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Toxicokinetic and Tissue distribution studies of Mercury in an Ayurvedic preparation- *Shila Sindur*

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ABSTRACT

Plan: *Shila sindur, an ayurvedic preparation containing mercury as chief ingredient was investigated for toxicokinetics and tissue distribution studies in laboratory animals.*
Methodology: *Shila Sindur at three doses i.e., 50 mg/kg, 300 mg/kg and 1000 mg/kg was studied. Single and repeated dose administration was used for toxicokinetics and tissue distribution studies, respectively and evaluated by using toxicokinetic and tissue distribution parameters.*
Outcome: *Toxicokinetics studies revealed low plasma clearance with high half life, this correlates with higher affinity of mercury to plasma proteins. Higher doses showed wide distribution by increased area under curve and volume of distribution. A dose dependent elevation in mercury concentrations and organ damage was found in kidneys followed by liver, lungs, spleen and brain. At higher doses, shila sindur found toxic, at moderate doses stringent monitoring was recommended for use. Present study concludes that low dose Shila sindur was found safe in terms of toxicological and tissue distribution patterns.*

Key words: *Shila sindur, toxicokinetics, tissue distribution, mercury*

INTRODUCTION

Ayurvedic medicine is a traditional system native to India and has been positioned as mainstream system of medicine since thousands of years. Globally, this system of medicine gained significance as an alternative and complementary medicine[1, 2]. This system uses natural plant based medicines and minerals including sulfur, arsenic, lead, copper and gold are often added to formulations with the belief that these metals are essential components of vital molecules within the human body[3].

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Indian Ayurvedic system and herbal medicines have attracted the global attention due to their slow and explicit cure (Anand Prem Rajan). Approximately 80% of India's 1 billion population use ayurveda[4,5]. This developing interest and usage raised up safety concerns about Ayurveda. Recent studies conducted by United States on this system of medicine found about 20% of ayurvedic treatments are containing toxic levels of heavy metals such as lead, mercury and arsenic[6].

Rasashastra, an intergral part of Ayurveda, deals with drugs of mineral origin comprehensively. In traditional Ayurvedic medicine as part of *Rasa Shastra*, practioners add minerals to plants/animal origin drugs and claim that such medicines are safe, when they are properly purified and detoxified. According to 'The Ayurvedic Formulary of India' mercury and lead are the most widely used heavy metals. As these metals are potent nephrotoxic, hepatotoxic, neurotoxic and hematotoxic agents [6, 7]. The toxicokinetics of these metals in ayurvedic preparations is geared up. Since there are no clear standards and recommendations are defined for metals contamination in ayurvedic preparations. In adequate scientific studies and clinical trials of many ayurvedic products lead to such circumstances[6].

This study was aimed to investigate toxicokinetic parameters, to detect and estimate mercury accumulation in various organs on a 28 days intake of Shila sindur, a herbo-mineral preparation composed of mercury, sulphur and arsenic disulfide (realgar) along with aloe vera. Shila sindur is indicated for all types of skin disorders, skin diseases associated with itching, skin conditioning and other diseases of infectious origin like fever, abscess, gonorrhea, cardio protection and neuro protection[8].

MATERIALS AND METHODS

Reagents and chemicals

Shila sindur was procured from the local market. Sodium Carboxy Methyl Cellulose, haemotoxylin, eosin, mercuric chloride, 2-mercaptoethanol and HPLC grade methanol were procured from SD fine chem., Mumbai. All reagents and chemicals used were analytical grade.

Animals

Male adult Wistar albino rats age of 6–7 weeks, weighing between 150 to 200g, were used in the study. They were procured from the Mahaveera enterprises (Reg no.146/1999 CPCSEA) Hyderabad, Andhra Pradesh. The animals were housed at CPCSEA Approved (Reg.no.1047/ac/07 CPCSEA) animal house of Vaagdevi college of Pharmacy. They were maintained under standard laboratory conditions at an ambient temperature of 25 ± 2°C and 50 ± 15% relative humidity, with a 12-h light/12-h dark cycle. Rats were fed with a commercial pellet diet (Hindustan lever Pvt. Ltd. Bombay, India.) and water *ad libitum*. They were fasted for 18 h prior to the experiment and during the experiment; the food and water were withdrawn. The animal experiments were performed after prior approval of the study protocol by the Institutional Animal Ethics Committee (1047/ac/07/CPCSEA, Dated 24/04/2007) of Vaagdevi college of Pharmacy, Warangal. Ethical norms were strictly followed during all experiments.

Toxicokinetic studies

Toxicokinetic studies were conducted as per OECD guideline 417[9]. The animals were grouped into 3 groups of 6 animals each. The doses were set as 50 mg/kg (low), 300 mg/kg (medium) and 1000 mg/kg (High). Shila sindur was administered orally (suspension with 0.1% Sodium CMC) to overnight fasted rats (OECD 417). Blood samples were taken at 1hr, 2hr, 4hr, 6hr, 12hr, 24hr and 48hr by retro-orbital puncture.

