https://doi.org/10.59459/1859-1655/JMM.402

ACUTE AND SUB-CHRONIC TOXICITY ASSESSMENT OF PYRIDOSTIGMINE BROMIDE 90 MG EXTENDED-RELEASE TABLETS IN EXPERIMENTAL ANIMALS

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ABSTRACT

Purpose: *This study aimed to assess the acute and subchronic toxicity of Pyridostigmine bromide 90 mg extended-release tablets in experimental animals.*

Subjects and methods: *Pyridostigmine bromide 90 mg extended-release tablets were prepared complying in-house specifications. Acute oral toxicity (LD50) was determined in white mice using the Litchfield-Wilcoxon method. Subchronic toxicity was assessed in rabbits following OECD guidelines.*

Results: *The acute toxicity study revealed an LD50 of 17.9 mg/kg when Pyridostigmine bromide 90 mg extended-release tablets were orally administered to mice. In the subchronic toxicity assessment, rabbits were treated with Pyridostigmine bromide tablets for 28 days. Doses of 7.2 mg/kg/day and 21.6 mg/kg/day showed no significant alterations in hematological parameters (red blood cells, hemoglobin, hematocrit, mean corpuscular volume, white blood cells, platelets), blood biochemical parameters (AST, ALT, total protein, creatinine, urea), and did not cause damage to the histopathology of the liver, spleen, and kidneys in rabbits. However, the dose of 21.6 mg/kg/day resulted in a statistically significant difference in rabbit weight gain compared to those administered a dose of 7.2 mg/kg/day and those in the control group*

Keywords: Pyridostigmine bromide 90 mg, extended-release, toxicity.

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Receipt date: November 10, 2023; scientific review: November 2023; accepted: December 20, 2023

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1. INTRODUCTIONS

Pyridostigmine bromide (PB) is a carbamate compound that reversibly inhibits cholinesterase enzyme activity, used in the treatment of myasthenia gravis. In military operations, PB can be used to prevent poisoning with nerve agents (Sarin, Soman, Tabun...) at low doses. However, due to PB's short half-life, when used prophylactically in this scenario, individuals must take it multiple times a day, resulting in inconvenience for soldiers engaged in battlefield operations. [2]. Hence, globally, several authors have noted the trend of investigating extended-release formulations of PB that require only once-daily administration in necessary situations.

In Vietnam, initial studies have been conducted to develop extended-release tablets of PB 90 mg to fulfill usage requirements, particularly in military operations. This study aims to evaluate the acute and subchronic toxicity in experimental animals of PB 90 mg extended-release tablets - a result of the production research that has been implemented previously.

2. SUBJECTS, EQUIPMENT AND METHODS

2.1. Subjects and equipment

- Subjects:

+ 60 adult Swiss albino mice, of undifferentiated gender, weighing between 18-22g each.

+ 24 New Zealand rabbits, of undifferentiated gender, weighing between 2.0-2.5 kg each.

- Experimental preparation: Extended-release tablets of Pyridostigmine Bromide 90 mg (each tablet containing 90 mg of PB and other excipients), meeting in-house standard specifications.

- Equipment and Chemicals: ABX Micros ES 60 hematology analyzer, Horiba Medica- France; Chem 5V3 Semi automated analyzer, Erba - Germany; and the necessary glassware, pipettes, and chemicals for testing (hematology, biochemistry) used in the experiments.

2.2. Methods

Determination of acute toxicity: 60 Swiss albino mice were evenly and randomly divided into 6 groups. Prior to the experiment, the mice fasted for 3 hours but had access to water. The administration of the drug was performed forcibly using a oral gavage needle. The drug was administered into the stomach of the mice at a volume of 0.2 ml/10g

body weight/dose, with escalating doses (10.24 mg/kg; 12.8 mg/kg; 16.0 mg/kg; 20.0 mg/kg; 25.0 mg/kg; and 31.25 mg/kg). General conditions and mortality rates of mice in each group were monitored for 72 hours. Following this, the highest dose that did not cause mouse mortality and the lowest dose that caused 100% mortality were determined. The general conditions of the mice were further monitored until the end of the 7th day post-administration. LD50 was calculated based on the mortality rate in each group using the Litchfield - Wilcoxon method [1].

