FEASIBILITY OF ZEBRAFISH LARVA MODEL AS A VIABLE SUBSTITUTE TO RAT NON-EVERTED SAC MODEL FOR PERMEATION EVALUATION OF BCS III DRUGS

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ABSTRACT

The oral route is the most convenient route of drug administration. Many drugs exhibit poor oral bioavailability. BCS III drugs exhibit high solubility and present a massive challenge due to poor permeability. Different permeation enhancers viz., nonionic Cremophor® RH 40, Tween® 80 and Lutrol® F68, anionic docusate sodium with sodium cholate, and anionic polymer sodium carboxymethyl cellulose were evaluated using rat non-everted sac method and zebrafish larva model. Maximum permeation enhancement was seen with docusate sodium for both drugs. The permeation enhancement ratio for netilmicin sulphate was 4.07±0.657, while for deferoxamine mesylate it was 1.482±0.378. Cremophor® RH 40 enabled augmented flux of netilmicin sulphate, and Tween® 80 showed enhanced permeation of deferoxamine mesylate. An excellent correlation was observed between apparent permeability and flux with drug absorbed per zebrafish larva (µg) (R^2= 0.938) for netilmicin sulphate and for deferoxamine mesylate (R^2= 0.9397). An important outcome of the study is the demonstration of the feasibility of the zebrafish larvae model as a viable substitute to the non-everted sac method, which could also enable screening of potential permeation enhancers for the development of orally bioavailable formulations of BCS III.

Keywords : Deferoxamine mesylate, netilmicin sulphate, oral absorption, permeation enhancers, rat non-everted intestinal sac, zebrafish larva model

INTRODUCTION

The oral route is the most convenient route of drug administration; nevertheless, many drugs exhibit poor bioavailability due to poor absorption, gastrointestinal metabolism, and/or instability in gastrointestinal pH. In particular, BCS III drugs that show high solubility present a massive challenge due to poor permeability[1]. Netilmicin sulphate (NTS)[2] and deferoxamine mesylate (DFM) are two such drugs commercially available only as injections. Limited studies have been reported on the non-invasive administration of DFM. Oral polymeric micelles using Poloxamer P 407, Tween® 80 and Span® 20 showed improved permeation through rat intestinal sac model[3].

While nano-formulations are beneficial for oral bioenhancement, a practical approach to improving permeability across the gastrointestinal membrane is using permeation enhancers (PE)[1,4]. Permeation of drugs in the presence of permeation enhancers has been studied using an intestinal segment of rats, namely jejunum or ileum, either everted[5] or non-everted[6] and in side-by-side diffusion cells separated by intestinal tissue, like the Ussing chamber[7].

P. Sharma et al. investigated the effect of different permeation enhancers for predicting oral absorption of cefotaxime sodium and ceftazidime pentahydrate using the everted rat intestinal sac model. Furthermore, permeation enhancement of both drugs occurred with sodium caprate, sodium caprylate, sodium cholate, and majorly with sodium deoxycholate, suggesting that enhancement was governed by the structure of the drug and the permeation enhancer[8].

The non-everted sac model is advantageous when compared to the everted sac model, due to ease of handling and lesser possibility of tissue damage, as the

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step of everting the intestine is bypassed. In addition, smaller intestinal segments can be used; hence, studies are possible with small amounts of a drug[6]. Ruan et al. evaluated the permeation of BCS III drugs acyclovir, ranitidine, acetaminophen and chloramphenicol using the NES model as a surrogate for oral absorption[6]. Olmesartan medoxomil nanoemulsion showed enhanced bioavailability using the non-everted sac method[9]. Similarly, a microemulsion of 5-fluorouracil (5FU) with Tween® 20 and Span® 20 showed a 7-fold increase in flux when evaluated in the rat non-everted sac (NES) model compared to the 5FU solution[10].

The effect of permeation enhancers on bioenhancement further confirms the vital role in enhancing the bioavailability of BCS III drugs. For instance, a hydrophobic ion-pair complex of insulin with the surfactant sodium deoxycholate showed improved oral bioavailability in diabetic rats[11]. Similarly, a SMEDDS comprising an ion-pair complex of BCS III drug doxorubicin with AOT and α-tocopheryl-polyethyleneglycol-1000-succinate revealed high oral bioavailability of doxorubicin and good anticancer efficacy in the rat fibrosarcoma model[12].

The everted and non-everted sac models, although well established, rely on animal sacrifice to obtain the intestinal tissue. However, the 3Rs of animal experimentation, namely reduce, refine and replace[13,14], propose a strong need for alternatives to animal-based experiments.

More recently, zebrafish embryos, larvae, and adult zebrafish have been proposed as models to predict the absorption of drugs. This model has similarities to humans in morphological, genetic and molecular features[15-17]. Zebrafish are small in size, easy to handle and entail fewer ethical issues. Such characteristics make it a model of choice in drug and formulation development[18]. Zebrafish larvae, in particular, have fully developed systems and are recommended as a model of choice for studying the absorption of various drugs[19,20]. The zebrafish larva model has been demonstrated as a feasible model for studying the absorption of BCS III antibiotics ceftazidime, cefotaxime, minocycline and netilmicin[21].

