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Toxicity and antidotal treatment by synthesized and optimized ferric(III) hexacyanoferrate(II) for thallium

Vivek Kumar[1,3], Dharam Pal Pathak[2,3] and Nidhi Sandal[1*]

Abstract

Background The active pharmaceutical ingredient (API) of USFDA-approved Radiogardase®-Cs capsule is ferric(III) hexacyanoferrate(II) which is commonly known as Prussian blue insoluble (PB). It enhances the elimination of radioactive or non-radioactive caesium/thallium (Cs(I)/Tl(I)) from the body. The API of Radiogardase®-Cs capsules is not available commercially; therefore, in-house API was synthesized and evaluated. The present study includes toxicity evaluation and in vivo Tl(I) removal efficacy of in-house synthesized and optimized PB prepared by direct (PB-1) and indirect (PB-2) synthesis methods. PB-1 and PB-2 were evaluated for acute and sub-acute oral toxicity in accordance with OECD guidelines in rats.

Results No significant changes were observed in treatment groups as compared to the control group of acute and sub-acute oral toxicity studies. The food intake, water consumption, body weight, clinical signs, organ weight and histopathological, biochemical and haematological parameters were monitored. The study found no evidence of mortality. The results indicated that the synthesized PB-1 and PB-2 were safe. As a result, the study further examined PB-1 and PB-2 for removal of Tl(I) in rats. A significant increase in Tl(I) removal was observed when PB-1 and PB-2 were administered orally to rats in comparison to no treatment group. The Tl(I) removal efficacy of PB-1 and PB-2 was comparable to Radiogardase®-Cs treated group. Results showed reduction in the body burden of Tl(I) as well as a higher level of elimination of Tl(I) in faeces and urine.

Conclusions The study provides substantial support regarding Tl(I) removal efficacy and safety of in-house synthesized PB-1 and PB-2 which can be used for formulation development.

Keywords Toxicity, Radiogardase®-Cs, Thallium, PB insoluble, Decorporation, In vivo

Background

Prussian blue insoluble (PB) is also known as ferric(III) hexacyanoferrate(II) (Bayzi IV et al. 2022). The crystal lattice of ferric(III) hexacyanoferrate(II), has been reported to play an important role in removal of Tl(I) and/or radioactive Cs(I) (Kraft 2018; Lin et al. 2019; Hodorowicz 2022). In early seventeenth century, ferric(III) hexacyanoferrate(II) was synthesized as a coloured compound which found extensive use in paints, textiles, pigments, dyes, electro catalyst, batteries, etc. (Carniato et al. 2020; Estelrich and Busquets 2021). The use of PB as a potential antidote of Cs(I) was first reported by Nigrovic in 1966 (Nigrovic et al. 1966).

*Correspondence:
Nidhi Sandal
nidhisandal@rediffmail.com; nidhisandal@inmas.drdo.in
[1] Division of CBRN Defense, Institute of Nuclear Medicine and Allied Sciences (INMAS), Defence Research and Development Organization (DRDO), Brig. S.K. Mazumdar Road, Timarpur, New Delhi 110054, India
[2] Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi, India
[3] Delhi Pharmaceutical Sciences and Research University (DPSRU), New Delhi, India

Since then, several researchers have reported the various aspects of PB insoluble such as its safety, efficacy, crystal lattice, chemical constitution and physicochemical properties with special reference to it antidotal potential for Cs-137 and Tl(I) poisoning (Sandal et al. 2023; Mohammad et al. 2015; Faustino et al. 2008). According to reports, the drug regulatory authorities of Japan, Germany and the USA have approved it as a decorporation agent for Cs(I)/Tl(I) (Goyer 2016; Dobbs 2009). The 500 mg capsules of PB under the trade name of Radiogardase®-Cs are available (Bishop and Catherine 2020). As per label claim, each capsules contains 68% of ferric(III) hexacyanoferrate(II) along with pharmaceutically acceptable ingredients including microcrystalline cellulose, sodium dodecyl sulphate, etc. The capsule formulation has maximum binding capacity (MBC) of more than 150 mg of Cs(I) per gram. The active pharmaceutical ingredient of Radiogardase®-Cs capsule is not available commercially. This is a proprietary product of HEYL Chem.-pharm. Fabrik GmbH & Co. KG. The capsules are available commercially, but access to API is a difficult area. Therefore, PB was synthesized in laboratory to yield the PB with MBC of more than 150 mg for Tl(I) per gm.

Use of PB has been reported in literature for Tl(I) poisoning cases (Dainiak and Albanese 2022). The drug has been extensively studied in animals and humans. It has been reported to reduce the biological half-life of Tl(I) by a factor of 1/3. Tl(I) is an odourless and tasteless heavy metal. It gets absorbed in the systemic circulation, after oral ingestion accidentally or incidentally. Tl(I) exposure occurs either accidentally (exposure by drug contamination or adulteration of herbal products) or intentionally (attempted homicides, likely nuclear terrorism through the use of dirty bombs (USFDA 2003). It can also get absorbed by inhalation of contaminated air, through skin contact and mucous membranes. Tl(I) mimics potassium ions and gets distributed throughout the body and gets accumulated in the liver, bones, brain, kidney, testes, stomach, lungs, spleen and skin of the scalp. Tl(I) crosses blood brain barrier, placental barrier, and it accumulated in hair, nails, tears and breast milk.

The mechanism of PB is based on the adsorption of the Cs(I)/Tl(I) ion and its ion exchange with the hydrogen ion ($H^{+)}$ and the water-bound hydronium ion (H_3O^+). Aside from serving as an ion exchanger, PB has a crystal lattice that makes it suitable for working with univalent cations. As a result of its high affinity to Tl(I) (ionic radius 0.147 nm) over potassium (ionic radius 0.133 nm), its binding affinity increases with cation ionic radius. Due to its binding abilities, PB reverses the concentration gradient and reduces the burden on the body by removing Tl(I) from the gut in faeces. PB binds with the enterohepatically circulating Tl(I), thereby inhibiting its reabsorption and enhancing its excretion in faeces (Hodorowicz et al. 2022). Due to the variety of structures formed by hexacyanoferrates(II, III), it is still difficult to explain how Tl(I) binds to PB. As a result, methods of removing Tl(I) remain speculative (Kumar et al. 2023).

A variety of synthesis methods are available for making PB, including simple methods and more complex ones. PB is generally produced by oxidation of ferrous ferrocyanide metal salts that exhibit an intense deep blue colour due to mixed-valence electrons (Volz et al. 2006). The spark discharge method has been used to produce PB in a simulated prebiotic environment of methane, among other gases, while brine aerosols of iron salts exist (Kraft A 2018). Access to therapeutic-grade ferric(III) hexacyanoferrate(II) can be very difficult in many areas of the world including the developed countries (Hall et al. 2015). A complex composition is present in PB despite its multifunctionality and synthesis methods play a major role in its Tl(I) binding efficacy (Faustino et al. 2008). Furthermore, there is variety of hexacyanoferrate analogue utilized as cosmetics, dyes, electrodes in batteries etc. (Bayzi IV et al. 2022). The PB of therapeutic grade utilized for decorporation of Tl(I)/Cs(I) has specific synthesis parameters which have been optimized during synthesis (Kumar et al. 2023). The PB used in the study was synthesized by direct and indirect method, and different synthesis parameters were optimized to obtain the desired in vitro properties within the limits like MBC, moisture content, iron content, cyanide content, etc.

