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Pharmacokinetics Profile of Chitosan Nanoparticles in Chronic Lead-induced Toxicity Rats Model

Aditya Marianti[1*], Nur Dina Amalina[2], Sri Mursiti[3], Faya Nuralda Sitompul[4], Shafira Septiana Futri[2], Legendra Gantar Negara[2], Intan Kharyna Sholehah[1], Dian Sri Asmorowati[3], Putri Dyah Astari[1]

[1]Biology Department, Faculty of Mathematics and Natural Science, Universitas Negeri Semarang, Indonesia
[2]Pharmacy Department, Faculty of Medicine, Universitas Negeri Semarang, Indonesia
[3]Chemistry Department, Faculty of Mathematics and Natural Science, Universitas Negeri Semarang, Indonesia
[4]Research Institute for Microbial Diseases, Osaka University, Japan

* Corresponding author: aditya.marianti.am@mail.unnes.ac.id

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Abstract. Chronic lead exposure induces ROS accumulation which causes physiological disorders. Chelation therapy has been widely used to overcome lead poisoning since it exerts only a few side effects. Nano chitosan prevents lead poisoning by inhibiting ROS. This study examined the pharmacokinetics of nano chitosan in chronic lead-induced toxicity animal models and the mechanism of action pathway using the bioinformatic approach, The area under the curve was estimated to be 12110.13 ± 7709.37 µg/mL hours using the pharmacokinetic model, and the Cmax was 82.34 ± 5.64 µg/mL. The Tmax and t½ calculations were 22.68 ± 11.67 and 80.47 ± 60.58 hours respectively. Chitosan nanoparticles regulated VEGFA, FGF2, and LGALS3 which plausibly played a substantial role in chronic lead exposure. However, chitosan is not suitable for oral administration due to its low gastrointestinal solubility. These characteristics make chitosan nanoparticles have the prospect of being developed as a supplement so that they can contribute to overcoming the negative impacts of chronic lead poisoning.

Keywords: chitosan nanoparticles; pharmacokinetic; lead poisoning

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INTRODUCTION

Lead is one of the most prevalent and prominent environmental toxins that is widely used in various industries (Wani et al., 2015). Aside from that, it also creates a public health concern (Boskabady et al., 2018). Chronic lead exposure has a cumulative effect on many physiological systems and organs, including hepar renal, nervous, hematopoietic, and hepatic systems. Prolonged exposure creates serious disorders in humans (Flora et al., 2012). As lead can accidentally enter the human body through dietary consumption, it triggers the emergence of many cancer types, including myeloma, ovary, kidney, lungs, intestine, and stomach(World Health Organization, 2021). Lead has also been reported to cause a reduction in the quality of semen in the male reproductive system (Marianti, Isnaeni, et al., 2020). In vitro study, lead could cause the impairment of sperm acrosome, while as in vivo study, lead will disrupt zinc ion in testes protection. Both will cause male infertility (Mukherjee et al., 2022).

According to WHO, lead exposure accounts for 1.5 percent of the global burden of disease and kills about 900.000 people each year (World Health Organization, 2021). Developing countries are particularly vulnerable to lead poisoning and are subjected to the highest burden of this hazard. In Indonesia, an estimated 400 children died due to chronic lead exposure in 2022 (Irawati et al., 2022). Chelation therapy with specialized drugs, such as dimercaprol, ethylenediaminetetraacetic acid, succimer, and d-penicillamine, is currently used to treat lead poisoning (Marianti et al., 2017). However, frequent treatment is limited due to the negative side effects, such as chronic kidney disease that enhances the result of renal dialysis (Sudjarwo et al., 2017), (Rozina, 2002), (Tang et al., 2021). Recent studies suggest that chitosan prevents lead poisoning due to the combined activity of reactive hydroxyl and amine groups that inhibit the formation of ROS and prevent lipid

peroxidation in a biological system (Marianti & Mahatmanti, 2018).