Hence, the objectives of the present study were dual. One was to evaluate the zebrafish larva model as a viable substitute for the rat non-everted sac model. Yet another aim was to identify an appropriate permeation enhancer for the BCS class III drugs NTS and DFM using the zebrafish larva model.

MATERIALS AND METHODS

Materials

Deferoxamine mesylate (DFM) was gifted by Novartis India Ltd. Netilmicin sulphate (NTS) was gifted by Emcure Pharmaceuticals, Pune, India. Sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), calcium chloride dihydrate ($CaCl_2.2H_2O$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), magnesium sulphate, methylene blue, glucose, trypan blue, sodium acetate, glacial acetic acid, formic acid, ferric chloride, anhydrous, hydrochloric acid, Tween® 80 (T80), docusate sodium (AOT) and sodium carboxymethyl cellulose (medium viscosity) (CMC) were procured from S. D. Fine-Chem Limited, Mumbai, India. Sodium cholate (SCHO) was purchased from Hi Media Laboratories Pvt Ltd., Mumbai. Lutrol® F68 (LF68) and Cremophor® RH 40 (CRRH40) were gift from BASF, India, Ltd. Double distilled water was used to prepare all solutions. All reagents and chemicals used were of analytical grade.

Methods

Analysis of NTS by UV-Visible spectrophotometry

10 mg of NTS was dissolved in 10 mL of distilled water to obtain NTS aqueous solution (1 mg mL^{-1}). Standard solutions of concentrations of 4 – 20 µg mL^{-1} were obtained by diluting 0.4 mL to 2 mL of NTS aqueous solution (1 mg mL^{-1}), respectively. To these aliquots, 4mL of aqueous trypan blue solution (0.02 % w/V) and 1 mL of acetate buffer solution (pH 2.9) were added[22]. The volume was made up to 10 mL with distilled water, shaken intermittently, and after 20 minutes, absorbance was determined at 684 nm against reagent blank using UV- visible spectrophotometer (JASCO V-630 spectrophotometer). The results of concentration vs absorbance were plotted to find slope and regression parameters.

Analysis of DFM by UV – Visible spectrophotometry

10 mg of DFM was accurately weighed and dissolved in purified water in a 10 mL volumetric flask. Standard solutions of concentration 25 to 200 µg mL^{-1} were obtained by diluting 1 mg mL^{-1} standard solution as 0.25 to 2 mL. To these aliquots of DFM, 1.2 mL of ferric chloride solution was added, and the volume was made to 10 mL respectively with distilled water[23]. The absorbance of these solutions was recorded at 485 nm against blank using UV-visible spectrophotometer (JASCO V-630 spectrophotometer). The results of concentration vs absorbance were plotted to find slope and regression parameters.

Non-everted sac method (NES)

All animal studies were performed as per protocol approved by the institutional animal ethics committee of ICT (ICT/IAEC/2017/P23), Mumbai. Sprague-Dawley rats 200 ± 30 g of either sex were used for the study. After sacrificing the rats by CO_2 asphyxiation followed by cervical dislocation, the abdomen was opened by a midline incision, and the intestine was cautiously handled to remove the intestinal segment. The mesenteric attachments were carefully removed without damage to the intestinal structure. The jejunal segment was removed and transferred to Tyrode's solution. (Tyrode's solution:139 mM NaCl, 3 mM KCl, 17 mM, $NaHCO_3$, 12 mM glucose, 3 mM $CaCl_2.2H_2O$, 1 mM $MgCl_2.6H_2O$). The jejunal segment was washed by gently blowing Tyrode's solution with a pipette and was cut into approximately 5 cm segments. Drug solutions (NTS: 2 mg mL^{-1}) (DFM: 2mg mL^{-1}) were prepared with and without permeation enhancer (10 µL or 10 mg mL^{-1}) in Tyrode's solution. One end of the cut jejunal segment was ligated using a thread and filled with 0.5 mL of drug solution (1mg mL^{-1}).

Table I: Permeation enhancement ratio (PER) in intestinal permeability in NES model for NTS and DFM in the presence of PE

Sr. No.	PE	PER(NTS)	PER(DFM)
1	AOT	4.07 ± 0.657	1.482 ± 0.378
2	CRRH40	3.207 ± 0.371	1.165 ± 0.076
3	T 80	2.809 ± 0.499	1.238 ± 0.158
4	SCHO	2.272 ± 0.468	1.091 ± 0.131
5	CMC	1.397 ± 0.150	1.043 ± 0.155
6	L F68	1.393 ± 0.216	#0.963 ± 0.048

#Indicates no enhancement

The filled sacs were suspended in test tubes containing 5 mL of Tyrode's solution each. Non-everted intestinal sacs filled with Tyrode's solution served as blank. During the experiment, the tubes were maintained at 37 ^{0}C under aeration (50 - 60 bubbles per minute). 2 mL medium was withdrawn from test tubes at the end of 15, 30, and 60 minutes and replaced with 2 mL aerated Tyrode's solution maintained at 37 ^{0}C to maintain constant volume throughout the experiment. Aliquots (1.5 mL) of the medium were analysed by UV-visible spectrophotometry as described for NTS and DFM, and concentration was extrapolated from the standard curve. All the experiments were performed in triplicate.