Additionally, the synthesized PB APIs were evaluated for their safety and efficacy studies. The toxicity studies were done to assess safety profile as per OECD (Organisation for Economic Co-operation and Development) guidelines in rats (Steinmetz and Edward 2009). To estimate toxic dose and therapeutic indices of medications, acute and 28 days repeated oral toxicity studies were performed (Al-Afifi et al. 2018). PB is reported to be a non-toxic compound due to its negligible or no absorbance in systemic circulations after oral administration. PB is a locally active drug which binds the enterohepatically circulating Cs(I)/Tl(I), thereby stopping its reabsorption in the circulation (Jain et al. 2023). The bound Cs(I)/Tl(I) gets excreted through faeces. Important consideration regarding PB toxicity is the presence of cyanide ions which are tightly held by iron group. The previous studies have reported insignificant release of cyanide ions from PB in the physiological pH conditions of gastrointestinal tract (Yang Y et al. 2008). However, in acidic conditions, the PB may release cyanide ions, which is highly toxic (Yang Y et al. 2007). Therefore, there was a need to investigate the oral toxicity of the in-house synthesized and optimized PB.

The in vivo removal efficacy of the synthesized PB APIs was studied in rats administered with Tl(I) and compared with standard treatment with Radiogardase®-Cs capsules. Thus, the current research work was conducted to evaluate the toxicity and in vivo efficacy of in-house synthesized and optimized PB (PB-1 and PB-2) so that the synthesized APIs can be considered for further formulation development research.

Methods

Chemicals

PB was synthesized by direct (PB-1) and indirect (PB-2) methods and Radiogardase®-Cs 500 mg hard capsules were obtained from Heyl Chem.-Pharm. Fabrik GmbH & Co. KG, Berlin, Germany. High-purity laboratory chemicals Pvt. Ltd., India, supplied 1000 mg/L Tl(I) in atomic absorption standard solution. Potassium ferrocyanide (Central Drug House (P) Ltd., India) and ferric chloride (Thermo Fisher Scientific India Pvt. Ltd.) for synthesis were used. Moreover, all other chemicals used in this study, such as nitric acid (Merck, India), diethyl ether (Central Drug House (P) Ltd., India) and thallium chloride (TlCl), were procured from Sigma-Aldrich Co., USA, which are of the highest quality.

Synthesis and optimization of ferric(III) hexacyanoferrate(II)

Direct method (PB-1)

The direct method for synthesis of PB-1 involved the preparation of solutions of 40 mM ferric chloride and potassium ferrocyanide in water separately. Potassium ferrocyanide solution was added over ferric chloride solution to yield ferric(III) hexacyanoferrate(II). The resulting mixture was constantly stirred and centrifuged, and the supernatant was decanted, followed by five washes with Millipore water and then dried at 80 °C for 2 h (Kumar et al. 2023).

Indirect method (PB-2)

The indirect method for the synthesis of PB-2 involved a two-step reaction. Separately, 0.3 M ferrous sulphate and 0.1 M potassium ferrocyanide were prepared. A solution of ferrous sulphate was added to potassium ferrocyanide solution in 1:1 ratio. After keeping for 2 h at 60 °C for ageing under atmospheric conditions, Berlin white was formed. Following ageing, hydrogen peroxide was added to Berlin white, oxidizing it to PB, and then washed with warm water 5–6 times and a tray dryer was used for drying at 80 ˚C for 2 h (Kumar et al. 2023).

Animals

For in vivo Tl(I) removal efficacy and toxicity, Sprague–Dawley rats (150–200 g) of either sex were used. A 12-h photoperiod of light and darkness was given to the animals, and they were also maintained at ambient temperature and humidity. A normal diet (rodent chow) and water ad libitum were maintained for at least one week to acclimatize the animals. Polypropylene cages (with a wire mesh top, which was regularly changed) and a hygienic husk bed (regularly changed) were used until the end of the experiment. In accordance with INM/IAEC/2018/07, dated 11 April, 2018), they were maintained in accordance with the principles and guidelines of the Institutional Animal Ethics Committee. The ethical guidelines were provided by the Committee for the Purpose of Control and Supervision of Animal Experiments (CPSCEA), New Delhi.

Acute (14 days of single-dose) oral toxicity study

PB-1 and PB-2 were tested for acute oral toxicity in rats following OECD 423 guidelines (OECD 2001) which have been developed to assess the health and environmental effects of chemicals, including pharmaceuticals. One of four fixed dose levels, 5, 50, 300 and 2000 mg/kg body weight, was chosen as the starting dose. Each group consisted of 3 female rats. Females were nulliparous and non-pregnant.

Treatment of test substance was administered as below:

Group I: Served as control and received vehicle only ($n=3$),

Group II: Served as test and received PB-1 with starting dose of 2000 mg/kg ($n=3$).

Group III: Served as test and received PB-2 with starting dose of 2000 mg/kg ($n=3$).

The animals of Group I-III were observed continuously for behavioural, neurological and autonomic profiles for 2 h and after a period of 24, 48, 72 h and thereafter up to 14 days for any lethality, moribund state or death. Cage side observations included changes in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern. All animals (Group I-III) were observed for morbidity and mortality twice daily. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Body weights of each animal were recorded at the start of study and thereafter at weekly intervals. Animals (Group I-III) were sacrificed by cervical dislocation under mild anaesthesia using diethyl ether on 15th day of the study and were subjected to a detailed post-mortem examination.

Sub-acute (28-day repeated dose) oral toxicity study

Sub-acute oral toxicity study was carried out in rats as per OECD guidelines 407 (OECD 2008). A limit test at dose level of 1000 mg/kg body weight was carried out in

control and test group as per guideline. Total of 30 rats were selected for study based on their body weight and randomly divided into three groups. Each group consisted of 10 animals (5 males and 5 females). Females were nulliparous and non-pregnant.

Group I: served as control group received vehicle only.

Group II: served as test group received PB-1 (1000 mg/kg body weight).

Group III: Served as test and received PB-2 (1000 mg/kg body weight).

The following observations were made during the course of study up to 28 days of study.

Rats were examined for clinical signs and mortality after application of drug, at 5–10 min, 30–45 min, 1, 2, 4, 6 and 24 h followed by once daily throughout the study period. Cage side observations included changes in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern. All animals were observed for morbidity and mortality twice daily. A detailed analysis of the signs and symptoms was conducted, with particular focus on the evaluation of seizures, drooling, gastrointestinal distress, fatigue, slumber and unconsciousness.

Observations and parameters for toxicity

Body weight: At the beginning of the study and every week thereafter, animal weights were recorded.

Feed and water consumption: Rats were recorded as to how much feed and water they consumed by comparing the amount of feed and water left over from one week to the next.