Tissue Distribution Studies

Tissue distribution studies guidelines were adopted from OECD guideline 417[9]. Animals were divided into six groups based on the dose and frequency of administration of drug i.e., High/repeated doses, High/single dose, Medium/repeated doses, Medium/single dose, Low/repeated doses, Low/single dose). In each group 6 animals were taken. In repeated doses groups, the shila sindur was administered for 28 days. After 24 hrs (for single dose) and 28th day (for repeated doses), the animals were sacrificed. Liver, lungs, kidneys, brain and spleen were collected and then weighed for wet weight and homogenized in phosphate buffer saline (PBS) of pH 7.4. The tissue homogenates were centrifuged at approximately 4000 rpm for 20 min and stored at -20^{0}C until analysis.

Clinical Bio-Chemistry Parameters

Clinical biochemical parameters like hematological parameters assessed by using Erba Heamo Lab-8 Heamaolog analyser (US Tech Inc, Washington MD, USA); liver function parameters i.e., SGOT and SGPT; Kidney function by assessing Serum Creatinine were conducted by using respective diagnostic kits (Crest Biosystems).

Histopathology

The rats were sacrificed by cervical dislocation. Complete necropsies were carried out on all animals. Tissues were preserved in 10% neutral buffered formalin. The tissues examined were brain, lungs, liver, spleen and kidney. Tissues were subjected to microscopic examination by embedding in paraffin wax, sectioned at five micrometers thickness by using Microtome (Rofin, Switzerland) and stained with haemotoxylin and eosin. Scanning Electron Microscopy (SEM) images were obtained for all the tissues (SEM- Joel, USA) to observe histopathology at various doses.

Analysis

The blood samples were analyzed using HPLC-ICP-MS. The kinetic parameters were estimated using Kinetica (version 5.0)

HPLC - ICP MS method validation for the presence of mercury

1. Equipment: Total mercury concentrations were determined using a Perkin Elmer SCIEX Elan DRC-e ICPMS (Table 1). Mercury species were determined using a Perkin Elmer series 200 HPLC pump with a Perkin-Elmer 3_mC8 (33mm×3mm) column coupled to a Perkin Elmer Sciex Elan 6000 ICPMS (Table 2). The lowest quantifiable mercury concentration was 0.004µg/ml.

2. Standards:

A stock solution (1000mg/l) of mercury (II) was prepared by dissolving the appropriate amount of mercuric chloride in de-ionized water. Stock solution was stored in airtight bottle and refrigerated. Working standards [(10ng/ml to 3 µg/ml) and (5, 10, 30, 50,100µg/ml)} were prepared daily from the stock solution by serial dilution using extracting solutions.

3. Reagents:

All reagents were analytical reagent grade and used without further purification. A 2-mercaptoethanol solution was prepared containing 0.5% (v/v) 2-mercaptoethanol in 5% (v/v) methanol and used as the extracting reagent and mobile phase for HPLC-ICPMS work.

4. Measurement of Hg concentration in Rat tissues and sediments by 2-mercaptoethanol extraction:

Freeze-dried samples (0.2 g) were weighed into 55 ml polytetrafluroacetate (PTFE) digestion vessels (CEM, USA) with 5ml and 2.5 ml of 0.5% (v/v) 2-mercaptoethanol for rat tissues and sediment samples respectively. The vessels were heated in a microwave oven at 120^0C for 15 min. The extracts were transferred to acid washed 10 ml polypropylene centrifuge tubes and centrifuged in an Eppendorf centrifuge 5804 for 20 min at 3000 rpm. For sediment samples, after the supernatant was separated, the extraction was repeated and extracts combined.

5. HPLC-ICP-MS:

All supernatants were filtered through Acrodisc LC 13-mm Syringe filters with 0.2_mPVDF membranes before analysis. Aliquots of extracts (100µl) were injected onto the HPLC–ICP-MS (Table 2). External calibration using the standards 0–100µg/l was used. The chromatography package Turbochrom Navigator (Perkin Elmer, Australia) was used to quantify mercury species by peak area.

Table 1: Operating conditions for ICP-MS.

Plasma conditions	
RF forward power	1200W
Plasma argon flow rate	15 L/min
Auxiliary argon flow rate	1.2 L/min
Nebulizer gas flow	0.92 L/min
Mass spectrometer settings	
Acquisition mode	Peak hopping
Isotopes monitored	202Hg, 201Hg, 200Hg, 198Hg, 181Ta, 184W, 103Rh, 115 In, 159Tb, 165Ho
Dwell time	50 ms
Sweep time	13.5 s
Sweeps per reading	15
Replicates	Total: 3
Integration time	750 ms per element

Table 2: Operating conditions for HPLC-ICP-MS.