- Determination of subchronic toxicity (according to A. Wallace Hayes [5] and guidelines from the OECD [8]): 24 rabbits were randomly divided into 3 groups. Rabbits were continuously administered the tablets or distilled water for 28 days, at a volume of 5 ml/kg/dose every 24 hours, as follows:

+ Control group: administered distilled water.

+ Experimental group 1 (EG1): administered PB tablets at a dose of 7.2 mg/kg of active ingredient (equivalent to the human dose, with a conversion factor of 4 times, the estimated human dose being 90 mg/50kg/day).

+ Experimental group 2 (EG2): administered PB tablets at a dose of 21.6 mg/kg of active ingredient (threefold the equivalent human dosage, adjusted accordingly).

Monitoring encompassed general condition surveillance (including abnormal manifestations and body weight changes), assessment of hematopoietic function (red blood cell count, white blood cell count, platelet count, hemoglobin concentration, hematocrit level, and mean corpuscular volume), as well as live function (AST, ALT, total protein), kidney function (urea, serum creatinine levels) in rabbits at baseline (D0), day 14 (D14), and day 28 (D28) post-administration. Gross anatomical and histopathological evaluations of the heart, liver, spleen, kidneys, and lungs were conducted on 30% of rabbits in each group at D28.

- Data analysis: conducted using medical statistical methods, employing Microsoft Excel software.

3. RESULTS AND DISCUSSIONS

3.1. Results of acute toxicity assessment

Table 1. Acute toxicity of orally administered PB tablets in mice

Group	Dose (mg/kg)	Volume of administration	Number of mice deceased within 72 hours
1 (n = 10)	10.24	0.2 ml/10g	0
2 (n = 10)	12.8	0.2 ml/10g	2
3 (n = 10)	16.0	0.2 ml/10g	3
4 (n = 10)	20.0	0.2 ml/10g	6
5 (n = 10)	25.0	0.2 ml/10g	9
6 (n = 10)	31.25	0.2 ml/10g	10

After administration, mice in the respective groups exhibited signs of agitation, increased secretion leading to wet fur, elevated urinary output, onset of dyspnea, muscular tremors, and convulsions. These manifestations varied in frequency and severity across groups administered different doses, with a tendency of escalation observed in the higher dose groups. Additionally, mortality was observed in groups administered higher doses. The number of deceased mice increased with escalating doses of tablet administration (table 1).

LD50 was determined using the Litchfield - Wilcoxon method and verified using Excel [1], yielding LD50 = 17.9 mg/kg with a confidence interval of 15.4-22.4 mg/kg at p = 0.05. This result is higher than the published LD50 for orally administered PB in mice in drug substance form (16 mg/kg) [9]. The discrepancy may stem from variations in formulation components, excipients impacting the bioavailability of PB in experimental animals [3].

3.2. Results of subchronic toxicity assessment

- Changes in body weight of experimental rabbits:

Table 2. Changes in body weight of experimental rabbits (mean ± SD, kg)

Group (n = 8)	Term of the study			p
	D0	D14	D28	
Control group [(a)]	2.24 ± 0.15	2.33 ± 0.18	2.52 ± 0.19	$p_{0-14} < 0.05$; $p_{0-28} < 0.05$
% Increase/decrease in body weight compared to pre-experiment		+ 4.02%	+ 12.5%	

EG 1[(b)]	2.27 ± 0.20	2.29 ± 0.16	2.60 ± 0.19	$p_{0-14} > 0.05$; $p_{0-28} < 0.05$
% Increase/decrease in body weight compared to pre-experiment		+ 0.88%	+ 14.54%	
EG 2[(c)]	2.18 ± 0.09	2.09 ± 0.19	2.27 ± 0.17	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$
% Increase/decrease in body weight compared to pre-experiment		- 4.13%	+ 4.13%	
p	$p_{a-b; a-c} > 0.05$	$p_{a-b; a-c} > 0.05$	$p_{a-b} > 0.05$; $p_{c-a; c-b} < 0.05$	

After 28 days of medication, the weight of rabbits in both the control group and the group administered a dose of 7.2 mg/kg/day showed a statistically significant increase ($p < 0.05$). However, at the higher dosage (21.6 mg/kg/day), the rabbits' weight showed no significant change compared to the baseline ($p > 0.05$).

