

## Pyridostigmine Bromide and Potassium Iodate: Subacute Oral Toxicity and Stability

Papiya Bigoniya<sup>1\*</sup>, Anil Kumar Singh<sup>2</sup>, Dharmesh Bigoniya<sup>3</sup> and Gopalan N<sup>2</sup>

<sup>1</sup>Department of Pharmacology, Radharaman College of Pharmacy, Radharaman Group of Institutes, Ratibad, Bhopal, M.P., India

<sup>2</sup>Bioprocess Scale up Facility, Defence Research and Development Establishment, Jhansi Road, Gwalior, India

<sup>3</sup>Food and Drugs Administration, Government of Madhya Pradesh, Idgah Hills, Bhopal, India

### Abstract

Subacute toxicity of pyridostigmine bromide (PB) and potassium iodate (PI) tablets upon forty five days administration were assessed by changes in body weight, hematology, serum biochemical parameters and histopathology of rat liver and kidney. LD<sub>50</sub> of PB was estimated to be 66.9 mg/kg. At dose of 45 mg/kg/day showed decrease in body weight and liver weight with cytoplasmic acidophilic bodies and vesicular steatosis signifying hepatocellular injury. Kidney tissue developed neutrophil polymorph infiltration with inflammatory glomerulonephritis, protein casts and glomerulosclerosis. Serum SGOT, SGPT, ALP and lipid profile were elevated. The oral LD<sub>50</sub> of PI was found to be 944.6 mg/kg. PI at 150 mg/kg dose showed anorexia and body weight loss. Serum SGOT, ALP, cholesterol and triglyceride level were elevated at 85 and 150 mg/kg dose and focal area of tubular injury and inflammatory cell infiltration occurred only at 150 mg/kg dose in kidney. The content of PB and PI has been found stable in accelerated (40 ± 2°C/75 ± 5% RH), intermediate (30 ± 2°C/65 ± 5% RH) and long term (30 ± 2°C/65 ± 5% RH) conditions. PB 30 mg/kg/day and PI 85 mg/kg/day doses are safe for rats, which are far in excess of human exposure levels.

**Keywords:** Pyridostigmine bromide; Potassium iodate; LD<sub>50</sub>; Subacute toxicity; Hematology; Histopathology

### Introduction

The research proposal aimed at validation of subacute toxicity and stability of pyridostigmine bromide (PB, 30 mg) and potassium iodate (PI, 85 mg) tablets. Pyridostigmine belongs to a family of carbamate compounds, is a reversible acetylcholinesterase (AChE) inhibitor. It is used to combat poisoning by nerve agents (e.g., Sarin, Soman, Tabun, Methylphosphonothioic acid), to be given prior to exposure in the context of chemical warfare or terrorism to increase survival. Nerve agents are lethally toxic compounds as they produce an irreversible inhibition of both AChE and pseudo-AChE enzyme which are responsible for metabolic degradation of acetylcholine. Preservation acetylcholine in nerve ending induce generalized muscarinic cholinergic response e.g. fall in blood pressure and heart rate, mental confusion and ataxia, vomiting and intestinal cramps, bronchoconstriction and excessive secretion, and finally respiratory paralysis and death. Pyridostigmine is a quaternary carbamate inhibitor of cholinesterase that does not cross blood brain barrier and is taken daily in anticipation of an attack, which carbamylates about 30% of peripheral cholinesterase. The carbamylated enzyme eventually regenerates by natural hydrolysis and an excess Ach level reverts to normal [1]. The median lethal oral dose for PB in literature is reported to be variable as 61.6 mg/kg and 80 mg/kg [2-4].

Radiation emitted as a result of nuclear explosion or terrorist attack related meltdown of nuclear power plant on exposure is absorbed by thyroid gland causing thyroid cancer. Potassium iodide/iodate protects the thyroid gland. PI is preferred over iodide for better taste and longer shelf life. The usual adult dose of pyridostigmine is 30 mg/kg but depending on severity dose can vary upto 120 mg and for PI upto 85-175 mg. Though pyridostigmine can decrease lethality in Nerve gas exposure, it can itself cause mild symptoms like diarrhoea, abdominal cramps, increased salivation, bronchial secretion and sweating, muscular weakness, nausea and vomiting, constricted pupil, all these are very rare in low dose. PI can cause skin rashes, swollen salivary

glands, headache, bronchospasm and gastro-intestinal disturbances can be mild or severe and may be dose dependent. Animals exposed for 4 weeks to iodate dissolved in drinking water showed conflicting evidence of toxicity. Mice showed marked acute toxicity such as hemolysis and renal damage upward 300 mg/kg with a no observable toxic effect at 120 mg/kg [5]. Dogs exposed orally from 66 to 192 days with dose upto 100 mg/kg were assessed for retinal damage which remained unchanged [6].

The functional assessment of physiological and histological toxicity in these experiments were not adequate, although in some animal experimental evidence of gastric toxicity and other minor abnormalities indicative of hemolysis were reported. All these studies are in part incomplete nonetheless, they may not be sufficient to derive adequate safety factors for overt organ toxicity by PB or PI. Evaluation of effect of PB and PI on serum biochemical parameters along with organ targeted toxicity especially on vital liver and kidney are required to determine the safety issue for subacute administration in graded doses. The goal of this proposal was to establish the pre-clinical safety and stability data of the PB (30 mg) and PI (85 mg) tablets. To meet these objectives the core approaches was determination of single dose acute toxicity (LD<sub>50</sub>), subacute toxicity upon forty five days administration by assessing changes in general behavior, body wt. gain, routine hematology, serum biochemical parameters and histopathology of liver and kidney. Stability carried out at more elevated temperature enables prediction to

**\*Corresponding author:** Papiya Bigoniya, Department of Pharmacology, Radharaman College of Pharmacy, Radharaman Group of Institutes, Ratibad, Bhopal-02, M.P., India, E-mail: [p\\_bigoniya2@hotmail.com](mailto:p_bigoniya2@hotmail.com)

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be made on the effective life of product at normal temperature at which the potency must be at least 95% of label claim. Stability tests were aimed at 6 months accelerated term, 6 month intermediate term and 12 month long term data as need for regulatory approval in conditions  $40 \pm 2^\circ\text{C}/75 \pm 5\%$  RH,  $30 \pm 2^\circ\text{C}/65 \pm 5\%$  RH and  $25 \pm 2^\circ\text{C}/60 \pm 5\%$  RH respectively.

## Material and Methods

### Experimental animals

Male Wistar Albino rats (150-200 g) were purchased from the Animal House, Radharaman College of Pharmacy, Ratibad, Bhopal, M.P., following standard guidelines of CPCSEA, India. The animals were allowed to free access of water and standard palette diet (Hindustan Lever Ltd.) and housed in paddy husk bedding with a controlled ambient temperature ( $24 \pm 2^\circ\text{C}$ ), humidity ( $50\% \pm 20\%$ ) and a 12 h light/dark cycle. All experiments were performed between 09:00 AM and 4:00 PM. All experimental procedures were conducted according to the CPCSEA guidelines for the Care and Use of Laboratory Animals. The experimental protocols were approved by Institutional Animal Ethical Committee of Radharaman College of Pharmacy, Bhopal, India (IAEC/RCP/July-2011/01).

### Dose preparation

The BP grade pyridostigmine bromide and potassium iodate tablets contain not less than 95.0% and not more than 105.0% of the labeled amount (British Pharmacopeia, 2008). Solubility of PB and PI is very good in water, but the tablet formulation is not totally soluble in water due to excipients and additives. To formulate different oral doses according to the weight of animal, drugs were weighed and formulated in a suspension with 2% carboxy methyl cellulose in water. Equivalent weight of drug in tablet was calculated and suspension was freshly prepared containing required amount of drug in mg/0.2 ml of suspension.