Chitosan is a linear and semi-crystalline polysaccharide. It is comprised of (1→4)-2-acetamido-2-deoxy-β-D-glucan (N-acetyl D-glucosamine), and (1→4)-2-amino-2-deoxyβ-D-glucan (D-glucosamine). Chitosan, which is produced from the deacetylation of chitin, can bind Pb^{2+} ions (Li et al., 2015). The deacetylation degree of chitosan affects its ability to bind metal ions (Mathaba & Daramola, 2020). Moreover, chitosan has the potential to be developed as an ideal biological material in a pharmaceutical formulation. It has improved biological activity, including high biocompatibility, biodegradability, immunogenicity, and low toxicity (Saleh et al., 2015). Furthermore, the higher the degree of deacetylation leading to the greater the chelation ability of chitosan (Sulistyani et al., 2017). Chitosan has a relatively large molecular weight, which makes its distribution to body tissues ineffective (Jhaveri et al., 2021). Chitosan irradiated with gamma Co-60 at a dose of 150 kGy, which had reduced viscosity and molecular weight (Marianti et al., 2020), was proven to have a protective effect on the small intestine of rats induced by chronic lead acetate, although it was not optimal (Marianti et al., 2022).

Chitosan nanoparticles were distributed more effectively, penetrated the intercellular space more quickly, and increased affinity for biological systems due to an increase in contact surface area by the same amount (Gao et al., 2022;Mohammed et al., 2017). Chitosan nanoparticles have been extensively utilized in the human body as an excellent medical biodegradable material (Zhao et al., 2018). The pharmacokinetics and bodily distribution from intraperitoneal administration of chitosan in mice have been investigated (Li et al., 2015). The degradation mechanism and metabolic pathway of water-insoluble chitosan nanoparticles were also investigated (Kim, 2018). However, the pharmacokinetics profile of chitosan nanoparticles in chronic lead exposure has not been documented yet. Therefore, this study evaluates the pharmacokinetic profile of chitosan nanoparticles in chronic lead exposure in an animal model and the pathway of action mechanism through a bioinformatic approach, namely the application of computational techniques to manage and analyze biological information.

METHODS

Chitosan nanoparticles solution

Chitosan nanoparticles ($C_6H_{11}NO_4$) were obtained from Nanoshell with purity 99, molecular weight 161 g/mol, and APS 80-100nm. It was dissolved in 1% (v/v) of CH_3COOH (acetic acid) solution in a concentration of 21 g/L.

Animal housing

Male Wistar rats weighing 150 – 200 g were put in a room with pre-arranged environmental aspects, including adjusted light, controlled humidity and temperature, and adequate water supply. All animal experiments were conducted under the approval of the National Research Council's Guide for the Care and Use of Laboratory Animals in were stated by the Medical Research Ethics Committee of the Faculty of Medicine, Sultan Agung Islamic University No. 342/VII/2021/Komisi Bioetik.

Lead poisoning model rat preparation

Twenty rats were induced with Pb acetate through oral administration on the dose of 200 mg/kg BW for 30 days. After 30 days, the rats were taken with 2 ml of blood through the orbital sinus. The whole blood was used to measure blood Pb levels using the Atomic Absorption Spectrophotometry (AAS) method. The measurement was performed at the Laboratory of Chemistry in Universitas Negeri Semarang, Indonesia. Rats were declared to have lead poisoning when the blood lead levels reached 10 µg/dL.

Pharmacokinetic study of chitosan in rats

Chitosan nanoparticles were dissolved in 1% (v/v) of CH_3COOH (acetic acid) solution with a concentration of 21 g/L. Chitosan nanoparticles were administered through the oral route to lead-poisoning rat models with a dose of 64 mg/kg body weight**.** Blood samples were obtained at 0, 0.25, 0.5, 1, 3, 5, 8, 10, 24, and 48 hours after treatment from the orbital sinus vein and were placed into a vacutainer plain tube.

