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Toxicity Assessment of Glycofurol in Zebrafish and *In Silico* Admet Profiling

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Abstract

This study assessed glycofurol's embryo and adult toxicity in zebrafish, a solvent widely used in pharmaceuticals. Despite its common use, limited toxicity data exists in rodents, prompting the examination of acute toxicity in adult zebrafish and developmental toxicity in embryos. Following OECD guidelines 236 and 203, Fish Embryo Acute Toxicity (FET) and Fish Acute Toxicity Testing were conducted. Results showed concentration-dependent embryo mortality with LC_{50} based on the Probit method of analysis is 0.36%. The adverse effects in embryos like coagulation, lack of somite formation and various such changes were observed at 0.4% and above. Adult zebrafish exhibited no mortality or abnormal signs at the 0.2 ml/L concentration of glycofurol. Based on the results obtained, glycofurol is safe at concentration of 0.2% in zebrafish FET and 0.2 ml/L in adult zebrafish acute toxicity test. *In silico* prediction of pharmacokinetics and toxicity profiles showed that the glycofurol has favourable PK (pharmacokinetics) properties and no serious toxicities were predicted.

Key words: Glycofurol, embryotoxicity, zebrafish, In silico prediction.

Glycofurol is a solvent which is used for parenteral administration of lipophilic compounds and is considered as less toxic than polyethylene glycol (PEG) and Dimethyl sulfoxide (DMSO). It exhibits amphiphilic properties, forming micelles with lipophilic drugs, thereby aiding in their dissolution in water. Glycofurol (GF) has been used as a solvent in drug-containing formulations such as skin depigmentation treatments, lotions, and ointments, as well as to administer benzodiazepines by nasal administration. It is used to make injectable solutions for versatile pharmaceuticals ranging from smallmolecules like diazepam or phenytoin to genes or proteins. Recent research examined the efficacy and tolerability of diazepam delivered intranasally in a GF and water blend, showed 75% bioavailability in humans (Ivaturi *et al.*, 2009). Methadone delivered rectally in a 20% GF solution also shown excellent absorption in humans (Boongird *et al*., 2011). The toxicity data of glycofurol in rodents through oral and intravenous route is limited. There are no studies availablestating clear safety data in the preclinical species about its NOAEL (No Observed Adverse Effect Level) or LOAEL (Lowest Observed Adverse Effect Level). There was no study available for glycofurol toxicity in zebrafish. Zebrafish (*Danio rerio*) is one of the lower sentient laboratory animal species and considered as one of the replacement models in the field of biomedical research. OECD (Organisation for Economic Co-operation and Development) has devised guidelines 236 and 203 to conduct Fish Embryo Acute Toxicity (FET) Test and Fish Acute Toxicity Testing. Using zebrafish for assessing the toxicity of chemicals will reduce the usage of rodents in the toxicity studies. Hence the adult zebrafish toxicity study and developmental toxicity of glycofurol in Zebrafish embryos were conducted.

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Materials and Methods

The test chemical Glycofurol 99 % extra pure was procured from Sisco research laboratories, Kolkata, India.

Fish acute embryo toxicity (FET) test was conducted by considering the OECD test guideline no. 236 (OECD, 2013). Adult male and female zebrafish in the ratio of 1:2 was kept in the spawning tank and provided light and darkness for 10 and 14 hours, respectively. Zebrafish starts laying eggs by 30-45 minutes after the dawn. The eggs were collected and kept in a clean petri dish with reverse osmosis purified and UV treated (RO) water. The eggs were cleaned with the help of a sieve under running RO water for 4 times and they were kept again in another clean petri dish. Glycofurol stock solutions of different concentrations were prepared in RO water and kept in each well of a sterile cell culture plate. The test concentrations of the solutions were 5, 2.5, 1.2, 0.4, 0.2, 0.1, 0.07 %. Each concentration was allocated to four wells and control was allocated to 4 wells and control consisted of RO water. 40 eggs were exposed under each specified concentration (with 10 eggs in each well) and were observed under the light microscope from 0 to 96 hours. Different stages of the embryo were observed with the help of a binocular microscope and photographs were taken with the help of a mobile camera fitted with the stand to the eye piece of the microscope.