The average length of the filled sacs (n >25) was 3.67 cm ± 0.42 cm, and the circumference was 1.57 cm ± 0.09 cm, equivalent to a diameter of ~0.5 cm. The surface area of the sac calculated, assuming it to be a cylinder, was 6.101 ± 0.31 cm^2. The flux and permeation enhancement ratio (PER) was calculated. The cumulative amount of the drug permeated through the sac per unit area (µg cm^{-2}) was plotted against time (min). Flux was calculated from the slope of the graph.

Table II: NTS and DFM absorbed in µg per zebrafish larva and % cumulative drug permeated in NES in presence of different permeation enhancers

PE	NTS		DFM	
	Drug absorbed µg per larva in 60 minutes	% Cumulative drug permeated from NES in 60 minutes	Drug absorbed µg per larva in 60 minutes	% Cumulative drug permeated from NES in 60 minutes
-	1.083±0.193	9.421±0.205	2.519±0.424	45.452±2.226
AOT	2.807±0.037	41.026±1.557	3.942±0.051	62.82±3.342
CRRH 40	2.185±0.024	35.962±2.002	2.713± 0.825	51.751±0.693
T 80	1.504±0.019	28.798±1.684	2.854±0.559	57.025±4.655
SCHO	#1.033±0.042	27.528±2.582	2.763± 0.483	46.098±5.535
CMC	#0.873±0.081	16.387±1.986	#2.455± 0.125	46.894±0.659
LF 68	#0.655±0.054	14.519±0.205	#2.016± 0.050	#42.971±3.307

Each value represents means ± SD, N= 3; PE= 1%w/V or 1%V/V

 # Indicates no enhancement

Apparent permeability (Kp) was calculated as shown in equation 1:

Apparent permeability = Flux/Initial concentration i.e.

$$Kp = (\frac{dQ}{dt})/A \times C_0 \quad \text{Eq. (1)}$$

where

dQ/dt = the change in concentration in the acceptor compartment with time

A = area of permeation

C_0 = initial concentration in the donor compartment

Enhancement in permeation of model drugs in the presence of PE was calculated as Permeation Enhancement Ratio (PER) as shown in equation 2:

$$PER = Kp(enh)/Kp(Plain\ solution) \quad \text{Eq. (2)}$$

where,

$Kp_{(enh)}$ is the apparent permeability of the model drug in the presence of PE and

$Kp_{(Plain\ solution)}$ is the apparent permeability of the model drug solution

% Cumulative drug permeated across the sac in 60 minutes was calculated for the plain solution of the drug and the drug solution in the presence of a permeation enhancer.

Cytotoxicity study in zebrafish larvae (ZFL)

The zebrafish wild-type (WT) embryos were procured from a local supplier. Embryo stock solution was prepared by dissolving sodium chloride 290 mg, potassium chloride 12.58 mg, calcium chloride 36.63 mg, magnesium sulphate 39.6 mg and 0.1 mL methylene blue in 100 mL distilled water in a volumetric flask[24,25]. The stock solution (10 mL) was diluted to 100 mL with distilled water to yield an embryo medium to rear the embryos. Embryos 14-day post fertilisation were used for the cytotoxicity study.

Larvae (N=20) were counted manually and transferred using a 3 mL plastic transfer pipette into 10 mL glass vials. Drug solutions (NTS:5 mg mL^{-1} and DFM: 5 mg mL^{-1}) were prepared in embryo medium. To vials containing larvae (N=20) embryo medium stock solution, drug solution was added to obtain concentrations of 250, 500, 1000, 1500, and 2000 µg mL^{-1}, and volume was made up to 5 mL with distilled water. Cytotoxicity was evaluated by monitoring the larvae for loss of motility. The sinking of larvae confirmed the death of larvae. Cytotoxicity was determined at the end of 30, 60, 90, and 120 minutes. The larvae (N=20) incubated in a 5 mL embryo medium served as controls. All experiments were conducted at a temperature of 25 ± 2^0 C. All the experiments were performed in duplicate. The safe concentration considered for the drug absorption study was lower than the maximum tolerated concentration (MTC), defined as the concentration at which no lethality was observed.