Chromatography	
HPLC column	PE C8, 3_m (33mm×3mm)
Mobile phase	0.5% (v/v) 2-mercaptoethanol in 5% (v/v) CH3OH, pH 5.3, flow rate, 1.5 ml min−1; temp, 25 ◦C
Sample volume	100µl
Plasma conditions	
RF forward power	1200W
Plasma argon flow rate	15 lmin−1
Auxiliary argon flow rate	1.2 l min−1
Nebulizer gas flow	0.84 l min−1
Mass spectrometer settings	
Acquisition mode	Peak hopping
Isotopes monitored	202Hg, 201Hg, 200Hg, 198Hg, 181Ta and 184W
Dwell time	100ms
Sweeps per reading	1
Replicates	1
Readings	500 (6.52 min)

RESULTS AND DISCUSSION

A. Blood Hg profile at various doses:

Table 3: Concentration of Hg in blood at different time points

Time (hrs)	*Concentration (µg/ml) of Hg in Blood*		
	1000mg/kg	*300mg/kg*	*50mg/kg*
0	0	0	0
2	4.128 ± 0.341**	3.451 ± 0.124**	2.480 ± 0.241
4	12.015 ± 0.651***	8.140 ± 0.412***	4.178 ± 0.176
6	5.018 ± 0.125**	4.312 ± 0.125**	2.451 ± 0.270
12	3.871 ± 0.345**	2.871 ± 0.315*	1.631 ± 0.311
24	2.151 ± 0.158**	1.021 ± 0.315	0.810 ± 0.134
48	1.891 ± 0.135**	0.984 ± 0.105*	0.503 ± 0.089

*All values are expressed as Mean ± SD, n=4, *p<0.05; **p<0.01 ***p<0.001 when compared to 50mg/ kg dose group. Statistical Comparision was performed by using ANOVA coupled with Dunnet's multiple comparision test.*

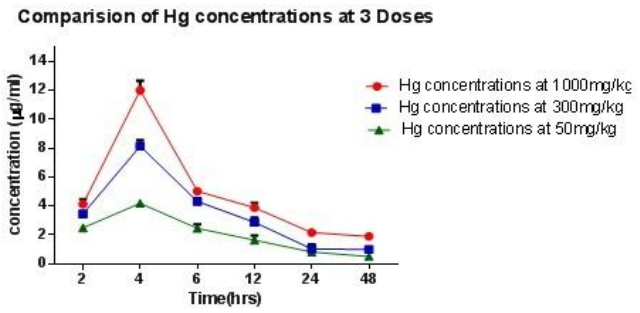


Fig 1: *Graph for Concentration Vs Time Profiles*

B. Toxicokinetic parameters

Table 4: Toxicokinetic Parameters

Sl. No	*Parameter*	*Units*	*1000mg/kg*	*300mg/kg*	*50mg/kg*
1	C_{max}	µg/ml	12.01±0.15***	8.14±2.19**	4.17±0.30
2	T_{max}	hr	4.0±0.00	4.0±0.00	4.0±0.00
3	HVD	hr	3.11±0.19**	4.588±0.80*	6.6±0.98
4	AUC last	µg/ml*hr	154.91±15.02***	103.093±9.25***	61.538±9.58
5	AUC extra	µg/ml*hr	83.52±6.37***	28.634±5.23***	13.784±3.61
6	AUC tot	µg/ml*hr	238.4±42.15***	131.727±16.29***	15.323±2.10
7	$T_{½}$	hr	30.61±5.16**	20.170±3.14*	18.994±4.13
8	MRT	hr	43.48±1.64**	28.149±3.11*	25.983±4.15
9	K_e		0.022±0.00015*	0.034±0.001	0.036±0.0018
10	V_d	ml	158.45±44.8***	45.4±6.24**	16±3.16
11	Cl	ml/hr	3.48±0.62**	1.54±0.25*	0.57±0.012

*All values are expressed as Mean ± SD, n=5, *p<0.05; **p<0.01 ***p<0.001 when compared to 50mg/ kg dose group. Statistical Comparision was performed by using ANOVA coupled with Dunnet's multiple comparision test.* ***C_{max}:*** *Peak plasma Concentration;* ***T_{max}:*** *Time to reach peak concentration,* ***HVD:*** *Half- Value Duration;* ***AUC:*** *Area under plasma concentration /time;* ***$t_{1/2}$****, terminal half life;* ***MRT:*** *Average mean residence time.* ***K_e:*** *Elimination rate constant;* ***V_d:*** *Volume of distribution,* ***Cl:*** *Clearance.*

