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RESEARCH ARTICLE

EFFECTIVE METHOD TO DELIVER TEST SUBSTANCE IN ADULT ZEBRAFISH (*DANIO RERIO*).

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Abstract

The present study has proposed an effective protocol for delivery of test substances via oral route to adult zebrafish (*Danio rerio*). The aim was to assess the accuracy of this feeding procedure based on bioaccumulation of GNPs in major organs of zebrafish (F_0 fish). For this purpose, a butterfly needle as a vector was used to administer GNPs which act as a test substance in the present study. Chemically synthesized GNPs of two different sizes (15±6.6 nm-Type I and 47±7.7 nm-Type II) were used to investigate size dependent pattern of accumulation in major organs. A concentration of 20 µg/g body weight/day was administered orally to male zebrafish for 28 (chronic) days. The efficiency of this protocol was determined by estimating the overall pattern of distribution of type I & II GNPs in major organs of F_0 fish using Inductive Coupled Plasma-Mass Spectrometry (ICP-MS). The analysis confirmed the presence of GNPs accumulated in major organs (brain, heart, liver, kidney and testes) of male zebrafish. Interestingly, a distinctive pattern of accumulation between both the types of GNPs was observed. The current protocol described in this article can effectively deliver the GNPs through gastrointestinal tract and further allow distribution through the blood stream in an adult zebrafish model.

Introduction:-

Selecting the route of administration to experimental animals require thorough planning and consideration to deliver the agent in an appropriate manner with minimal adverse effects from the procedure. There are different routes available for administering test substances to laboratory animals. It may be oral, topical inhalation or injection.

Although human pathologies and targeted drug delivery mechanisms have typically been studied using mammalian systems, the smaller vertebrate 'zebrafish' has received huge attention as a model system. The advantages of zebrafish over standard vertebrate models are multifactorial including high genetic and organ system homology to humans, high fecundity, external fertilization, ease of genetic manipulation, and transparency through early adulthood that enables powerful imaging applications (Tavares and Lopes, 2013). The use of zebrafish in scientific research has increased over the decade. Initially, it was a popular model for developmental biology but recently, use of zebrafish has extended to additional areas of research such as pharmacology, clinical research, and drug discovery.

In order to use zebrafish in scientific research, efficient methods need to be developed and practiced to deliver compounds or agents into the body. While immersion of the drug into water is the most popular drug administration technique in zebrafish research, there are other effective alternatives to this. Intraperitoneal injection proves beneficial when drugs are insoluble in water or which needs substantially small amount of drug to be delivered to an

individual animal. Stewart A. et al outlined a simple protocol for the intraperitoneal injection of drugs in adult zebrafish (Stewart et al., 2011). Intravenous injection of drugs to zebrafish has been a challenge because of the small vessel diameter. Traditionally, injections were given directly into the heart. However, the heart gets often punctured during the intra-cardiac injection leaving the fish prone to infection, massive blood loss or fatal organ damage. This results in a very high mortality rate. A new injection procedure in the zebrafish was developed where the site of injection is behind the eye and into the retro-orbital venous sinus. This retro-orbital (RO) injection technique was effectively employed for injection of drugs in the adult fish as well as transplantation of whole kidney marrow cells (Pugach et al., 2009). Currently, researchers have come up with novel methods for administering compounds orally to adult zebra fish using gluten as a vector to administer chemicals to adult zebrafish (Zang et al., 2011). Also, diets incorporating gelatin as a binding agent have been used successfully for decades to feed fish (Spotte, 1973 and Spence, 2008). Benjamin et al., (2014) tested the acceptability of a commercially available gelatin diet by zebrafish and determined a gender-based food assessment in order to establish dose levels of drug. However, the idea to administer drug through diet to zebrafish gives an inaccurate indication of the actual dose due to variability in voluntary consumption by the fish. Adding to the technology, a novel gavage procedure was developed to deliver substances in precise amounts to zebrafish for studies in biomedical research (Collymore et al., 2013). However, major drawback of this technique incorporates the use of anesthesia every time the fish is fed with the catheter. Recently, a micropipette based method for conducting oral drug administration in zebrafish has been developed (Kulkarni et al., 2014).