Blood collection: In the sub-acute oral toxicity study, blood samples were collected on day 29 to assess haematological and biochemical changes. Diethyl ether was used to anesthetize and collect blood samples from the rats after 4–6 h of fasting. In order to carry out the haematology test, blood was collected from the retro-orbital area in potassium ethylene di-amine tetra acetate (K_3EDTA) tubes. The blood collected was analysed for changes in the blood cell composition in a fully automated three-part haematology analyser (Celltac-α; Nihon Kohden, Germany) (Dahiya A et al. 2022). A heart puncture was done during necropsy to collect blood for biochemical analysis. A centrifuge (REMI R-12C Plus, India) was used to separate serum, and they were kept at 80°C until analysis. Based on the manufacturer's instructions, serum biochemistry estimations were performed on a full-automatic biochemistry analyser (Erba Chem 7, Transasia Biomedicals Ltd.) (Dutta et al. 2021).

In vivo efficacy of optimized PB for Tl(I) in rats

Treatment schedule Sprague–Dawley rats were divided into five groups: The animals received a single dose of Tl(I) (20 mg/kg) except Group I.

Group I: Animals received food (rat chow) and water ad libitum daily.

Group II: Positive control (received Tl(I) 20 mg/kg body weight).

Group III: The PB-1 (3 g/70 kg of human weight) in 1 mL water was administered orally through cannula after 24 h of Tl(I) administration for 28 days.

Group IV: The PB-2 (3 g/70 kg of human weight) in 1 mL water was administered orally through cannula after 24 h of Tl(I) administration for 28 days.

Group V: Radiogardase®-Cs (3 g/70 kg of human weight) in 1 mL water was administered orally through cannula after 24 h of Tl(I) administration for 28 days.

During the period of the study, the animals health and behaviour were monitored closely in metabolic cages for 28 days, and urine and faecal samples were collected after 1st day of the administration of Tl(I) (shown in Fig. 1). Blood samples were collected through retro-orbital. Thereafter, samples collection was done according to the schedule presented in Fig. 1. The collected samples were digested using concentrated HNO_3, and estimation of Tl(I) content was done by Graphite furnace atomic absorption spectrometry (GF-AAS) Labindia AA 8000, Mumbai, India.

Estimation of Tl(I) using graphite furnace atomic absorption spectrometry (GF-AAS)

In situations where high-sensitivity analysis is required, the furnace method is preferable. The GF-AAS method was used to measure Tl(I) traces. The instrument uses a graphite tube with pyrolytic coating for the introduction

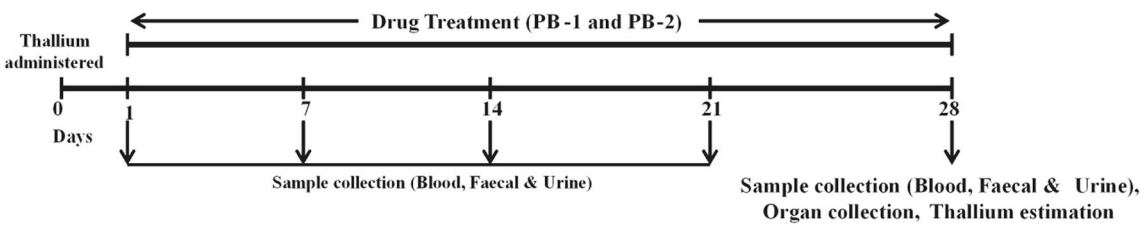

Fig. 1 Diagrammatic representation of experimental schedule: in vivo study

of samples, which is heated to 3000 °C. In addition to setting the heat program, a hollow cathode lamp containing Tl(I) elements was selected and the instrument parameters were set. By using a graphite auto sampler or a hand pipette with a maximum volume of 20 µL, Tl(I) was detected at an emission line of 276.8 nm. The standard/test sample or blank was injected into the graphite tube. A triplicate analysis of standard samples, test samples and blanks was performed using GF-AAS. To assess carry over, blank samples and sensitivity corrections were run between every ten test samples. Validation of the method was carried out in terms of range, linearity, precision, accuracy and specificity. The limit of detection (LOD) and lowest limit of quantification (LOQ) of the experiment were determined.

Statistical analysis

GraphPad Prism 5.00 software was utilized for statistical analysis of the data. ($n=3$–5). One way ANOVA were performed with Tukey's post-hoc analysis, where appropriate and two-way ANOVA were performed with Bonferroni tests, if necessary. All cases were considered significant if the $p<0.05$.

Results

Toxicity: single-dose (acute) oral toxicity study

In single-dose acute oral toxicity, test group animals (Group II and Group III) received PB-1 and PB-2 once at high dose, i.e. 2000 mg/kg body weight, and observed for 14 days. In the drug-treated, control and vehicle control groups, all haematological parameters were within normal ranges after treatment with PB. At high doses, PB has no significant influence on both cellular elements and non-cellular elements of the blood. There was no evidence of toxicity or mortality. Animals in test group (Group II and Group III) were also normal in terms of all parameters recorded in cages, compared to animals in control group. In the test group, the body weight of animals was significantly different from the vehicle control group animals at intervals of 1, 7 and 14 days (Additional file 3: Fig S1). During the study, no moribund stages were observed. A necropsy of treated animals revealed no obvious morphological alterations (Table 1).

Sub-acute oral toxicity study

PB was given to test group animals (Group II and Group III) repeatedly for 28 days, while vehicle was given to control group animals. There was no sign of toxicity, moribund stage or mortality in test group compared to control. Body weight differences between control and experimental groups were statistically insignificant ($p>0.05$). After 28 days of repeated doses of PB-1 and PB-2, no changes were observed in parameters in the cages, such as skin, fur, eye movements, etc. In both test and control groups, feed intake and water consumption were consistent during the entire 28 study days (Additional file 1: Table S1). Comparing the treated test groups (Group II and Group III) to vehicle control groups (Group I), no statistically significant differences were found in haematological parameters such as complete blood count (CBC profile) and differential leukocyte count (DLC). Biochemical parameters were not statistically significant ($p>0.05$) in the test group animals, including liver function tests, kidney function tests, electrolytes, blood glucose and total cholesterol (Table 2). It was found that there was no significant difference in organ weight between the treated rat and the vehicle control group rat (Additional file 2: Table S2). The internal organs of the sacrificed animals showed no pathological changes during the sub-chronic toxicity study. When compared with the control group, the macroscopic analysis of the organs of the test group treated animals did not show any change in colour, texture, swelling and atrophy/hypertrophy (Fig. 2). Based on the above findings, it can be concluded that repeated oral toxicity studies in rats over 28 days did not cause drastic effects on haematological and biochemical parameters (Table 3).

Table 1 Parameters of analytical method validation for GF-AAS analysis of Tl(I)

Parameters	Tl(I)
Linearity range (µg/L)	5–100
Regression coefficient (R^2)	0.9989
Linearity equation	$y=0.0034x+0.0652$
Accuracy (% mean recovery)	99.5–101.5
Precision (Inter-day) %RSD	< 2%
Precision (Intra-day) %RSD	< 2%
LOD (µg/L)	0.664
LOQ (µg/L)	2.214

In vivo antidotal effect of PB-1, PB-2 and marketed formulation Radiogardase®-Cs for Tl(I)

Urine and faeces Tl(I) elimination in rats

Rats were given a single dose of TlCl followed by the administration of PB-1, PB-2 and Radiogardase®-Cs daily for 28 days. In this study, Tl(I) levels were monitored in urine and faeces on 1, 7, 14, 21 and 28th day of study. A comparison was made between PB-1, PB-2 and the marketed formulation Radiogardase®-Cs with positive control group.