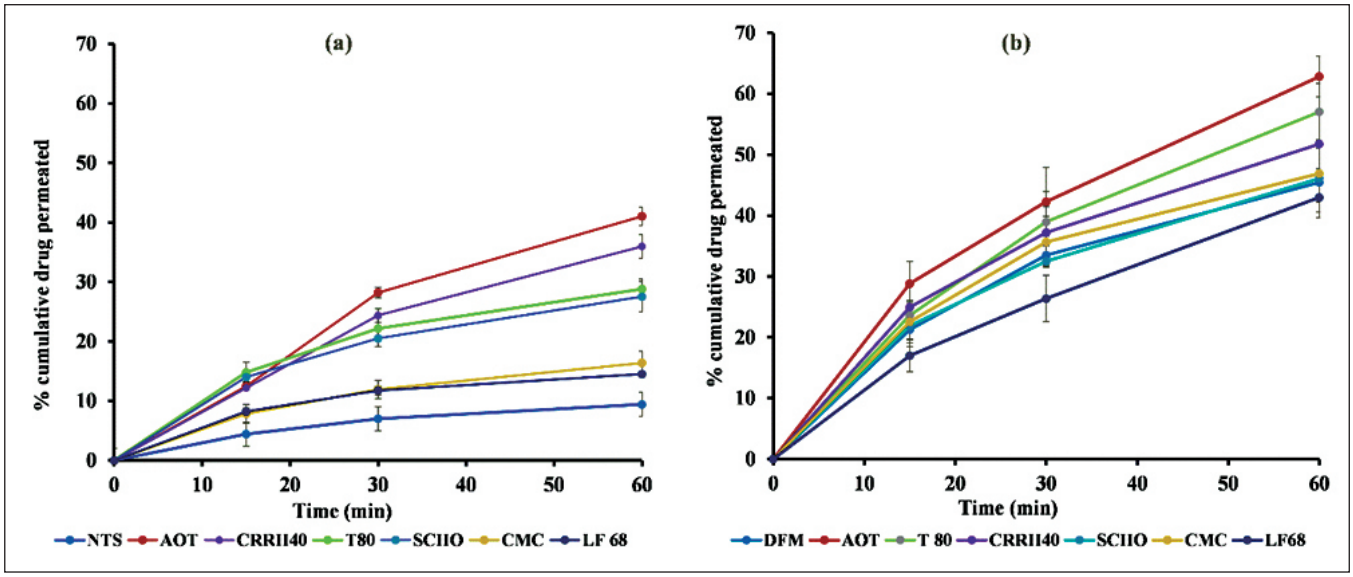

Fig. 1: Cumulative drug permeated (%) vs time(min) with and without permeation enhancer for (a) NTS (b)DFM (n=3, mean± SD)

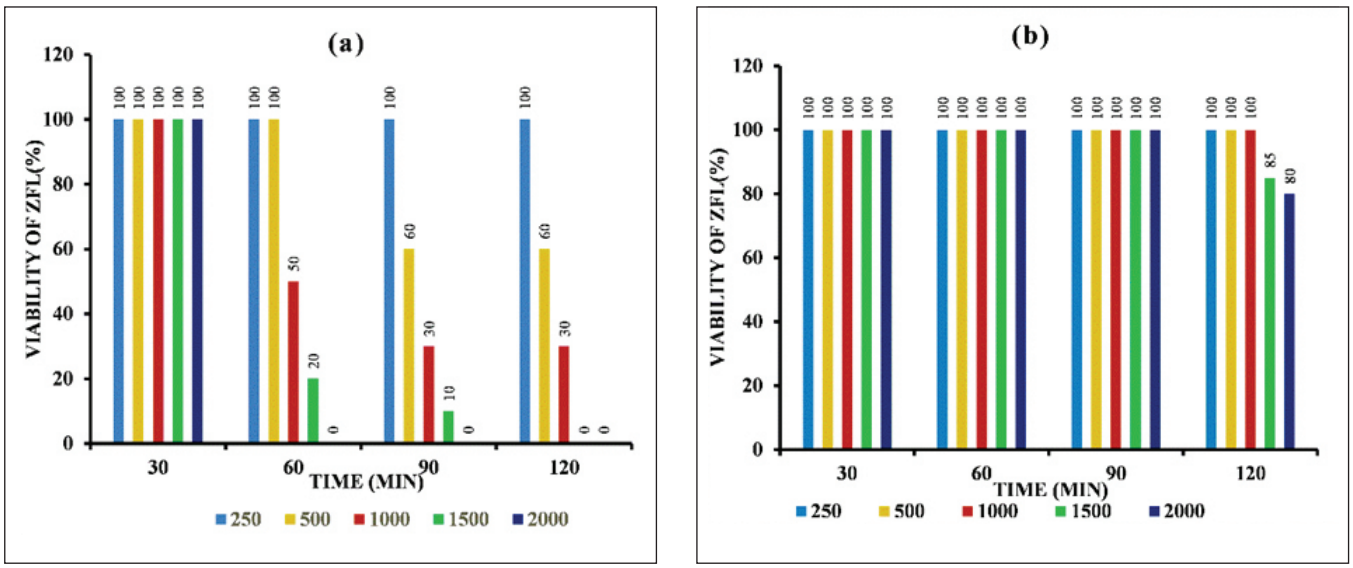

Fig. 2:Viability of ZFL (%) vs time (min) (a) NTS (b) DFM (n=2)