In the present paradigm, a repeated dosing schedule was employed. Preliminary experiments showed no significant accumulations of NPs on acute exposure (4 days). Thus, the exposure duration for this particular experiment was continued for 28 days in order to study the distribution pattern of NPs administered. Control group received an equal volume of distilled water while for the test groups; GNPs (respective to type I or II) were administered orally at a repeated dosing of 20 µg/g for 28 days. This dose was calculated based upon the LC_{50} value obtained from zebrafish embryo toxicity assay. Our study has slightly modified the technique of oral administration developed by Kulkarni P. et al for oral dosing by making use of a butterfly needle. This helps the test drug suspension to pass at a faster flow rate which enters directly into the esophagus of the fish thus avoiding the administered solution to regurgitate.

Methods:-

Synthesis of 10-20 nm GNPs (Type I GNPs):-

Small nanoparticles were produced following the procedure of the Turkevich et al., (1951). Briefly, 10ml of 1mM tetrachloroaurate is heated to near boiling (96°C) followed by addition of 1 ml of 41mM trisodium citrate. Solution was stirred vigorously on a magnetic stirrer with heating mantle till about 8–10 min and then allowed to cool at room temperature.

Synthesis of 40-50 nm GNPs (Type II GNPs):-

To produce nanoparticles comparatively bigger in size, the procedure of Abdelhalim et al,, (2012) was used with slight modification. Briefly, 10ml of 0.5mM tetrachloroaurate is heated to 56°C followed by addition of 1 ml of 5mM trisodium citrate. Solution was stirred vigorously on a magnetic stirrer with heating mantle till about 8–10 min and then allowed to cool at room temperature.

Characterization of GNPs:-

The formation of GNPs was monitored using double beam UV/Vis spectrophotometer (Thermo Scientific, Evolution 201 series). The size and shape of the nanoparticles were confirmed using Transmission Electron Microscopy (Philip, Model No.CM200, Operating voltages: 20–200 kv resolution 24 A°). This solution was stored at 4°C for further use. Stability of the suspension was monitored every week using UV-Visible spectrophotometer and was found to be stable for 2 months.

Husbandry of zebrafish:-

All animal experiments were conducted with prior approval from MGM's Medical College, Institutional Animal Ethics Committee. Indigenous wild type zebrafish strains were maintained at the Zebrafish facility of MGM Central Research Laboratory. All procedures for maintenance and care of zebrafish were as per The Zebrafish Book (Westerfield, 2000). Adult male zebrafishes (weighing between 0.3 to 0.5gm body weight) were used in the age group of 4-5 months. Fishes were fed twice a day by local fish feed and once with live artemia cysts. They were maintained on a 14:10 h light:dark cycle in a room with controlled temperature (28 ± 2ºC).

Experimental groups:-
A total of 30 male fishes were randomly divided into three groups comprising of one control group and two test groups for two different sizes of GNPs. Each group included ten fishes. The detailed description of experimental groups is as follows:

Group 1-Control group (n=10 fishes)

Group 2-Test groups
Group 2A-15 nm GNP test group (n=10 fishes)
Group 2B-47 nm GNP test group (n=10 fishes)

Fishes from the test groups received oral administration of approximately 100 µl of Type I & II GNPs solution at the doses of 20 µg/g/day as per the designed schedule.

Protocol for oral dosing:-
Materials:-
Holding tank, small sized fish net, micropipette (100-1000 µl), test solution (type I & II GNPs) to be administered, butterfly needle (Becton Dickinson India Ltd.), 1000 µl microtips, 10µl microtips edged at approximate 45° angle, burette stand, tissue paper wicks and recovery tank.

The dosing schedule was of 28 days in duration, so chosen as it signifies the standard exposure durations for chronic studies using zebrafish as per the OECD guidelines (Test no. 229, 2012). The idea behind the present study was to validate the technique for oral administration of test substance to adult zebrafish in order to study the toxic effects of GNPs on reproductive system.