### Determination of LD<sub>50</sub> of pyridostigmine bromide tablet

**Limit test:** Pyridostigmine, a carbamate cholinesterase (ChE) inhibitor, was been used clinically for decades to treat myasthenia gravis. Pyridostigmine is a polar chemical at physiological pH (containing a quaternary ammonium group) and therefore should be largely prevented from entering into the central nervous system by the blood-brain barrier. Generally, anti-ChE effects of pyridostigmine are considered to be limited to the peripheral nervous system [1]. Pyridostigmine has a relatively short inhibitory action on ChE compared to organophosphorus inhibitors such as sarin, a nerve agent of concern in the Persian Gulf War. The temporary occupation (carbamylation) of the active site serine of AchE by pyridostigmine can prevent the long-term inactivation (phosphorylation) caused by nerve agents, therefore pyridostigmine was used prophylactically to protect soldiers from possible nerve agent exposures during the Gulf War [7,8]. Rats were treated with 23, 30, 39 and 50 mg/kg doses of pyridostigmine and observed for functional signs of toxicity and lethality for 24 h. Lethality was noted in rats treated with 39 mg/kg but not 30 mg/kg pyridostigmine, thus 30 mg/kg was defined as the maximum tolerated dose (MTD) by Song et al. [9]. The median lethal oral dose for PB is reported to be variable between 61.6 to 80 mg/kg [2-4]. The LD<sub>50</sub> is expected to be less than 100 mg/kg so limit test was not performed.

**Main test:** The selected starting dose 40 mg/kg was administered to a single animal and observed. A dose propagation factor of 1.55 (antilog of 0.20) was selected for further dosing and observation for mortality.

### Determination of LD<sub>50</sub> of potassium iodate tablet

**Limit test:** The oral LD<sub>50</sub> of iodates have been reported to be in the range of 500 to 1100 mg/kg and parenteral are in the range of 100 to 120 mg/kg in mice as reviewed by Webster et al. [10]. The lowest parenteral lethal doses in rabbits were 75 mg/kg. Mice apparently tolerated a single dose of 250 mg/kg [11]. Based on these informations the limit test was started at 1200 mg/kg dose. Animals were administered the test dose individually one at a time, observed for 72 h for mortality and for further 14 days to check if there are any late deaths.

**Main test:** Single animal was dosed in sequence usually at 48 h intervals, however the time intervals between dosing is determined by onset, duration and severity of toxic sign. For selecting starting dose the results of the Limit dose is taken into consideration. The first animal was dosed a step below the toxicological best estimate LD<sub>50</sub> value. The selected dose 625 mg/kg was administered to a single animal and observed. As there is no information about slope of the dose-response curve, a dose propagation factor of 1.2 (antilog of 0.08) was selected. The test was continued on 900 and 1080 mg/kg dose in the same manner.

### Subacute toxicity study

In subacute tests animals are dosed daily with PB and PI starting at around expected therapeutic levels for 45 days once a day oral dosing with one dose on ED<sub>50</sub> level, one dose above safe level and one dose below safe level are generally used. The animals are observed for toxic signs, hematological and biochemical observations. The purpose of this test is to determine the maximum tolerated dose and to determine the nature of toxic reaction. No single test can establish the biochemical effect of any drug when used for a long time, in view of multiplicity and complexity of the body functions it is obvious that a number of parameters should be assessed. These tests are determination of body weight, relative vital organ weight, hematological parameters and histological study of liver and kidney. Biochemical analysis of blood was done for marker enzymes like alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), total proteins, cholesterol, triglycerides, high density lipoprotein, low density lipoprotein and glucose.

### Animal grouping

Male Wistar albino rats weighing 150-200 g were divided into seven groups, each containing ten animals. The drugs were administered to the animals as follows:

- Group I- Control (2% CMC suspension)
- Group II- Pyridostigmine bromide (20 mg/kg, p.o)
- Group III- Pyridostigmine bromide (30 mg/kg, p.o)
- Group IV- Pyridostigmine bromide (45 mg/kg, p.o)
- Group V- Potassium iodate (50 mg/kg, p.o)
- Group VI- Potassium iodate (85 mg/kg, p.o)
- Group VII- Potassium iodate (150 mg/kg, p.o)

All the drugs were given orally in the form of suspension for 45 days. Body weight, food consumption and hematology were assessed on every 7<sup>th</sup> day and biochemical parameters and histology was evaluated on 45<sup>th</sup> day two hours after administration of last dose on ten animals of each group.

### Evaluation parameters

**Body weight and relative organ weight:** Pretreatment body weight

of all the animals were noted and percent increases in body weight compared to initial weight were calculated. Liver, kidney, spleen and heart were removed after sacrifice and washed with cold saline solution, pressed between filter paper pads and weighed. Relative organ weight (Weight of organ/100 g of body weight) was calculated and recorded. A part of liver and kidney were preserved in Aqua Bouine's fluid for histopathology.

**Hematological parameters:** Blood samples were collected by retro-orbital puncture on the schedule dates and 2 hrs after the last dose of drugs on 45<sup>th</sup> day. The parameters such as hemoglobin (Sahli's Hemoglobinometer) concentration, total White Blood Cell (WBC) count, total Red Blood Cell (RBC) count (Neubauer hemocytometer; Feinoptik, Germany) and differential WBC count (Neutrophil, Eosinophil, Basophil, lymphocyte and monocyte by Leishman's staining method) was done [12].

**Biochemical parameters:** The animals were sacrificed by carotid bleeding on 45<sup>th</sup> day. Two ml of blood was collected in a clean and dry test tube. The blood was allowed to coagulate for 30 min and centrifuged at 3000 rpm for 5 min. The supernatant serum was separated and used for estimation of biochemical parameters i.e. glutamate pyruvate transaminase and glutamate oxaloacetate transaminases, alkaline phosphatase, cholesterol, triglyceride, total protein, HDL, LDL and glucose [13-20].

**Histopathological studies:** Preparation of permanent tissue slides and staining (Hematoxyline and Eosin) were based on method of Nanji et al. [21].

### Stability testing

The samples are taken out from the storage (accelerated, intermediate and long term) on the planned testing date and kept at 25°C until the time of analysis [22]. The analysis was conducted within 3 days after the samples had been taken out from the stability chamber. Detailed study parameters, methods and specifications are given in table 1 in reference with British Pharmacopoeia [23].

### Stability indicating assay method of pyridostigmine bromide tablet

**Sample preparation:** The samples consisted of pyridostigmine tablets (DRDE, Gwalior). The declared content of PB is 30 mg per tablet. A sample of 3 tablets were crushed in a mortar and accurate weight of a 2.5 tablet (912.5 mg), which was transferred to a 50 ml volumetric flask and diluted with the mobile phase. The solution was

filtered through a 0.45 µm filter. A volume of 1 ml was transferred to a 25 ml volumetric flask and diluted with the mobile phase and the sample was then injected three times each.

**Standard curves:** A five-point standard curve with 1.66, 3.00, 5.00, 7.00 and 10.00×10<sup>-2</sup> mg/ml of PB was used. Each standard was injected six times. All the standards were diluted with the mobile phase.

**Equipment:** Shimadzu HPLC system SPD-M20A Japan (Prominence DIODE ARR) which include Quaternary pump 680, auto sampler ASI-100, Injector with a 200 µl loop, column oven, photodiode array detector (PDA-100), and data system (LC Solution). The detector, an LC-20 AD Prominence Diode Array Detector (PDA-100) was operated at 270 nm. The column was connected to an oven and the temperature was set at 35°C. The separation was achieved by reverse phase Luna 5 µC-18 (2), 100A, 150×4.6 mm column. Run time was set to 10 min.

**Sample testing:** The mobile phase consisted of acetonitrile:water (7.8:92.2, v/v) mixture with 0.015 M hexanesulfonic acid sodium salt. The pH in the mobile phase was adjusted to 2.6 with 1 M H<sub>2</sub>SO<sub>4</sub>. The injection volume was 20 µl and the flow rate was 1 ml/min. The data was recorded on a PDA detector. Calculation was performed by software LC solution system provided by Shimadzu, Japan. The system used a weighted regression line in computing the results [24].

**External standard calibration:** By injecting solution in different concentrations, peak response is plotted vs. concentration. Unknown samples are analyzed in similar manner and their concentrations determined from the calibration curve. The calibration curve must cover the range of unknown sample [25].

**Assay method:** Equivalent weight of 230 mg of PB from tablets were dissolved in 10 ml of anhydrous acetic acid, 40 ml of acetic anhydride were added and titrated with 0.1 M perchloric acid to determine the end point.

1 ml of 0.1 M perchloric acid is equivalent to 26.11 mg of pyridostigmine bromide [23].