The Tl(I) content in urine samples was higher during the initial phase of the study wherein higher amount of Tl(I) was excreted due to its inherent pharmacokinetics.

Table 2 Effect on biochemical parameters during sub-acute toxicity study of PB -1 and PB -2

Biochemical parameters	Male			Female		
	Control	PB -1	PB-2	Control	PB-1	PB-2
Liver function test						
Alkaline phosphatase (ALP) (U/L)	95.100 ± 3.580	95.040 ± 3.205	95.010 ± 2.025	94.840 ± 2.196	94.040 ± 1.992	94.780 ± 1.296
Aspartate transaminase (AST) (U/L)	152.900 ± 2.738	153.200 ± 2.723	153.200 ± 2.232	153.300 ± 2.912	152.900 ± 1.467	152.7 ± 2.135
Alanine transaminase (ALT) (U/L)	61.930 ± 1.985	62.580 ± 1.394	62.630 ± 1.264	62.420 ± 1.055	62.560 ± 0.932	62.640 ± 1.215
Globulin (g/dL)	3.400 ± 0.397	3.380 ± 0.3564	3.380 ± 0.3564	3.350 ± 0.284	3.470 ± 0.318	3.470 ± 0.318
Albumin (g/dL)	3.608 ± 0.269	3.600 ± 0.158	3.610 ± 0.1653	3.680 ± 0.254	3.770 ± 0.226	3.730 ± 0.208
Cholesterol (mg /dL)	70.603 ± 4.963	70.420 ± 3.563	70.360 ± 2.791	68.490 ± 7.100	69.890 ± 3.170	69.790 ± 2.204
Total Protein (g/dL)	6.540 ± 0.209	6.560 ± 0.241	6.560 ± 0.241	6.522 ± 0.259	6.576 ± 0.278	6.558 ± 0.151
Kidney function test						
Calcium (mg/dL)	8.520 ± 0.345	8.600 ± 0.245	8.596 ± 0.2356	8.790 ± 0.2498	8.830 ± 0.141	8.810 ± 0.079
Phosphorus (mg/dL)	5.740 ± 0.3058	5.620 ± 0.192	5.646 ± 0.233	6.070 ± 0.3878	6.012 ± 0.162	6.112 ± 0.318
Uric acid (mg/dL)	4.340 ± 0.289	4.340 ± 0.2074	4.348 ± 0.234	4.620 ± 0.2599	4.786 ± 0.217	4.770 ± 0.155
BUN (mg/dL)	12.430 ± 0.351	12.440 ± 0.355	12.500 ± 0.145	12.470 ± 0.2411	12.480 ± 0.304	12.470 ± 0.340
Blood urea (mg/dL)	26.930 ± 0.035	26.260 ± 0.654	26.330 ± 0.352	26.58 ± 0.314	26.420 ± 0.248	26.440 ± 0.294
Creatinine (mg/dL)	0.780 ± 0.045	0.760 ± 0.055	0.7720 ± 0.0303	0.77 ± 0.033	0.790 ± 0.029	0.780 ± 0.026
Electrolytes						
Chloride (mmol/L)	102.300 ± 1.838	101.900 ± 1.301	101.800 ± 1.421	103.700 ± 1.365	103.700 ± 1.761	103.500 ± 0.029
Blood glucose (Random) (mg/dL)	129.000 ± 5.082	130.200 ± 4.811	130.800 ± 3.871	131.700 ± 4.666	130.500 ± 2.667	130.300 ± 2.361
Sodium (mmol/L)	144.200 ± 1.225	144.200 ± 1.225	144.000 ± 1.288	150.400 ± 3.921	151.100 ± 2.074	151.500 ± 1.279
Potassium (mmol/L)	4.478 ± 0.359	4.400 ± 0.200	4.436 ± 0.281	4.890 ± 0.115	4.940 ± 0.054	4.950 ± 0.093

The Tl(I) levels of urine decreased on the 7th day and thereafter in all the groups. In Radiogardase®-Cs, Tl(I) content in urine was 2394.118 ± 0.138 µg/L on day 1st which decreased to 734.691 ± 0.082 µg/L on 7th day. Similarly, in PB-1 from 2484.088 ± 0.355 µg/L to 641.000 ± 0.044 µg/L and in PB-2, the value was found to be reduced from 2236.270 to 729.231 ± 0.043 µg/L on 7th day, and in positive control group (Tl(I)), the Tl(I) content was found to be 2540.437 ± 0.286 µg/L at first day and 998.000 ± 0.180 µg/L on 7th day. On subsequent treatment days up to the 28th day, a further decrease in Tl(I) content was recorded in urine samples. The results are shown in Fig. 3.

As shown in Fig. 4, Tl(I) content in faeces increased after 24 h of TlCl administration and on 7th, 14th, 21st and 28th days of treatment with Radiogardase®-Cs, PB-1 and PB-2, the Tl(I) content in faeces was significantly higher than the no treatment group. The higher levels of Tl(I) in faeces indicate the effect of PB therapy. PB binds enterohepatically circulating Tl(I) and removes it in faeces**.** Figure 4 shows the Tl(I) level in faeces during 28th days of treatment.

Tl(I) estimation in blood samples

Radiogardase®-Cs, PB-1 and PB-2 reduced the blood Tl(I) levels within 7th day of treatment. Significant difference was found in treatment groups (Groups III-V) as compared to positive control group (Group II) (Tl(I)); however, Groups III-V exhibited similar blood Tl(I) profile. Blood Tl(I) levels decreased further on successive sampling days up to the 28th day. Figure 5 shows the blood Tl(I) levels for each day of sampling for all groups along with their level of significance. In all treatment groups and positive control groups, Tl(I) were administered at a single dose of 20 mg/kg and blood Tl(I) levels were monitored. Various pharmacokinetic parameters, such as $t_{1/2}$ and clearance, were calculated (Table 4).

(See figure on next page.)