Sample preparation

Blood samples were centrifuged for 10 minutes at 3000 rpm and 4℃. The supernatant was obtained as the serum sample and stored at -4℃ for analysis.

Chitosan nanoparticle concentration in rats' serum

Chitosan nanoparticle levels in the serum samples were analyzed using visible spectrophotometers based on the methods developed by Badawy, with slight modification (Badawy, 2012). Briefly, the chitosan nanoparticles suspension was produced by liquifying 100 mg chitosan nanoparticles by dissolving them in 100 mL of 1% (v/v) acetic acid (CH_3COOH). 0.5 ml of serum in the tube was added with 100 µL of 0.5 M NaNO2 and was homogenized by the vortex. To complete the depolymerization-deamination process, the arranged solutions were incubated for 30 minutes in 80°C water. The acidity level was then arranged into 8 by 0.1 M NaOH addition by stirring which was then combined into 0.5 mL of 0.04 M thio barbituric acid solution. The tubes were then incubated again inside a water bath for 10 minutes at 80°C. The solution from each tube was stirred for absorbance measurement at 555 nm using an ELISA reader against a blank.

Pharmacokinetics parameters

The maximum time was calculated to maintain Cmax (maximum concentrations) by using the computation of pharmacokinetic parameters with the formulation of time curve (Tmax) and drug concentration. The trapezoid method was used to calculate the area under the chitosan curve (AUC). The slope of the curve with a focus on the linear regression of drug concentration (ln vs time) was used for the elimination constant (Ke). The volume distribution (Vd) was calculated by using linear regression interception (ln Co). The following formulae were used to calculate Vd, half-life (t½), and clearance (Cl): Vd = F dosage/Co, t½ = 0.693/ke, and Cl = 0.693 Vd/ t½.

Analysis of Bioinformatics

Direct protein target (DTPs) of chitosan was collected from SEA prediction target and Swiss Target Prediction using canonical SMILES of chitosan (PubChem ID 71853). The proteins that role in chronic lead exposure was collected from NCBI-gene with the keyword "chronic lead exposure". STRING-DB v11.0 was chosen to create the PPI network (Cahyono, et al. 2021; Hermansyah, et al. 2021; Mursiti et al., 2021; Suzery et al. 2021). The confidence level of > 0.4 was significant. The PPI network was visualized using Cytoscape software. Cytohubba was used to analyze and choose three genes with the highest degree of scoring as hub genes. The physicochemical and pharmacokinetic profile of chitosan was studied using SwissADME, a free-access online web tool developed by (Daina et al., 2017). (http://www.swissadme.ch/index.php) which requires submission of their respective SMILES notations.

Statistical analysis

The results from the research were reported as mean ± standard deviation (SD). Statistical analysis was ANOVA (one-way analysis of variance) with P value of < 0.05 was a significant difference. Further testing of difference was Tukey's HSD *post hoc* test.

RESULTS AND DISCUSSION

Chitosan nanoparticles are extensively applied in several pharmacological and biomedical fields for their prominent nontoxic property, high biocompatibility, and biodegradability. A previous study indicated that the primary function of chitosan group -OH and $N\text{-}H_2$ affects the formation of heavy metal complexes, including Pb which results in decreasing blood and tissue Pb accumulation (Silitonga & Siswanta, 2019). In the present study, the pharmacokinetic properties of chitosan nanoparticles in chronic lead toxicity were investigated in an in vivo model. As for the molecular mechanism, biological activities owned by chitosan were determined using a bioinformatic approach.

Optimation of Wavelength Spectral analysis

For the first step, the maximal absorbance of chitosan nanoparticles was evaluated. The absorbance scan was performed on 15µg/mL chitosan nanoparticles in 1% (v/v) aqueous acetic acid solution within the range of 425 -695 nm. The same solution without chitosan nanoparticles was used as a control. After 10 minutes, the reaction with thiobarbituric acid gave rise to a pink-colored derivative with an absorbance at 555 nm (Figure 1). Therefore, all absorbance measurements were carried out at the same wavelength due to the high sensitivity of the chemical reactions and the performance of colored complexes.

