ZEBRAFISH, A MODEL FOR EVALUATING THE PHARMACOKINETICS OF THE DRUGS: A COMPREHENSIVE REVIEW

By

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Bachelor of Pharmacy (Hons)

School of Pharmacy
Brac University
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Declaration

It is hereby declared that

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Approval

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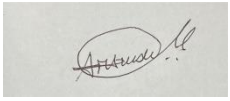
Supervisor: 

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Ethics Statement

The study does not involve any kind of animal trial and human trial

Abstract

To facilitate the broad use of zebrafish in drug discovery, it is essential to know the pharmacokinetic since how the body reacts with a molecule and the drug concentration at the designated site depends on it. The potential effect of a molecule can be revealed in a short time through zebrafish with high accuracy and precision. Zebrafish is considered the second most popular animal in research however; a limited scope of zebrafish research is currently available in Bangladesh. More research is required focusing on zebrafish-based pharmacokinetics, toxicology, gene editing studies to extrapolate the conducted data into mammalian models. This review summarizes the recent research that has been conducted on zebrafish whether from current medicine or xenobiotics: environment contaminants, crude chemicals, etc. to demonstrate and compare the working procedure and extrapolation on human model.

Keywords: Zebrafish, Pharmacokinetics, Absorption, Distribution, Metabolism, Excretion xenobiotics.

Dedication

Dedicated to my parents specially departed soul of my father and my supervisor, Tanisha Momtaz.

Acknowledgement

Alhamdulillah, all praises to Almighty Allah who bless me with patience, strength, courage and self-confidence which helped me most to get this project done properly.

First and foremost, to my most esteemed supervisor (Tanisha Momtaz, Lecturer, School of Pharmacy, Brac University), who have directed me from the idea of topic to last line of my project. I would like to express my deepest gratitude and adoration. Working under her supervision enabled me to understand and expand my knowledge. That project would not come to light without her valuable input and monitor. Her precious feedback, rapid communication, and hard working for my thesis helped a lot to finish the project accurately and quickly. I am highly grateful to have had the opportunity to work under her supervision and wish to work with her in future.

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Moreover, I would like to show mark of reverence towards my mother, Kazi Nasrin Akter a former teacher of BRAC primary school who taught me how to get things done in tough situation. Surely, I would not come this far without her prayers and unconditional love.

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List of Acronyms

CNS	Central Nervous System
DPF	Days Post Fertilization
HPF	Hours Post Fertilization
ADME	Absorption, Distribution, Metabolism and Excretion
KD	Kilo Dalton
PC	Pericardial Cavity
IP	Intra-Peritoneally
IY	Intra Yolk
CYP	Cytochrome P450
ABC	ATP–Binding Cassette
COMT	Catechol-O-methyltransferase
PFOA	Perfluorooctanoic Acid
PS NPs	Polystyrene Nanoparticles
BCF	Bioconcentration Factor
SOD	Superoxide Dismutase
CAT	Catalase
GST	S-transferase
MMA	Methylated Metabolites Arsenic

Chapter 1

Introduction

1.1 General Information about Zebrafish

Fish are the most abundant and phylogenetically diversified vertebrate, and widely used in drug discovery, pharmacokinetics, and environmental toxicology studies. Zebrafish (Danio rerio) have been using in research since 1972 when George Streisinger introduced it as a potential biological model organism for its high predictivity (Specificity:89%, Sensitivity: 68% and Accuracy: 78%) with humans ((Bozkurt, 2020; Cornet et al., 2017; Zabegalov et al., 2021). It is a tiny (3–5 cm in length), torrid, freshwater, teleost fish, which is native to the Indian subcontinent and originated from the Himalayas (India) (*Figure 1A*). The name came from its particular colorful stripes which is similar to Zebra (Katoch & Patial, 2021). The taxonomy of the zebrafish is:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Danio*

Species: *Danio rerio* (Sarvaiya et al., 2014)

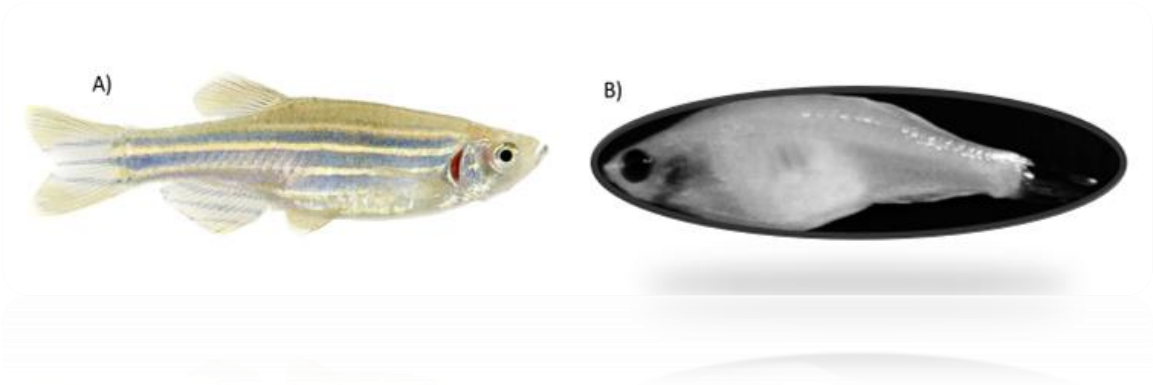

Figure 1: A) An Adult Zebrafish; B) Crisper Zebrafish

Zebrafish has now become the second most used animal model after mice because of its salient features and special characteristics. For example, it has a high fecundity rate which enables them to lay hundreds of eggs per week. It is also less expensive compared to rodents such as mice, guinea pigs, frogs, dogs, etc. As they are small in size, hundreds of larvae can be experimented within a small volume. Zebrafish larvae are transparent which enables the researchers for optical manipulation to visualize the distinct cells and tissues. Moreover, they can absorb drugs through diffusion, microinjection as well as can tolerate foreign modified genes. Zebrafish embryos develop quickly compared with other mammalian models resulting in shorter experimental time. Zebrafish embryos develop quickly compared with other mammalian models resulting in shorter experimental time (Matos et al., 2020; Molina et al., 2021). Zebrafish larvae are favored over adult fish for size, color, and ethical permission. They exhibit three types of pigments on their screen: black melanophores, reflective iridophores, and yellow xanthophores. These pigments can be removed by gene editing technology resulting in Casper zebrafish *(Figure 1B).* Casper zebrafish are mutated for *nacre* and *roy* genes which are responsible for black and yellow pigment respectively. Another distinctive feature of zebrafish is that it can be used as both *in-vivo* and *in-vitro* experiments. For *in-vitro* experiments, highest 5 days post-fertilization (dpf) is acceptable as before 5 days they are not aware of suffering and pains (Bhusnure et al., 2015). During these days, most of