Fish acute toxicity test was conducted by considering the OECD test guideline no. 203 (OECD, 2019). After an acclimatization period of 9 days, the limit test was conducted with 0.2 ml of glycofurol per litre of water in one tank containing 7 adult fishes and the other tank contains only water which served as control. As per the OECD test guideline 203, the limit test should be performed with 100 mg/L. As the test substance, glycofurol is a liquid and the specific gravity of glycofurol is 1.08 g/mL at 20 °C (Chemspider, 2024), the test concentration taken was 200 mg/L (0.2 ml/L), which was approximately twice the limit test concentration mentioned in the OECD guideline 203. The fish were exposed to the test solution for 96 hours, under static conditions. The temperature and pH of the water in both the control and test were monitored and were around 6.8 in the test and 7 in the control. The temperature of the water was maintained at around 25 °C. The fish were provided with 12 h of alternative light and dark periods. Both the experiments were done after

Table I : List of *in silico* tools used in the prediction of ADME and Toxicity Properties for Glycofurol

In silico Tool Name	Principle of Prediction	Website/Reference
ToxTree	Prediction of presence of structural alerts for the toxicities	Patlewicz*et al.,* (2008) Standalone free tool
ToxRead	Prediction of toxicities based on read-across principle	Gini *et al.,* (2014) Standalone free tool
VEGA-QSAR	Prediction of toxicities based on the QSAR principle	Benfenati *et al*., (2013) https://www.vegahub.eu/portfolio-item/vega-qsar/
T.E.S.T	Toxicity Estimation Software Tool (TEST) estimate toxicities of a molecule based on QSAR methodologies	Martin, (2016) https://www.epa.gov/comptox-tools/toxicity-estimation-software-tool-test
ProTox-II	Toxicity prediction using fragment similarity based CLUSTER cross-validation machine learning	Banerjee *et al., (*2018) https://tox-new.charite.de/protox_II/
SwissADME	Prediction of ADME or pharmacokinetics properties	Daina *et al*., (2017) http://www.swissadme.ch/
ADMETSAR 2.0	Prediction of ADME or pharmacokinetics and toxicity properties	Yang *et al*., (2018) http://lmmd.ecust.edu.cn/admetsar2
ADMETLAB 2.0	Prediction of ADME or pharmacokinetics and toxicity properties	Xiong *et al.,* (2021) https://admetmesh.scbdd.com/

the Institutional animal ethics approval (19/SA/IAEC/2022).

In silico ADMET predictions for glycofurol was performed using various online and standalone tools as mentioned in the Table I below. Glycofurol's canonical SMILES notation was taken from the Pubchem CID 110717 and used in all the tools for the prediction of ADMET properties.

Results and Discussion

Fish embryo acute toxicity (FET) test

It was observed that the death of the embryos was dependent on increase in time and concentration of the GF. Coagulation of embryo, lack of somite formation and no heartbeat of embryo were considered as the endpoint for the evaluation of embryo mortality. After 24 hours, embryo mortality rates were 100% at 5% GF concentration, 50% at 2.5%, and 15% at 1.2%. No mortality was observed from 0.4 % to 0.07 % concentration of GF at the end of 24 h. Table II depicts the number of live and dead embryos at the end of 96 h of exposure to GF at different concentrations.

Table II : Cumulative data on live and dead embryos after 96 h of exposure to GF at different concentrations.

Concentration of GF in different wells	Nature of the embryo		Mortality %
	live	dead	
control	37	3	7.5
0.07 %	37	3	7.5
0.1 %	36	4	10
0.2 %	32	8	20
0.4 %	31	9	22.5
1.2 %	26	14	35
2.5 %	0	40	100
5.0 %	0	40	100

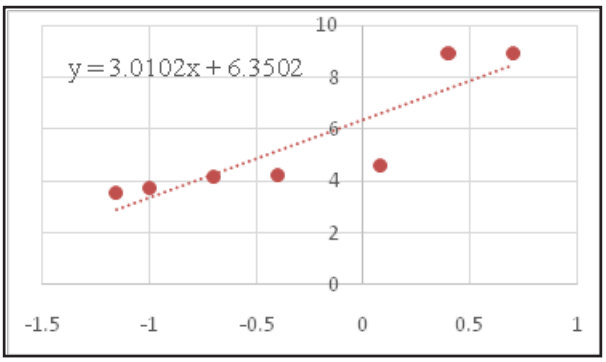


Fig 1: Probit vs log concentration of glycofurol (%)

Median lethal concentration (LC_{50})Calculation using Probit method:

Probit of 50% Mortality is 5; replace y with 5 in the slope equation.

5 = 3.0102x+6.3502

(5-6.3502) = 3.0102x

x= (5-6.3502)/3.0102

x=-0.4485

LC_{50} = Antilog of x

LC_{50} = 0.356 %

The calculated LC_{50} of glycofurol based on the Probit method of analysis is 0.36%.