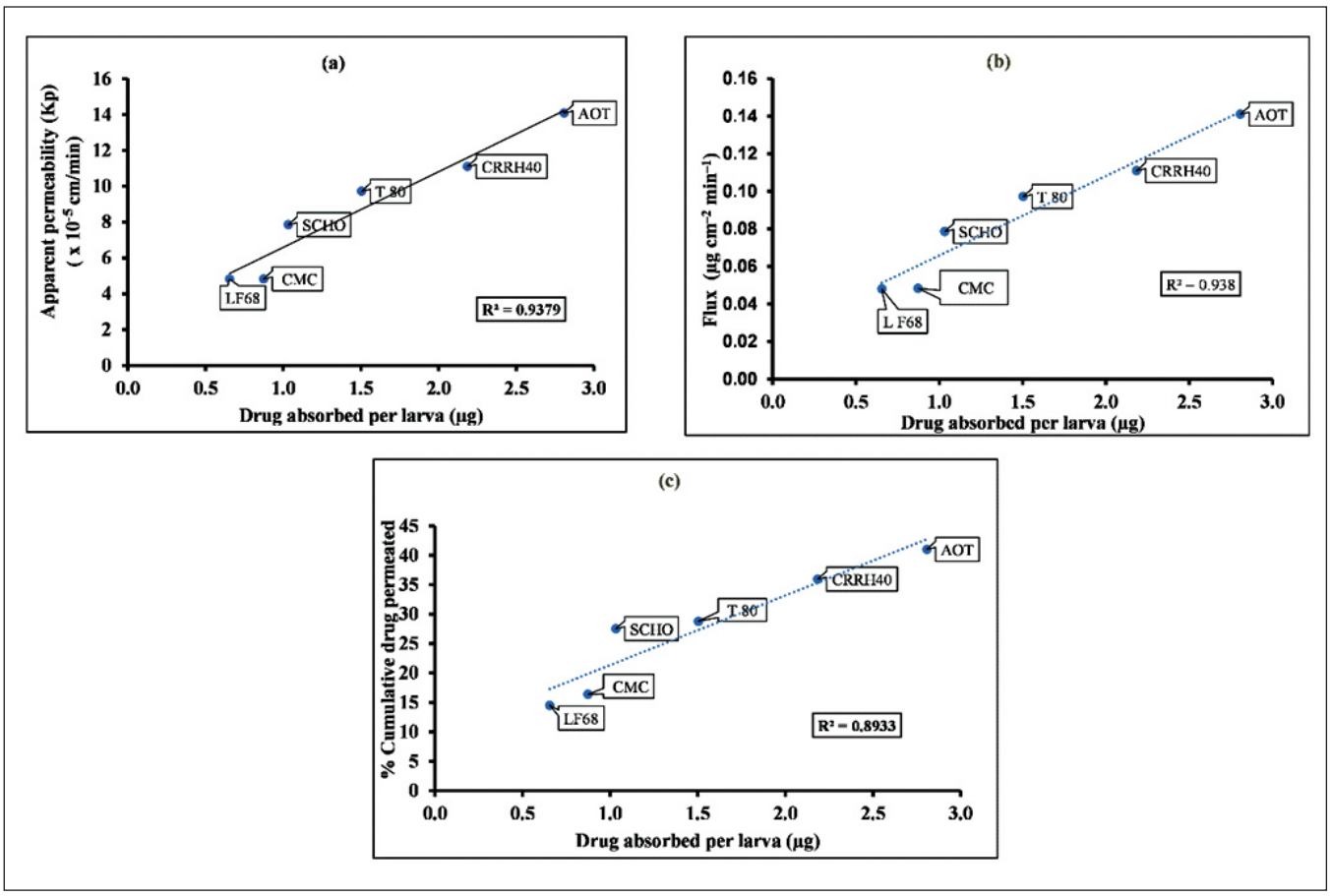

Fig. 3: Correlation between a) apparent permeability (Kp) (x10^{-5} cm min^{-1}) vs drug absorbed per larva (µg) b) flux (µg cm^{-2} min^{-1}) vs drug absorbed per larva (µg) c) cumulative drug permeated (%) in NES model and drug absorbed per zebrafish larva (µg) for NTS (n=3, mean ± SD)