C. Tissue distribution studies

Table 5: Concentration (µg) of Hg in different tissues

Doses	*Brain*	*Liver*	*spleen*	*lungs*	*Kidney*
High / repeated dose	0.015 ±0.001	0.514±0.003**	0.514±0.002	0.714±0.0021***	25.781±2.347***
High / single dose	0.001±0.0003	0.07±0.003	0.003±0.0003	0.09±0.0005**	2.151±0.098*
Medium / repeated doses	0.014±0.002	0.505±0.145**	0.505±0.07	0.405±0.003***	6.215±1.23**
Medium / single dose	0.015±0.001	0.05±0.002	0.002±0.0001	0.05±0.004**	1.315±0.089
Low / repeated doses	0.012±0.004	0.124±0.013*	0.0211±0.003	0.012±0.002*	2.012±0.015*
Low / single dose	< LLOQ	0.015±0.0002	< LLOQ	0.001±0.0001	0.129±0.005
High / repeated dose	0.015 ±0.001	0.514±0.003**	0.514±0.002	0.714±0.0021***	25.781±2.347***

*All values are expressed as Mean ± SD, n=4, *p<0.05; **p<0.01 ***p<0.001 when compared to Low single dose group. Statistical Comparision was performed by using ANOVA coupled with Dunnet's multiple comparision test.*

C. Ratio of tissue - blood concentrations

Table 6: *Ratio of tissue to blood concentrations at different doses*

Doses	*Brain*	*Liver*	*spleen*	*lungs*	*Kidney*
High / repeated dose	4.128	0.003634	0.124516	0.100291	0.172965
High / single dose	3.84	0.00026	0.018229	0.000781	0.023438
Medium / repeated oses	3.451	0.004057	0.146334	0.146334	0.117357
Medium / single dose	2.93	0.005119	0.017065	0.000683	0.017065
Low / repeated doses	2.48	0.004839	0.05	0.008508	0.004839
Low / single dose	0.98	0	0	0	0.00102
High / repeated dose	4.128	0.003634	0.124516	0.100291	0.172965