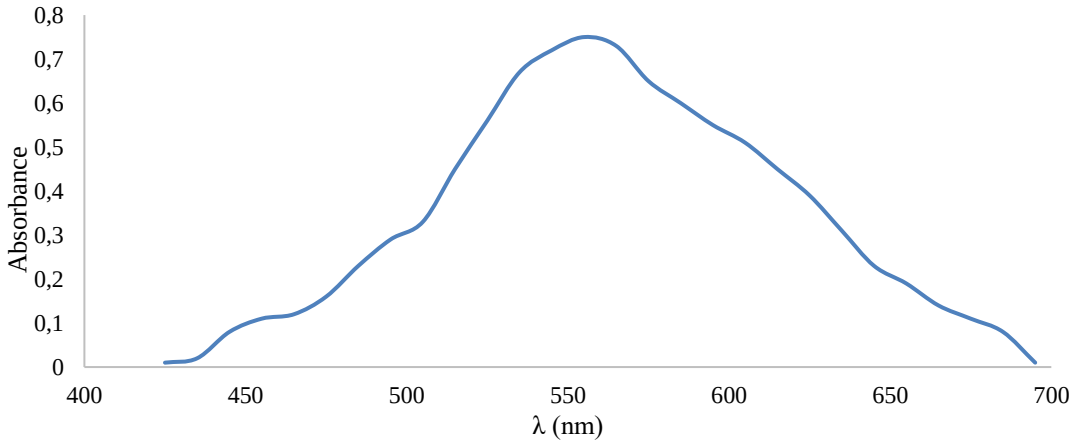

Figure 1. Absorbance spectrum of the chitosan nanoparticles with thio-barbituric acid. The spectrum between 425 to 695 nm is plotted and exhibits 555 nm as the maximum wavelength which is required for the pink color to be formed.

Pharmacokinetic profile of nano chitosan in chronic lead exposure rat's model

Following the administration of chitosan nanoparticles at the dose of 64mg/kg BW on chronic lead exposure rat models, the chitosan nanoparticles concentration was detected in rat serum after 15 minutes (0.1286 µg/mL). The maximum plasma concentration (Cmax) was achieved 8 hours after the administration (Figure 2).

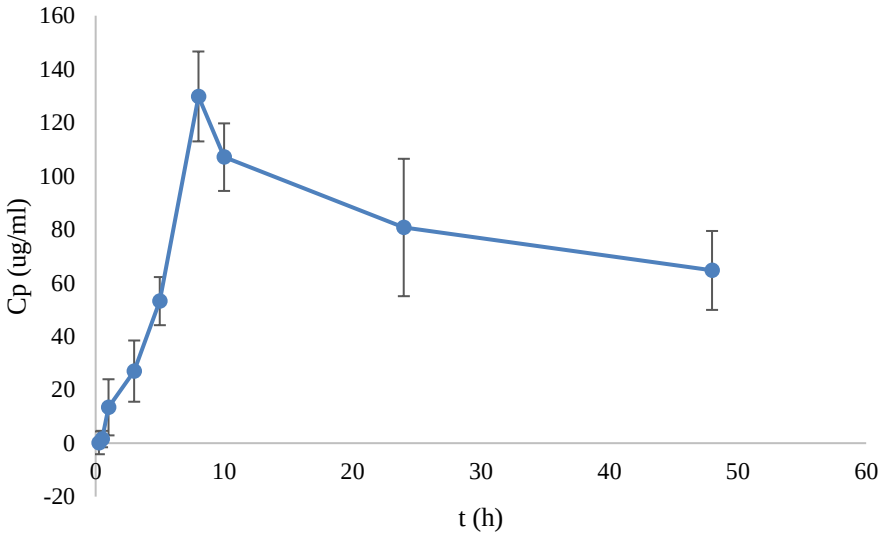

Figure 2. Chitosan nanoparticle concentrations in serum from 0-48 h after oral chitosan administration. Data are presented as mean±SD.