Preparation of dosing tanks:-
a. 10 L glass aquarium tanks were obtained. Experiments were conducted for two major groups (control and test) with ten healthy male zebrafish in each set.
b. The tanks were filled with reverse osmosis water at optimum temperature (for zebrafish: 25-29 °C).

Preparation of test solutions:-
a. Type I & II GNPs were chemically synthesized and characterized.

Preparation of administration schedule:-
a. An administration schedule is prepared such that the fishes are dosed in the same environment during the same time of the day.
b. For test groups, type I & II GNPs were administered orally (with respect to the test group) at a repeated dosing of 20µg/g for duration of 28 days. At the same time, control groups were administered with equal volume of distilled water.

Detailed procedure for oral administration of test substance:-
The procedure given below is a slight modification of the previously described protocol by Kulkarni et al., (2014). It allows the oral delivery of precise volume of solution reliably, safely and efficiently to adult zebra fish.
a. Arrange the set up as described in Fig 1(A) including following items. Holding tank, small sized fish net, injector consisting of 1000 µl micropipette holding appropriate amount of test substance to be administered, butterfly needle (Becton Dickinson India Ltd.) and 10 µl microtip edged in near 45° angle, tissue paper wicks and recovery tank.
b. The butterfly needle is inserted to a 1000 µl micropipette holding the GNP solution. The pointed end of the needle is covered by a 10 µl microtip. This acts as an injector as shown in Fig 1(B).
c. The injector is clamped to a holder such that it is held in a fixed position.
d. With the use of a damp tissue (in order to keep the body moist), the zebrafish is held in a position such that the tip of the injector is gently inserted into the mouth of zebrafish and appropriate volume of the test solution/suspension is then slowly released into the oral cavity of the fish as described in Fig 1(C).
e. After successful administration, the fish is released back to the recovery tank.

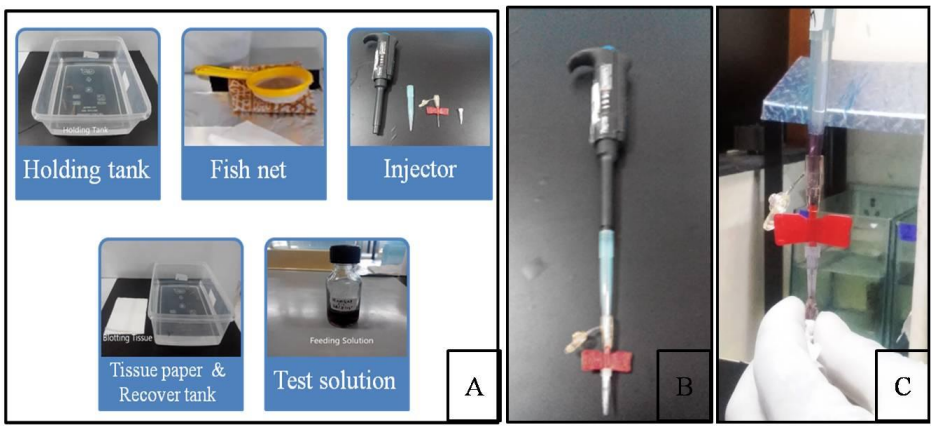

Fig 1:- Stepwise representation of equipments required for oral delivery of test substance to adult zebrafish

The entire procedure is summarized in Fig 2.