Concentration of Mercury in Various Organs

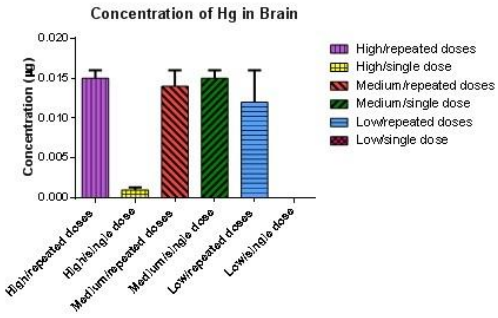


Fig 3: *Concentration of Mercury in Brain*

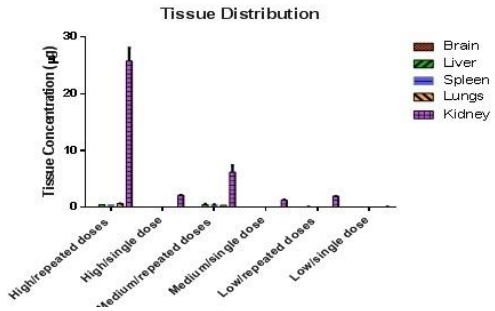


Fig 2: *Tissue distribution*

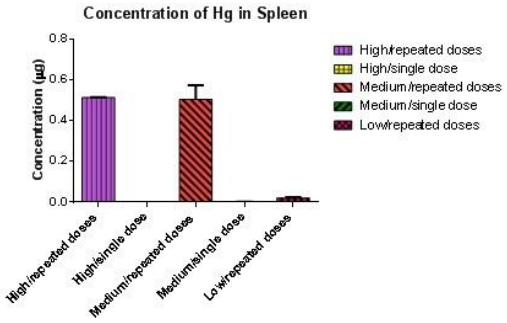


Fig 5: *Concentration of Mercury in Spleen*

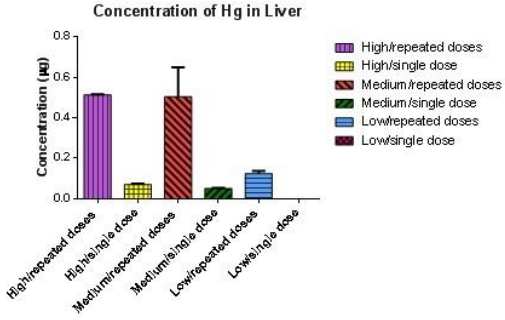


Fig 4: *Concentration of Mercury in Liver*

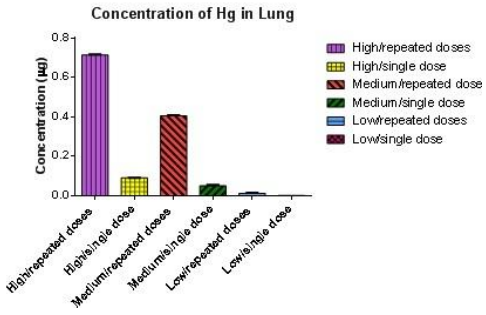


Fig 6: *Concentration of Mercury in Lung*

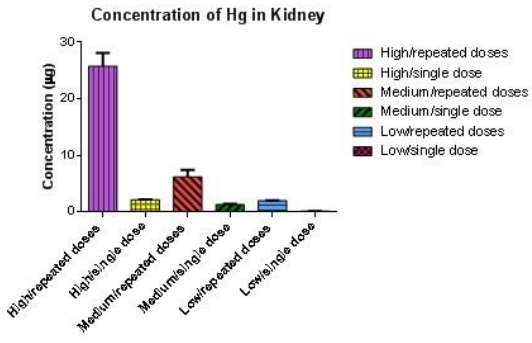


Fig 7: *Concentration of Mercury in Kidney*

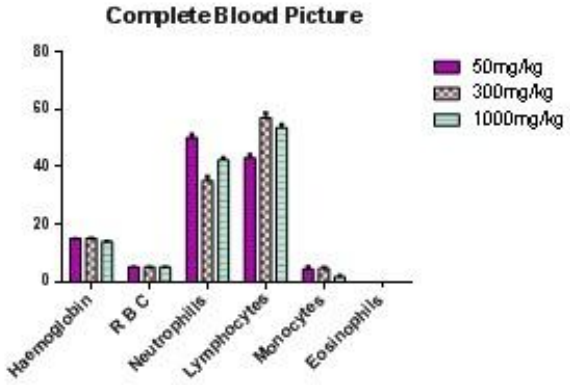
D. Bio-chemical parameters

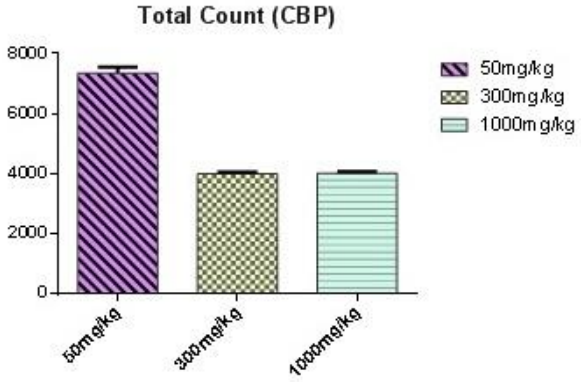
1. Complete blood picture

Table 7: *Complete blood picture of 28 day- treated animals at various doses*

Cells	*1000mg/kg*	*300mg/kg*	*50mg/kg*
Haemoglobin(gms%)	13.9±0.18	14.95±0.12***	14.95±0.07***
R.B.C(m/cmm)	4.98±0.03	4.95±0.07	4.96±0.04
Total count(TC) (m/cmm)	4008.33±60.66	3991.66±60.66	7341.66±204.97***
Neutrophils(%)	42.33±0.74	35±1.63***	50±1.29***
Lymphocytes(%)	53.5±1.25	57±1.63***	43±1.29***
Monocytes(%)	1.66±0.47	4.5±0.5***	4.33±0.74***
Eosinophils(%)	0±0	0±0	0±0

All values expressed as Mean ± SD, (n=4); *p<0.05 significant, **p<0.01 highly significant, ***p<0.001 very highly significant when compared with 50mg/kg dose group. Statistical Comparision was performed by using ANOVA coupled with Dunnet's multiple comparision test.





2. *Liver function tests: SGOT and SGPT estimation*

Table 8: *Liver Function Tests of 28 day- treated animals at various doses*

Group	SGOT (U/L)	SGPT (U/L)
1000mg/kg	189.33±4.60***	178.50±5.02***
300mg/kg	93.33±2.42**	80.66±1.69**
50mg/kg	59.16±1.57*	68.83±2.26*
Control	38.23±1.20	36.69±1.89

*All values expressed as Mean ± SD, (n=4); *p<0.05 significant, **p<0.01 highly significant, ***p<0.001 very highly significant. Statistical Comparision was performed by using ANOVA coupled with Dunnet's multiple comparision tests.*