### Stability indicating assay method of potassium iodate tablet

**Potassium iodate:** Equivalent weight of 300 mg of PI from tablets was dissolved in q.s to 50 ml of water. 25 ml was taken in an iodine flask and added 3 g of PI, 100 ml of water and 10 ml of HCl. The flask was closed and kept in dark for 5 min. The solution was titrated with 0.1 M sodium thiosulphate to a light straw colour and then titration was completed to a colourless end point using starch mucilage as indicator.

Attributes	Pharmacopoeial reference	Test method	Specificatio	
			Pyridostigmine bromide tablet	Potassium iodate tablet
Weight uniformity	BP Vol. IV (Appendix XII C/A3 02, 2008)	% error of each tablet from average weight	<5%	<5%
Dissolution time	BP Vol. IV (Appendix XII B/A2 86-88, 2008)	Basket type Dissolution test apparatus	T <sub>80%</sub> 60 min	t <sub>75%</sub> 15 min
Disintegration time	BP Vol. IV (Appendix XII A/A2 83, 2008)	Disintegration test apparatus	≥ 15 min	≥ 15 min
Hardness test	BP Vol. IV (Appendix XVII H/A4 24, 2008)	"Monsanto" Tablet hardness tester	≥ 70 N	≥ 70 N
Friability test	BP Vol. IV (Appendix XVII G/A4 23, 2008)	BP standard tablet friability tester	≤ 1%	≤ 1%
Moister content	Not in Monograph (Musa et al., 2008)	Water absorption ratio	>10%	>10%
Pyridostigmine Bromide Assay	Waehler et al. 1993 [24] BP Vol. II (Page no. 1785, 1980)	HPLC (% LC) And Titrametry	95.0-105.0%	95.0-105.0%
Potassium Iodate Assay	BP Vol. II (Page no. 1785, 2008)	Titrametry	95.0-105.0%	95.0-105.0%

**Table 1:** Stability tests parameters and specifications for pyridostigmine bromide and potassium iodate tablet according to British Pharmacopoeia (BP).



Each ml of 0.1 M sodium thiosulphate is equivalent to 3.567 mg of  $KIO_3$  [23].

## Result

### LD<sub>50</sub> of pyridostigmine bromide tablet

The test substance could be classified in the hazard classification as class 3,  $LD_{50} > 50 \leq 300$  mg/kg in the Globally Harmonized System (GHS). The criterion for classification of test substance is expected  $LD_{50}$  value below 300 mg/kg. So the Limit test was not repeated at 300 mg/kg dose. These categories of drugs ( $LD_{50}$  between 50-300 mg) are considered moderately toxic and the probable lethal dose for man is estimated to be near 4 g. The result is represented in figure 1 and exact  $LD_{50}$  was estimated as 66.9 mg/kg from log dose-response curve and regression analysis.

### LD<sub>50</sub> of potassium iodate tablet

The  $LD_{50}$  is lesser than the test dose 1200 mg/kg. The test substance could be classified in the hazard classification as class 4,  $LD_{50} > 300 \leq 2000$  mg/kg in the Globally Harmonized System (GHS). The criterion for classification of test substance is expected  $LD_{50}$  value below 2000 mg/kg. From the log dose-response curve and regression analysis the  $LD_{50}$  was found to be 944.6 mg/kg (Figure 2).

### Subacute toxicity of pyridostigmine bromide tablet

**Body weight and relative organ weight:** Vehicle treated animals showed 26.50% increase in body weight after 45 days. Pyridostigmine treatment at 20 and 30 mg/kg dose showed 6.42 and 4.26% increase in body weight, whereas 45 mg/kg dosing decreased the body weight gain by 15.62% after 45 days of continuous treatment (Table 2). Table 3 compiles the effect of daily 45 days oral administration of PB on relative organ weight of rats. Effect on kidney, heart and spleen weight were nonsignificant but at 30 and 45 mg/kg dose liver weight had been decreased significantly ( $p < 0.001$ ).

**Hematological parameters:** Pyridostigmine treatment at 45 mg/kg dose significantly ( $p < 0.01-0.05$ ) decreased RBC total count upon 15 days of treatment and significant decrease in hemoglobin content was observed only after 45 days of treatment. Total WBC count was

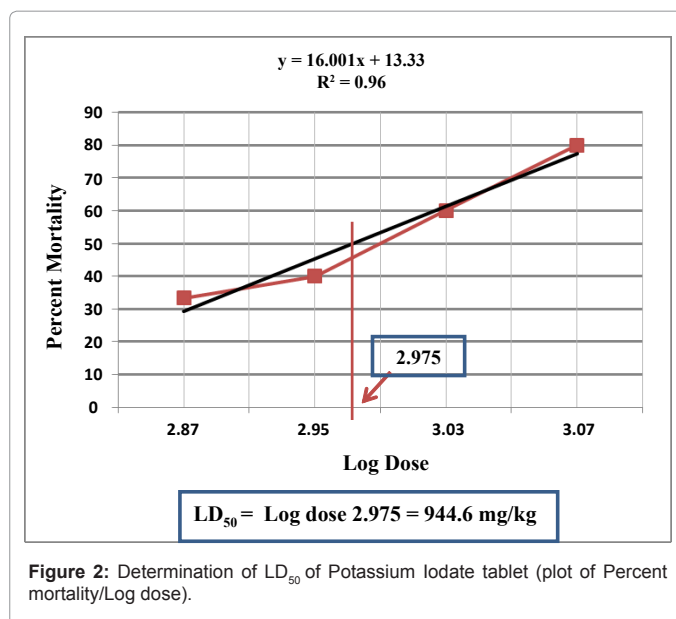
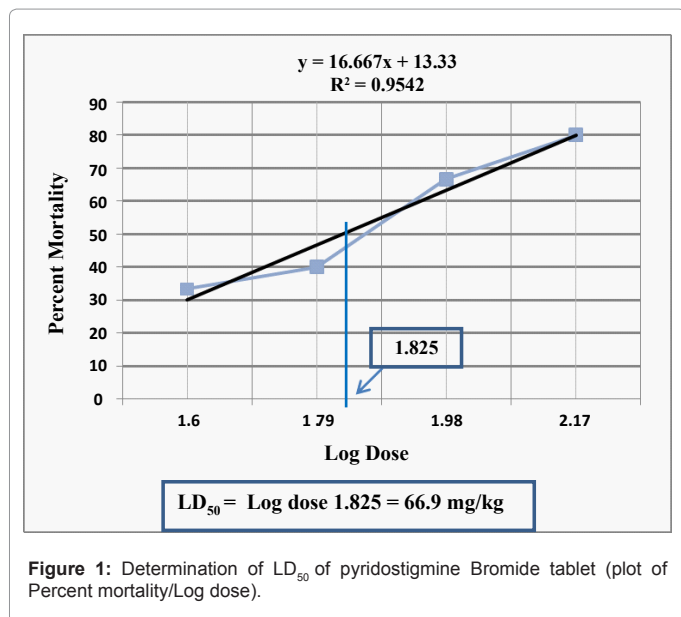


Figure 2: Determination of LD<sub>50</sub> of Potassium Iodate tablet (plot of Percent mortality/Log dose).

significantly ( $p < 0.01-0.05$ ) increased on 30 and 45 mg/kg dose after 15 days with extremely significant ( $p < 0.001$ ) increase in differential eosinophil count (Table 4).

**Biochemical parameters:** The animals treated with 45 mg/kg dose of PB tablet showed highly significant ( $p < 0.001-0.05$ ) increase in serum SGOT, SGPT, ALP, cholesterol, triglyceride and LDL, and decrease in HDL level (Table 5). PB has nonsignificant effect on serum total protein and glucose content at all the tested doses.

**Histopathology of liver and kidney:** PB tablet showed cloudy swelling of some hepatocytes with focal inflammatory piecemeal necrosis at 20 mg/kg dose in histopathology slides of liver. Pyridostigmine 30 mg/kg dose treated animals showed hepatocytes with multiple, prominent and enlarged nuclei and cytoplasmic acidophilic bodies whereas at 45 mg/kg dose prominent acidophilic bodies were seen in most hepatocytes with micro- and macro vesicular steatosis (Figure 3).

Photomicrograph of kidney tissue from pyridostigmine 30 mg/kg dose treated animals showed diffuse tubular injury with neutrophil polymorph infiltration, tubular dilatation, tubular atrophy, interstitial inflammatory cells infiltration and fibrosis, whereas at 45 mg/kg dose protein casts, glomerulosclerosis and thickening of basement membranes were also found (Figure 4).