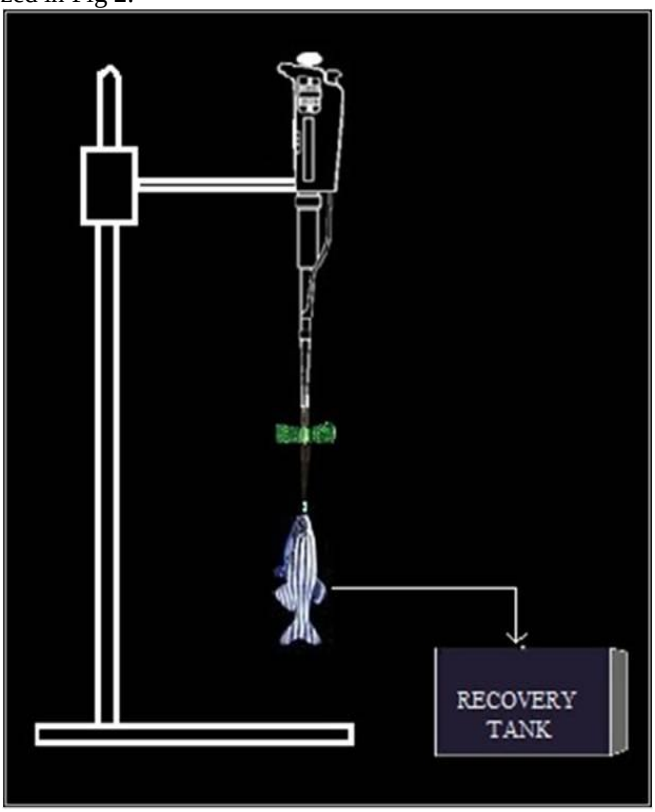

Fig 2:- Schematic representation of oral dosing to adult zebrafish.

Distribution of AuNPs in tissues after repeated administration:-

To determine the distribution of GNPs in major organs (brain, heart, liver, kidney and testes) of male zebrafish, the ICP-MS technique was used. At the end of exposure duration, dissection was performed to obtain desired tissue samples. The organs from three fishes were pooled. Samples were dried, weighed and subjected to acid digestion as described by Moor et al., (2001).

Statistical analysis:-

Statistical analysis were performed using Statcalc3 version 4.0 software. One way ANOVA was applied to calculate significance of difference between the control and test groups and between two test groups.

Results:-

Synthesis and Characterization of GNPs:-

Colloidal GNPs were prepared from tetrachloroaurate in aqueous solution using trisodium citrate by chemical reduction method. On reaction, the solution resulted in change of colour from yellowish to wine red for GNPs 10-20 nm and purple pink for GNPs 40-50 nm as shown in Fig 3(A) and (B); thus indicating the formation of GNPs. Both the nanoparticle suspensions were found to be stable for over 2 months.

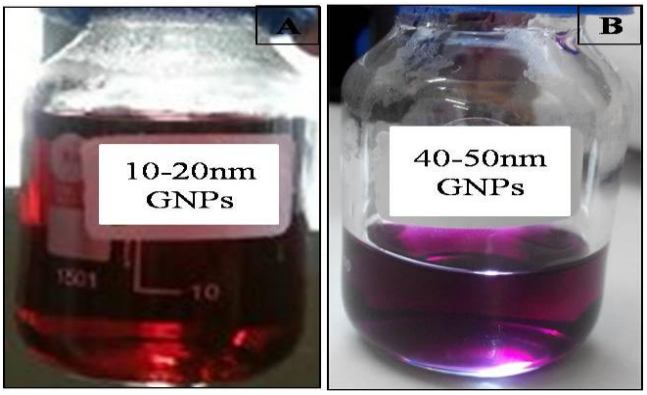

Fig 3:- Colloidal GNPs synthesized through chemical reduction method. (A) GNPs 10-20nm and (B) GNPs 40-50nm.

Fig 4 shows the absorption spectra of the synthesized gold nanoparticles of two different dimensions revealing the characteristic surface plasmon bands. UV-vis spectras were acquired with a UV/Vis spectrophotometer (Thermo Scientific, Evolution 201 series). Surface Plasmon Resonance band for GNPs results in a strong absorbance in the visible region (500 nm-600 nm). The characteristic absorbance peak was obtained at 520 nm for 10-20 nm GNPs (Fig 4A) and 530nm for 40-50nm GNPs (Fig 4B).