the organs developed except CNS thus need not require ethical permission (Sarvaiya et al., 2014). The genetic screening on zebrafish is conducted either forward genetic or reverse genetics. In forward genetics, initially phenotypes or characteristics are identified and then the reason is being found for the mutation of genes. Conversely, in reverse genetics which can be conducted by the *Crispr/Cas* system, the particular gene is chosen and then it is used to reveal the phenotype (Yoganantharjah & Gibert, 2017). For all these reasons, they are widely used in assessing pharmacokinetic parameters, checking drug-drug interaction, finding novel drug administration routes, investigating drug toxicity, gene editing by CRISPR CAS9, and so on (Katoch & Patial, 2021; Zabegalov et al., 2021).

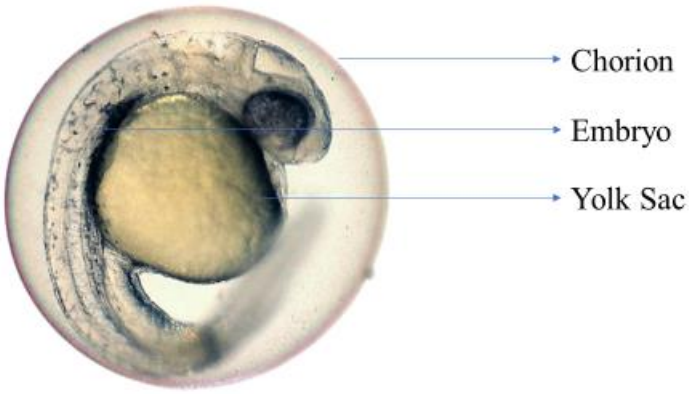

Figure 2: Zebrafish embryo of 27hpf surrounded by chorion.

1.2 Physiology and Anatomy of Zebrafish

Zebrafish are widely used in biomedicine, human disease modeling, assessing drug toxicity, pharmacokinetics, drug discovery, and so on because of their huge genetic similarities with humans (Katoch & Patial, 2021; Zabegalov et al., 2021). It carries 80-82% disease responsible proteins similarity with humans (Matos et al., 2020; Molina et al., 2021). That is why it can also resemble some human physiology like the skin, blood, digestive system,

heart, brain, liver, etc. Unlike mammals, the full body of the fish is covered with mucous membrane though there a little keratinized epithelium is seen on the jaw and fins (Menke et al., 2011). Beneath the mucus it contains epidermis, dermis and hypodermis layer through which it can absorb molecules except the eye (Morikane et al., 2020). The eye of zebrafish is quite smaller than human, but there are basic similarities in both human and zebrafish eyes. The eye of zebrafish contains three layers: 1) the tunica fibrosa, which covers the flat cornea and the sclera. The cornea of zebrafish made of five layers which is also seen in humans (Hong & Luo, 2021), 2) the tunica vasculosa, that encompasses the choroid, the choroid rete, and the iris, 3) retina through which it gives signals to brain, and some drugs show fine distribution in both eyes and brain (Menke et al., 2011). The zebrafish brain is very simple compared to higher mammals and its Central Nervous System (CNS) developed after 5 dpf. However, it holds a variety of neurogenic niches such as spinal cord, optic tectum, retina, cerebellum and olfactory bulbs compared with mammals which have only hippocampus dental gyrus, lateral subventricular zone, and olfactory bulbs. As a result, it provides a wide space to monitor distribution of drugs in various places. The brain can be divided into five parts: the telencephalon, the diencephalons, the mesencephalon, the metencephalon, and the myelencephalon. Brain also controls the heart rate. Heart of a zebrafish has two chambers whereas in mammals it is four. Heart can be divided into six parts: (a) Sinus venosus; (b) atrium; (c) ventricle; (d) bulbus arteriosus; (e) ventral aorta; (f) thyroid follicles. Pulse rate of a zebrafish heart is 120-180 beats per minute which is very close to humans rather than mice that have heart rate 300-600 beats per minute (Bournele & Beis, 2016). So, blood flow of zebrafish and mammals are almost at the same rate as well as the regulation of hematopoietic stem cells which allows the researchers to conduct anti-hematopoietic disorders on zebrafish (Molina et al., 2021). Hematopoietic tissue of zebrafish contains three types of cells: erythrocytes which transport oxygen and carbon-dioxide, thrombocytes that prevent blood

clot, and leukocytes that help in the defense system. Blood carries the metabolic molecules and metabolites which are modified in the liver. The main role of zebrafish liver is to produce metabolic enzymes such as cytochrome P450, monooxygenase, monoamine oxidase, alcohol dehydrogenase, and aldehyde dehydrogenase as well as serum protein such as: albumin, fibrinogen, complement factors, and acute-phase proteins. The metabolites are excreted through the kidney. At larval stage (11-48 hours post fertilization, hpf), mesonephros works as a kidney and during the hatching period (48-72, hpf) zebrafish have pronephros which act like kidneys. Later, after the larval period (72hps- 30dps), mesonephros along with pronephros start working together. Similar to the mammalian kidney, it has nephrons with a glomerulus, proximal tubules, distal tubules, and collecting ducts *(Figure 3).* Importantly, zebrafish has well developed digestive system where all the molecules are passing through. Despite a lack of stomach and caecum, it can run its digestive system through the pharynx, esophagus, intestinal bulb, mid-intestine, and posterior intestine very well. At the posterior part of the intestine, it releases somatostatin and glucagon hormones which helps in growth and metabolism respectively. The pancreas of zebrafish is composed of two parts, dorsal posterior which the endocrine cells of the Islet of Langerhans, and another part is ventral anterior anlage that has exocrine cells, the pancreatic duct, and a small number of endocrine cells. Three lobes are seen around zebrafish liver (Menke et al., 2011; Verbueken, 2019).