### Subacute toxicity of potassium iodate tablet

**Body weight and relative organ weight:** Vehicle treated animals showed 29.62% increase in body weight after 45 days. PI treatment at 50 and 85 mg/kg dose showed normal body weight gain with average 26.75 and 25.86% increase in body weight, whereas 150 mg/kg dosing showed reduced weight gain by 15.71% only after 45 days of continuous treatment (Table 6). Effect of PI treatment at 50, 85 and 150 mg/kg dose has nonsignificant effect on relative weight of kidney, heart, liver and spleen of rat (data not tabulated).

**Hematological parameters:** PI treatment for 45 days on 50, 85 and 150 mg/kg dose have nonsignificant effect on RBC and WBC total count, hemoglobin content and also on WBC differential count (data not tabulated).

Treatment (mg/kg, p.o)	% change in body weight						
	7 <sup>th</sup> day	14 <sup>th</sup> day	21 <sup>st</sup> day	28 <sup>th</sup> day	35 <sup>th</sup> day	42 <sup>nd</sup> day	45 <sup>th</sup> day
Vehicle control	+4.16	+7.33	+8.71	+12.87	+17.42	+21.59	+26.50
Pyridostigmine (20)	+2.67	+6.25	+4.04	+4.15	+8.33	+8.33	+6.42
Pyridostigmine (30)	+1.43	+0.94	+3.56	+2.45	+5.34	+5.83	+4.26
Pyridostigmine (45)	-5.27	-6.82	-7.46	-10.40	-11.48	-14.05	-15.62

**Table 2:** Effect of daily oral administration of Pyridostigmine Bromide tablet up to 45 days on relative body weight of rats.

Treatment (mg/kg, p.o)	Relative organ weight on 45 <sup>th</sup> day (gm/100 gm of body weight) M ± SEM			
	Liver	Kidney	Heart	Spleen
Vehicle control	5.374 ± 0.104	1.223 ± 0.027	0.483 ± 0.029	0.304 ± 0.036
Pyridostigmine (20)	5.066 ± 0.082 <sup>ns</sup>	1.174 ± 0.052 <sup>ns</sup>	0.516 ± 0.090 <sup>ns</sup>	0.272 ± 0.045 <sup>ns</sup>
Pyridostigmine (30)	3.915 ± 0.040 <sup>***</sup>	1.104 ± 0.025 <sup>ns</sup>	0.432 ± 0.053 <sup>ns</sup>	0.202 ± 0.017 <sup>ns</sup>
Pyridostigmine (45)	3.407 ± 0.282 <sup>***</sup>	1.101 ± 0.090 <sup>ns</sup>	0.345 ± 0.055 <sup>ns</sup>	0.194 ± 0.054 <sup>ns</sup>

n=10. \*\*\*P<0.001 and ns=not significant when compared to respective control group

**Table 3:** Effect of daily oral administration of Pyridostigmine Bromide tablet up to 45 days on relative organ weight of rats.

Day	Pyrido-stigmine (mg/kg, p.o)	Hb (gm/dl) M ± SEM	RBC (10 <sup>9</sup> /mm <sup>3</sup> ) M ± SEM	WBC (10 <sup>3</sup> /mm <sup>3</sup> ) M ± SEM	Differential leukocyte count in % (M ± SEM)				
					N	L	E	B	M
0	control	12.70 ± 1.50	4.78 ± 0.13	5.02 ± 0.87	38.25 ± 2.27	52.28 ± 1.94	1.77 ± 0.07	1.57 ± 0.11	6.13 ± 0.88
	20	12.40 ± 1.84 <sup>ns</sup>	4.91 ± 0.85 <sup>ns</sup>	5.42 ± 0.23 <sup>ns</sup>	40.45 ± 2.45 <sup>ns</sup>	48.14 ± 1.62 <sup>ns</sup>	1.25 ± 0.11 <sup>ns</sup>	2.04 ± 0.01 <sup>ns</sup>	8.12 ± 0.67 <sup>ns</sup>
	30	12.82 ± 1.36 <sup>ns</sup>	5.75 ± 0.24 <sup>ns</sup>	5.18 ± 0.45 <sup>ns</sup>	44.09 ± 2.68 <sup>ns</sup>	46.61 ± 1.69 <sup>ns</sup>	2.40 ± 0.13 <sup>*</sup>	1.18 ± 0.02 <sup>ns</sup>	5.72 ± 0.93 <sup>ns</sup>
	45	13.15 ± 1.12 <sup>ns</sup>	5.91 ± 0.42 <sup>ns</sup>	5.09 ± 0.42 <sup>ns</sup>	42.13 ± 3.67 <sup>ns</sup>	46.70 ± 1.58 <sup>ns</sup>	2.52 ± 0.22 <sup>*</sup>	2.58 ± 0.58 <sup>ns</sup>	6.07 ± 0.50 <sup>ns</sup>
15	control	12.56 ± 1.97	5.11 ± 0.25	5.20 ± 0.79	40.22 ± 1.51	50.17 ± 1.60	1.98 ± 0.02	0.92 ± 0.14	6.71 ± 0.84
	20	13.42 ± 1.23 <sup>ns</sup>	5.78 ± 0.41 <sup>ns</sup>	8.32 ± 0.35 <sup>*</sup>	34.57 ± 2.30 <sup>ns</sup>	52.70 ± 3.97 <sup>ns</sup>	3.80 ± 0.27 <sup>ns</sup>	1.09 ± 0.22 <sup>ns</sup>	7.84 ± 0.97 <sup>ns</sup>
	30	11.08 ± 1.11 <sup>ns</sup>	4.72 ± 0.21 <sup>ns</sup>	8.97 ± 0.36 <sup>*</sup>	32.43 ± 2.39 <sup>ns</sup>	53.52 ± 2.11 <sup>ns</sup>	7.51 ± 0.32 <sup>***</sup>	1.44 ± 0.33 <sup>ns</sup>	5.10 ± 0.56 <sup>ns</sup>
	45	9.86 ± 1.14 <sup>ns</sup>	3.71 ± 0.20 <sup>**</sup>	9.34 ± 1.34 <sup>**</sup>	34.45 ± 3.12 <sup>ns</sup>	52.90 ± 4.22 <sup>ns</sup>	8.00 ± 0.97 <sup>***</sup>	1.22 ± 0.11 <sup>ns</sup>	3.43 ± 0.33 <sup>*</sup>
30	control	12.80 ± 1.56	5.45 ± 0.56	5.35 ± 0.78	39.24 ± 2.67	48.57 ± 1.55	1.26 ± 0.09	1.13 ± 0.01	9.80 ± 0.39
	20	11.50 ± 1.22 <sup>ns</sup>	4.55 ± 0.43 <sup>ns</sup>	8.95 ± 1.02 <sup>ns</sup>	31.08 ± 2.97 <sup>ns</sup>	52.93 ± 3.97 <sup>ns</sup>	5.32 ± 0.81 <sup>**</sup>	1.33 ± 0.03 <sup>ns</sup>	9.34 ± 0.49 <sup>ns</sup>
	30	9.83 ± 1.27 <sup>ns</sup>	4.10 ± 0.62 <sup>ns</sup>	9.76 ± 1.21 <sup>*</sup>	30.25 ± 2.17 <sup>ns</sup>	53.07 ± 4.97 <sup>ns</sup>	8.12 ± 0.94 <sup>***</sup>	1.05 ± 0.07 <sup>ns</sup>	8.51 ± 0.94 <sup>ns</sup>
	45	9.02 ± 1.31 <sup>ns</sup>	3.02 ± 0.55 <sup>*</sup>	10.94 ± 1.46 <sup>**</sup>	30.86 ± 4.07 <sup>ns</sup>	52.48 ± 3.84 <sup>ns</sup>	8.65 ± 1.05 <sup>***</sup>	1.30 ± 0.06 <sup>ns</sup>	6.71 ± 0.82 <sup>*</sup>
45	control	12.76 ± 1.23	5.29 ± 0.56	5.60 ± 0.41	37.62 ± 3.40	50.25 ± 1.90	2.14 ± 0.07	1.42 ± 0.03	8.93 ± 1.11
	20	9.34 ± 1.36 <sup>ns</sup>	3.83 ± 0.25 <sup>ns</sup>	9.35 ± 1.33 <sup>*</sup>	30.97 ± 2.67 <sup>ns</sup>	51.50 ± 3.43 <sup>ns</sup>	5.35 ± 0.97 <sup>*</sup>	1.58 ± 0.02 <sup>ns</sup>	10.60 ± 1.45 <sup>ns</sup>
	30	9.42 ± 1.99 <sup>ns</sup>	3.38 ± 0.62 <sup>ns</sup>	10.33 ± 1.17 <sup>**</sup>	27.80 ± 2.53 <sup>ns</sup>	52.28 ± 3.27 <sup>ns</sup>	10.35 ± 1.09 <sup>***</sup>	1.45 ± 0.03 <sup>***</sup>	8.22 ± 1.06 <sup>ns</sup>
	45	8.09 ± 1.07 <sup>*</sup>	3.04 ± 0.77 <sup>*</sup>	10.90 ± 0.69 <sup>**</sup>	27.03 ± 2.07 <sup>*</sup>	54.44 ± 3.17 <sup>ns</sup>	10.45 ± 1.22 <sup>***</sup>	1.04 ± 0.07 <sup>***</sup>	7.04 ± 1.02 <sup>ns</sup>

n=10. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 and ns=not significant when compared to respective control group