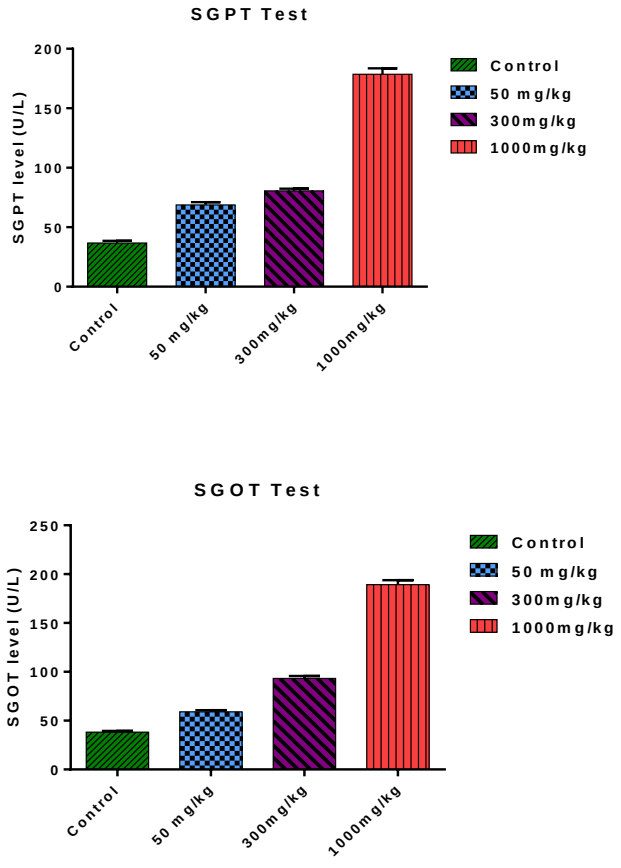


Fig 9*: Liver Function Tests*

3. Kidney function test: serum creatinine estimation

Table 9: *Serum Creatinine levels of 28 day- treated animals at various doses.*

Group	*Serum Creatinine (mg/dl)*
1000mg/kg	3.06 ±0.33***
300mg/kg	2.28 ±0.19**
50mg/kg	1.75 ±0.10*
Control	0.89±0.01

All values expressed as Mean ± SD, (n=4); *p<0.05 significant, **p<0.01 highly significant, ***p<0.001 very highly significant. Statistical Comparision was performed by using ANOVA coupled with Dunnet's multiple comparision test.

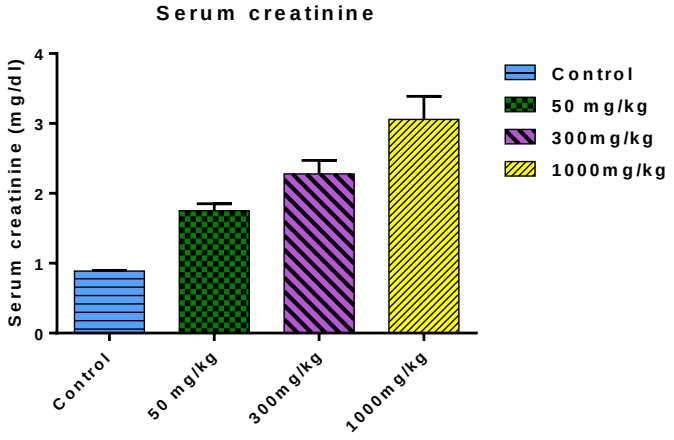
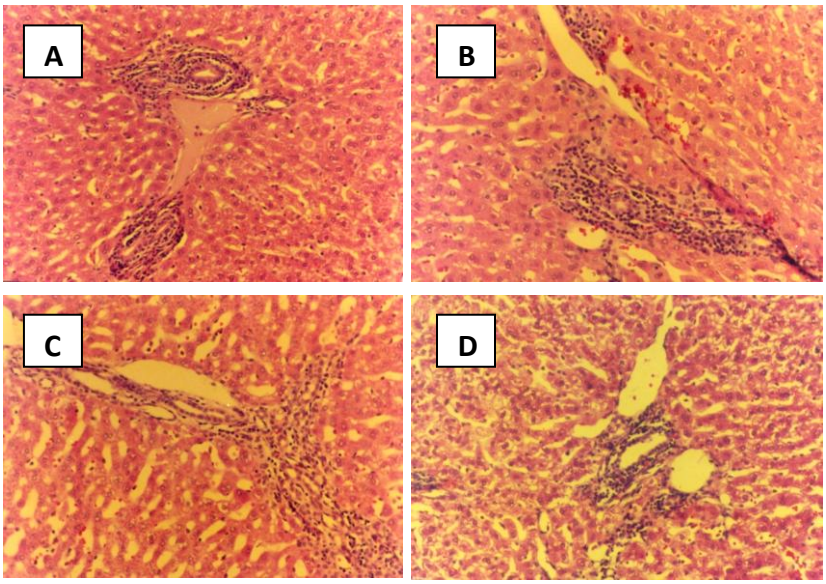


