.65	1.18 ± 0.10	14.5 ± 0.19	8.8 ± 0.21	195 ± 3.9
Treated	148 ± 1.07	1.22 ± 0.09	13.8 ± 0.17	9.5 ± 0.18	186 ± 4.6

Histological examination was carried out on preparations of the principal organs fixed in formalin and stained with hematoxylin-eosin. No signs were found of toxicity or poor tolerance of the preparation given.

The relevant findings in the treated animals can be summarized as follows:

Liver: normal structure of the hepatic lobules. Normal liver cells with rounded nucleus in the centre of the cytoplasm. Normal vascular network and biliary ducts.
Kidney: renal pelvis clear. No cloudy swelling seen in the renal glomeruli or in the proximal or distal tubules. No interstitial infiltration with small cells seen.
Heart: nothing remarkable.
Adrenal: normal cortex and medulla.
Stomach and intestine: no lesions seen.

CONCLUSIONS:

From the results reported above it can be seen that continued treatment for 180 days with the preparation CERNILTON has no inhibitory effect on the growth and development of the rabbit. The blood pictures is not affected, nor are renal of hepatic function. No manifestations of toxicity are seen in the principal organs.

It is concluded that the preparation CERNILTON is not toxic to the New Zealand rabbit when given orally even for as long as 180 days.

c) Foetal toxicity

The evaluation of foetal toxicity and the search for possible teratogenic properties of CERNILTON were carried out by means of two experiments: the first on the Sprague-Dawley rat, and the second on the New Zealand rabbit. Treatment was given by mouth during the organogenetic period of pregnancy, the test preparation being administered by gastric intubation in three different doses.

Examination of the foetuses was carried out at birth. Account was taken of their number, their vitality and their body weight. The skeleton was examined after rendering the soft tissues transparent and staining the bony tissue with alizarin red. After the end of pregnancy all the animals were sacrificed in order to look, by direct examination of the walls of the uterine cornua, for possible signs of resorption.

A. Test on the rat

The test was carried out on 40 female and 20 male adult Sprague-Dawley rats. The animals were distributed in 20 cages each containing 2 females and one male. The mating period lasted for 5 days during which the males were rotated daily between the cages in such a way that during the mating period two females were in the company of different males. By this procedure a percentage of pregnancies of 50 to 70% were obtained.

The treatment with the test preparation was started immediately after the end of the mating period when the females were divided into 4 groups of 10 each. The four groups were constituted as follows:

Group 1: Controls
Group 2: Treated with CERNILTON in a dose of 1/2 tablet/kg by gastric intubation.
Group 3: Treated with CERNILTON in a dose of 1 tablet/kg by gastric intubation.
Group 4: Treated with CERNILTON in a dose of 1.5 tablets/kg by gastric intubation.

The treatment was carried out daily from the first to the 15th day of pregnancy.

TABLE 15

Foetal toxicity of Cernilton in the rat.

Group	1		2		3		4	
Number of Animals per group	10 F	5 M	10 F	5 M	10 F	5 M	10 F	5M
Number of Pregnancies	6		7		5		7	

Findings:

Total number of foetuses	67	84	56	77
Mean number of fetuses per litter	11	12	10	12
Mean foetal weight in G	5.97	6.07	6.15	6.09
Number of live births	67	82	56	76
Number of stillbirths	0	2	0	1
Resorptions	2	0	1	2
Number malformed	0	0	0	0

The results set out in table 15 were subjected to statistical analysis by carrying out a comparison between the various respective incidences in the 4 experimental groups according to the X^2 test. The following table shows the comparisons made.

TABLE 16

Statistical comparison of the results obtained in the 4 experimental groups.

	Parameters Compared	Calculated X^2	Significance
-	Pregnancy animals versus non-pregnant animals	1.7333	Not sig.
-	Foetuses per pregnancy	0.0285	Not sig
-	Live births versus stillbirths	2.7665	Not sig.
-	Resorptions	2.3315	Not sig.

Critical values for 3 degrees of freedom:

P 0.05 = 7.815 P 0.01 = 11.345

No significant differences were seen in the number of pregnancies, total number of fetuses, number of live births and still births, and number of resorptions in the 4 experimental groups. The preparation CERNILTON therefore shows no foetal toxicity or teratogenic activity in the Sprague-Dawley rat.

B*. Test on the rabbit*

The test was conducted on 20 adult female New Zealand rabbits that after mating were divided into 4 experimental groups of 5 each. The 4 groups were subjected to the following treatments:

Group 1: Control
Group 2: Treated with CERNILTON in a dose of 1/2 tablet/kg
Group 3: Treated with CERNILTON in a dose of 1 tablet/kg
Group 4: Treated with CERNILTON in a dose of 1.5 tablet/kg.

The treatment was carried out daily be gastric intubation and continued from the 1st to the 20th day of pregnancy. The results obtained are reported in the following table:

TABLE 17

Foetal toxicity of CERNILTON in the rabbit.

Group	1	2	3	4
Animals per Group	5	5	5	5
Number of Pregnancies	5	4	4	5
Findings:				
Total number of foetuses	43	34	31	37
Mean number of fetuses per litter	8	9	8	7
Mean foetal weight	51.5	49.7	51.8	50.2
Number of live births	43	33	31	36
Number of stillbirths	0	1	0	1
Number of resorptions	1	0	0	2
Number of malformations	0	0	0	0

In this experiment also the results obtained were subjected to statistical analysis by making a comparison between the various respective frequencies in the 4 experimental groups according to the X^2 test. The following table reports the comparisons made.

TABLE 18

Statistical comparison of the results obtained in the 4 experimental groups.

Parameters Compared	Calculated X^2	Significance
Pregnant animals versus non-pregnant animals	2.2222	Not sig.
Foetuses per pregnancy	0.0665	Not sig.
Live births versus stillbirths	2.1210	Not sig.
Resorptions	3.2468	Not sig.

Critical values for 3 degrees of freedom:

P 0.05 = 7.815 P 0.01 = 11.345

No significant differences were observed in the number of pregnancies, the total number of fetuses, the numbers of live births and stillbirths and the number of resorptions in the 4 experimental groups. It is therefore concluded that the preparation CERNILTON exerts no foetal toxicity or teratogenic activity on the New Zealand rabbit.

d) Anaphylactic action

The anaphylactic action was studied by means of the technique described by Pasteur – Valery Radot on page 83 of the treatise “Maladies allergiques”. This technique consists of the intravenous injection of a suspension of the test preparation, finely dispersed, 48 hours after sensitization with the same suspension given subcutaneously. A positive response is indicated by hypotensive collapse and haemorrhagic extravasations in the internal organs of the guinea-pig. The study was carried out on 30 adult guinea-pigs weighing around 500 G. Three subcutaneous injections of suspension of CERNILTON were given on 3 consecutive days. Subsequent injection intravenously of the suspension diluted 1/100 did not give rise, in the pre-treated guinea-pig, to manifestations of collapse. The internal organs of the animals, on examination at autopsy, did not show manifestations of haemorrhage.

In consequence of this sensitization phenomena on the part of the test preparation can be excluded, inasmuch as no greater incidence of anaphylactic phenomena is to be expected than may be found with any category of drug.

CONCLUSIONS

Given the consistently negative results of the tests of acute, chronic and foetal toxicity, the absence of anaphylactic action and the consistent inhibitory activity on the inflammation produced in two tests (carrageenin-induced oedema and cotton pellet granuloma) it can be concluded that the preparation CERNILTON can provide excellent results in its proposed clinical use as an anti-inflammatory agent.

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