We hypothesize that the feeding conditions may have contributed to the weight gain observed in both the control group and the group administered a dose of 7.2 mg/kg/day; whereas rabbits in the higher dosage group may have experienced adverse effects on their feeding capacity, resulting in insignificant weight gain. These results are consistent with the findings reported by Bigoniya et al. [4].

- Effects of PB on rabbit hematological parameters:

Table 3. Effects of PB on rabbit hematological parameters

Parameters	Term of the study	Mean ± SD values (n = 8)			p
		Control group [(a)]	EG 1[(b)]	EG 2[(c)]	
RBC ($\times 10^6/mm^3$)	D0	10.23 ± 2.63	11.26 ± 2.93	12.18 ± 2.56	$p_{a-b; a-c} > 0.05$
	D14	10.31 ± 2.66	11.63 ± 1.47	11.10 ± 3.33	
	D28	10.72 ± 1.22	10.80 ± 1.39	11.54 ± 1.39	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
WBC ($\times 10^3/mm^3$)	D0	9.98 ± 2.14	9.10 ± 1.85	9.63 ± 2.05	$p_{a-b; a-c} > 0.05$
	D14	12.69 ± 3.39	11.40 ± 3.74	12.65 ± 4.64	
	D28	12.03 ± 3.35	10.94 ± 2.14	13.08 ± 3.72	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
PLT ($\times 10^3/mm^3$)	D0	291.88 ± 88.31	312.38 ± 106.03	259.75 ± 99.87	$p_{a-b; a-c} > 0.05$
	D14	250.88 ± 98.85	299.25 ± 84.60	222.13 ± 95.53	
	D28	247.75 ± 60.62	258.13 ± 100.11	222.25 ± 78.67	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
Hematocrit (%)	D0	48.91 ± 8.56	48.16 ± 7.80	49.96 ± 8.83	$p_{a-b; a-c} > 0.05$
	D14	45.64 ± 8.09	50.09 ± 6.91	47.88 ± 8.36	
	D28	49.15 ± 4.53	50.84 ± 4.34	49.84 ± 6.50	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
HGB (g/dL)	D0	15.66 ± 4.01	17.53 ± 3.23	18.93 ± 3.85	$p_{a-b; a-c} > 0,05$
	D14	15.95 ± 4.22	17.34 ± 2.63	17.29 ± 4.50	
	D28	16.53 ± 2.42	16.73 ± 1.39	17.76 ± 1.12	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
MCV (μm^3)	D0	67.38 ± 2.62	67.63 ± 2.56	68.38 ± 3.25	$p_{a-b; a-c} > 0,05$
	D14	66.88 ± 3.14	67.25 ± 2.19	69.13 ± 1.96	
	D28	68.13 ± 2.47	68.00 ± 1.69	68.50 ± 2.67	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				

The results indicate that rabbits continuously administered the tablet for 28 days at both doses of 7.2 and 21.6 mg/kg/day showed no significant changes in hematological parameters compared to the control group at each assessment time point ($p > 0.05$).

Morgan's investigation into the subchronic oral toxicity of PB in rats, utilizing doses ranging from 1 mg/kg to 90 mg/kg over a period of 90 days [7], and Kluwe's examination of subchronic oral toxicity in dogs, employing a maximum PB dose of 20 mg/kg for 3 months [6], did not observe alterations in hematological parameters among the subjects. Similarly, in the study by Bigoniya et al. involving rats treated with PB doses of 20 mg/kg, 30 mg/kg, and 45 mg/kg for 45 days, only the group administered 45 mg/kg showed a decrease in red blood cell count after 15 days of treatment; a decrease in hemoglobin after 45 days of treatment; and an increase in white blood cell count after 15 days of treatment in the groups administered PB at doses of 30 mg/kg and 45 mg/kg [4].

- Effect of PB on rabbit blood biochemical parameters:

Table 4. Effect of PB on AST, ALT, and total protein in rabbit blood.