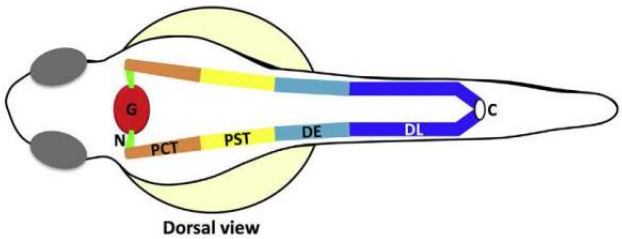

Figure 3: The structure of pronephros at 48 hpf comprises a central fused glomerulus (G) which is connected to the bilateral tubules by short neck (N) segments. Here, PCT is for proximal convoluted tubule; PST refers to proximal straight tubule; DE means distal early tubule; DL for distal late tubule, and C denotes to cloaca (Verbueken, 2019)

1.3 Pharmacokinetic: Absorption, Distribution, Metabolism and Excretion (ADME)

Pharmacokinetic refers to the fate of the drug in our body which includes ADME–processes, which include initial absorption, then distribution, followed by metabolism and excretion. The pharmacokinetics of zebrafish is straightforward as they have a simple physiological structure. Absorption is the movement of drugs from the site of administration to the body compartment by active or passive process. Zebrafish can take foreign particles into their body in many ways, diffusion, oral ingestion, and by injection. Before the hatching period, zebrafish are protected by a thin acellular permeable layer called chorion. Chorion contains small pores which allow the transfer of molecules less than 3 kilo Dalton (KD) into the larvae and from there larvae can uptake the drug through skin by passive diffusion. At the time of larval period (72hps- 30dps), it can take drugs orally. Moreover, the researchers can directly push xenobiotics into the yolk sac or the vasculature from which it spreads throughout the body *(Figure 2B).*

Distribution is referred to transport the drugs from one compartment to another. Distribution is followed either by one or two compartment models or by non-compartment models. As zebrafish have a closed blood system, it can follow both compartment models (Gore et al., 2012). Whole body is considered as one compartment model and blood and tissues are considered as two compartment models.

Metabolism or biotransformation is the process of converting one chemical so that it can easily excrete from the body. It can be classified as phase I, phase II and phase III. Phase I consists of some simple reaction such as: oxidation, reduction, hydrolysis, hydration etc. reaction occurred. In phase II, the reactive is quite complex like: sulfonation, acetylation, methylation glucosidation etc. Liver secretes various enzymes like cytochrome P450 (CYP),

monooxygenase (FMO), monoamine oxidase (MAO), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (ALDH) in phase 1 and uridine 5'–diphospho (UDP)–glucuronosyltransferase (UGT), sulfotransferase (SULT) glutathione *S*–transferase (GST), etc. in phase- II metabolism. Phase III metabolism is an active transport system that removes drugs from the cell with help of ATP–binding cassette (ABC). ABC carrier need ATP to transport the molecules against concentration gradient (Verbueken, 2019) *(Figure 4).*

Elimination is the irreversible process of removal of the drugs from the body. The main route of excretion is kidney by the process of glomerular filtration. Moreover, elimination also occurred by liver through biliary excretion. Mainly, small molecules that have less than 200 Da weight are eliminated through the kidney. On the other hand, large molecules are eliminated via liver (Clarke & Marzinke, 2020).

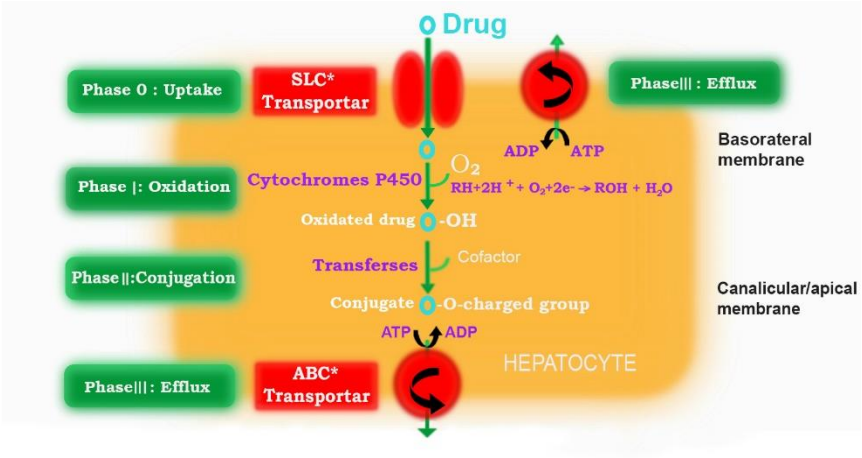

Figure 4: Metabolism pathway of Zebrafish (Verbueken, 2019).

1.4 Rationale of the study

This research was conducted to overview and compile the information and data of the known drug kinetics on zebrafish and compare with mammalian value so that we can predict the closest outcome while screening of new drugs or in pathology which will provide more accurate and precise value. At present, scientists are trying to conduct a phase I clinical trial on zebrafish. Without having a proper knowledge on kinetic it's tough to understand how body reacts with the molecules. This study will assist to understand the pharmacokinetic profile of xenobiotics on zebrafish. Moreover, the study will refer to future works in Bangladesh.

