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Short communication

Oral dosing in adult zebrafish: Proof-of-concept using pharmacokinetics and pharmacological evaluation of carbamazepine

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ABSTRACT

Background and methods: We describe a method for obtaining pharmacokinetics (PK) and pharmacology data from adult zebrafish in terms of mg/kg using a novel method of oral administration. Using carbamazepine (CBZ) as a test drug, we employed dried blood spot (DBS) cards to enable drug quantification for PK; and we evaluated the pharmacological anxiolytic effect using novel tank test.
Results: The PK study confirmed the presence of CBZ in both blood and brain and the behavioural study showed dose dependent anxiolytic effect. The reproducibility of oral dosing was confirmed by the fact that the results obtained in both the experiments had negligible errors.
Conclusions: This report enables a novel approach for optimizing the utility of zebrafish in drug discovery and drug delivery research.
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Introduction

Zebrafish (*Danio rerio*) has gained popularity as a pre-clinical screen in pharmaceutical research owing to its small size, large fecundity, rapid development, affordable maintenance and a complex vertebrate system conserved within its sequenced genome (approximately 75% similarity with human genome) [4]. Even though embryonic and larval stages of the fish are more popular in research, adult zebrafish are increasingly being reported as model organisms for studies for cardiac safety pharmacology [8], abuse liability [11], different kinds of neuroscience studies [10] and many other toxicity and pharmacology models [9]. However, conventional studies in adult zebrafish are conducted by addition of the chemicals to the aquarium water at different concentrations and assessing their effects. This requires large amounts of drugs in order to meet therapeutic concentrations and the actual oral dosage of the drug in terms of milligrams per kilogram (mg/kg) of body weight cannot be ascertained as drug exposure is *via* multiple bodily routes.

Oral drug administration in adult zebrafish has been unexplored, with a single report [15] suggesting the use of gluten granules for oral administration of Felbinac, in which successful delivery of the drug in adult zebrafish blood was confirmed using a high performance liquid chromatography (HPLC)-based method. However, all test chemicals cannot be retained in such granules, and the quantification of chemicals in the granules to confirm retention and dose delivered is rather approximate and imprecise.

The present study describes a novel method of oral administration in adult zebrafish, combined with that of dried blood spot (DBS) cards for preparation and storage of whole blood and whole brain homogenate, enabling low-volume sample extraction and analysis using liquid chromatography tandem–mass spectrometry (LC-MS/MS) for drug quantification. We used the above method for evaluating effects of the anxiolytic drug carbamazepine in the novel tank test (a popular paradigm for anxiety measurement [3]). We believe that the proposed paradigm will contribute to the use of zebrafish as a model in preclinical pharmacokinetics and pharmacology research by reducing compound requirement, holding space requirement and increased sample size.

Abbreviations: BBB, blood–brain-barrier; CBZ, carbamazepine; DBS, dried blood spot; LC–MS/MS, liquid chromatography tandem–mass spectrometry; PK, pharmacokinetics.

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Materials and methods

Aquaculture

All procedures for experimentation were as per Guidelines for Use of Zebrafish in the NIH Intramural Research Program (http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf) and the Zebrafish Book [13]. Indigenous wild type male adult zebrafish strains (5–6 months old) were used for this study (obtained from Vikrant Aquaculture, Mumbai, India). Fish were maintained in a recirculation system with polysulphone housing tanks containing purified water (Millipore ELIX system grade) with 0.2% sea salt at 28 °C under a 14:10 h light and dark cycle (Westerfield M, 2000). Fish were fed three times daily with live hatched brine shrimp and dry food.

All fish (27 for the pharmacokinetic study and blood–brain-barrier-permeability evaluation, and 20 for the evaluation of anxiolytic effect) were fasted overnight and weighed prior to the study. On the day of dosing, fresh CBZ in 1% DMSO was administered orally at the required dose with the dose volume of 10 μl/kg body weight of zebrafish. The dose volume was rounded-off to the nearest single decimal of the micro-litre.

Chemicals, instruments and materials

Carbamazepine (CBZ), DMSO and Thioridazine were purchased from Sigma–Aldrich, USA. HPLC-grade acetonitrile and methanol were purchased from Merck (Mumbai, India). Heparin, and other routine chemicals for fish water were purchased locally. The HPLC system consisted of an Agilent 1200 quaternary pump, auto sampler with thermostat, column oven, and online degasser, triple quadrupole mass spectrometer (Mass hunter software version B.03.01) with multimode source (Agilent Technologies, Inc., 2850 Centerville Rd., Willington, DE 19808-1644, USA). The analytical column used was reverse phase symmetry C18 (75 mm × 4.6 mm, *id* 3.5 μm). FTA® Elute blood spot cards (DMPK type-B cards) were supplied by Whatman (Sanford, USA), Ultrasonic bath from Bandelin sonorex sonicator, centrifuge from Eppendorf (model# Centrifuge 5810), and Milli Q Water system from Millipore (model #Gradient A10). A digital Stop watch was used to keep track of various timed observations during the novel tank test.