Fig. 2 Histology organ images of male control; in-house synthesized and optimized PB treated male; Female control and in-house synthesized and optimized PB-1 and PB-2 treated female in sub-acute oral toxicity study. Figure represents photomicrographs of brain (**a1**–**a6**), trachea (**b1**–**b6**), lungs (**c1**–**c6**), heart (**d1**–**d6**), spleen (**e1**–**e6**), testis (**f1**–**f3**), ovary (**f4**–**f6**), liver (**g1**–**g6**), adrenal gland (**h1**–**h6**), stomach (**i1**–**i6**), kidney (**j1**–**j6**), ileum (**k1**–**k6**), jejunum (**l1**–**l6**), duodenum (**m1**–**m6**), colon **n1**–**n6**, respectively. At 10X magnification, histologic cross sections (H&E stained) of treated groups showing comparable histological features with the control sections

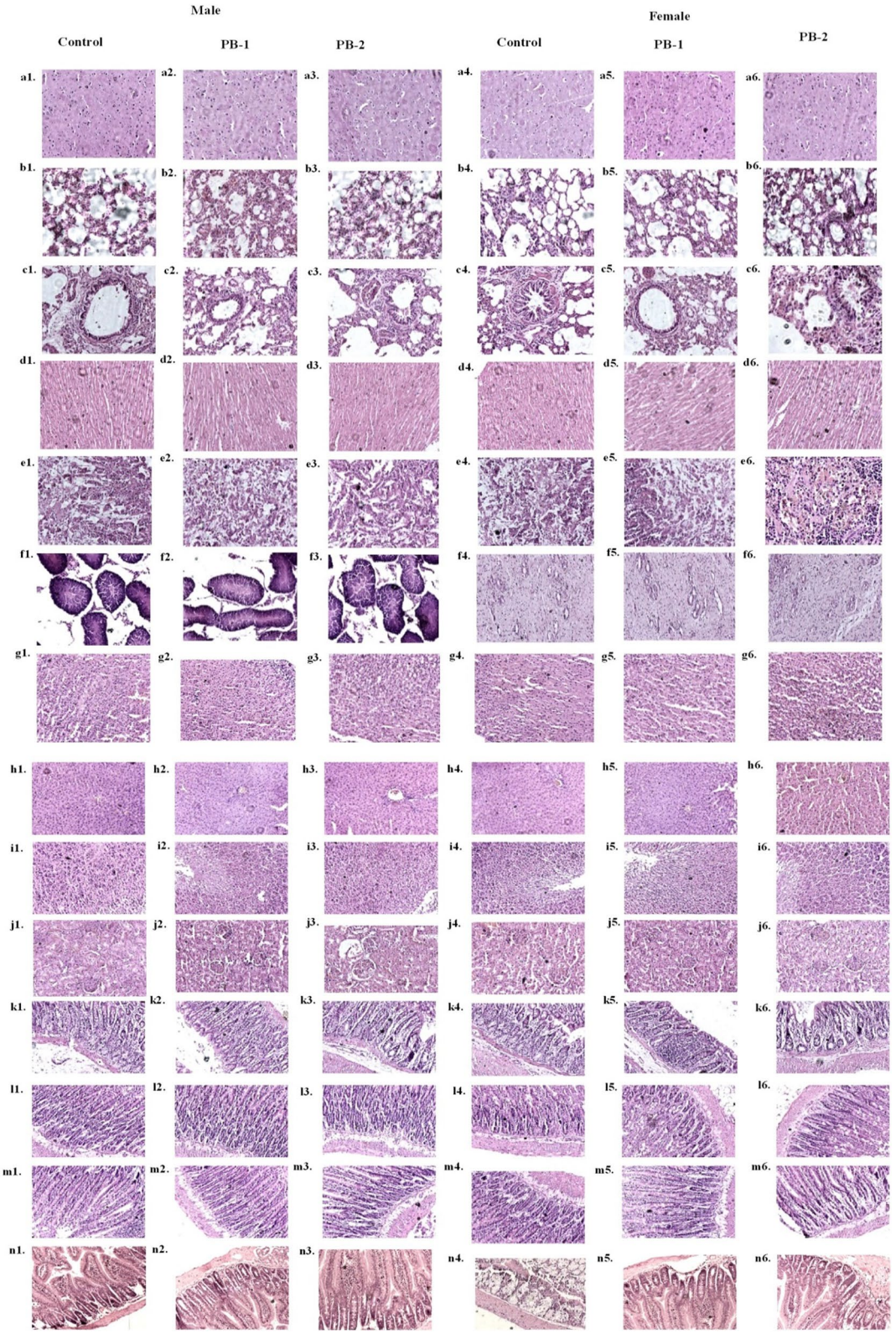

Fig. 2 (See legend on previous page.)

Table 3 Effect on haematological parameters during sub-acute toxicity study of (PB -1 and PB -2)

Parameters	Male			Female		
	Control	PB-1	PB-2	Control	PB-1	PB-2
WBC (10^3µL)	7.460 ± 0.321	7.860 ± 0.288	7.920 ± 0.377	6.260 ± 0.555	6.360 ± 0.365	6.380 ± 0.389
RBC (10^6µL)	8.740 ± 0.182	8.842 ± 0.118	8.800 ± 0.265	8.360 ± 0.336	8.580 ± 0.311	8.480 ± 0.303
HGB (g/dL)	14.660 ± 0.473	15.050 ± 0.432	15.090 ± 0.462	14.260 ± 0.416	14.540 ± 0.428	14.560 ± 0.343
HCT (%)	44.520 ± 1.397	44.120 ± 1.724	44.720 ± 1.547	43.420 ± 1.377	43.900 ± 1.342	43.620 ± 0.861
MCV (fL)	56.700 ± 1.350	57.500 ± 1.576	56.940 ± 1.083	56.920 ± 2.317	57.200 ± 2.690	57.080 ± 3.360
MCH (pg)	19.300 ± 0.652	19.520 ± 0.854	19.310 ± 1.007	19.030 ± 0.634	19.110 ± 0.537	19.100 ± 0.474
MCHC (g/dL)	33.720 ± 0.444	33.840 ± 0.591	33.840 ± 0.591	33.100 ± 1.122	32.760 ± 1.834	32.980 ± 1.665
PLT (103µL)	1221.000 ± 23.820	1217.000 ± 87.15	1217.000 ± 68.200	1179.000 ± 34.440	1177.000 ± 39.690	1175.000 ± 42.750
LYMPH (%)	71.600 ± 1.140	72.400 ± 1.517	71.800 ± 1.304	68.600 ± 2.702	69.400 ± 1.817	69.200 ± 2.490
MONO (%)	6.200 ± 0.837	6.600 ± 0.548	6.400 ± 0.894	5.200 ± 1.483	5.600 ± 1.140	5.400 ± 1.140
GR (%)	24.800 ± 1.920	25.400 ± 1.140	25.600 ± 1.140	23.400 ± 2.074	24.400 ± 2.074	24.000 ± 1.580
RDW	23.540 ± 0.994	24.040 ± 1.043	24.040 ± 1.043	13.500 ± 1.281	14.020 ± 1.180	14.060 ± 0.472
PCT	0.900 ± 0.044	0.944 ± 0.049	0.950 ± 0.037	0.736 ± 0.091	0.720 ± 0.106	0.718 ± 0.025
MPV (fL)	7.600 ± 0.224	7.740 ± 0.182	7.760 ± 0.207	7.780 ± 0.164	7.940 ± 0.230	7.800 ± 0.509
PDW (fL)	16.300 ± 0.692	16.820 ± 0.366	16.740 ± 0.323	15.580 ± 0.476	15.620 ± 0.487	15.640 ± 0.343