Chapter 2

Methodology

The component of this systematic review is carried out by screening of some renowned journals from the Google scholar and PubMed search engine. Almost all the articles were taken from the last 10 years ranging from 2012 to 2022 to find out the recent pharmacokinetic experiment of zebrafish. To catch the most related articles, ‘pharmacokinetic’, ‘Zebrafish’, ‘ADME’, ‘Absorption’, ‘Distribution’, ‘Metabolism’, ‘Excretion’ is utilized to extract the articles that really describe the kinetic profile. Most of the articles were excluded based on the title and abstract. Moreover, articles were excluded if they describe pharmacodynamics rather than kinetics. In case of lack of full text, BracU Athens account as well as Sci-hub play a vital role to access the publications. Finally, the selected articles were analyzed individually and made connections between them so that the kinetics profile of zebrafish can easily be understood.

Chapter 3

Pharmacokinetics study on Zebrafish

3.1 Absorption

First step of pharmacokinetic is absorption. The ways of absorption of drugs for zebrafish is quite vast such as by orally, skin diffusion from the aqueous medium, or by micro-injection into chorion, pericardial cavity (PC), intraperitoneally (IP) and intra yolk sac (IY) (Guarin, Faelens, et al., 2021; Guarin, Ny, et al., 2021; Verbueken, 2019). When a molecule is mixed in aqueous solution for absorption, pH is an important factor because that molecule can alter the pH of the solvent. Hence, a suitable buffer needs to be used according to the nature of the molecule to protect the fish (Verbueken, 2019). When a zebrafish larva is kept in aqueous medium, most of the drug is absorbed through chorion as the larvae is covered with it (Morikane et al., 2020). The absorption of drugs in aqueous immersion depends on the lipophilicity and growth of larvae (Guarin, Ny, et al., 2021). The more developed the larvae, the better absorption is observed (Zhang et al., 2015). For instance, it is seen that during 3 to 4 dpf, 106% absorption is observed due to the opening of GIT. In these days, the larvae can not only absorb drugs trans dermally or trans-gilly but also take drugs orally. No increasing absorption value is seen from 4 dpf to 5 dpf because no more major physiological changes occur at that moment (van Wijk et al., 2019). Although immersion in aqueous medium results in good absorption for less lipophilic drugs, very minute amount of absorption is observed for lipophilic drugs whose log D value is greater than 1. For lipophilic compounds, IP, IY or PC is best to get results in a short time. However, some difficulties in administration are seen when PC microinjection is used for larvae. Alternatively, IP microinjection is most suitable that can be applied in larvae, juvenile and adult zebrafish (Guarin, Ny, et al., 2021). By using IY micro-injection; DNA, morpholino and other pathogenic cells like bacteria or viruses can

be delivered to the infected embryos. Though, in IY administration, lipophilic drugs initially remain in the yolk sac, it is redistributed after some hours as yolk is also lipophilic in nature. Nevertheless, yolk has a tendency to accumulate molecules from surrounding and it has low accuracy and precision for small molecular weight compounds. Therefore, microinjections are not always feasible in all cases. In this circumstance, lipophilic drugs can be used to absorb drug molecules by immersing the larvae for 48 hours (Guarin, Faelens, et al., 2021; Guarin, Ny, et al., 2021). Most of the cases, the concentration of the drugs is kept low so that it cannot create any toxicity. Zebrafish are also assessed for toxicity tests (Zhang et al., 2015). Zhang, 2015 found that for toxicity tests, 6hpf is best to evaluate embryotoxicity because that moment is similar with adult toxicity, and 3df is most suitable to compare with mammalian toxicity.

An adult zebrafish can absorb drug molecules by absorbing through skin as well as orally. To absorb into the skin, molecular weight of the drug should be less than 500 Dalton and (log octanol-water partition coefficients, log P = 1–3 is required. Zebrafish skin have much similar with human skin such as they both bear upper epidermis, lower dermis and hypodermis layers. However, the average 1-month old zebrafish skin thickness is only 14 nm compared to humans whose skin thickness is almost 3mm which can lead to false -positive results. The flow of drug from the upper skin to circulation is shown in *(Figure 5).* To perform a test on zebrafish, the fish is anesthetized to stop motility and kept in a liquid solution to absorb molecules in an upside-down position covering with agarose gel to ensure that no drugs can leak through mouth or gill**.** Apart from liquid solution, zebrafish can absorb drugs from transdermal coating around it, which is more advantageous than liquid solution (Zhang et al., 2015). Morikane et al., 2020 shows that felbinac acid, an NSAID, can absorb through skin by maintaining first order kinetics, and showed that zebrafish are qualified to absorb drugs through skin.

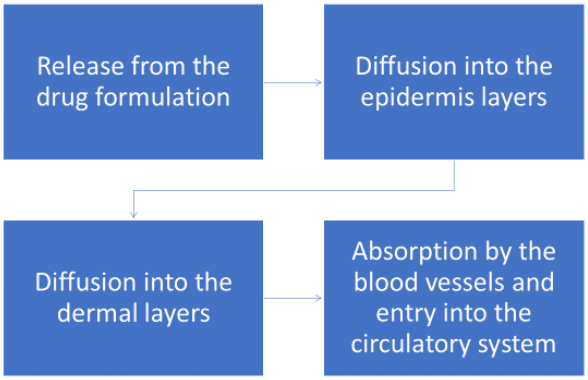

Figure 5: The flow of drug from the upper skin to circulation (Morikane et al., 2020).

3.2 Distribution

In recent years, distribution of molecules in the zebrafish is well studied with the help of florescence and the concentration of the molecules in specific tissue can be visualized through autoradiography (Diekmann & Hill, 2013; Guarin, Faelens, et al., 2021; Guarin, Ny, et al., 2021). Distribution depends on the route and site of administration of the drugs and also the development of the fish. Zebrafish Blood Brain Barrier (BBB) become developed between 3 to 8dpf. Distribution of drugs can assess to check how much drug is penetrated into BBB. For example, Diphenhydramine, haloperidol and scopolamine can cross BBB in human and also have equal distribution between head and trunk of zebrafish. On the other hand, scopolamine N-butyl bromide and desloratadine that cannot cross BBB in human, showed low concentration in head of zebrafish (Diekmann & Hill, 2013). An enzyme found in zebrafish named Catechol-O-methyltransferase (COMT) includes all molecules that contains catechol moiety, catechol estrogens, catecholamine neurotransmitters such as: dopamine, adrenaline and noradrenaline. Activity of *comt* genes was found in several areas of the zebrafish brain: the olfactory bulb, the areas near the telencephalic and diencephalic ventricles, the periventricular grey zone of the diencephalon, and the periglomerular nucleus which denotes fine distribution of the in the brains (Semenova et al., 2017). Zebrafish exhibit