Fig 10*: Kidney Function Test*

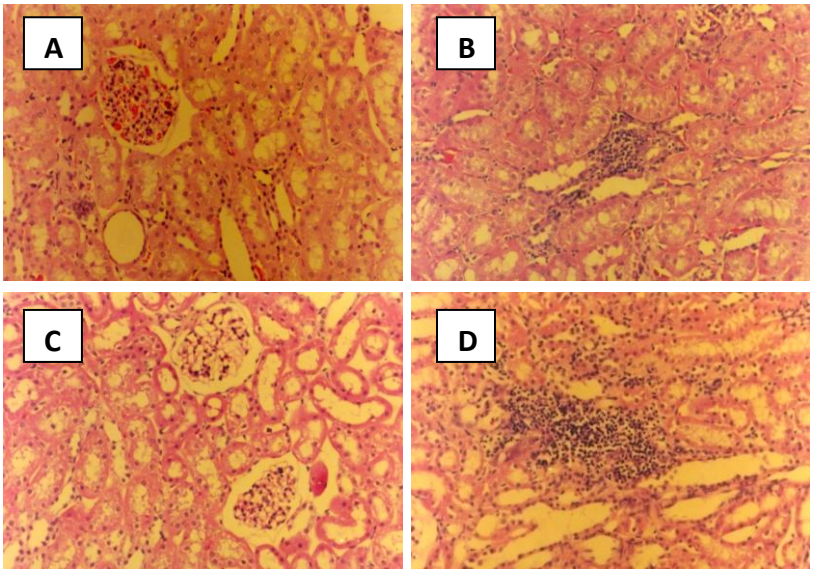
E. HISTOPATHOLOGICAL STUDIES

Fig: 11 HISTOPATHOLOGY OF LIVER



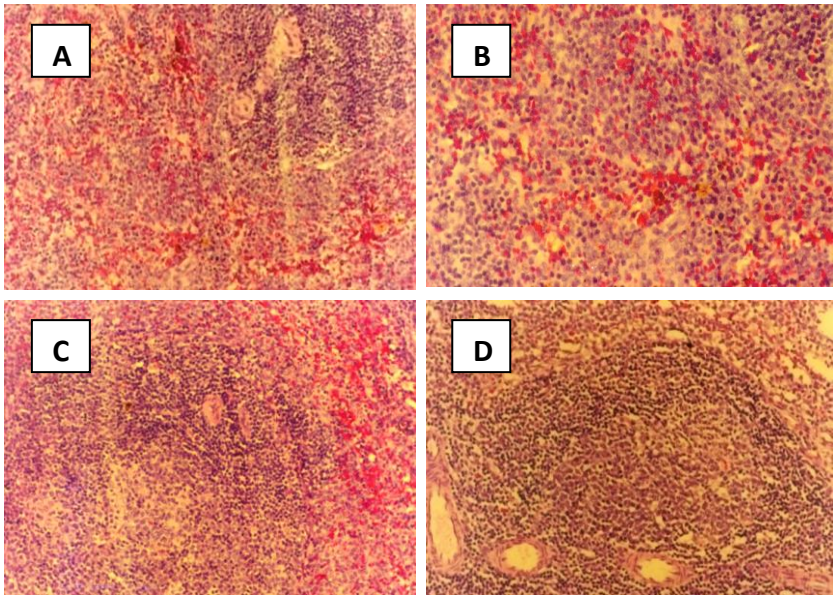
Control (A): Liver showing normal architecture
Low dose (B): Showing mild degenerative changes with few focal mono nuclear cell infiltration
Medium dose (C) showing mild-moderate degenerative changes with dilatation of hepatic venule with focal mono nuclear cell infiltration
High dose (D): showing dilated bile duct and portal tract with a proliferative changes and occasional necrotic changes

Fig.12: HISTOPATHOLOGY OF KIDNEY



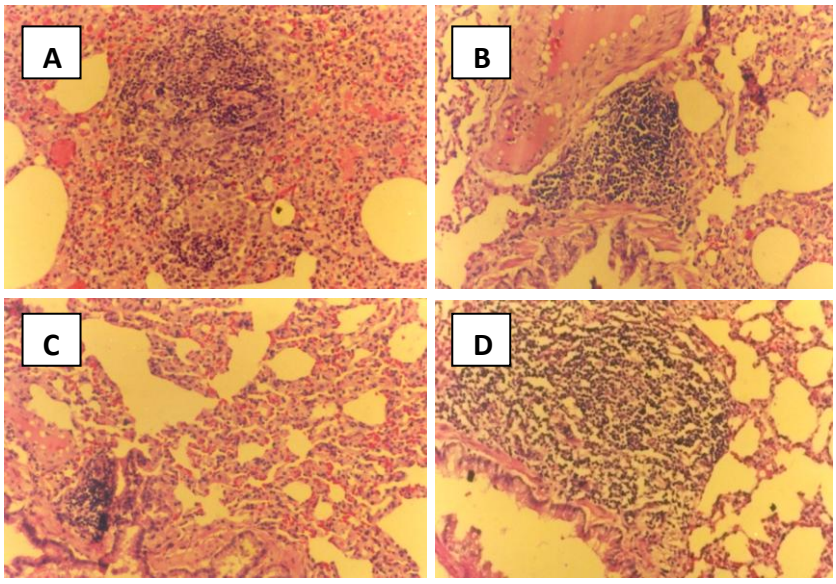
Control (A): Showing normal renal cortical parenchyma
Low dose (B): Showing normal renal cortical parenchyma with focal mild mono nuclear infiltration in Bowman's capsule
Medium dose (C): Shows mild degenerative changes with mono nuclear infiltration in the renal parenchyma
High dose (D): Show diffuse infiltration of mono nuclear cells with focal necrotic debris