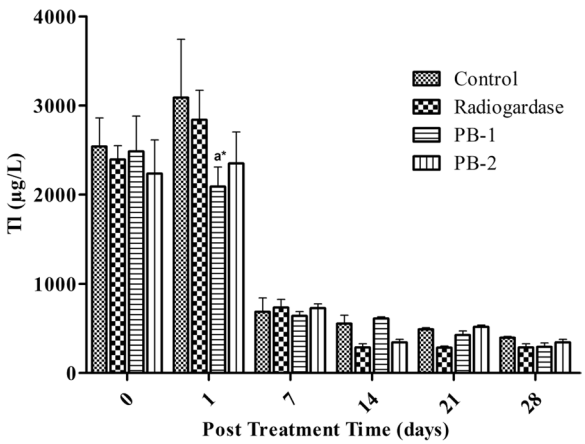

Fig. 3 Amount of Tl(I) excreted through urine during treatment (µg/L). Significance is shown as $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to positive control group

Tl(I) estimation in organs

Tl(I) content were estimated in major organs on 28th day of treatment after dissecting the animals and processing their organs for Tl(I) estimation by GF-AAS. Table 5 shows the Tl(I) content in organ of rats. It has been observed that Radiogardase®-Cs, PB-1 and PB-2 enhances the elimination of Tl(I) thereby reducing the Tl(I) content in each organ significantly as compared to positive control group. Tl(I) levels in organs of PB-1 and PB-2 treated groups were compared with Radiogardase®-Cs. Radiogardase®-Cs treated group showed an average of two fold decreases in Tl(I) levels in every organ except kidneys as compared to PB-1 and PB-2.

Discussion

Intoxication caused by Cs-137 and Tl(I) can be treated with PB safely and effectively. USFDA approved PB-containing Radiogardase®-Cs capsules in the year 2003, and since then there is only one manufacturer of PB capsules. In case of an emergency, these capsules are required on urgent basis and in large numbers as the dose of PB is 3–10 g per day (Bishop and Catherine 2020). In most of the countries, these capsules are not available and the import of these capsules is a time-consuming and expensive task. Therefore, there is a need for R&D and commercial production of this drug. Further, PB is available from different sources wherein the safety and effectiveness are the grey area and poorly known even among physicians and health professionals (Altagracia-Martinez et al. 2012).

PB is a well-established antidote used in the treatment of Tl(I) poisoning (Anaya-Ramos et al. 2021). PB enhances the faecal excretion of Tl(I) ions undergoing enterohepatic circulation. The authors have been working towards the development of PB API which is safe and efficacious. After a thorough optimization of process parameters, PB-1 and PB-2 were synthesized. The in vitro characterization of PB-1 and PB-2 was performed (data not shown here). Especially the in vitro maximum binding capacity (MBC) of PB was found to be more than 150 mg of Tl(I) per gram of PB which is as per the

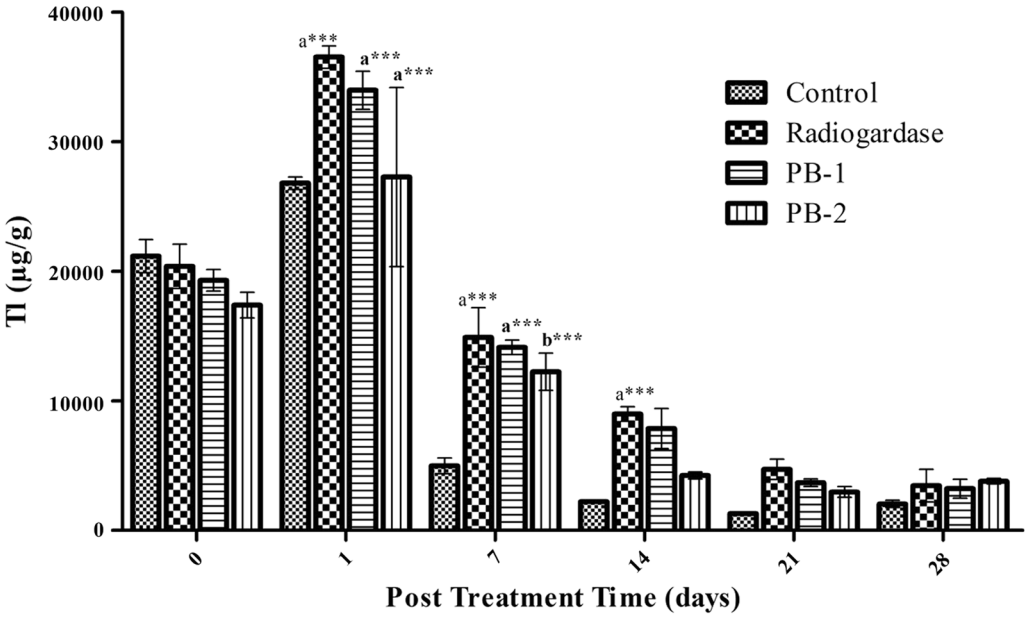

Fig. 4 Excretion of Tl(I) (µg/g) through faeces. Significance is shown as $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to positive control (Tl(I)) group, PB-1, PB-2 and marketed formulation Radiogardase®-Cs group

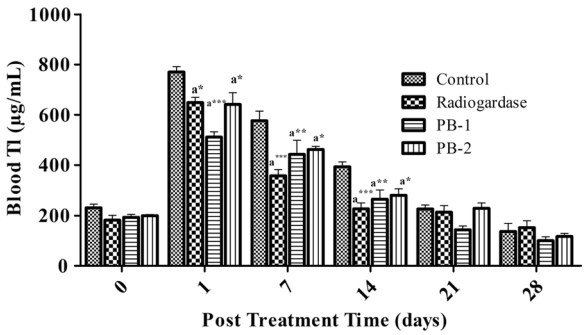

Fig. 5 Blood Tl(I) concentration (µg/ml) in rats during treatment. Significance is shown as $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to positive control group

Table 4 Pharmacokinetic parameter for Tl(I) in blood of rats

Pharmacokinetic parameter	Tl(I)	PB-1	PB-2	Radiogardase®-Cs
$t_{1/2}$ (days)	41.930	30.690	34.760	27.940
Clearance (L/days)	0.710	1.520	1.320	1.160

minimum requirement set forth by USFDA. Thereafter to evaluate the safety and efficacy study of synthesized APIs, acute and sub-acute oral toxicity according to OECD guidelines, and in vivo Tl(I) removal efficacy was performed in rats.

Acute oral toxicity study: Acute toxicity studies in rodents gives an insight into its harmful effects and is an essential requirement for regulatory submissions. The acute oral administration of PB-1 and PB-2 at 2000 mg/kg doses did not show any toxicity. No mortality was observed in either group during the study period. Throughout the study, cage side observations were made to observe normal animal behaviour. An appropriate index for assessing toxicity is body weight change (Senthilkumaran et al. 2017). The body weight change of all treated animals was gradual, as in vehicle control group animals and on applying statistical analysis, it comes out to be non-significant. As a result, PB is not toxic. Neither any macroscopic lesions nor gross morphological changes were found in test group animals during euthanasia, indicating that the NOAEL of PB could be greater than 2000 mg/kg body weight. Thus, the developed formulation can be considered safe on acute exposure.