fine distribution on eyes. While testing with known drugs such as: digoxin, gentamicin, ibuprofen, minoxidil, and quinine results some adverse effect on vision (Hong & Luo, 2021). When an adult zebrafish is kept under neonicotinoid (an insecticide) containing water, it is seen that the molecule is well distributed in muscle, liver, intestine, gill, brain and kidney. Khazaee & Ng, 2018 worked with perfluorooctanoic acid (PFOA) and found same distribution in those compartments. Being liver and intestine as a primary site of metabolism, concentration of molecules is greater than other organs. The molecule also penetrates into BBB and causes neurotoxicity (Yang et al., 2022).

When IY injection implemented, due to dynamic ooplasmic pace, some drugs molecules started to distribute towards embryonic cells. However, most of the drugs remain into the yolk and after a while it redistributes into the non-yolk sites. Therefore, experiment shows that almost 20–30% of the drugs become distributed into the non-yolk parts after 72h of injection which denoted independency of the lipophilicity of the compounds (Guarin, Faelens, et al., 2021; Guarin, Ny, et al., 2021). A quick distribution into the whole body is observed when PC, and IP microinjection is utilized (Guarin, Faelens, et al., 2021).

Zebrafish is also used for assessing environmental toxicity. While fluorescent polystyrene nanoparticles (PS NPs) were used it is seen that initially during 24hpf, the foreign particles remain in the yolk sac and later (from 48-120 hpf) it distributed to GIT, gallbladder, liver, pancreas, heart, and brain. This result varies depending on the PS NPs concentration, particle size and synthesis method (Pitt et al., 2018). (Yao et al., 2017) examined with fifteen compounds and found that some antitumor drugs such as Obatoclax mesylate, Vinblastine, Atractylodin, Embelin are well distributed into eyes, Gall bladder and Intestine *(Table 1, figure: 6).*

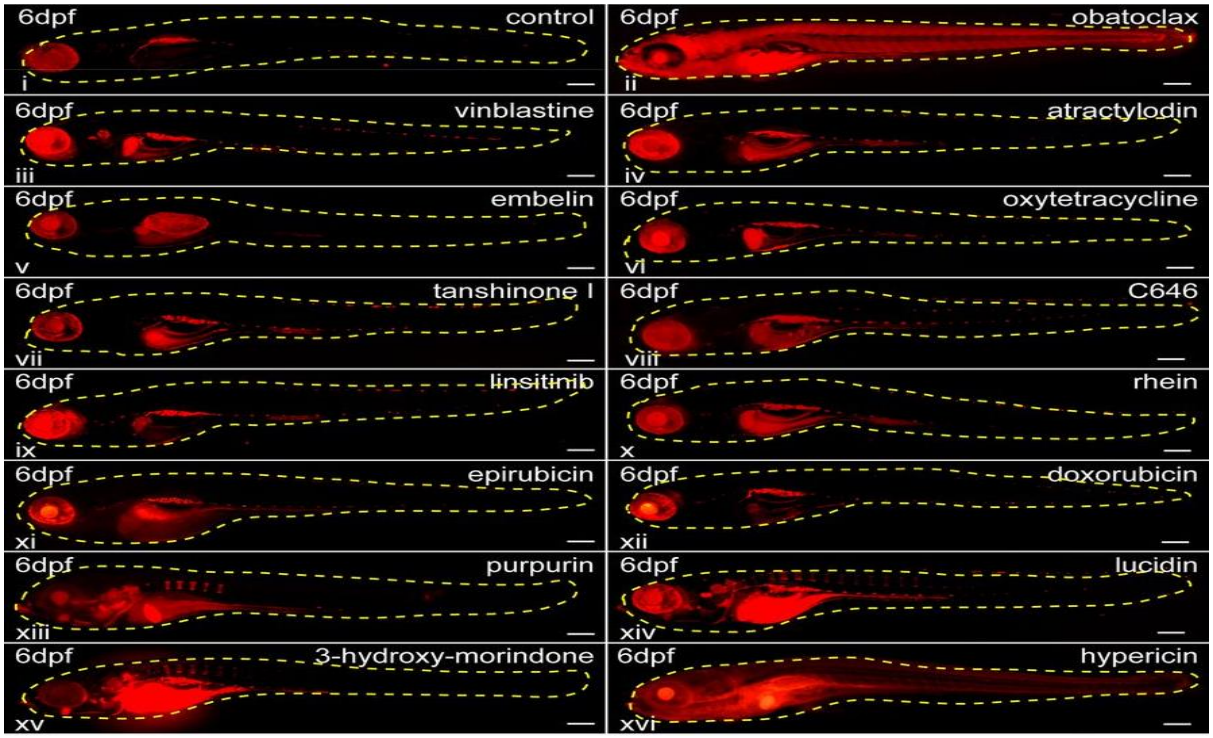

Figure 6: Distribution of red fluorescent compounds in different tissues in Zebrafish larvae (Yao et al., 2017).

Table 1: Distribution of molecules in different tissues (Yao et al., 2017).