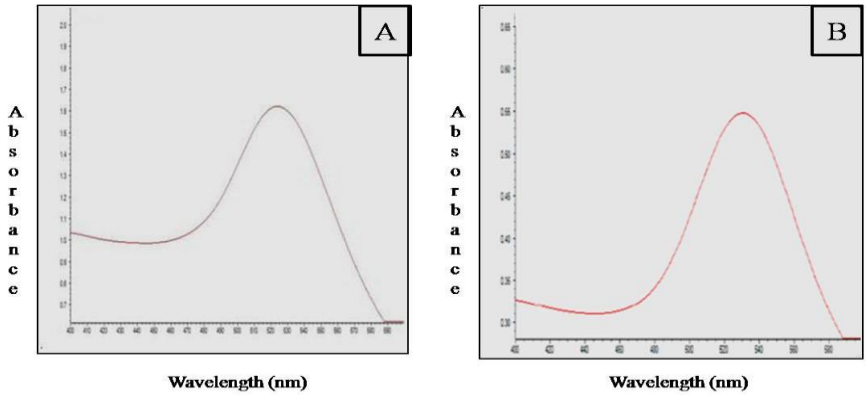

Fig 4:- UV/Vis spectra of GNPs of different sizes. (A) The characteristic absorbance peak was obtained at 520nm for 10-20nm GNPs (B) The characteristic absorbance peak was obtained at 530nm for 40-50nm GNPs

The analyses of size and shape of as prepared GNPs was confirmed using Transmission Electron Microscopy and is shown in Fig 5(A) and (B). Measurements shown by histograms (Fig 5C and 5D) reveal that the average particles sizes were 15±6.6 nm and 47±7.7 nm respectively.

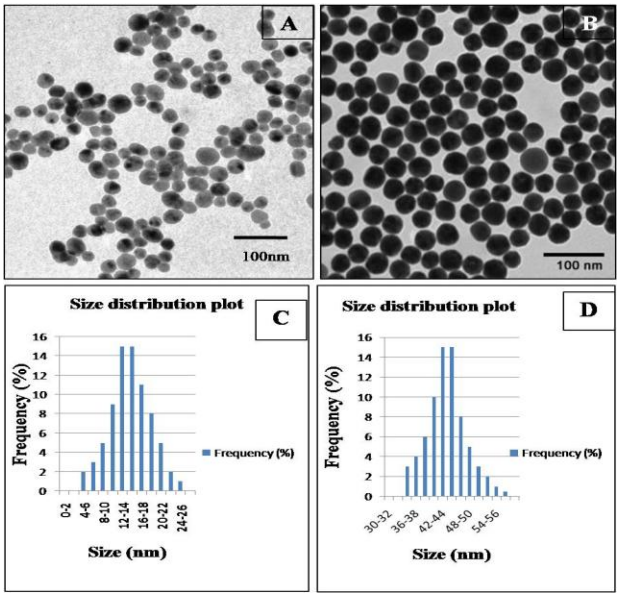

Fig 5:- TEM images of GNPs at scale bar 100nm and Size distribution plots. (A) And (B) indicate spherical GNPs with an average diameter of 15nm and 47nm respectively (C) and (D) Corresponding size distribution plots indicating distribution of particles within the range of 15±6.6nm and 47±7.7nm respectively

Oral drug administration:-

All fishes were administered GNPs orally and none of the fish showed any spillage either through the oral cavity or gills. The total time taken for administering GNPs per fish was approximately about less than 1 min. Furthermore, the fish did not show any symptoms of bleeding, damage to the oral cavity, distress, discomfort or any sign of trauma proving this to be a non-traumatic, painless and ethical method of oral administration to adult zebrafish.

Distribution of GNPs in tissues after repeated administration:-

The efficiency of protocol for oral administration to adult zebrafish was determined by estimating the overall pattern of distribution of type I & II GNPs in major organs of F_0 fishes using Inductive Coupled Plasma-Mass Spectrometry (ICP-MS).

ICP-MS data is obtained in triplicate to calculate standard error and standard deviation. On analysis using ICP-MS, no detectable gold metal was estimated in any of the major organs from control groups. In contrast, a significant size dependent absorption and deposition of NPs was estimated in major organs of zebrafish (One way ANOVA, $p<0.05$). The pattern of NP accumulation was maximum in testes and comparatively lower in rest of the organs in the order of testes>heart>brain>liver>ovaries>kidney. Results are described in Fig 6 as mean±SE.