Parameters	Term of the study	Mean ± SD values (n = 8)			p
		Control group [(a)]	EG 1[(b)]	EG 2[(c)]	
AST (UI/L)	D0	50.38 ± 8.53	58.00 ± 10.14	47.38 ± 9.26	$p_{a-b;\ a-c} > 0.05$
	D14	55.63 ± 13.43	60.13 ± 19.32	49.13 ± 14.43	
	D28	67.13 ± 21.18	73.13 ± 20.86	63.00 ± 15.29	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
ALT (UI/L)	D0	51.63 ± 5.68	61.13 ± 15.61	54.38 ± 8.81	$p_{a-b;\ a-c} > 0.05$
	D14	51.25 ± 14.09	50.25 ± 12.21	49.38 ± 8.81	
	D28	64.13 ± 18.33	62.63 ± 19.45	64.75 ± 14.57	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
Total protein (g/L)	D0	5.13 ± 0.40	5.21 ± 0.59	5.25 ± 0.52	$p_{a-b;\ a-c} > 0.05$
	D14	4.66 ± 0.38	4.83 ± 0.21	4.81 ± 0.43	
	D28	4.88 ± 0.22	4.89 ± 0.24	4.83 ± 0.28	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
Creatinine (µmol/L)	D0	115.38 ± 10.38	115.13 ± 11.17	131.50 ± 20.85	$p_{a-b;\ a-c} > 0.05$
	D14	113.43 ± 13.64	115.25 ± 10.91	116.88 ± 18.30	
	D28	126.13 ± 6.94	123.88 ± 8.31	130.25 ± 11.59	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				
Urea (mmol/L)	D0	5.33 ± 0.16	5.41 ± 0.12	5.46 ± 0.80	$p_{a-b;\ a-c} > 0.05$
	D14	5.50 ± 0.37	5.43 ± 0.17	5.21 ± 0.30	
	D28	5.03 ± 0.33	5.13 ± 0.27	5.24 ± 0.45	
	$p_{0-14} > 0.05$; $p_{0-28} > 0.05$				

Comparisons of hematological indices (AST, ALT, total protein, creatinine, urea) across rabbit groups at concurrent time points and within each group across experimental intervals revealed no statistically significant variations ($p > 0.05$). These results are consistent with prior investigations by Morgan and Kluwe [6], [7]. Similarly, a study conducted by Bigoniya observed non-significant elevations in AST and ALT levels in rats orally administered PB at a dose of 45 mg/kg for 45 days, with no discernible effect on blood protein levels [4].

- The influence of PB on histopathological changes in the rabbit experimental model: Examination of gross morphology, structural integrity, and tissue coloration of the heart, liver, kidneys, lungs, and intestines in both the experimental and control groups yielded no discernible anomalies or significant differences. However, histopathological analysis revealed scattered inflammatory cell infiltration and signs of degeneration in some liver tissue specimens across all three study groups, with no clear distinction

between the control and experimental groups. Notably, renal histopathology images did not display any observable lesions. Nevertheless, to establish definitive conclusions, further investigation with larger sample sizes and higher dosage regimens is required

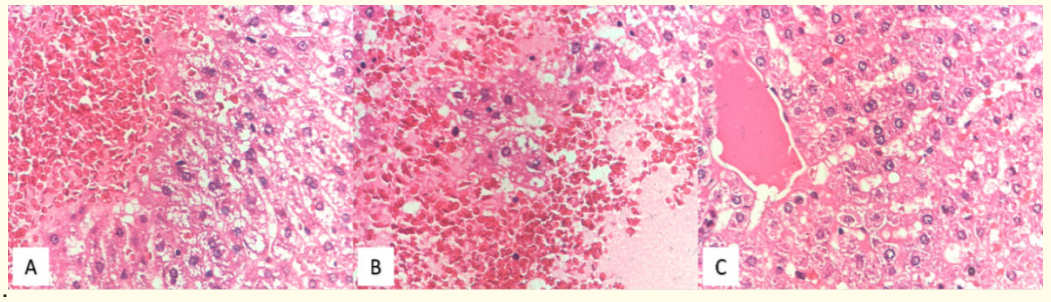

Fig. 1. Hepatic histological images: A) Control group (PD1); B) Experimental group 1 (PD3); C) Experimental group 2 (PD6)