No	Name of the Compound	Function	Distribution
1	Obatoclax mesilate	Antitumor	Whole body, Eye, Gall bladder
2	Vinblastine	Antitumor	Eye, Gall bladder, Intestine
3	Atractylodin	Antitumor	Eye, Gall bladder, Intestine
4	Embelin	Antitumor	Eye, Gall bladder, swim bladder
5	Oxytetracycline	Amine biding inhibitor	Eye, Gall bladder
6	Tanshinone I	Memory enhancement	Eye, Gall bladder, Intestine
7	C646	P300 inhibitor	Eye, Gall bladder, Intestine
8	Linsitinib	Insulin receptor inhibitor	Eye, Gall bladder

9	Rhein	Glucose metabolism regulation	Eye, Gall bladder, Intestine
10	Epirubicin	Chemotherapeutic	Eye, Intestine, Liver
11	Doxorubicin	Chemotherapeutic	Eye, Intestine, Liver
12	Purpurin	Art dye	Eye, Gall bladder, Bone, intestine
13	Lucidin	Traditional Chinese herb against kidney stones.	Eye, Gall bladder, Bone, intestine, Swim bladder.
14	3-hydroxy-morindone	A novel compound	Eye, Intestine, Bone
15	Hypericin	Photosensitizer	Whole body, Eye, Gall bladder

3.3 Metabolism

Several adult and larval zebrafish metabolism mimics mammalian metabolism. Like humans, zebrafish also show phase I, phase II metabolism. A key player in phase I metabolism- Cytochrome P450 (CYP) regulates 60% oxidation reaction in zebrafish in abundance of monooxygenases enzyme (Poon et al., 2017; Verbueken, 2019). In many cases, phase I metabolism in Zebrafish is homologous to human phase I metabolism. It has been said that the zebrafish *Cyp3a65* gene is homologous to human CYP3A4. However, lacking other enzymes, it cannot fully demonstrate the human CYP. To replicate accurate human metabolism, a transgenic humanized zebrafish was developed that contains CYP3A4 enzyme which will give the same detoxification. Experiments showed that these transgenic zebrafish exhibit the same metabolism of Ketoconazole as mammals (Poon et al., 2017). That is why zebrafish can be consider to be a good model to assess the metabolism of drugs.

Bioaccumulation refers to the stockage of chemicals in the body if metabolism is altered or the rate of excretion is decreased. The bioconcentration factor (BCF) defines how prone the

molecule is to accumulate in a living organism. For fish it is evaluated as the ratio of the concentration of the tested substance between the fish (mg/kg) and water (mg/L) at a stable state (*Bio-Accumulation*, n.d.). A molecule will bioaccumulate if it has a BCF factor more than 1000. Research found that bioaccumulation factors of Chlorfenapyr is 864.6 and 1321.9 when treated with 1.0 and 10 µg/L which will eventually cause liver and brain damage. Similarly, the value of *n*-octanol/water partition ratio or coefficient (log K_{ow}) is another indicator of bioaccumulation. According to the European Chemical Agency (ECHA), the value of log K_{ow} should be less than or equal to 3 in order to avoid bioaccumulation. For example, Tralopyril, a metabolic product of Chlorfenapyr (insecticide) rapidly accumulates in zebrafish having log K_{ow} = 4.7 (Chen et al., 2021). Therefore, these insecticides should not be used in the field because they are polluting the aquatic environment. The log K_{ow}, and BCF value of a molecule can be found by experimenting in zebrafish and predict whether the drug will be accumulated in the body or not.

It is essential to know the mechanism of metabolism so that the researchers can find out the hidden toxicity of pharmaceuticals (Jones et al., 2012). With help of scientific tools such as LC-MS, HPLC-MS analysis, scientists are finding out the metabolites of the drugs such as hydroxyibuprofen; a metabolite of Ibuprofen (Jones et al., 2012). Moreover, N-demethylation and nitro-reduction was found in zebrafish while tested with neonicotinoid insecticide clothianidin. Effect of metabolism depends on the isomers of molecules, especially enantiomers. As most of the drugs are organic in nature, chirality makes those molecules more effective. For instance, metabolism of Metolachlor (herbicide) shows inhibitory effects of superoxide dismutase , catalase and glutathione S-transferase enzymes as well as causing liver damage but its enantiomer didn't (Yang et al., 2022). It can be noticed in *figure 7* that, at low concentration, S-metolachlor absorbs more drugs than others. On the contrary, when the dose is increased, S-metolachlor has a lower absorption rate than metolachlor. Furthermore,

concentration of drugs decreased linearly because of continuous metabolism. Notably, both of the drugs show rapid fall out during the washing period. Thus, it can be said that isomerism affects drug absorption and metabolism (Ou-Yang et al., 2022).

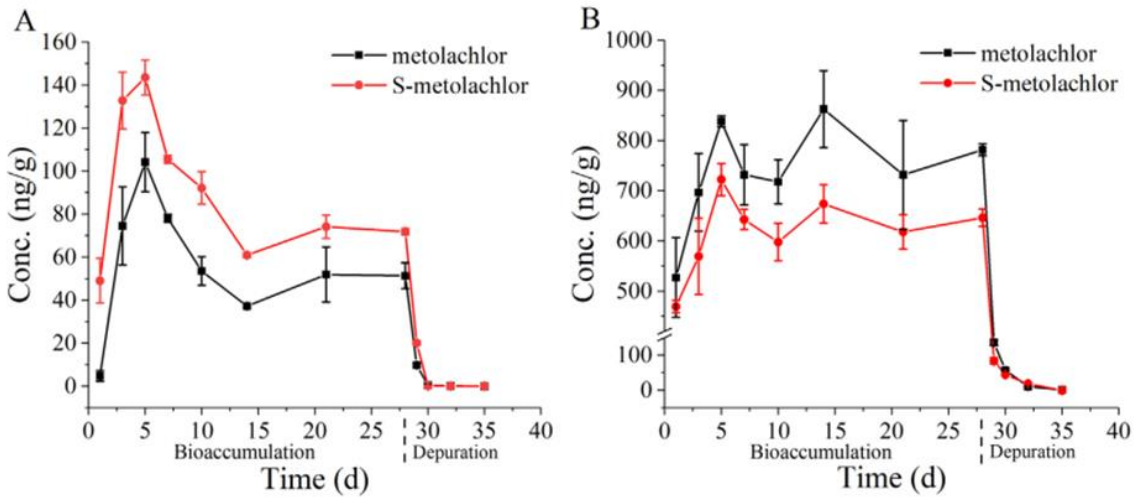

Figure 7: Absorption and elimination of metolachlor and S- metolachlor at different concentration (Ou-Yang et al., 2022).