Fig. 13: HISTOPATHOLOGY OF SPLEEN



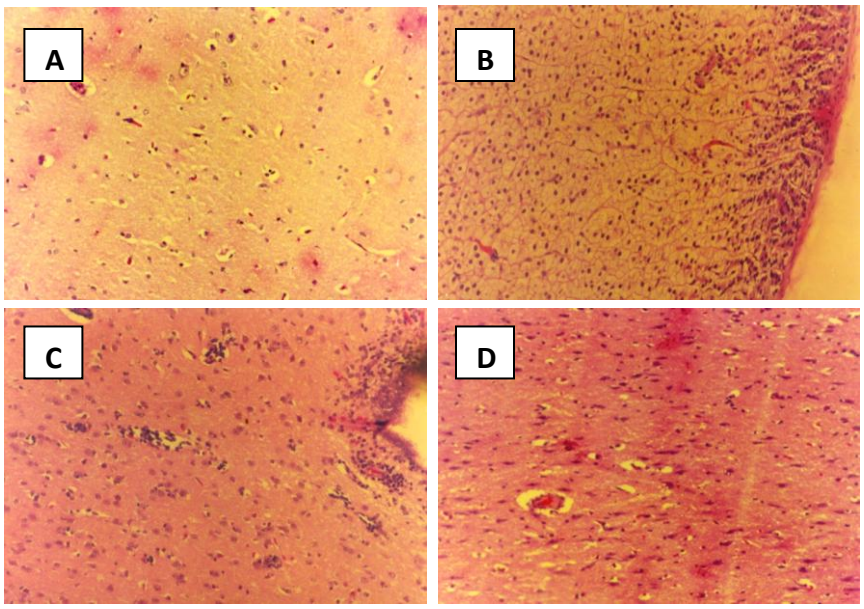
Control (A): Shows normal lymphoid masses (White pulp)
Low dose (B): Shows mild proliferative changes in the sphlenic lymphoid masses
Medium dose (C): Shows mild-moderate proliferative changes in the sphlenic lymphoid masses
High dose (D): Spleen showing mild-moderate proliferative changes in the sphlenic lymphoid masses

Fig.14: HISTOPATHOLOGY OF LUNG



Control (A): Shows normal bronchioles and alveoli
Low dose (B): shows alveoli with mild inflammatory mono nuclear infiltration
Medium dose (C): Shows foci of mono nuclear aggregates in the lung parenchyma
High dose (D): the alveoli adjacent to bronchiole a large aggregates of mono nuclear cell infiltration and no degenerative changes

Fig 15: HISTOPATHOLOGY OF BRAIN



Control (A): Shows normal white matter of cerebral cortex
Low dose (B): shows normal white matter of cerebral cortex
Medium dose (C): Shows normal white matter of cerebral cortex
High dose (D): Shows normal white matter of cerebral cortex

DISCUSSION

Repeated dose toxicity studies categorically suggest that with increasing doses there were significant changes taken place in the clinical/ biochemical parameters evaluated. Though the changes are not intense, but changes occurred in the complete blood picture were significant at higher doses. The elevated serum creatinine levels indicating an extensive damage to the kidney at higher doses. The glomeruli in the kidneys are those which were extensively damaged and inflammation mediated necrotic tissues were mainly seen in the medium and high dose study groups. A significant damage to the liver is indicated by the elevations in the SGPT and SGOT levels. The changes in the clinical/ biochemical parameters were strongly supported by the histopathological studies. There was also level II (medium) damage in lungs and liver in higher repetitive doses.

There was a strong positive correlation between the clinical parameters, histopathological studies and the tissue concentrations in the study groups which is pivotal for these present investigations. Mercury concentrations in the kidney are very high followed by the liver and lungs. Some accumulation of mercury is also observed in spleen and the least content observed in brain. There was 15 to 20 fold increase in the kidney mercury concentrations in high dose compared to low dose groups. Tissue to blood concentrations is also high, suggesting a strong association and high affinity binding that led to damage of glomeruli leading to elevation in creatinine levels. In liver there was 5 fold increase in mercury levels between the low dose and high dose groups and hence necrosis in the hepatocytes and subsequent elevation in SGPT and SGOT levels.

There was dose dependent increase in the plasma concentrations. From the toxicokinetic parameters, Plasma clearance (CL) was extremely low with high half life ($T_{1/2}$). This strongly correlates with the high plasma protein binding/affinity of mercury. Increase in AUC and V_d was also observed with increasing doses. But it is quite evident from the study that plasma concentrations and subsequent PK analysis doesn't supinely speak about the toxicity of mercury as evident from the tissue concentrations even at low doses. Saturation kinetics of the mercury at high dose needs further investigations.

CONCLUSION

Above investigations categorically conclude that low single dose or even low repeated dose of mercury could be safe and may not lead to accumulation in the tissues especially in kidney - the target organ prone to damage. Medium dose administration needs stringent monitoring of the clinical/biochemical parameters for optimization of the dose. At this dose the administration of the drug or its continuation is at the discretion of the medical practitioner. High single dose and high repeated doses have showed very deleterious effects, further continuation may lead to severe nephrotoxicity, thus shila sindur at higher doses are not advisable for administration. Therefore, the process of purification and preparation of the product-shila sindur need to be improved.

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