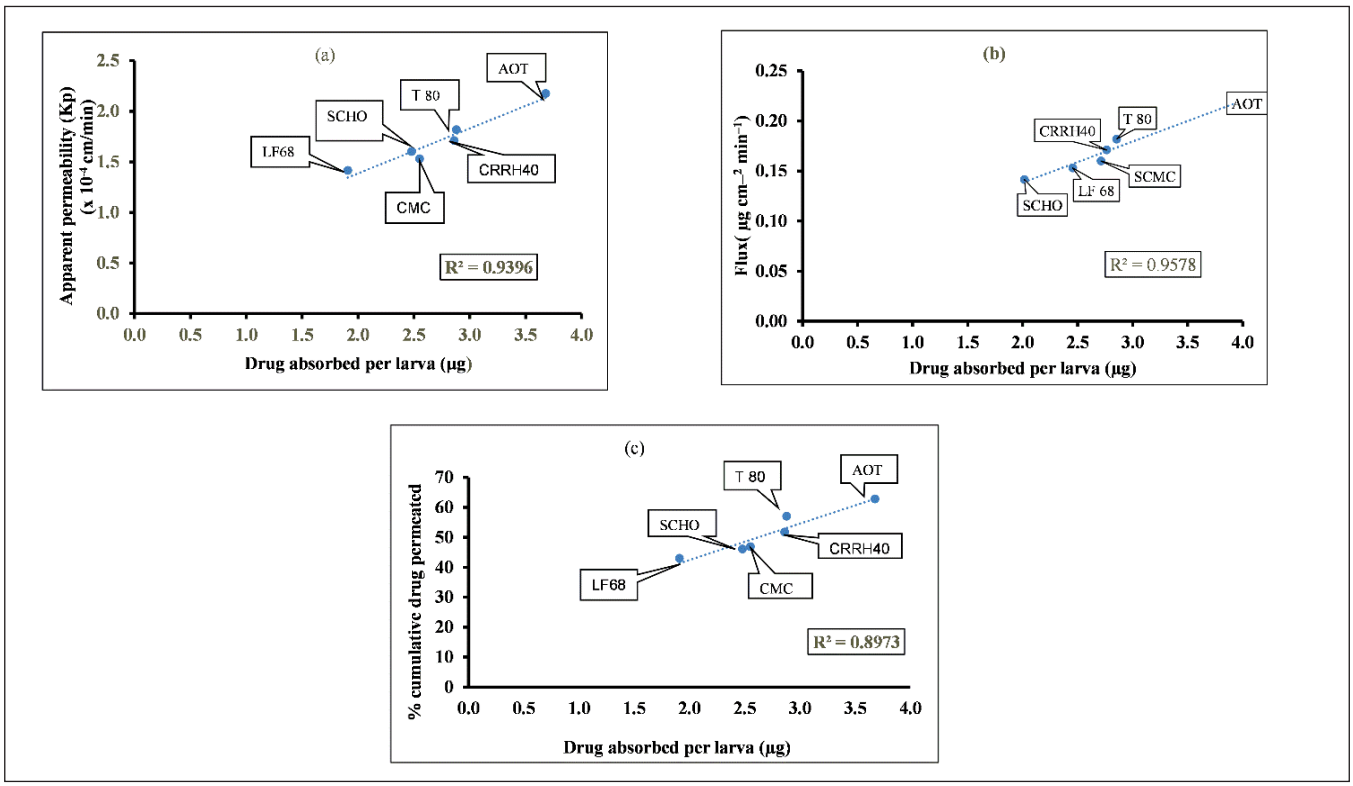

Fig. 4: Correlation between a) apparent permeability (Kp) (x 10^{-4} cm min^{-1}) vs drug absorbed per larva (µg) b) flux (µg cm^{-2} min^{-1}) vs drug absorbed per larva (µg) c) cumulative drug permeated (%) in NES model and drug absorbed per zebrafish larva (µg) for DFM (n=3, mean ± SD)

Drug absorption in zebrafish larvae (ZFL)

Larvae (N=20) 14 days post fertilisation were counted manually and transferred using a 3 mL plastic transfer pipette into 10 mL glass vials. Drug solutions (NTS: 1 mg mL^{-1} and DFM: 5 mg mL^{-1}) were prepared with and without PE (10 µL or 10 mg mL^{-1}) in embryo medium. To vials containing larvae (N=20) with embryo medium stock solution, drug solution/drug solution plus PE (10 µL or 10 mg mL^{-1}) were added, and volume was made up to 5mL with distilled water. For the drug absorption study in ZFL, the concentration of NTS was 100 µg mL^{-1}, while in the case of DFM, the concentration was 1000 µg mL^{-1}. The larvae (N=20) incubated in a 5 mL embryo medium served as controls. All experiments were conducted at a temperature of 25 ± 2^0 C. The larvae were maintained in the drug solutions for 1 h. The vials were placed in an ice bath at the end of 1 h to anaesthetise the zebrafish larvae. The solution was carefully aspirated using a micropipette without damaging the larvae. The larvae were gently washed with distilled water (1 mL) to remove the drug solution adherent to the surface. This procedure was repeated twice.

The anaesthetised zebrafish larvae were triturated with 1 mL of water in a 2 mL Eppendorf tube using a glass rod. Then 1 mL of formic acid (5% V/V) in water was added to precipitate proteins[21]. The Eppendorf tubes were vortexed for 2 minutes, sonicated using a bath sonicator for 15 minutes, and centrifuged (Remi RM-12C Micro Centrifuge) for 15 minutes at 9056 x g. The supernatant was carefully aspirated out and collected in different Eppendorf tubes. The weight of the zebrafish WT (N=20) (12.08±2.84 mg) larvae pellet settled at the bottom of the Eppendorf tube was noted. Aliquots (1.5 mL) of the medium were analysed by UV- visible spectrophotometry as described for NTS and DFM, and the concentration was extrapolated from the standard curve. The concentration of drug absorbed (µg) per zebrafish larva was estimated. All the experiments were performed in triplicate.

Statistical analysis

All values were expressed as mean value ± standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using Student's t-test, and $P < 0.05$ was the criterion for statistical significance.

Statistics of the correlation between models tested

The statistics of correlations between two variables were tested by the linear correlation coefficient (R^2).