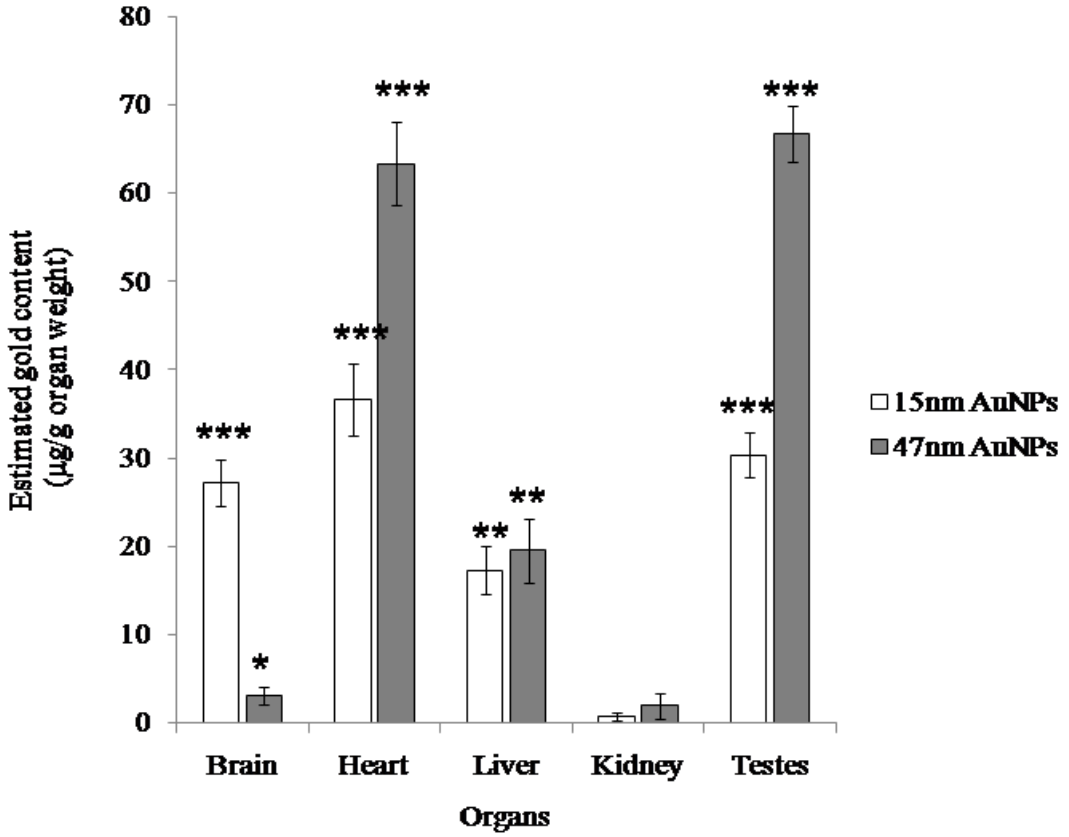

Fig 6:- Histogram indicating accumulation of GNPs in major organs of zebrafish after oral administration. Gold content estimated in control groups were below detectable limits. Data is represented as mean±SE. One way ANOVA was applied using Statcalc3 version 4.0 software to determine statistically significant differences between control and test groups (*p<0.05, **p<0.01 and ***p<0.001).

Discussion:-

Currently, immersion dosing is the commonly used method for dosing zebrafish in embryonic, larval as well as the adult stage. The technique involves dosing the water in static well plates or tanks and subsequently replacing the water and test compound at regular intervals throughout the study period (Kily et al., 2008; Lam et al., 2008; Lien et al., 2006; Oppedal and Goldsmith 2010). This results in wastage of vast quantities of costly pharmaceutical ingredients, as well as voids the utility of small sized zebrafish. In case of intraperitoneal injections to zebrafish, they are first anaesthetized and then chemical solutions are injected into the peritoneum in a small volume (generally < 10 µl) (Esbaugh et al., 2009; Liu et al., 2008; Zodrow and Tanguay, 2003). Methods of oral administration for fish have also been reported (DeKoven et al., 1992; Kulkarni et al., 2014).