3.4 Excretion

Excretion is essential in order to avoid toxicity. In zebrafish the main route of excretion is kidney (pronephros). These pronephroses contain single glomerulus which act as human glomeruli (Kotb et al., 2014). When chemical concentration of the aqueous medium is kept constant, excretion of the drugs from the fish can be calculated by $C_f(t) = C_f \times e^{-kd \times t}$ and elimination half-life ($t_{1/2}$) is calculated by $t_{1/2} = ln2/kd$ where C_f (µg/ g, wet weight) is the average concentrations of drugs in whole fish or tissue at time t and k_d is the bio-uptake rate constant (Yang et al., 2022). To check the glomerular activity and barrier function zebrafish is treated with 10-kDa Dextran and it has been observed that 10-kDa Dextran is quickly visible in the pronephric lumen of zebrafish, and rapidly eliminated at the cloaca. However, when the dose is increased (500-kDa), zebrafish fails to excrete the Dextran which indicates the active barrier function of zebrafish (Kotb et al., 2014). Several studies found that

zebrafish can eliminate drug like mammals such as, hydroxy-ibuprofen and carboxy-ibuprofen, metabolites of ibuprofen are readily excreted through zebrafish and mammalian kidneys (Jones et al., 2012). Similarly, O-desmethyltramadol (M1) and N-desmethyltramadol (M2), metabolites of tramadol eliminate slowly from zebrafish eyes, muscle and grill but rapidly eliminates from heart and brain tissue (Zhuo et al., 2016). Humans produce mono, Di and tri-Methylated Metabolites Arsenic (MMA) by biotransformation. By exposing Arsenite As (III) to zebrafish, it is also metabolized rapidly in liver by methylation and produce tri-methylated arsenic metabolism MMA(III), and MMA(V) which is rapidly excreted through kidney (Hamdi et al., 2012). These data help to understand potential toxicological effects of drugs.

A key feature of kidney is reabsorption which is necessary to maintain body homeostasis. In freshwater fish most of the osmoregulatory activities are performed by the gills. Moreover, gut epithelia absorb most of the vital ions, and kidney reabsorb the rest of the necessary ions from glomerular filtrate (Kersten & Arjona, 2017). In mammals, ClC-K channels play an important role in salt and water transport (Jentsch & Pusch, 2018). It has two isoforms: ClC-Ka and ClC-Kb. On the contrary, Zebrafish have only one clc-k isoform that helps in reabsorption. In the kidney reabsorption is carried out by clc-k/barttin channels, renin or the uro-guanylin receptor. Among them, clc-k/barttin channels zebrafish maintain most of its chloride ions. Experiment showed that knockout of clc-k/barttin channels causes death of the fish due to low chlorine concentration although other channels are working. From above description it can be said that zebrafish model could be used to understand better structure-function relationships of ClC-K and barttin proteins by which we can assess the reabsorption of drug (Pérez-rius et al., n.d.).

Chapter 4

Discussion

To assess a drug, it is crucial to know the pharmacokinetic in the body. From in vitro, we may predict the kinetic pattern but most often it is not matched with the real physiological environment. The experiments can be done in mice, rats, pigs, monkeys however, they require ethical permission and the number of permitted spices is quite low. Any miscalculation or mistake could be lethal to the organism. In these circumstances, Zebrafish is an excellent organism which can provide more accurate results upon *in-vivo* experiment. Moreover, to get more accurate value, over 5dpf fish can be tested. To check the kinetic of a potential or known molecule, first of all we need to make sure the drug is properly absorbed in the fish. Nature of the drug, maturity of the fish, ways of insertion etc. are common factors for absorption. When zebrafish larvae are kept in the aqueous medium containing drugs, it spontaneously starts absorbing the molecules through chorion or skin. However, it is time consuming and the lipophilic drug cannot be absorbed by this route. As a result, we may get false positive results. For example, erythromycin and D-sotalol causes QT prolongation in mammals but it did not alter zebrafish heart rate because of poor absorption. Similarly, due to slow absorption rate, sodium valproate did not cause any hepatotoxicity in zebrafish but in mammals do (Morikane et al., 2020). Here, microinjection is used to reduce the time as well as to have good bioavailability. Interestingly, if absorption from aqueous medium and the microinjection are applied together, the percentage of false positive and false negative value is decreased and the percentage of accuracy and precision become increased (Guarin, Faelens, et al., 2021; Guarin, Ny, et al., 2021). As insertion of live attenuate like virus or bacteria is successfully done on zebrafish, we can assess potential vaccines and antibiotic on it (Lacava Bailone et al., 2020). Moreover, adult zebrafish can be used to evaluate ointment

preparation because of having good absorption through skin. Additionally, pharmacokinetics assessment on zebrafish leads us the way of noble route of administration like releasing of drugs from beads or nanoparticles.

Distribution comes after absorption. If the drug remains in a single tissue, therapeutic effect will not show. When the drug candidates are assessed for distribution, prediction to deposit the drugs in specific tissue or organs as well as side effects and toxicity can be known. Sometimes, IY microinjection insertion did not show proper distribution so we can use it for aqueous environment because of the nature of the drug (Guarin, Ny, et al., 2021). PC and IP results were comparable showing that IP microinjections are a proper alternative to pericardial exposure in the zebrafish eleuthero-embryo as it shows good distribution (Guarin, Faelens, et al., 2021). Drug distribution depends on the route and site of the drug administration as well as the development of the fish. It has been seen that many compounds that pass through the BBB in zebrafish also pass through the BBB in humans. Similarly, molecules that do not pass through the BBB in zebrafish also do not pass through humans.