**Table 4:** Effect of daily oral administration of Pyridostigmine Bromide tablet up to 45 days on peripheral blood parameters of rats.

Day	Treatment (mg/kg, p.o)	SGOT (IU/L) M ± SEM	SGPT (IU/L) M ± SEM	ALP (U/I) M ± SEM	Cholesterol (mg/dl) M ± SEM	Triglyceride (mg/dl) M ± SEM	Protein (gm/dl) M ± SEM	HDL (mg/dl) M ± SEM	LDL (mg/dl) M ± SEM	Glucose (gm/dl) M ± SEM
45	Vehicle control	44.72 ± 2.66	42.21 ± 3.15	105.74 ± 4.34	119.70 ± 3.22	63.50 ± 3.70	7.63 ± 0.14	40.67 ± 2.86	76.33 ± 2.11	109.72 ± 5.30
	Pyridostigmine (20)	47.23 ± 2.29 <sup>ns</sup>	44.25 ± 2.56 <sup>ns</sup>	112.49 ± 3.04 <sup>ns</sup>	126.86 ± 5.75 <sup>ns</sup>	116.90 ± 5.10 <sup>***</sup>	7.95 ± 0.81 <sup>ns</sup>	39.06 ± 2.76 <sup>ns</sup>	76.42 ± 3.14 <sup>ns</sup>	110.26 ± 2.88 <sup>ns</sup>
	Pyridostigmine (30)	56.46 ± 3.73 <sup>ns</sup>	52.87 ± 2.64 <sup>ns</sup>	120.80 ± 3.50 <sup>*</sup>	141.32 ± 5.16 <sup>***</sup>	146.97 ± 5.22 <sup>***</sup>	9.55 ± 0.68 <sup>ns</sup>	38.77 ± 3.22 <sup>ns</sup>	3.15 ± 3.82 <sup>ns</sup>	115.80 ± 3.03 <sup>ns</sup>
	Pyridostigmine (45)	62.72 ± 3.45 <sup>**</sup>	54.24 ± 3.11 <sup>*</sup>	132.56 ± 4.33 <sup>***</sup>	150.87 ± 5.12 <sup>***</sup>	161.15 ± 3.33 <sup>***</sup>	10.08 ± 1.23 <sup>ns</sup>	27.34 ± 1.12 <sup>**</sup>	91.30 ± 3.56 <sup>*</sup>	120.03 ± 3.25 <sup>ns</sup>

n=10. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 and ns=not significant when compared to respective control group

**Table 5:** Effect of daily oral administration of Pyridostigmine Bromide tablet up to 45 days on serum biochemical parameters of rat.

**Biochemical parameters:** The animals treated with 50, 85 and 150 mg/kg dose of PI tablet showed extremely significant (p<0.001) increase in serum SGOT and ALP level after 45 days. Cholesterol and triglyceride level was significantly (p<0.001) increased at 85 and 150 mg/kg dose and serum glucose decreased only at 150 mg/kg dose treatment. HDL was increased and LDL decreased only at 50 mg/kg dose and at higher doses levels were unchanged. The SGPT and total protein concentration of serum was unaltered by PI at all the tested doses (Table 7).

**Histopathology of liver and kidney:** PI tablet treatment at 50, 85 and 150 mg/kg dose had no adverse effect on liver and kidney

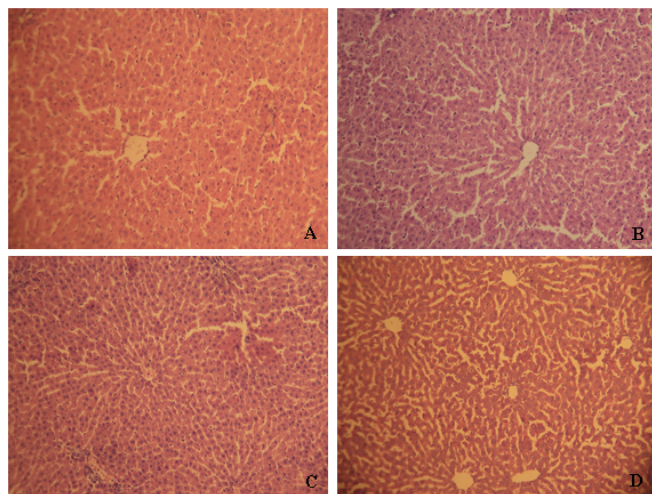
architectures. Liver slide showed clear hepatic lobule, radial liver cell cord and hepatic sinusoid in all the treated groups. Kidney slides showed focal area of tubular injury with neutrophil polymorph infiltration and interstitial inflammatory cell infiltration only at 150 mg/kg dose (Figures 5 and 6).

### Stability under accelerated conditions

**Physical stability:** The physical stability of PB 30 mg and PI 85 mg tablet proved to be unchanged after storage upto 3 months in accelerated storage condition at 40 ± 2°C / 75 ± 5% RH. The results obtained for the test item appearance had not changed significantly.

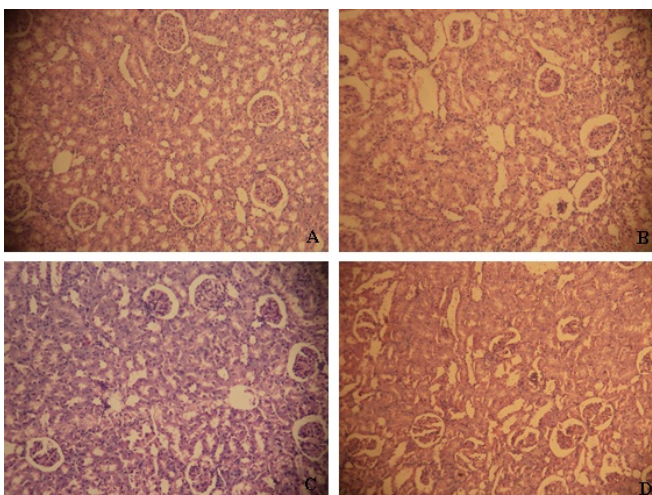
**Chemical stability:** Storage upto 3 months under accelerated





**Figure 3:** Photomicrograph of liver tissue from pyridostigmine bromide treated animals.

(A) vehicle control showing clear hepatic lobule, radial liver cell cord and clear hepatic sinusoid; (B) pyridostigmine 20 mg/kg dose treated animals showing cloudy swelling of some hepatocytes with focal inflammatory piecemeal necrosis; (C) pyridostigmine 30 mg/kg dose treated animals showing hepatocytes with multiple, prominent and enlarged nuclei started to appear and cytoplasmic acidophilic bodies and (D) pyridostigmine 45 mg/kg dose treated animals showing prominent acidophilic bodies in most hepatocytes with micro- and macro vesicular steatosis (10 $\times$ ).



**Figure 4:** Photomicrograph of kidney tissue from pyridostigmine bromide treated animals.

(A) vehicle control animals and (B) pyridostigmine 20 mg/kg dose treated animals showing normal kidney architecture and histology; (C) pyridostigmine 30 mg/kg dose treated animals showing diffuse tubular injury with neutrophil polymorph infiltration, tubular dilatation, tubular atrophy, interstitial inflammatory cells infiltration and fibrosis and (D) pyridostigmine 45 mg/kg dose treated animals showing microcystic tubular dilatation, tubular casts and glomerulosclerosis. Thickening of basement membranes, dilated tubules, protein casts and interstitial inflammation was also found (10 $\times$ ).