The plasma level results of nano chitosan from 0 to 48 h after oral administration are available for measurement. The concentration maximum (Cmax) value of nano chitosan is 82.34 ± 5.64 µg/mL. The Tmax and the elimination half-life are 22.68 ± 11.67 and 80.47 ± 60.58 hours, respectively. The volume of distribution (464.88 ± 71.64 L/kg) was higher than clearance (5.78 ± 3.15 mL/hours) (Table 1). Pharmacokinetic parameters are shown in Table 1. In general, nanoparticle-based drugs have high oral bioavailability and may increase the absorption of a compound, resulting in high plasma concentration, as shown by the high AUC level (12110.13 ± 7709.37 µg/mL hours). The result indicates that chitosan nanoparticles stay inside the body for a long duration with a fast onset of 15 minutes. A typical subject has an absorption rate constant (Ka) of 0.16 ± 0.13 hours^{-1} and an elimination rate constant (K) at 0.01 ± 0.01 hours^{-1}.

Table 1. Pharmacokinetics parameters of chitosan nanoparticles

Pharmacokinetic parameter	value
Ka (hours^{-1})	0.16 ± 0.13
K (hours^{-1})	0.01 ± 0.01
Cmax (µg/)mL	82.34 ± 5.64
Tmax (hours)	22.68 ± 11.67
Vd (L/kg)	464.88 ± 71.64
Cl (mL/hours)	5.78 ± 3.15
T1/2 elimination (hours)	80.47 ± 60.58
AUC -~ (µg/mL hours)	12110.13 ± 7709.37
T1/2 abs (hours)	7.46 ± 6.71

Bioinformatic analysis

Based on the pharmacokinetics data, we investigated the potential target genes related to the effect of chitosan as lead poisoning therapy. To determine the potential therapeutic target of chitosan toward lead poisoning, we collected 466 genes that represented chronic lead exposure according to the PubMed-NCBI database. Direct protein target (DTPs) of chitosan was also acquired using SEA prediction targets and Swiss Target Prediction to obtain more comprehensive data. It was shown that a total of 34 genes from the database were encoded as chitosan DTPs. Venn diagram was then utilized to cross the genes between the DTPs of chitosan and chronic lead exposure. Five overlapping genes were detected and then used for further analysis of chitosan potential target genes (CPTGs) (Figure 3A). CPTGs were curated to identify the interaction among each gene and visualized the network using the STRING online tools. In the PPI network, we found 5 nodes with 3 edges. We also discovered an average node at the degree of 1.2 (Figure 3B). The top three genes with the highest degree score were vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), and galetic-3 (LGALS3) (Figure 3C). Moreover, three genes with the highest score are involved with each other for inhibiting lead poisoning. A previous study reported the association between chronic lead exposure and elevated levels of several specific pro-inflammatory cytokines that lead to promote angiogenesis (Machoń-Grecka et al., 2018). Short-term lead exposure induces VEGF expression and angiogenesis that correlate with triggered FGF levels (LaBreche et al., 2011). Another study also suggests that lead exposure may module angiogenesis via induction of VEGF gene expression using the mitogen-activated protein kinase (MAPK) pathway (Saghiri et al., 2016). Our results reveal that chitosan has a direct interaction with VEGF and FGF2 that can prevent angiogenesis due to chronic lead poisoning. This data also supports the concept that the effects of lead exposure can be minimized by inhibiting angiogenesis (Machoń-Grecka et al., 2018). On the other hand, lead concurrently dysregulates angiogenesis-triggering pro-and antiangiogenic pathways which promotes angiogenesis. Chitosan may influence the suppression of VEGF and FGF expression by direct binding targets.