Table III depicts, zebrafish embryos hatched at 36-38 h for GF concentrations of 0.2%, 0.1%, and 0.07%, similar to the control group. A delayed hatching time of 72 h was noted at 1.2% GF concentration, while hatching occurred at 48 h for 0.4% GF concentration.

Table IV displays images of zebrafish embryo stages at different GF concentrations and time interval.

Zebrafish embryo morphological changes were monitored closely from 0 to 96 hours and

Table III : Time of hatching of zebrafish embryos at different concentrations of glycofurol

Concentration of GF	Time of hatching
Control	after 36-38 h
0.07%	after 36-38 h
0.1%	after 36-38 h
0.2 %	after 36-38 h
0.4 %	72 h
1.2 %	96 h

Table IV : Images of different stages of zebrafish embryo with respect to time at different concentrations of GF

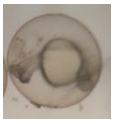
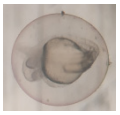
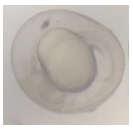
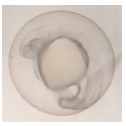
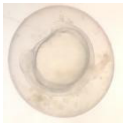
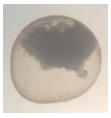
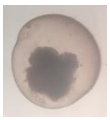
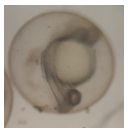
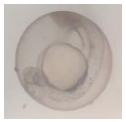
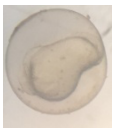
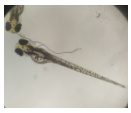
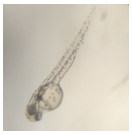
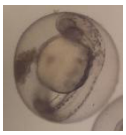
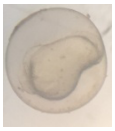
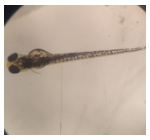
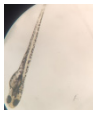
Time	control	0.07 %	0.1 %	0.2 %	0.4 %	1.2 %	2.5 %	5 %
24 h								
48 h								
72h								
96 h								

Table V : Normal morphological changes observed in Zebrafish embryo

Normal morphological changes observed	Concentration of GF in different wells							
	control	0.07 %	0.1 %	0.2 %	0.4 %	1.2 %	2.5 %	5 %
Somite formation	O	O	O	O	O	O	O	N
Appearance of eye spots	O	O	O	O	O	D	N	N
Detachment of tail	O	O	O	O	D	D	N	N
Hatching	O	O	O	O	SD	D	N	N
Appearance of melanin pigment	O	O	O	O	D	D	N	N
Resorption of yolk	O	O	O	O	O	N	N	N

O-Observed, N- Not observed, D-Delay in the occurrences of morphological changes, SD- Slight delay in the occurrences of morphological changes

mentioned in Table V, detailing somite formation, eye spot appearance, detachment of tail, melanin pigment development, hatching, and yolk resorption. Notably, yolk resorption was absent after 96 hours at a 1.2% GF concentration. At the 96-hour mark, lateral fin movement, mouth opening, and yolk resorption were observed only in the control and GF concentrations of 0.2%, 0.1%, and 0.07%.

The heart rate of zebrafish was measured by visualization of heartbeat and counting after the video recording for 20 seconds. The heart rate was normal in all the concentrations, except that there was an increase in the heart rate in 1.2% concentration. However, the values were foundwithin the normal range(121.6 ± 11.8 beats per minute)as reported by Chan *et al.*, (2009).

Fish acute toxicity test:

A limit test was done to assess the maximum tolerable concentration and LC_{50} of GF by adult zebrafish as per the OECD test guideline no. 203. No death of fish was seen initially after setting up the control and test. Observations were made regularly once in 8 h from 24 h to 96 h. No death of fish was recorded in both the test and control, at the end of 96 h. No abnormal clinical signs were also expressed by the fish in both control and test. These findings suggest that the LC_{50} of

Table VI : Heart rate of zebrafish embryos at 96 hpf of exposure to GF at different concentrations

Concentration of GF (%)	Heart rate in bpm (beats per minute)
Control	100.5 ± 4
0.07	95 ± 9
0.1	98 ± 5
0.2	97 ± 8
0.4	101 ± 7
1.2	143 ± 7

Values are Mean ± SEM; n=10

GF is higher than the test concentration of 0.2 ml/L.

In Silico Prediction of ADME and Toxicity Properties of Glycofurol:

In Table VII, the predicted pharmacokinetics properties are listed, and the prediction showed that the glycofurol has got good pharmacokinetics profile with good oral absorption, moderate distribution, with low protein binding and low half-life. It did not have any inhibitory effect on metabolic enzymes and have low half-life. These PK properties are considered as the favourable properties of any solvent used in the formulation and less likely to get accumulated or does not have possibility of drug-drug interactions, hence considered safe.