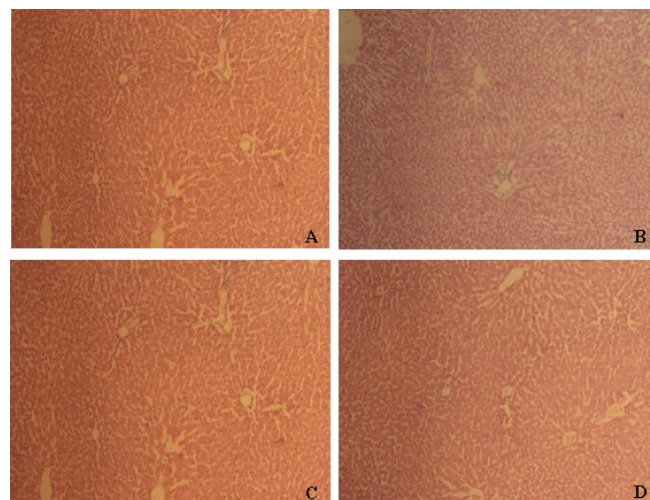
conditions ( $40 \pm 2^\circ\text{C}/75 \pm 5\% \text{RH}$ ) had no significant effect on the chemical stability of the drug product. With regard to organic impurity only slight changes were observed (HPLC chromatogram). The content of PB and PI has not been changed significantly compared to the initial values.

### Stability under intermediate and long term conditions

Storage for 6 months in intermediate term condition ( $30 \pm 2^\circ\text{C}/65 \pm 5\% \text{RH}$ ) and for 12 months in long term ( $25 \pm 2^\circ\text{C}/60 \pm 5\% \text{RH}$ ) condition do not have any adverse effect on stability of the products (Tables 8 and 9).

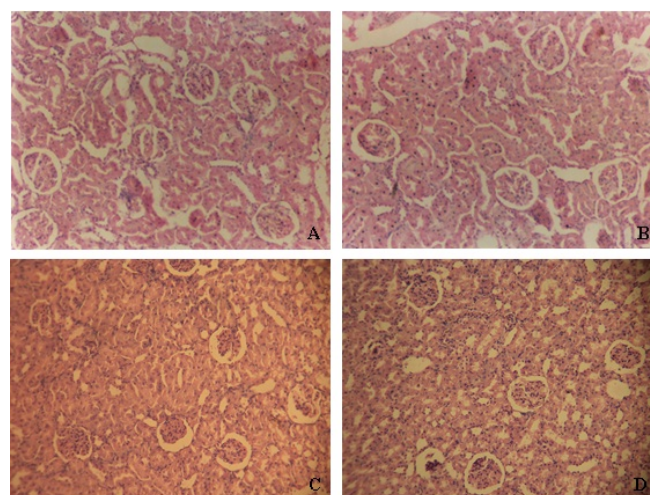
### Discussion

PB is used for more than 50 years in the routine treatment



**Figure 5:** Photomicrograph of liver tissue from potassium bromide treated animals.

(A) Vehicle control animals showing clear hepatic lobule, radial liver cell cord and clear hepatic sinusoid; (B) potassium iodate 50 mg/kg; (C) potassium iodate 85 mg/kg dose treated animals and (D) potassium iodate 150 mg/kg dose treated animals all showing normal liver architecture and hepatocytes (10 $\times$ ).



**Figure 6:** Photomicrograph of kidney tissue from potassium iodate treated animals.

(A) Vehicle control animals; (B) potassium iodate 50 mg/kg dose treated animals and (C) potassium iodate 85 mg/kg dose treated animals showing normal kidney architecture and histology; (D) potassium iodate 150 mg/kg dose treated animals showing focal area of tubular injury with neutrophil polymorph infiltration and interstitial inflammatory cell infiltration (10 $\times$ ).

Treatment (mg/kg, p.o)	% increase in body weight						
	7 <sup>th</sup> day	14 <sup>th</sup> day	21 <sup>st</sup> day	28 <sup>th</sup> day	35 <sup>th</sup> day	42 <sup>nd</sup> day	45 <sup>th</sup> day
Vehicle control	+4.16	+7.33	+8.71	+12.87	+17.42	+21.59	+26.50
Potassium Iodate (50)	+4.71	+7.78	+10.32	+12.05	+19.95	+22.71	+26.75
Potassium Iodate (85)	+3.15	+6.84	+10.40	+13.29	+17.11	+20.46	+25.86
Potassium Iodate (150)	+4.70	+5.84	+7.69	+8.90	+9.23	+10.34	+15.71

Table 6: Effect of daily oral administration of Potassium Iodate tablet up to 45 days on relative body weight of rats.

Day	Treatment (mg/kg, p.o)	SGOT (IU/L) M ± SEM	SGPT (IU/L) M ± SEM	ALP (U/l) M ± SEM	Cholesterol (mg/dl) M ± SEM	Triglyceride (mg/dl) M ± SEM	Protein (gm/dl) M ± SEM	HDL (mg/dl) M ± SEM	LDL (mg/dl) M ± SEM	Glucose (gm/dl) M ± SEM
45	Vehicle control	44.72 ± 2.66	42.21 ± 3.15	105.74 ± 4.34	119.70 ± 3.22	63.50 ± 3.70	7.60 ± 0.11	40.67 ± 2.86	76.33 ± 2.11	109.72 ± 5.30
	Potassium Iodate (50)	111.23 ± 2.69***	49.15 ± 4.46 <sup>ns</sup>	262.28 ± 4.21***	132.95 ± 5.25 <sup>ns</sup>	67.58 ± 3.10 <sup>ns</sup>	8.72 ± 0.41 <sup>ns</sup>	57.16 ± 3.76**	62.27 ± 3.14**	102.23 ± 4.28 <sup>ns</sup>
	Potassium Iodate (85)	137.93 ± 9.03***	56.89 ± 4.64 <sup>ns</sup>	397.02 ± 14.02***	153.76 ± 5.60***	123.85 ± 3.20***	7.63 ± 0.45 <sup>ns</sup>	44.45 ± 3.22 <sup>ns</sup>	85.71 ± 2.82 <sup>ns</sup>	98.34 ± 5.03 <sup>ns</sup>
	Potassium Iodate (150)	208.96 ± 12.45***	54.42 ± 4.11 <sup>ns</sup>	377.05 ± 14.33***	167.36 ± 5.12***	91.94 ± 3.33***	8.69 ± 0.23 <sup>ns</sup>	46.19 ± 2.12 <sup>ns</sup>	84.71 ± 3.56 <sup>ns</sup>	73.28 ± 4.95***

n=10. \*\*P<0.01, \*\*\*P<0.001 and ns=not significant when compared to respective control group

Table 7: Effect of daily oral administration of Potassium Iodate tablet up to 45 days on serum biochemical parameters of rat.

Drug product: Pyridostigmine Bromide			Dosage: 30 mg				Packaging: Amber glass bottle			
Storage		Appearance	Average weight (%)	Hardness [N]	Friability [%]	Disintegration time [%] 15 min	Dissolution time [t <sub>80%</sub> ] 60 min	Moisture content [water absorption ratio]	Content (Assay) [% LC]	
Months	Conditions									
Specifications		White, round, flat tablet	<5%	≥ 70 N	≤ 1%	≥ 15 min	≥ 70%	>10%	95.0-105.0%	
Initial	--	Complies	4.34%	116.4 N	0.27%	3.05 min	82.55%	77.98%	98.73%	
1	40 ± 2°C/ 75 ± 5% RH	Complies	4.68%	114.2 N	0.27%	2.46 min	81.95%	91.42%	98.52%	
3		Complies	4.75%	111.5 N	0.35%	2.35 min	85.05%	96.22%	97.45%	
6		Complies	4.98%	112.3 N	0.62%	1.47 min	83.91%	124.02%	95.74%	
3	25 ± 2°C/ 60 ± 5% RH	Complies	4.50%	114.4 N	0.32%	2.00 min	82.35%	92.02%	97.28%	
6		Complies	4.98%	113.6 N	0.34%	1.56 min	81.52%	91.09%	96.52%	
9		Complies	4.89%	114.2 N	0.32%	1.59 min	81.50%	91.90%	96.16%	
12		Complies	5.00%	114.5 N	0.27%	2.09 min	73.98%	95.04%	96.33%	
3	30 ± 2°C/ 65 ± 5% RH	Complies	4.68%	115.8 N	0.29%	2.75 min	78.22%	89.05%	98.52%	
6		Complies	4.59%	112.7 N	0.32%	2.22 min	76.60%	91.56%	96.80%	

Table 8: Summary of stability study results for pyridostigmine bromide 30 mg tablet.