Pharmacokinetic Predictions

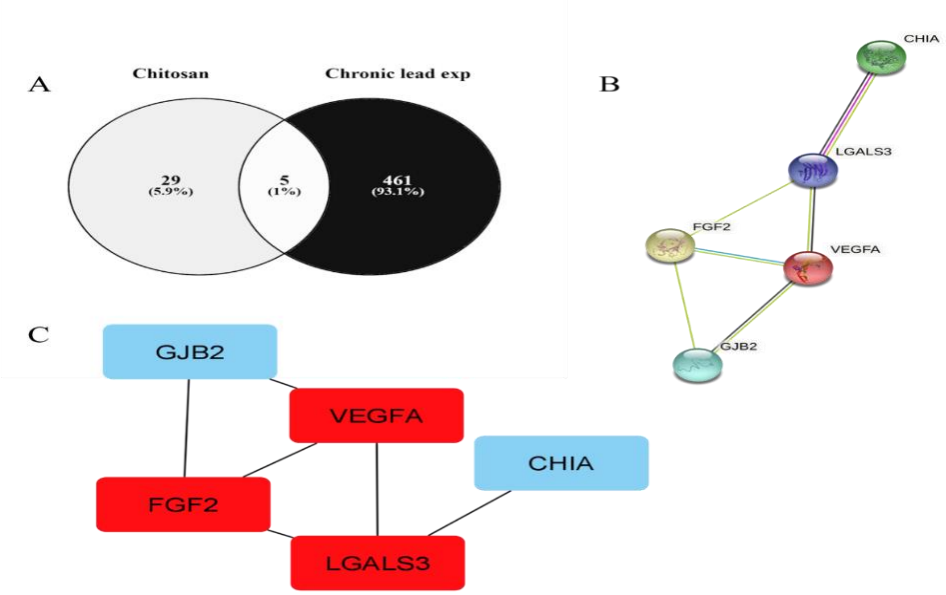

Figure 3. Bioinformatic profile of chitosan direct protein target.

We utilized SwissADME, a free-access web tool for evaluating the pharmacokinetics and drug-likeness scores of medicinally essential compounds. It can also conduct ADME studies on chitosan compounds. To publish pharmacokinetics data, this program interprets the molecular fingerprint (FP) of the submitted query structures and searches for the presence or chemical properties within a molecule. In this study, we used chitosan structure with PubChems ID 71853 (Table 2) for evaluation of the pharmacokinetics parameters prediction using SwissADME.

Table 2. General characteristics of chitosan

Small molecule	Chitosan
PubChem ID	71853
Molecular formula	C56H103N9O39
Molecular weight (in g/mol)	1526.45

The physiochemistry properties of topological prediction water solubility were evaluated using the ESOL model, Ali model, and Silicos-IT model (Table 3). Chitosan has highly soluble properties that support better oral absorption. In addition, medicine produced for oral and parenteral administration must be highly soluble in water to transport a sufficient number of active components to the active site targets.

Furthermore, to evaluate the absorption property of chitosan we predicted gastrointestinal absorption (GI absorption) and P-glycoprotein substrate (Pgp substrate) (Table 3). It is found that chitosan has low GI absorption and acts as a drug transporter of Pgp substrate. A previous study reported an improvement in GI absorption due to inhibition of Pgp (Hoosain et al., 2015). Pgp is an ABC superfamily membrane transporter found in both the intestinal epithelium and the blood-brain barrier (BBB), where it plays a dynamic role in the bioavailability of orally administered therapeutics (Ronaldson & Davis, 2022). On the other hand, Pgp substrate may cause pharmacokinetics-related drug-drug interactions, resulting in toxic or other undesired side effects due to decreased clearance and accumulation of the drug or its metabolites (Elmeliegy et al., 2020). BBB permeability was the parameter considered in the distribution of chitosan. Chitosan is predicted not to be distributed to BBB. Chitosan is predicted not to interact with CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 (Table 3). Drug interactions are frequently caused by interactions at the CYP3A level. These predictions indicated that chitosan has high solubility but is not suitable for oral administration due to its low GI solubility. Chitosan is also suitable to be combined with various types of drugs since there is no drug interaction.