Sub-acute oral toxicity study: For assessing the sub-acute oral toxicity, rats were administered PB for an extended period of time (28 days) and a wider range of parameters were monitored throughout the study period. Body weight, cage side observations, behavioural pattern, haematological parameters and biochemical parameters, histological changes in organs and weights of collected organs were observed (Sandal et al. 2023). During the 28 days of this study, no mortality was recorded in test group animals. There was no change in behavioural pattern of test group animals as well as control group. No

Table 5 Tl(I) content in various organs of the rat after 28 days dosing interval

Tissue	Tl(I) content (µg/g)			
	Positive control Tl(I)	PB-1	PB-2	Radiogardase®-Cs
Liver	0.220 ± 0.011	0.127 ± 0.009[a***]	0.116 ± 0.006[a***]	0.068 ± 0.009[a***]
Kidney	1.230 ± 0.089	0.282 ± 0.064[a***]	0.488 ± 0.064[a**]	0.046 ± 0.016***
Heart	0.464 ± 0.029	0.434 ± 0.045	0.564 ± 0.018	0.386 ± 0.038
Stomach	0.237 ± 0.04	0.104 ± 0.007[a*]	0.108 ± 0.023[b*]	0.054 ± 0.025**
Bone	0.220 ± 0.012	0.103 ± 0.021[a***]	0.179 ± 0.035[a***]	0.074 ± 0.026[a***]
Colon	0.453 ± 0.016	0.144 ± 0.028[a***]	0.154 ± 0.016[a***]	0.047 ± 0.016[a***]
Intestine	0.545 ± 0.038	0.247 ± 0.031[a***]	0.392 ± 0.040[a***]	0.226 ± 0.011[a***]
Brain	0.282 ± 0.024	0.215 ± 0.011[a***]	0.212 ± 0.019[a***]	0.126 ± 0.011[a***]
Muscles	0.370 ± 0.094	0.105 ± 0.003[a*]	0.141 ± 0.022[a*]	0.051 ± 0.003**
Spleen	1.060 ± 0.055	0.288 ± 0.041[a***]	0.379 ± 0.037[a***]	0.114 ± 0.004[a***]
Adrenal gland	21.900 ± 1.960	14.400 ± 0.898[a**]	15.500 ± 1.141[ba*]	8.460 ± 0.131[a***]
Whole body retention	26.980 ± 2.243	16.234 ± 1.153	18.233 ± 1.584	9.628 ± 0.314

[a] Control group

Values are presented as Mean ± SEM. (N = 5) after 28 days of treatment. Significance is shown as ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to positive control group (Tl(I))

signs of toxicity were observed. Body weight gain pattern of treated animals was found statistically insignificant ($p > 0.05$) with respect to vehicle group. Another important assessment of toxicity was done by analysing animals feed/water consumption. The results showed statistically insignificant change in feed/water consumption of PB treated group with respect to vehicle control group.

The haematological parameters like RBC, WBC count, platelets count, packed cell volumes (PCV) and haemoglobin concentrations of all treated and control group animals were found in reference range (Table 3). The minor changes were not statistically significant ($p > 0.05$) with respect to vehicle control group. This gives a conclusive result that repeated dosing with PB-1 and PB-2 did not exert any toxicity, abnormalities or any hazardous effects on important blood parameters.

OECD guidelines recommend estimation of biochemical parameters which are important markers to assess toxicological effects of a test agent (Sojakova et al. 2015). Effect on renal and hepatic function is of prime importance in sub-acute oral toxicity studies (Steinmetz and Edward 2009). In the present study, no effect of repeated dosing of PB-1 and PB-2 was observed on biochemical parameters related to kidney and liver. Plasma urea levels, total serum protein and plasma creatinine levels which are indicative parameters of glomerular filtration rate and thus the health of kidney were well within the reference ranges in all groups and insignificantly differ upon statistically analysis among the groups (Sojakova et al. 2015). Alterations in level of ALT, ASP and ALP enzymes especially elevation in their levels indicate diseased liver condition. No statistically significant alterations were found in levels of ALT ASP; ALP was found indicating normal hepatic function of treated animals as in control group. Thus, no hepatotoxicity as well as renal toxicity was observed and the PB can be considered safe. Insignificant statistical change in levels of electrolytes such as sodium, potassium, chloride, etc., of treated group with respect to control group was observed. These intracellular ions are key players in maintaining homeostasis within body (Al-Afifi et al. ATSDR 1992). In this study, the histological results of the organs at higher dose levels did not result in any changes in the cytoarchitecture of the brain, liver, heart, spleen, kidney, lung, stomach, intestine, testis, ovary, adrenal gland and trachea (Fig. 2) (Pearce 1994). These results were also found incoherence with results of haematological and biochemical analysis. It can be concluded from the present study that PB showed no signs or symptoms of acute toxicity at a single dose of 2000 mg/kg in rats. At 1000 mg/kg dose, repeated-dose toxicity indicated no toxic effects of the test drug. PB-1 and PB-2 have no toxic effects, and it can be used as an antidote in case of Cs(I)/Tl(I) poisoning.

In vivo Tl(I) removal efficacy: Tl(I) Removal efficacy of PB has been reported by several researchers in animals. Multiple investigations suggest that PB reduces the half-life of Tl(I) and also reduces the Tl(I) levels in critical organs like heart, brain, kidney, lungs, etc. (Zhao et al. 2018). Leung and Ooi (2000) reported that PB significantly reduced both brain Tl(I) concentrations and mortality in Rats administered orally 30 mg/kg of Tl(I) (Leung and Ooi 2000). It was reported that treatment with PB increased the survival of rats from 0 to 50% administered with lethal dose of Tl(I) with threefold

increase in excretion (Manninen et al. 1976; Aaseth et al. 2019). In addition to preclinical use of PB in Tl(I) poisoning, several clinical reports suggest a significant role of PB in reducing the Tl(I) levels (Wang 21 et al. 2021; Wallbridge et al. 2023). Thus, in the present study, in–vivo Tl(I) removal efficacy of PB-1 and PB-2 was evaluated in rats administered with 20 mg/kg of Tl(I). Tl(I) removal significantly increased from faeces as PB binds with enterohepatically circulating Tl(I), thereby inhibiting its reabsorption. The faecal elimination of Tl(I) enhanced significantly in rats treated with Radiogardase®-Cs, PB-1 and PB-2 in comparison to control groups.

The changes in Tl(I) excretion through urine remained insignificant in between the control and treated groups; this could be due to the mechanism of action of PB. PB binds with Tl(I) in the lumen of intestine which enhances its excretion through faeces and thereby mobilizing the intracellular Tl(I) in the circulation which leads to maintain the Tl(I) levels in blood and also the urine. Therefore, insignificant changes were observed in Tl(I) excretion through urine in all the groups (Agrawal et al. 2021). Even then, faecal excretion of Tl(I) was three times more than urine excretion during PB therapy. However, there was no significant variance in the treatment groups III-V in faecal excretion. The blood and urine Tl(I) content of treatment groups decreased significantly as compared to positive control group II.