After distribution, the molecule needs to excrete from the body. Molecules need to modify such a way so that they can easily excrete from the body. Liver enzymes modify those molecules so that it can be easily removed from the body. zebrafish liver secretes lots of enzymes such as monooxygenase, monoamine oxidase, alcohol dehydrogenase, sulfotransferase, glucuronosyltransferase etc. These enzymes mainly perform phase I metabolism. Phase II and phase III metabolism also seen in zebrafish but in minor quantities. For example, Tramadol (a centrally acting analgesic) undergo metabolism and produce M1, and M2 metabolites which is also similar with mammals (Zhuo et al., 2016). To have more accurate result, transgenic liver enzyme is introduced in zebrafish that have same metabolism like human. Introducing transgenic liver enzymes isn't new. In the past, it was introduced in

mice because normal mice may not predict human drug metabolism since mice have its own P450 expression and catalytic activity (Poon et al., 2017). Interestingly, when comparing an antitumor drug epiribicin with rats, it showed that the molecule is astronomically accumulating in both zebrafish and in rat liver, and excreted through bile. From there we can say, it partially mimics the mammals' pharmacokinetics to assess the kinetics and their application of novel drug candidates (Yao et al., 2017). So, using fish is more convenient in terms of time and cost. In addition, the activity of prodrug can be evaluated by this mechanism since pro drugs become active after metabolism.

Failure of metabolism will create bioaccumulation that will result in toxicity. Through zebrafish, we can check the accumulation of drugs, and modify it so that it can readily be metabolized for elimination. Nowadays, zebrafish is widely used for evaluating environmental toxicity. There are thousands of molecules used around us for various reasons. These chemicals may enter our food chain and may not be metabolized or eliminated from our body. We need to identify them and stop using them. By utilizing zebrafish, we can identify these molecules. For instance, Chlorfenapyr is widely used as an insecticide which is mostly accumulated in zebrafish. Although some countries like the USA, Canada banned chlorfenapyr, many others are still using them because of its cross-resistance to other classes of insecticides (Chen et al., 2021). Furthermore, many chemicals are used as a mixture of their isomers. Not all the isomers are environment friendly. Through zebrafish, most active and environment friendly isomers can be detected. Like, S-metolachlor, and metolachlor, S-metolachlor do not cause any side effects, on the other hand metolachlor destroys the antioxidant system of zebrafish at high concentration (Ou-Yang et al., 2022). So, it can be said that zebrafish is an excellent tool to find novel eco-friendly molecules. Furthermore, transgenic zebrafish liver enzymes become popular day by day and by this technique the evaluation of prodrugs can be assessed since pro-drug become active after metabolism.

Metabolic drugs can be eliminated from the body through kidney or bile. In zebrafish a study found that after 72hpf pancreas is formed, no metabolic enzyme is secreted from there resulting in no bile elimination (Dhillon et al., 2019; Menke et al., 2011). So, the kidney is one and only way to eliminate molecules. It is seen that zebrafish have excellent elimination of drugs and also have good barrier function.

Chapter 5

Limitation of Zebrafish

For centuries, mice have been used in laboratories for drug discovery due to its enormous genetic similarities with humans. Yet, because of some drawbacks sometimes it is not preferable for drug discovery, and pharmacokinetic assay. For example, maintenance of mice is expensive, it takes time to grown-up, it is difficult to manipulate genes, it is also not translucent (Hedrich, 2012). Although study with zebrafish solves all the above mentioned problems, it persists some disadvantages as well. The zebrafish lacks many human organs, including breast tissue, lungs, and the prostate. So, assessment of drugs in these organs cannot be possible in zebrafish. Some cellular components found in human skin are absent in zebrafish such as stratum corneum which enables quick absorption of drugs compared with humans (Morikane et al., 2020). The zebrafish larvae is surrounded by chorion which also prevents the absorption of high molecular weight compounds (de Souza Anselmo et al., 2018). Infectious illnesses such as microsporidiosis and mycobacteriosis are frequently seen in zebrafish. As a results, it hinders absorption and metabolism. The metabolizing enzymes of the liver (e.g., CYP450s) are not completely defined in Zebrafish, thus we need to introduced humanized zebrafish enzymes (Poon et al., 2017; Rajan et al., 2020). Because of an evolutionary gene duplication event, teleost fish have two copies of many human genes. In addition, the issue of skewed sex ratios in zebrafish cohorts, may interfere with natural breeding and hinder investigations such as carcinogen or other toxicant bioassays that need balanced sex ratios in control groups *table 2* (Katoch & Patial, 2021; Molina et al., 2021; Sarvaiya et al., 2014).

Table 2: The pros and cons of zebrafish (Molina et al., 2021)

Pros	Cons
High reproductive rate	Differences in cellular structure
Transparent embryos	Different hepatic anatomy and structure
Fewer ethical/legal restrictions	Poorly developed cell culture and embryonic stem cell technology
Inexpensive and easy maintenance	Diverse physiology compared to mammals
Short maturation time	Poor delivery in embryonic stem cell technology
External fertilization and large clutch size	Less genetic conservation and Develop at lower temperatures than mammals
Tractable for forward (mutagenesis, high throughput screens) and reverse genetics (gene knockdown, gene editing)	Unavailability of well-characterized inbred strains
Fully mapped and small sized genome that enables easy genetic manipulation	Lack of specific antibodies

Chapter 6

Conclusion

In conclusion, zebrafish serve as excellent tools to evaluate pharmacokinetic profiles *in-vivo* platforms because of their genetic similarities with humans. To assess toxicity and drug discovery, it is essential to have adequate knowledge of kinetics. Absorption and distribution can be potentiated either using aqueous exposure or microinjection. Metabolism and excretion can be detected through various spectroscopy. Alike rats and mammals, it is proved that zebrafish exhibit similar pharmacokinetic profiles. Although not always desired kinetics is observed, we should find out the effective concentration to extrapolate the data into rodents or humans. In spite of being native to Bangladesh, very few experiments are conducted on zebrafish. Recently, the department of fisheries of the public university as well as some private organizations such as: 'Institute for Pharmaceutical Skill Development and Research' conducted some morphological, and pharmacological research on zebrafish. Rabbane et al., 2016, demonstrated that the culture, reproduction, and embryogenesis of zebrafish in laboratory conditions in Bangladesh is quite easy. It took very little space, less hazardous, and less personnel to maintain the husbandry of zebrafish compared with mice. Moreover, Azad et al., 2022 found four types of wild zebrafish in Bangladesh which allow the development of more effective and efficient management techniques. So, in universities we can start using zebrafish to conduct pharmacokinetic researches as they are cost effective, time consuming, free from ethical burden etc.

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