The experimental set up in the present study is an effective technique of oral drug administration in adult zebrafish using a method that is simple, non-invasive, non-traumatic and can allow accurate delivery of test substance through the oral route. This technique is an improvement over the previously described techniques by Collymore et al., (2013) and Kulkarni et al., (2014) for administration of drugs via oral route to adult zebrafish. This procedure is also efficient as it takes approximately less than one minute to feed per fish. Itis rapid, requiring about 10 min to administer test substance to 7-8 fish, with an average time of 30-45 sec per fish. Other advantages of our oral administration include ease of handling, minimal use of drugs, high drug bioavailability and use of cost effective set up. It is safe, as we have observed zero mortality. Collymore et al. (2013) developed a gavage procedure to deliver precise amount of substances to zebrafish which took about 10 min to administer 3-4 fish. However, the drawback of this procedure is that in order to gavage fish, it is mandatory to anaesthetize the fish using buffered MS-222. Though the concentration of anaesthesia used was 150 mg/l which is within the acceptable limits, unfortunately it

may not prove beneficial in studies involving repeated dose administration of test substance as the minimum anesthetic concentration (MAC) value may increase on repeated anaesthetizing the fish (Posner et al., 2013). The present study, however, does not require the use of anesthesia as it uses a damp tissue to hold the zebrafish in order to keep its body moist. Moreover, procedure by Collymore et al. (2013) recommends that zebrafish should be gavaged with not more that 5 µl of test solution as the success rate decreased. Fortunately, in our study with the help of butterfly needle, we are able to administer up to 100 µl of test solution orally without expelling liquid through the gills or mouth. The butterfly needle helps the test substance to pass at a faster flow rate which enters directly into the esophagus of the fish thus avoiding the administered solution to regurgitate. On the other hand, administering drug to individual zebrafish is more effective in order to avoid competition and maintain a hierarchy among the fish than group feeding.

To the best of our knowledge, this is one among very few reports on systematic bioaccumulation of GNPs in adult zebrafish. The biodistribution studies carried out in male zebrafish using the currently described oral method of administration resulted in distinctive size dependent pattern of NP accumulation in major organs. It would be expected that small NPs have a higher probability to be internalized by passive uptake than large. However, in the present study, type II AuNPs showed increased accumulation than type I AuNPs in almost all major organs except for brain tissue. A size-dependent uptake in different cell lines has been observed for gold (Chithrani and Chan, 2007; Wang et al., 2010), mesoporous silica (Lu et al., 2009), polystyrene (Varela et al., 2012) and iron oxide NPs (Huang et al., 2010) with the maximum cellular uptake at a NP core size in the range of 30–50 nm. This suggests that there is an optimal size for active uptake.

Conclusion:-

Oral administration represents a useful technique for pharmacological research in zebrafish. The protocol discussed here enables one to obtain a reliable data related to dosage requirements in relation to body weight of the subject (viz.mg/kg, ml/kg, etc.). Advantages of this protocol include a methodology for quick oral delivery of low concentration of drugs which proves to be highly successful and cost effective. This protocol could effectively be used towards therapeutics, research involving studies related to drug delivery, uptake and permeability. With the advent of this technique, zebrafish may be an excellent experimental model organism for studying oral pharmacokinetics.

The protocol of oral administration described in the present study was validated based upon the distribution and uptake of NPs in major organs. The higher or preferential accumulation of NPs with respect to size observed could be due to mode of endocytosis, accessibility through blood brain barrier cellular uptake mechanisms, etc. Such differences in the uptake response depending upon the size of the NP could have major applications in drug delivery and imaging studies. From the present data, it is evident that GNP in the size range of 10-50 nm could penetrate the sertoli cell barrier in zebrafish and enter the testes. Among the major organs, about 6% of type 1 GNPs and about 12% of type II GNPs appeared to be accumulated in the testicular tissue. The pattern of NP accumulation was maximum in testes and comparatively lower in rest of the organs. Therefore, it would be of interest to investigate if GNPs in this particular size range could also gain access to the female reproductive system (ovarian tissue).

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