Oral drug administration

We are the first laboratory to develop a method for conducting oral drug administration in zebrafish (Provisional Indian patent: CBR No. 10389, Application No. 3644/CHE/2011: Oral drug administration in the zebrafish (*Danio rerio*)). This method briefly involves the use of a micropipette with a small tip that is gently inserted into the mouth and pharynx of zebrafish. The test drug solution/suspension is then gently released into the fish ensuring that the administered solution does not regurgitate. During training sessions of dosing personnel, permitted food grade-coloured solutions were used to ensure no spillage occurs either through the oral cavity or gill filaments.

Pharmacokinetics and blood–brain-permeability measurements

In the present study, CBZ was administered at a dose rate of 5 mg/kg body weight. Blood was collected by modification of a reported method [6] wherein fish were anaesthetized with tricaine (MS-222) and blood (7–10 μl/fish) was collected by puncturing the heart using heparin rinsed insulin syringes and dispensed into eppendorf tubes containing heparin. A ratio of 1:2 of heparin to blood was maintained. Brain was collected based on a reported method [5] wherein fish were sacrificed after collecting the blood, to harvest their brain; eyes of fish were removed carefully, the skull portion flipped with forceps and the brain collected without disrupting the tissue. Then the brain tissue was processed with Dulbecco's Phosphate Buffer Saline (DPBS) to obtain brain homogenate by using Qiagen's Tissue homogenizer. All calculations were corrected as ng/ml of the analyte with respect to the original blood and brain homogenate volumes. Three adult male fish were sacrificed per time point. Collection of blood and brain samples was carried out before drug administration (pre-dose) and at time points 5 min, 15 min, 30 min, 60 min, 120 min, 240 min, 360 min and 480 min post drug administration.

Whatman FTA® DMPK Cards were used for drug analysis in zebrafish blood and brain using Dried Blood Spot (DBS) methodology. An FDA listed medical devices, DMPK cards are being used in discovery stage pharmacokinetics regularly. Various drugs have been analyzed using DBS technique and this technology has given good results in the rapid identification and quantification of drugs [5]. However, there exists no reported method for the pharmacokinetic analysis of drugs using DBS in zebrafish. In our experiments we report for the first time the use of DBS for zebrafish wherein the blood and homogenized brain samples have been analyzed in the present study using Whatman FTA® DMPK cards (type-B). 7 μL aliquots of nine calibration standards, six sets of quality control samples and samples from the study in blood and homogenized brain were directly spotted onto the DMPK type-B card in this study. For extraction, a 3 mm diameter disc was punched out from the centre of each DBS and taken into a clean eppendorf tube. CBZ was extracted with acetonitrile:methanol (1:1, v/v) (200 μL) containing Thioridazine as Internal Standard (IS) in an eppendorf tube and vortex mixed for approximately 3 min and centrifuged for 10 min at 14,000 rpm [14]. Internal standard working solution (5 ng/mL) was prepared from its primary stock solution using acetonitrile and methanol (1:1, v/v). The blood and brain samples were quantified using a validated LC–MS/MS based method by comparison with a calibration plot prepared of freshly spiked DBS samples.

Pharmacokinetic parameters of CBZ were calculated using PK solver for excel (Non-Compartmental analysis) of blood and brain data after extravascular input.

Novel tank diving test to measure anxiety behaviour in adult zebrafish

This method was used for proof-of-concept of the method of oral drug administration as it is simple and does not require irreversible chemical modulation or euthanasia. This experiment was conducted based on a reported protocol [3]. In brief, fish were placed in an experimentation room for 2 h prior to the experiment to allow acclimatization. Acclimatized fish (5 males per dose group) were treated with 1 mg/kg, 3 mg/kg and 6 mg/kg CBZ, administered orally (*po*) and transferred into their native tank. After 10 min of treatment, zebrafish were placed individually into a novel tank (13.5 height × 26.5 top × 22 bottom × 8 cm width) maximally filled with aquarium water. Tank was divided into two equal horizontal portions by a line drawn on the external surface of tank walls. Zebrafish swimming behaviour was recorded for 8 min using a digital camera and analyzed manually by expert observer. The following endpoints were analyzed: latency to upper half (s), number of transitions to upper half, time spent in upper half (s), erratic movements and freezing bouts. Anxiety behaviour of adult zebrafish was assessed by the qualitative scoring (1–5).

Statistical analysis was performed using GraphPad Prism® software. Behavioural effects of drug and vehicle treatments were evaluated statistically using analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. The qualitative scoring of anxiety like behaviour was evaluated statistically using Kruskal–Wallis test followed by Dunnett's *post hoc* test. Statistical significance was set at the $p < 0.05$ level.

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