In a human intravenous pharmacokinetic study with glycofurol, authors showed a clearance of 0.6 L/h/Kg, half-life of 1.1h and Vd of 0.5 L/kg for glycofurol in the healthy volunteers (Bury *et al.,* 1984), these findings are closer to the prediction by the *in silico* tools.

The literature on toxicity profile of glycofurol is limited, hence predictions was performed using various *in silico* tools with the principles of presence of structural alert, read across and QSAR models, predicted that the glycofurol is non-genotoxic, non-carcinogenic, non-teratogenic and the oral acute toxicity is more than 2000 mg/kg (Table VIII).

The zebrafish embryo AC_{50} was predicted as 318.73 µg/L or 0.0003g/L in VEGA-QSAR model, which is approximately 10000 times less than the IC_{50} of glycofurol obtained in the current embryo-toxicity study which is 0.356% equivalent to 3.56 ml/L or 3.56 g/L. The fish acute toxicity was predicted in VEGA-QSAR

Table VII : Prediction of Pharmacokinetics Properties for Glycofurol using the Online *In Silico* Tools

Properties	SwissADME	ADMETSAR 2.0	ADMETLAB 2.0	Summary
Absorption				
GI absorption	High	+ (0.94)	+++	Good gastrointestinal absorption is predicted
Caco-2	n/a	+ (0.71)	+	
Human Oral BA	n/a	+ (0.51)	+++	
Distribution				
Plasma Protein Binding	n/a	40%	12.7%	Low to Moderate protein binding
Fraction unbound	n/a	n/a	77.4%	
Volume of Distribution	n/a	n/a	1.5 L/Kg	Moderate Vd
BBB Permeant	No	+ (0.75)	+	May cross BBB
P-gp substrate/ inhibitor	No	No	No	No effect on P-gp
Metabolism	No CYP activity	No CYP activity	CYP2C19 substrate	No overall effect on CYP activity
Excretion				
Clearance	n/a	n/a	4.98 ml/min/kg	Moderate to high clearance
Half-life	n/a	n/a	0.75 h	Less half-life could be due to moderate clearance and Vd

n/a = module is not available in the tool; + = positive; values in the parenthesis indicates probability

Table VIII : Toxicity Prediction of Glycofurol with the *In silico* Tools

Parameters	ToxTree	ToxRead	VEGA	T.E.S.T	ProToxII	Summary
Ames Mutagenicity	-ve	-ve	-ve	-ve	-ve	Non-mutagenic
						Equivocal -
Micronucleus	+ve	n/p	-ve	n/a	n/a	Glycofurol contains a structural alert to cause micronucleus, whereas the QSAR model is negative for micronucleus
Chromosomal Aberration	n/a	n/a	-ve	n/a	n/a	Negative for chromosomal aberration
Carcinogenicity	-ve	n/p	-ve	n/a	+ (0.55)	Non-carcinogenic, though ProToxII model showed positive prediction however, the probability is very less
Developmental Toxicity	n/a	n/p	-ve	+ (0.59)	n/a	Negative for developmental toxicity though the TEST tool showed positive prediction however, the probability is very less
Oral Acute Toxicity (mg/kg)	n/a	n/a	n/a	2713.46	2940	Predicted to have acute toxicity > 2000 mg/kg

-ve = negative; n/p = not predicted; n/a = module is not available in the tool; values in the parenthesis indicates probability

was more than 100 mg/L which is similar to the experimental acute LC50 of zebrafish, more than 0.2 ml/L or 200 mg/L. Therefore, it is concluded that the glycofurol is non-toxic to zebrafish at the concentrations studied.

Conclusion

At the conclusion of the study, the assessment of glycofurol toxicity in zebrafish embryos unveiled crucial information regarding its concentration-dependent effects on mortality and certain morphological changes. Probit analysis of LC_{50} was 0.36% solution of glycofurol. These findings highlighted the potential toxicity of glycofurol on zebrafish embryos, warranting further investigation to comprehend the underlying mechanisms.

Conversely, the adult zebrafish displayed tolerance to the tested concentrations, indicating a LC_{50} higher than 0.2 ml/L. This juxtaposition of results emphasized the necessity of understanding glycofurol impact on zebrafish models comprehensively. The study's implications extended beyond its immediate findings, underlining the importance of further research to ascertain the safety profile of glycofurol and its potential implications in pharmaceutical applications and beyond.

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