The area under the curve (AUC) of blood profile with respect to time represents that total amount of drug/test agent in the body. The AUC of blood Tl(I) profile was considerably lower in Radiogardase®-Cs, PB-1 and PB-2 during the first 14 days of therapy, as shown in Table 6. This can be explained by the fact that Tl(I) is getting excreted via its inherent route of excretion i.e., urine as well as in faeces due to PB therapy. Therefore, the rate of removal of Tl(I) in the initial phase of 14 days was faster (Altagracia-Martinez et al. 2012). After 14 days, due to the deposition of Tl(I), its urine excretion decreases and the rate of Tl(I) elimination decreases. In this phase the PB therapy is extremely important. Although the overall elimination of Tl(I) decreases, PB helps in triggering the movement of deposited Tl(I) by rapidly removing it in faeces. Therefore, Tl(I) level in blood, faeces and urine decreases during the later phase of therapy. Continuous treatment with PB hastens the elimination of Tl(I) and brings these levels in the reference ranges.

In the present experiment, PB therapy was continued up to 28 days and then animals were dissected and the Tl(I) levels were determined in major organs. It was observed that Tl(I) levels were significantly higher in Group II as compared to treatment Group III-V. The whole-body retention of ingested Tl(I) was calculated as the sum of Tl(I) content per gram of each organ which was significantly lower in treatments groups. This suggested that PB-1, PB-2 and Radiogardase®-Cs treatment was effective in hastening the elimination of Tl(I). However, it is pertinent to mention here that insignificant change was observed among the Group III, IV and V suggesting that PB-1, PB-2 and Radiogardase®-Cs were equally effective in their Tl(I) removal efficacy.

The desired removal of Tl(I) is can be achieved when PB is synthesis by an optimized method. In process quality control of PB by monitoring its physicochemical parameters is essential to achieve the desired quality of PB. The physicochemical parameters of PB affect its maximum binding capacity for Tl(I) which in turn affects it's in vivo Tl(I) removal efficacy. This parameter includes moisture content, iron content, particle size etc. Moisture content has been reported to affect Tl(I) MBC directly. Lower moisture content reduced MBC; therefore, drying of PB is a critical optimizing parameter. Also the iron content, which defines its purity, is an important parameter to be monitored for safety as well as efficacy. Therefore, it is critical to evaluate and compare the PB synthesized by different methods to ensure its safety and efficacy..

Conclusions

The acute and sub-acute toxicity studies confirmed that in-house synthesized and optimized PB (PB-1 and PB-2) had no toxic effects on rodents. There were no indications of death, significant behavioural changes, gross pathology in the rats, and no differences in body weight, organ weight, or other blood parameters among treated and control rats given the prescribed doses. Furthermore,

Table 6 Area under curve for Tl(I) in blood

Treatment	Time in days			
	0–7	7–14	14–21	21–28
Tl(I)	2.820 ± 0.155	2.930 ± 0.377	2.170 ± 0.062	1.270 ± 0.141
PB-1	1.870 ± 0.511	1.520 ± 0.319	1.380 ± 0.265	1.410 ± 0.305
PB-2	1.520 ± 0.498	1.680 ± 0.386	1.890 ± 0.357	1.880 ± 0.340
Radiogardase®-Cs	1.390 ± 0.221	1.330 ± 0. 0.267	1.650 ± 0.269	1.850 ± 0.186

the H&E staining clearly demonstrated that the anatomical structure of organs was similar in the treated and control groups. The NOAEL of PB was found to be more than 1000 mg/kg. Due to the fact that PB-1 and PB-2 are not absorbed in the gut and do not get metabolized in the intestine, it has no toxicity. Additionally, the in vivo experiments for the Radiogardase®-Cs, PB-1 and PB-2 showed that the treatment with these drug samples significantly reduced the Tl(I) as compared to no treatment group. PB-1 and PB-2 exhibited similar in vivo Tl(I) removal efficacy which is comparable to commercially available Radiogardase®-Cs. The effects of PB, in conjunction with supportive measures, can lead to full recovery with timely treatment. Therefore, it may be concluded that oral administration of PB-1 and PB-2 has no adverse effects and good in vivo Tl(I) removal efficacy.

Abbreviations

AAS	Atomic absorption spectrometry
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APIs	Active pharmaceutical ingredients
AUC	Area under the curve
CBC	Complete blood count
Caesium	Cs(I)
CPSCEA	Committee for the Purpose of Control and Supervision of Animal Experiments
DLC	Differential leukocyte count
GR	Glomerular filtration rate
GF-AAS	Graphite furnace atomic absorption spectrometry
H&E	Haematoxylin and eosin stain
HCT	Haematocrit
HGB	Haemoglobin
HNO_3	Nitric acid
K_3EDTA	Potassium ethylene di-amine tetra acetate
MBC	Maximum binding capacity
MCH	Mean cell haemoglobin
MCV	Mean cell volume
MPV	Mean platelet volume
NaOH	Sodium hydroxide
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PB	Prussian blue insoluble
PCT	Procalcitonin
PCV	Packed cell volumes
PLT	Platelet count
RBC	Red blood cells
RDW	Red cell distribution width
Thallium	Tl(I)
TlCl	Thallium chloride
USFDA	United States Food and Drug Administration
WBC	White blood cells

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42269-023-01086-x.

Additional file1. **Table S1**. Effect on neurological behaviour water/feed consumption during sub-acute oral toxicity study of (PB -1 and PB -2) Results are mentioned in mean, $n = 5$. Statistically insignificant differences were found between groups at $p > 0.05$

Additional file2. **Table S2**. Effect on organ weight during sub-acute toxicity study of PB -1 and PB -2

Additional file3. **Figure S1**. Effect on body weight during **A.** Acute toxicity study at high dose (2000 mg/kg), **B.** Sub-acute toxicity study at 1000 mg/kg dose of PB-1 and PB-2. Results are mentioned in mean ± SEM, $n = 3$ and $n = 5$. Statistically insignificant differences were found between groups at $p > 0.05$ (SEM: Standard error of mean)

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Author contributions
All authors have read and approved the manuscript. All authors contributed to the study conception and design. Material preparation, literature review and data collection and analysis were performed. "Methodology was performed by VK which involves the systematic analysis of methods applied to a field of study. This includes research design, data collection and analysis techniques. The author has carefully considered the methodology used to ensure that the work is valid, reliable and relevant to the field. VK analysed and interpreted the animal data regarding the haematological, biochemical parameters and in vivo removal efficacy of PB for thallium. RH performed the histological examination of the animal involved in toxicity study and was a major contributor in writing the manuscript. The original draft preparation and Editing was done by Vivek Kumar. Editing is a crucial stage in the writing process and greatly enhance the quality and impact of a written work. DPP has supervised the study. He reviews and provided feedback on the author's work, ensuring that it meets the necessary standards of quality and accuracy. NS was responsible for conceptualizing the project and developing in overall direction. This involves identifying key research questions, outlining the scope of the project and determining the method that was used to collect and analyse data. The statistical methods and other analytical data interpretation during the research process were performed by NS. Formal analysis, investigation and proof reading were done by Nidhi Sandal. All authors read and approved the final manuscript."

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Availability of data and materials
The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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