Table 3. SwissADME pharmacokinetics parameters prediction of chitosan

ESOL class	Highly soluble 6.03
Ali class	Highly soluble 5.69
Silicon-IT class	Soluble 15.00
GI absorption	Low
BBB permeation	No
Pgp substrate	Yes
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Synthetic accessibility	10.00

The physicochemical property for pharmacokinetics drug discovery was evaluated under lipophilicity characteristics. We predict lipophilicity using the partition coefficient between water (iLOGP) and n-octanol (Estrada-Tejedor et al., 2013). XLOGP3 (an atomistic method that includes corrective factors and a knowledge-based library) (Riyadi et al., 2022). WLOGP (our own design of a pure atomistic technique based on Wildman and Crippen's fragmental system), MLOGP (an archetype of topological method based on a linear relationship with 13 molecular), and SILICOS-IT (topological solubility).(Daina et al., 2017). These results suggest that chitosan has low GI absorption and, consequently, the metabolism could not involve different types of CYP enzymes (Cytochrome P450 system). This phenomenon could depend on their chemical structure and lipophilicity degree (Table 4).

Table 4. Lipophilicity of the chitosan

iLOGP	1.31
XLOGP3	-21.40
WLOGP	-20.53
MLOGP	-17.69
SILICOS-IT	-21.13
Consensus Log Po/w	-15.89

Furthermore, Veber and Egan's criteria were used to support Lipinski's RO5 oral bioavailability analysis. PAINS (Pan-assay interference compounds), Brenk, and synthetic accessibility scores were also predicted in the test (Table 5). During this test, according to the Veber and Egan guidelines, chitosan has poor drug-likeness. In

addition, Chitosan likewise had a bioavailability score of 0.17 based on the SwissADME software. This result indicates that the ligand has low bioavailability. The accessibility score for chitosan was 10. Furthermore, chitosan lacked any molecular fragment with PAINS characteristics or a Brenk fragment. In general, our expected outcomes might become a valuable input for advanced experiments in the future.

Table 5. Drug likeness and medicinal chemistry of chitosan.

Lipinski	No; 3 violations: MW>500, NorO>10, NHorOH>5
Ghose	No; 4 violations: MW>480, WLOGP<-0.4, MR>130, #atoms>70
Veber	No; 2 violations: Rotors>10, TPSA>140
Egan	SA>140 Egan
Muegge	No; 7 violations: MW>600, XLOGP3<-2, TPSA>150, #rings>7, Rotors>15, H-acc>10, H-don>5
Bioavailability score	0.17
PAINS	0 alert
Brenk	0 alert
Leadlikeness	No, 2 violations; MV>350, Rotors>7
Synthesis accessibility	10.00

The novelty of this research is the pharmacokinetic profile of chitosan nanoparticles on chronic lead exposure rat models. Knowing the pharmacokinetic profile of a drug or compound is very useful for determining doses, predicting residues in tissue, seeing the correlation between drug concentration and its pharmacological and toxicological activity, evaluating the level of drug availability in the body, as well as looking at its physiological and pathological activity.

Chitosan nanoparticles have the prospect of being developed as a supplement that is effective, safe, and easy to use so that it can contribute to overcoming the negative impacts of chronic lead poisoning. Chitosan nanoparticles are expected to be useful for people who cannot avoid chronic exposure to Pb from their work or living environment.

CONCLUSION

In conclusion, our study found that chitosan nanoparticles can be detected in the serum and had a duration time of more than 80 hours with an onset of 15 minutes. Overall, using a bioinformatic and pharmacokinetic prediction approach, we predict that the genes VEGFA, FGF2, and LGALS3 play a major role in chronic lead poisoning. These data suggest that chitosan nanoparticles may be a good candidate for the treatment of chronic lead poisoning. For further research, it is recommended to examine the effectiveness of chitosan nanoparticles to prevent or overcome the toxic effects of chronic heavy metal Pb poisoning in vivo, using an animal model of heavy metal poisoning.

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