RESULTS

Non-everted sac method (NES)

The rat non-everted intestinal sac model (NES)[6] is a preferred model for the prediction of oral absorption due to its simplicity and no morphological damage being caused to the intestinal tissue, which are major concerns with the everted sac model. The permeation of NTS and DFM was determined in the presence of some nonionic and anionic surfactants and polymers, namely nonionic CRRH40, T80, and LF68, anionic surfactants AOT, SCHO, and anionic polymer CMC.

The percent cumulative drug permeated against time is depicted in Fig. 1, while the permeation enhancement ratios are reported in Table I.

Cytotoxicity study in zebrafish larvae (ZFL)

As shown in Fig. 2, cytotoxicity studies revealed the 100 % viability of ZFL at 250 µg mL^{-1} for NTS and 1000 µg mL^{-1} for DFM. Based on the data, DFM was found to be well tolerated compared to NTS, so the concentration selected for drug uptake studies in ZFL was 100 µg mL^{-1} for NTS and 1000 µg mL^{-1} for DFM.

Drug absorption in zebrafish larvae

The results of drug absorption in ZFL at the end of 60 minutes are reported for NTS and DFM in Table II, which also presents data on drug permeated at 60 minutes in the NES model.

Statistics of the correlation between models tested

To understand the correlation between the two models, drug absorption data obtained using the zebrafish larva model was compared with the following parameters of the NES data: apparent permeability (Kp), flux and the cumulative amount of drug permeated at 60 minutes.

The plots of apparent permeability (Kp), flux and the % cumulative amount of drug permeated at 60 minutes vs drug absorbed per ZFL are shown in Fig. 3 and Fig. 4 for NTS and DFM, respectively. For NTS, an excellent correlation was observed between apparent permeability and drug absorbed per zebrafish larva (R^2= 0.9379) (Fig. 3a) and flux and drug absorbed per zebrafish larva (R^2= 0.938) (Fig. 3b). The plot of % cumulative drug permeated vs drug absorbed per zebrafish larva revealed a lower correlation (R^2= 0.8933) (Fig. 3c).

In the case of DFM, an excellent correlation was observed between Kp and drug absorbed per zebrafish larva (R^2= 0.9396) (Fig. 4a) as well as flux and drug absorbed per zebrafish larva (R^2= 0.9397) (Fig. 4b). The plot of % cumulative drug permeated vs drug absorbed per zebrafish larva showed a lower correlation (R^2= 0.8973) (Fig. 4c).

DISCUSSION

The drugs selected in this study were of class BCS III, exhibiting good aqueous solubility but poor permeability after oral administration. Typically, BCS III drugs are known to be high molecular weight (>500 Da), as seen with NTS (1441.6 Da) and DFM (656.8 Da). Netilmicin sulphate is used to treat infections caused by aerobic and anaerobic Gram-negative bacteria or aerobic Gram-positive bacteria, cocci, etc[26]. Deferoxamine mesylate is used for acute iron intoxication and chronic iron overload due to transfusion-dependent anaemia[27].

Both drugs exhibit poor oral bioavailability and are currently available for administration by the unfriendly route for a patient, namely injection into the vein or muscle. Hence NTS and DFM were considered apposite drug candidates for the present study.

A major challenge with BCS III drugs is poor permeation and hence limited *oral* absorption. Strategies for permeation enhancement are, therefore, an important consideration. In this study, the non-everted sac (NES), a well-established model for the evaluation of drug permeation, was compared with the zebrafish larvae (ZFL) model to establish a correlation between the two models and also augmenting permeation of NTS and DFM using permeation enhancers.

Permeation enhancers used in this study were chosen from nonionic and anionic surfactants and polymers. Cationic surfactants were not considered due to their toxicity[28] and the cationic nature of both drugs, which could entail drug permeation enhancer repulsion. Nonionic surfactants are preferred due to the wide versatility of applications, high biocompatibility, moderate interaction with biological barriers, high degree of compatibility with other components, good physicochemical stability, low toxicity, and are less affected by pH and changes in ionic strength[29]. Nonionic surfactants are known to entrap hydrophilic drugs in micellar cores to enable oral

bioenhancement[30]. The nonionic surfactants evaluated included CRRH40, T80 and LF68. The ability of the cationic drugs to interact with the anionic surfactant/polymer to form a lipophilic complex was relied on for possible bioenhancement. Accordingly, the anionic surfactants AOT[12], SCHO, and anionic polymer CMC, which reported enhancing the permeation of hydrophilic drugs across the gastrointestinal membrane, were evaluated[8].

The rat non-everted intestinal sac model (NES)[6] is a preferred model for the prediction of oral absorption. The NES model is simpler to maintain the continuity of experiments, with the additional advantage of collecting analytically clean samples. Studies using the NES model report use of the duodenum, jejunum, and ileum[7]. The jejunum, which has moderate length and is known to be a major absorptive region for many drugs, was used in the study[31].

As reported in Table I, amongst nonionic surfactants, CRRH40 revealed maximum permeation enhancement of NTS (~3 fold), followed by T80 for NTS, corroborating reported data that cremophors are more effective permeation enhancers than Tweens[32].