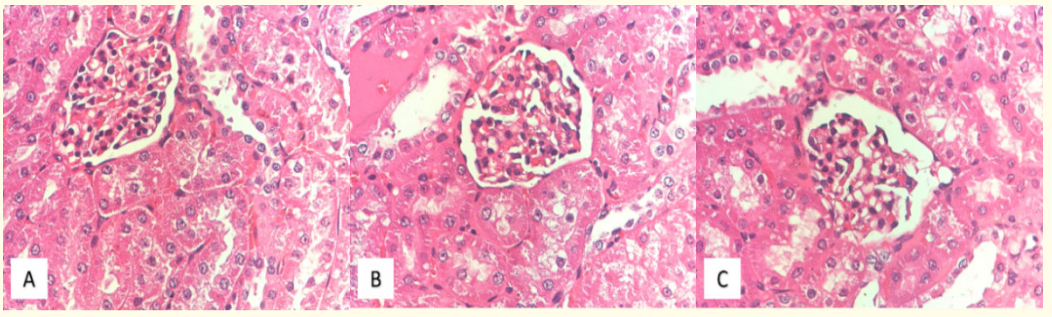

Fig. 2. Renal histological images: A) Control group (PD2); B) Experimental group 1 (PD3); C) Experimental group 2 (PD5)

4. CONCLUSIONS

The study has determined the acute toxicity and sub-chronic toxicity of PB 90 mg sustained-release tablets in experimental animals, as follows:

- Acute toxicity: The oral LD50 of PB 90 mg sustained-release tablets in white mice is determined to be 17.9 mg/kg, with a confidence interval ranging between 15.4 to 22.4 mg/kg, p = 0.05.

- Sub-chronic toxicity: Administration of PB at a dosage of 21.6 mg/kg/day over a 28-day period resulted in observable effects on rabbit weight gain compared to dosages of 7.2 mg/kg/day and the control group. Furthermore, continuous administration of the drug at dosages of 7.2 mg/kg/day and 21.6 mg/kg/day over 28 days did not elicit alterations in hematological parameters (including red blood cells, hemoglobin, hematocrit, mean corpuscular volume, white blood cells, and platelets), blood biochemistry (AST, ALT, total protein, creatinine, urea), or induce histopathological changes in the liver, spleen, or kidneys of rabbits.

REFERENCES

1. Do Trung Dam (2014), *Methods for Determining Drug Toxicity*, Medical Publishing House, pp..102-110, 132-142.
2. Anonymous (2000), "Gulf War and Health, Depleted Uranium, Pyridostigmine Bromide, Sarin, and Vaccines", *Institute of Medicine (IOM)*, pp. 207.
3. Aquilonius SM, Hartvig P (1986), "Clinical pharmacokinetics of cholinesterase inhibitors", *Clin Pharmacokinet*, 11: 236-249.
4. Bigoniya P., Singh A.K., Bigoniya D., Gopalan N (2013), "Pyridostigmine bromide and potassium iodate tablet: Subacute oral toxicity and stability", *Journal of Drug Metabolism & Toxicology*, 4 (2), pp. 1-11.
5. Hayes A.W (1994), "Principles and methods of toxicology", *Raven Press Ltd*., New York, USA.
6. Kluwe W.M, Page J.G, Toft J.D, Ridder W.E, Chung H (1990), "Pharmacological and toxicological evaluation of orally administered pyridostigmine in dogs", *Fundam Appl Toxicol,* 14, pp. 40-53.
7. Morgan E.W, Zaucha G.M, Waring P.P, Le Tellier Y, Seewald J.B, et al. (1990), "Ninety-Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats", *Letterman Army Institute of Research Presidio*, San Francisco, CA, pp. 550.
8. OECD 423 (2001), *OECD guideline for testing of chemicals - Acute oral toxicity - Acute toxic class method*.
9. Science Lab.com (10/09/2005 06:14 PM), *Material Safety Data Sheet Pyridostigmine Bromide MSDS*, Chemicals & Laboratory Equipment, pp. 2,4. ❑