Drug product: Potassium Iodate			Dosage: 85 mg				Packaging: PVC blister			
Storage		Appearance	Average weight (%)	Hardness [N /kg/cm <sup>2</sup> ]	Friability [%]	Disintegration time [Sec%]	Dissolution time [t <sub>75%</sub> ] 15 min	Moisture content [ratio]	Content (Assay) [% LC]	
Months	Conditions									
Specifications		White, round, flat tablet	<5%	≥ 70 N	≤ 1%	≥ 15 min	≥ 70%	>10%	95.0-105.0%	
Initial	--	Complies	4.35%	113.2 N	0.31%	1.85 min	87.8%	124.66%	97.30%	
1	40 ± 2°C/ 75 ± 5% RH	Complies	4.60%	112.2 N	0.62%	1.80 min	88.7%	167.02%	96.74%	
3		Complies	4.55%	109.6 N	0.74%	1.72 min	89.5%	174.55%	95.75%	
6		Complies	4.57%	104.5 N	0.86%	1.78 min	90.0%	1.82.34%	95.43%	
3	25 ± 2°C/ 60 ± 5% RH	Complies	4.49%	114.5 N	0.34%	1.86 min	78.9%	130.40%	98.22%	
6		Complies	4.40%	112.2 N	0.44%	1.72 min	80.0%	151.33%	97.02%	
9		Complies	4.89%	110.5 N	0.56%	1.56 min	83.9%	160.24%	97.11%	
12		Complies	4.92%	106.8 N	0.60%	1.45 min	86.7%	167.29%	95.30%	
3	30 ± 2°C/ 65 ± 5% RH	Complies	4.50%	112.8 N	0.42%	1.75 min	88.5%	165.47%	96.90%	
6		Complies	4.67%	111.5 N	0.55%	1.56 min	89.3%	171.20%	95.30%	

Table 9: Summary of stability study results for potassium iodate 85 mg tablet.

of myasthenia gravis and following surgery in the reversal of neuromuscular blockade. In the treatment of myasthenia gravis the average oral dose is 600 mg per day in divided doses however the dose must be adjusted to the needs of the individual patient [26]. PB was used during the Gulf War as a pretreatment to protect troops from the harmful effects of nerve agents. PB, a carbamate compound is the dimethylcarbamate ester of 3-hydroxy-1-methylpyridinium bromide. After returning from Gulf war thousands of U.S. military personnel's

complained of fatigue, sleep disturbance, muscle and joint pain and cognitive dysfunction. PB is used safely in patients of myasthenia gravis in dose as high as 600 mg/day though it could have different action in normal person without these disorders [2].

Bowman et al., reported alterations in muscle morphology after subchronic oral administration of PB for 15 days at 90 mg/kg dose, continuously in meal. This treatment causes 74–91% inhibition



of cholinesterase activity demonstrating shrunken, disruption of myofilaments, mitochondrial changes consistent with accumulation of calcium and nuclear alterations [27]. Beagle dogs of both sexes were administered PB orally once daily at 5, 10, or 20 mg/kg for 3 months as part of preclinical safety assessment. Daily doses of 10 or 20 mg/kg were lethal to some of the dogs when given up to 14 days and caused severe intestinal distress, including diarrhea, emesis, and reddened feces in all animals but these signs appeared to reverse upon discontinuation of the drug. The cause of death was intestinal intussusception. Systemic toxicity included hypersalivation, tremors and inhibition of plasma cholinesterase activities. AchE activities were also inhibited as reported by Morgan et al. [28]. These data suggest that prolonged oral administration of pyridostigmine at 20 mg/kg dose sufficient to cause profound and sustained inhibition of AchE activity (i.e., as high as 70%) cause mainly local, gastrointestinal distress related to altered intestinal motility.

Levine et al., evaluated the oral toxicity of PB in rats at doses of 0, 5, 15, 30, or 60 mg/kg/day administered for 13 weeks by daily gavage [29]. At dose of 15 mg/kg/day or greater exaggerated cholinergic stimulation with tremors and inhibition of AchE were found. Transient muscular weakness has occurred in 10-20% of neonates whose mothers received anticholinesterase drugs for the treatment of myasthenia gravis, although similar symptoms have also been reported in infants whose mothers were not treated with these drugs [30]. Haaren et al. [31] evaluate the behavioral effects of PB in male and female rats in the context of development of a syndrome in afflicted military personnel who served during the Gulf War [31]. The results of this experiment showed that small doses of PB disrupt well-established, schedule-controlled behavior in male and female rats in a schedule- and gender-dependent manner.

Number of studies has been performed to evaluate acute and subchronic oral toxicity of PB on muscle, autonomic nervous system and gastrointestinal track along with behavioral effect. Studies were essentially required to establish safety profile of military personals exposed to PB for long duration focusing on gross body parameters, hematology, vital organs and biochemical aspects. PB, which has a steep dose-response curve, is poorly absorbed by the gut. It is possible, therefore, that an increase in the bioavailability of PB could cause increased lethality seen in this study. PB, and other carbamates, are reversible inhibitors of both AchE (true cholinesterase) and nonspecific esterase (pseudocholin-AchE). Quaternary carbamates do not easily pass the blood brain barrier and their effect, therefore, is primarily on the peripheral nervous system. Metabolic and kinetic studies indicate that PB is poorly absorbed from the gut and, in order to produce a pharmacological effect, high levels must be given orally [32,33]. LD<sub>50</sub> of PB tablet was estimated to be 66.9 mg/kg higher than 61.6 mg/kg reported by Pool and Hane [3] lower than 80 mg/kg reported by McCain et al. [2], Latven and Sloane [4]. Bioavailability of PB after oral administration have ranged from a low of 3-4% to a high of nearly 30%, with a large variability between subjects, the mean bioavailability was 29.1% with a range of 14.7 to 51.1% as reported by Aquilonius and Hartvig may be possible reason of variable LD<sub>50</sub> in different setting [34].

PB is biotransformed in the blood and in the liver. The main metabolite 3-hydroxy-N-methylpyridinium is formed from reaction with plasma cholinesterases in the blood; this metabolite is then rapidly glucuronidated in the liver [33]. PB administration for 45 days at dose of 45 mg/kg/day showed body weight loss. Decrease in liver weight with enlarged nuclei, cytoplasmic acidophilic bodies and vesicular steatosis was found. Drug induced phospholipidosis causes

neutrophilic infiltration, steatosis, where as acidophilic bodies signify acute hepatocellular injury [35]. Kidney tissue developed neutrophil polymorph infiltration signifying inflammatory glomerulonephritis, tubular dilatation, protein casts, glomerulosclerosis and thickening of basement membranes at 45 mg/kg dose. The liver is the largest organ in the body and serves many vital functions in human body such as remove damaged RBCs from the blood in co-ordination with spleen, produces bile, clotting factors, stores vitamins, minerals, protein, fats and glucose from diet [36,37]. The most important task of the liver is detoxification substances like alcohol and, different medications such as chemotherapeutic drugs, antibiotics and toxicants. Chemical agents and toxins impose excess stress on the liver filtering function. Liver removes harmful chemical agents and toxins through the bile or urine and if accumulation of toxins is faster than the liver metabolizing ability, hepatic damage may occur [38].

It also showed decrease in RBC and hemoglobin content in blood with associated increase in eosinophil count. PB causes 49% AchE inhibition in erythrocytes at 10 mg/kg dose for 180 days and at 90 mg/kg dose 95% inhibition for 90 days. The addition of pyridostigmine to the diet resulted in dose-related decreases in plasma cholinesterase and erythrocyte AchE activity. Toxic signs were found associated with the decrease in cholinesterase activity included muscarinic (perianal, perioral, and periocular stains or material, diarrhea, and increased salivation) and nicotinic (hypertonia and tremors) effects [39,40]. Location of AchE at or near the outer cell membrane surface of RBC might have relevance for basic disease processes at the cellular level, and alterations in RBC activity associated with pathogenic conditions [41]. In Operations Desert Shield and Desert Storm approximately 40,000 who took the drug, about half experienced mild gastrointestinal problems and 28 had to discontinue its use because of more severe adverse effects, including exacerbation of asthma and allergy, hypertension, and severe gastrointestinal complaints. Increased neutrophil count as seen in the study may be due to allergic reaction to pyridostigmine or to bromide.