Membrane perturbation caused by nonionic surfactants coupled with micellar entrapment of the hydrophilic drugs could have improved permeation by enabling transcellular transport across the intestinal membrane[29]. CRRH40 and T80 are reported to enhance permeation by affecting tight junction permeability, thus enhancing paracellular transport. A combination of both the transcellular and paracellular routes may have contributed to the overall passive transport.

Polymeric surfactants like Lutrol are safer and a convenient alternative to classic surfactants due to their low CMC values, good biocompatibility profile and high drug entrapment efficiency, as they possess a large hydrophobic inner core[33]. Although ~1.3-fold enhancement was seen in the case of NTS, no improvement was observed in DFM permeation in the presence of LF68. Lower enhancement with LF68 could be attributed to the high molecular weight and higher viscosity of LF68.

Anionic surfactants act by disrupting lipid membranes and denaturing proteins[34]. Among the ionic surfactants, docusate sodium (AOT) revealed maximum permeation, even greater than that seen with CRRH40.SCHO (~ 2fold) and CMC (~1.3fold) showed enhancement with NTS (mol. wt. 1441.6 Da). This is ascribed to the nature of the ionic complex. Considering that the HLB of AOT is 10, the NTS-AOT ion-pair complex would be hydrophobic compared to the complex of NTS with SCHO, which has a high HLB of 18. Furthermore, CMC, a hydrophilic polymer, would have resulted in an even more hydrophilic complex and limited permeation by imparting viscosity to the medium. As seen with NTS, high permeation was seen with the DFM-AOT complex (Fig. 1). Further, all three anionic permeation enhancers revealed enhancement with NTS. With DFM, showed rank order for permeation with the anionic surfactants was AOT>SCHO>CMC which is similar to NTS. The enhancement with AOT, SCHO, and CMC was statistically significant (*$P < 0.05$) for NTS, but DFM did not show significant improvement ($P>0.1$). It could be due to the formation of a weaker ion pair complex with DFM may be leading to no significant enhancement in permeation.

Maximum permeation enhancement seen with AOT for both drugs is attributed to the enhanced lipophilicity of the drug AOT complex. The PER for NTS was 4.07 ± 0.657, while the PER for DFM was 1.482 ± 0.378, which was significantly lower ($P<0.05$). Among the nonionic permeation enhancers, while CRRH40 enabled augmented flux of NTS, T80 showed better permeation enhancement of DFM.

Permeation enhancement of NTS was significantly higher than DFM for the same PE. However, for the hydrophilic high molecular weight PEs, although the PER of NTS was higher, no significant difference was seen between the PEs for these PE with the same drug and between the chosen model drugs (Table I). This proposed that highly hydrophilic PEs may not be suitable PEs for BCS class III drugs.

The zebrafish larvae model (ZFL) helps to reduce and replace the use of higher animals in experiments. Such studies can eliminate the need for *in vivo* animal studies or at least enable a reduction in the number of animals required for the study. Zebrafish larvae, 14 days post fertilisation, have a length of ~6.2 mm and have well-developed cardiovascular and nervous systems. The digestive system is also well-developed, including the intestinal tissues involved in drug absorption[35]. When exposed to the drug solution, larvae absorb the drug, which can then be extracted and analysed[36]. Zebrafish larvae being a vertebrate model, a close correlation can be expected with studies involving *in vivo* whole animals like rats and mice[37].

The ZFL has been reported as a model for studying drug toxicity and drug absorption. However, this is probably the first report of the application of the zebrafish larva as a model to study absorption enhancement using permeation enhancers, and more particularly of BCS III drugs.

Correlating with the NES model, maximum absorption in the zebrafish larva model was seen with the anionic surfactant AOT for NTS and DFM. Further, the rank order for absorption in ZFL matched the NES data for NTS and was AOT> CRRH 40 > T80> SCHO>CMC>LF68.

In the case of DFM, too, the rank order between NES data matched with absorption in ZFL model and was AOT> T80 > CRRH 40 > CMC > SCHO >LF68.

Rat is used as an animal model in NES and closely resembles paracellular spaces and the metabolism of humans[6,38]. The strong correlation between the NES model and zebrafish larvae models, as seen in Fig. 3 and Fig. 4, indicates that the mechanism of drug absorption by zebrafish larvae and NES are comparable. So, it can be used as an initial model for shortlisting excipients for formulation development. A simple-to-handle zebrafish larvae model can be a suitable alternative model which can be used to predict enhancement in oral absorption using permeation enhancers as a strategy for highly hydrophilic NTS and DFM, model BCS class III drugs. The results obtained by the zebrafish larva model can be translated to the development of suitable drug delivery systems for poorly permeable drugs of BCS class III.

CONCLUSION

A significant outcome of the study is the demonstration of the feasibility of the zebrafish larvae model as a viable substitute to the non-everted sac method, which could also enable screening of potential permeation enhancers for the development of orally bioavailable formulations of BCS III drugs. In addition, the use of the zebrafish larvae model will enable a reduction in animal experimentation during formulation development. Additional studies with more BCS III drugs could further validate the model.

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