

AUTOMATED ANALYSIS OF CARDIOTOXICITY IN ZEBRAFISH

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Abstract

The zebrafish embryo model is gaining relevance in biomedical research. Characteristics of the embryo (optical transparency, small size, easy manipulation and low cost) make the model an ideal option for high throughput screening. The zebrafish shows great potential to be incorporated into preclinical drug screening pipelines. Drug discovery is a tedious, time consuming process that requires a long-term investment. Of all the compounds entering the R&D project less than 1% will be launched to the market. To increase productivity in drug discovery the necessity of automation arises.

In this study, we have selected two groups of drugs: sexual hormones and statins. The first group is used in hormone replacement therapy (testosterone, progesterone, estradiol) and the second in cholesterol lowering treatment (lovastatin, pravastatin and mevastatin). Heart rate effect in embryos is assessed in a manual assay and in a fully automated system. Our results show that automated assessment of drug-induced heart rate variations not only allows for a higher number of compounds to be tested but also improves quality of data, with decreased variability and increased reproducibility compared to manually performed assays. Thus, automation increase productivity and reduce cost in drug discovery field.

Introduction

High-Throughput-Screening (HTS) technology is a fundamental tool for pharmaceutical companies to test chemical compounds with potential cardiotoxic activity. It has been successfully applied to cell-based studies where robotics allows testing of thousands of compounds in a few hours. Examples of these studies have been published by Willumsen N.J. et al. 2003 (assay for ion channel dysfunction); Bremer S. et al 2001 (reporter gene assay); Netzer R et al (review for ion channel screening technologies). However, no fully automated HTS cardiotoxic studies *in vivo* have been reported so far.

Biobide has developed a HTS system that allows fully automated testing of libraries of compounds in zebrafish. The zebrafish model possesses many features that facilitate large-scale experimental approaches. It has the strengths of invertebrate models such as small size, large number of offspring, and a short generation time. Plus, its embryos are transparent and they develop rapidly and synchronously.

Our aim is to offer a rapid method for testing the cardiac toxicity of potential new drugs in a highly specific system at very reasonable price.

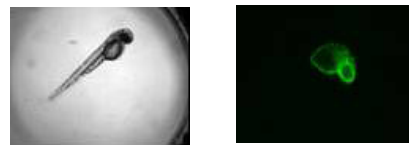
Here we have tested two groups of compounds used in clinical therapies by using a traditional manual method and our novel automated system. We demonstrated that the automated method provides higher quality, less dispersion and increased reproducibility of the data.

Method

For manual assays: 4 groups of 8 embryos at 48 hpf were dispensed into a 24 well plate. 60 µM of the indicated compound was then added to each well. The plate was incubated for 3 hours at 28.5 °C. Finally, heart beats were counted for 15 seconds. For manual results, a representative experiment of a total of 5-8 individual experiments is shown.

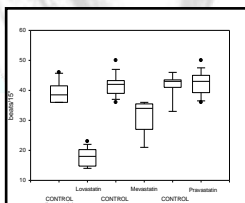
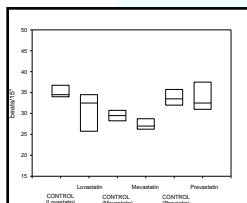
For automated assays: embryo sorting and dispensing in 96 well plates, drug dispensing at a final concentration of 60 µM and incubations were done automatically with the HTS platform of Biobide. Heart beat alterations were automatically analyzed with algorithms specially developed. For automated results, a representative experiment is shown.

Statistical analysis: The differences between the treatments, specificity and sensitivity for each method were analyzed using SPSS software.

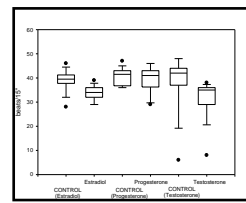
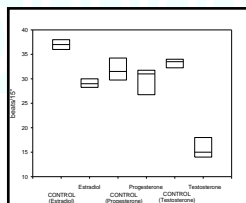


Images obtained by the automated system.
 The brightfield image (left) is used for the localization of the embryo. Heart rate analysis is performed in the fluorescent image (right).

Results

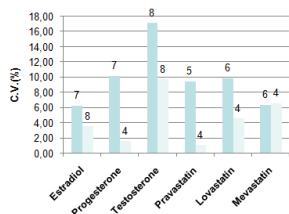


Effects of statins on zebrafish heart rate. The manual (A) and automatic (B) assays show that Pravastatin has no effect on heart rate ($p_{\text{manual}}=0.505$; $p_{\text{automatic}}=0.978$). Both assays indicate that Lovastatin ($p_{\text{manual}}=0.028$; $p_{\text{automatic}}<0.001$) and Mevastatin ($p_{\text{manual}}=0.015$; $p_{\text{automatic}}=0.001$) induce bradycardia in zebrafish.



Effects of sexual hormones on zebrafish heart rate. The manual (A) and automatic (B) assays show that Progesterone has no effect on zebrafish heart rate ($p_{\text{manual}}=0.197$; $p_{\text{automatic}}=0.456$). Both assays indicate that Estradiol ($p_{\text{manual}}<0.001$; $p_{\text{automatic}}<0.001$) and Testosterone ($p_{\text{manual}}<0.001$; $p_{\text{automatic}}=0.001$) induce bradycardia in zebrafish.

The coefficient of variation is decreased in the automatic assays.



Coefficient of variation for manual and automated assays. Mean and standard deviation were calculated using data from the number of plates shown on the graph. The manual experiments (blue bars) gave a higher value for coefficient of variation compared to automated assays (red bars), indicating that automation leads to an increase in the reproducibility of the data.

For similar sensitivity, the number of false positives is decreased in the automatic assays.

Drugs (60µM)	MANUAL			AUTOMATIC		
	100-specificity (%)	Sensitivity (%)	Area under the curve	100-specificity (%)	sensitivity (%)	Area under the curve
Pravastatin	-	-	0.5	-	-	0.46
Lovastatin	37	41	0.5	0	100	0.99
Mevastatin	36	85	0.80	15	90	0.93
Estradiol	20	84	0.88	14	86	0.93
Progesterone	-	-	0.6	-	-	0.5
Testosterone	9	83	0.94	14	82	0.91

False Positives and True Positives (%) were calculated using SPSS software. The data shown in the table indicates an increased sensitivity in the automatic method and a higher capacity for data discrimination. 100-specificity= % False Positives; Sensitivity= % True positives; Area under curve = capacity of data discrimination.

Conclusions

1. Summary of effects

Drug	Solubility	Bradycardia in zebrafish	Bradycardia in higher vertebrate
Progesterone	Water	No	No data
Testosterone	DMSO	Yes	In rat (Ward and Abdel-Rahman 2006)
Estradiol	DMSO	Yes	In rat (Mohamed et al. 1999)
Mevastatin	DMSO	Yes	No commercialized
Pravastatin	Water	No	No (Chen et al. 2003)
Lovastatin	DMSO	Yes	In rat (Bravo et al. 1998)

2. The automation allows:

- Rapid large-scale experiments
- Increased reproducibility of the data
- Better distinction between positive and negative results
- Highly informative results
- Similar effects to those observed in higher vertebrates for these compounds