Serum lipid profile was elevated along with increase in marker enzyme concentration viz. SGOT, SGPT and ALP. Necrosis or membrane damage releases the enzyme SGOT, SGPT and ALP into circulation, Estimation of these enzymes in the serum is useful quantitative markers of the extent and type of cellular damage. High levels of SGOT indicate the loss of functional integrity of liver, such as that of viral hepatitis, as well as cardiac infraction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner [42]. Liver toxicants cause disturbances in synthesis and metabolism of triglycerides, cholesterol and lipoproteins, the increased triglyceride content in the blood is in correlation with the fatty degeneration of the liver [43]. Increases in aspartate aminotransferase, lactate dehydrogenase, and creatine phosphokinase were observed at 210 days of pyridostigmine administration but the changes could not be attributed to compound administration/withdrawal [39].

Kluwe et al. [39] reported absence of pathological changes in clinical chemistry, hematology, or urinalysis parameters, associated with PB administration for up to 3 months at maximum 20 mg/kg dose, there was no drug-related lesions observed upon gross necropsy and microscopic evaluation of the major tissues and organs [28]. PB at this dose is sufficient to cause as high as 70% inhibition of AchE activity with mainly local, gastrointestinal distress related to altered intestinal motility. This study shows that PB above 40 mg/kg is toxic in concern to hematology, serum biochemical parameters and physiology of liver



and kidney when administered for over one month continuously. It is important to realize that PB is not an antidote and it has no value when administered after nerve-agent exposure [44]. PB is an antidote enhancer rather than a pretreatment capable of acting by itself in the absence of antidotes [45]. However, the pre-exposure use of PB has become entrenched in medical and military settings. Pyridostigmine at 30 mg/kg/day dose is safe in rats which are equivalent to average of 60 tablets in a day considering a 30 mg tablet so the required dose 30 mg thrice a day a total of 90 mg/day is safe for human use.

The oral acute median lethal of PI was found to be 944.6 mg/kg on rat. In earlier studies oral LD<sub>50</sub> was found to be 531 mg/kg in fasted mice and 1177 mg/kg in nonfasted mice [5]. Webster et al. [46] reported that average daily exposure of 1231 mg/kg PI for 2 weeks in drinking water was without much toxic effect and little mortality indicating a marked increase in toleration of iodate when given in divided, small doses with food. Single-dose, acute toxicity experiments the iodates cause intoxication and death attributed to renal damage with retention of non-protein nitrogen as reported by Webster et al. [46]. Hemolytic effects are evidenced by hemoglobinuria, and histologically by hemoglobin casts and hemosiderin deposits in the kidneys. Fatty visceral changes appear within 24 h after ingestion of iodates. Similar changes occur in fasted animals. Oral administration of PI at level above 140 mg/kg often increases the pH of the gastric contents with frequent accompanying degenerative changes in the parietal cells [47].

PI only at 150 mg/kg dose showed anorexia and loss in body weight on 45 days of continuous administration with nonsignificant effect on relative weight of kidney, heart, liver and spleen whereas at 50 and 85 mg/kg dose apparent devoid of any adverse effect. Hematological parameters were normal in all the groups treated. Above 250 mg/kg/day exposure in mice PI significantly lowers RBC and Hb concentration [46]. Interference of iodate or iodine respectively with iron uptake or utilization attribute to toxic signs which were either iron deficiency or general iodine toxicity. Webster et al. reported the minimum lethal dose for oral PI to be 200-250 mg/kg in dogs which invariably caused emesis [46]. At 200 mg/kg dose showed fatty changes in the viscera, necrotic lesions in the liver, kidney, and mucosa of the gastrointestinal tract and urinary bladder was found. Retinal degeneration was sometimes present after such high doses. In subacute toxicity study repeated doses of PI between 6-100 mg/kg were given in milk for several months. Emesis followed by anorexia and listlessness sometimes occurred in these animals. Pathologic changes in the sacrificed animals were confined largely to deposits of hemosiderin in the spleen, liver and kidneys, and mild to moderate inflammation of the mucosa of the gastrointestinal tract. Hematologic changes indicating a mild anemia were also found [47]. In this study serum SGOT, ALP, cholesterol and triglyceride level were elevated at 85 and 150 mg/kg dose of PI and serum glucose decreased only at 150 mg/kg dose. Pathologic changes occurred only at 150 mg/kg dose in kidney having focal area of tubular injury with neutrophil polymorph inflammatory cell infiltration.

Swiss mice received 0.05 to 0.65% PI in drinking water for 15-16 weeks. All mice that received iodate in the drinking water appeared healthy and showed no toxic symptoms at the end of the experiment, however hematologic and pathologic studies indicated hemolysis in animals getting 0.25% (540 mg/kg/day) and higher concentration of PI. Hemosiderin deposition attributable to hemolysis by iodate in the renal convoluted tubules were found in few of the 0.25% PI exposed group and nearly in all mice of the 0.50% and 0.75% (1256 mg/kg/day) groups. Similar subacute toxicity experiments carried out for 4 weeks on guinea pigs received 0.25 and 0.05% of PI. Both iodate groups

remained in apparent good health and showed no significant alterations in the blood picture and no gross or microscopic changes attributable to iodate. The daily consumption of PI in the drinking water by some mice and guinea pigs at times exceeded the estimated oral LD<sub>50</sub> values for single doses of iodate given by stomach tube [46].

Acceleration of chemical decomposition can be achieved by raising the temperature of drug product in an environment of high relative humidity and controlled temperature in its original packing. The results of accelerated storage tests carried out at more elevated temperature enables prediction to be made of the effective life of product at normal temperature. Maximum and minimum time at which the potency must be at least 90% of label claim (meet all specification), at the temperature indicated in order to predict shelf life for two years at room temperature. At least 6 months accelerated and 6 months long term data is a need for regulatory approval. Storage under accelerated testing conditions caused insignificant change of PB and PI assay results in concern to physical and chemical stability. The content of PB and PI has not been changed significantly compared to the initial values in intermediate and long term storage.

## Conclusion

PB (30 mg) and PI (85 mg) tablets were prepared as per British Pharmacopoeial specification. Validation of stability parameters showed product stability in accelerated condition for 3 months at 40°C and long term for 12 months at 25°C. The median lethal dose of PB and PI was 66.9 and 944.6 mg/kg respectively on rat. PB showed bodyweight loss and decrease in liver weight and increased neutrophil count may be due to allergic reaction to pyridostigmine or bromide. Liver and kidney showed cytoplasmic acidophilic bodies, vesicular steatosis and neutrophil polymorph infiltration signifying inflammatory changes at 45 mg/kg dose. Serum marker enzyme level and lipid parameters were elevated indicating loss of functional integrity of liver and muscle injury. PI only at 150 mg/kg dose showed anorexia, loss in body weight, increased serum SGOT, ALP, cholesterol and triglyceride level, and focal area of tubular injury with neutrophil polymorph inflammatory cell infiltration in kidney.

Pyridostigmine at 30 mg/kg/day and PI at 85 mg/kg/day dose are safe in rats which is far in excess of conceivable human exposure levels. For example for an average 70 kg soldier to become exposed to the highest doses used in this study (PB=45 mg/kg, PI=150 mg/kg) this person would have to simultaneously ingest 105 PB tablets (30 mg each) or 123.5 PI tablets (85 mg each). Human exposure would most likely occur at low levels over an extended period of time and by differing routes, in addition, we need to point out that soldiers may actually have been exposed to a host of other chemicals and/or stressors that may or may not have affected on their health. Biopharmaceutic and pharmacokinetic studies would identify the exact correlation between blood levels of PB or PI with occurrence of different serum and organ related toxicities as well as alterations in clearance rates for compounds and metabolites. Neuropharmacological, neuropathological and neurobehavioral assessment is also necessary in order to determine if nonlethal endpoints are neurological in nature.

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## Conflict of Interest

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