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FOCUS ARTICLE

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Reviewing toxicokinetics with a focus on metabolism of new psychoactive substances in the zebrafish (larvae) model

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Abstract

Zebrafish (*Danio rerio*) share around 70% of their genome with humans and many important enzymes for drug metabolism such as some cytochrome p450s have direct orthologs in zebrafish. To date, several studies showed a similar metabolism to humans in general. Furthermore, although using adult fish as model organism requires approval by an Ethics Committee, using larvae until 120 h postfertilization does not necessarily need any approval at least in the European Union. All these aspects seem to be beneficial for using zebrafish (larvae) in toxicokinetic studies for the so-called new psychoactive substances. These compounds are expected to have similar effects as traditional drugs of abuse but are often not listed as controlled drugs when they first appear on the market. However, no information about their toxicokinetics is available when they appear, which is particularly critical concerning their biotransformation. This knowledge is important for example, for developing urine-based screening procedures or for predicting drug–drug interactions. This focus article aims to briefly introduce into the topic of using zebrafish (larvae) in the context of toxicokinetic studies, particularly metabolism studies, and will highlight some aspects such as the route of administration, which are important to consider when using this model.

This article is categorized under:

Toxicology > New Psychoactive Substances

Toxicology > Analytical

KEYWORDS

new psychoactive substances, toxicokinetics, zebrafish

1 | INTRODUCTION

1.1 | New psychoactive substances

New psychoactive substances (NPS) or occasionally also called novel psychoactive substances are a heterogenous group of compounds that are consumed as replacement for drugs of abuse to overcome legal issues among others. They often

show similar effects as their traditional analogs but are usually not listed as controlled drugs when they first appear on the market. There were approximately between 50 and 100 NPS first-time detected per year over the last 10 years. Most of them disappear again after several weeks but some can be observed over years even after they were scheduled. The United Nations Office on Drugs and Crime (UNODC) and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) annually report about the NPS situation (EMCDDA, 2021; UNODC, 2020).

To keep pace with this high number of NPS in the context of forensic and clinical toxicology as well as doping control, it is important to steadily integrate them into analytical procedures. The gold standard for such screening procedures in these fields are mass spectrometry-based methods. They are highly flexible and new compounds, which might be NPS but also new therapeutic drugs as well as new doping agents, can be added to the mass spectral databases easily. However, before the compounds are added to these libraries, their reference spectra need to be recorded. This is in case of the parent compounds not a big issue if a reference standard is available. The analytical confirmation of an NPS intake is often performed in urine, due to the enlarged detection window compared to blood, and many compounds (NPS, therapeutics, etc.) are completely metabolized prior to their elimination or only trace amounts of the parent compound can be found in urine. Therefore, the metabolic fate and the elimination kinetics of a NPS needs to be known. Furthermore, the mass spectral information of the (main human) metabolites need to be available.

1.2 | Zebrafish (larvae)

One emerging technique to study the toxicokinetics of NPS is the use of zebrafish (larvae). They are increasingly used as alternative to mammal models although mammals are expected to show a better comparability to humans in terms of toxicokinetics. Zebrafish studies have certain advantages including ease of handling, lower monetary costs, and that zebrafish younger than 120 h postfertilization are not considered as animal experiments (EU directive, 2010/63/EU). Although zebrafish are non-mammals, they still share around 70% of their genome with humans (Howe et al., 2013) and many human cytochrome P450 enzymes have direct orthologs in zebrafish, such as some CYP1 and CYP3 (Goldstone et al., 2010). In contrast, zebrafish have much more CYP2 genes, compared to human, with only two (CYP2R1 and CYP2U1) recognized as orthologous based on sequence (Goldstone et al., 2010).

Thus, several studies showed a similar metabolism to humans (MacRae & Peterson, 2015). Different routes of administration can be used to expose NPS to zebrafish. Indirect exposure via tank water is possible but direct administration into each fish larvae via advanced microinjection techniques into the caudal vein, heart ventricle, or hindbrain led to systemic distribution, which can be beneficial (Park et al., 2020).

The current article focuses on the metabolism shown in zebrafish of NPS and some methodological issues will be discussed. Aspects such as toxicity, behavioral effects, metabolomic alterations, embryotoxicity, and teratogenicity of NPS on zebrafish larvae belong more to the toxicodynamics of NPS and will thus not be discussed.

2 | TOXICOKINETICS OF NPS STUDIED BY USING ZEBRAFISH LARVAE

The knowledge of the metabolic fate and thus the main excretion products into urine are crucial to develop thorough screening procedures for NPS. There are several examples available where NPS for example, synthetic cannabinoids were completely metabolized prior to excretion and thus an analysis using the parent compound as sole target will not discover an abuse (Diao & Huestis, 2019). Furthermore, some metabolites for example, of the synthetic cannabinoids JWH-018 and AM-2201 were shown to retain affinity and activity at the cannabinoid 1 receptor (Chimalakonda et al., 2012). This observation might also be true for other NPS making metabolism studies essential.

2.1 | Metabolism studies using adult zebrafish

When using zebrafish as metabolism model, one can either use adult fish or the larvae. Using adult fish requires approval by an Ethics Committee whereas using larvae until 120 h postfertilization does not necessarily need any approval. Drug application can be done via different routes. The easiest way, in case of adult fish, is the application via the tank water as shown by Sardela et al. (2018). They diluted sibutramine, JWH-073, hexarelin, or selegiline (ranging from 1 to 15 mg) with water and then introduced them into the four-liter zebrafish tanks containing 18 fish each. The

hydroxy and carboxy metabolites of the synthetic cannabinoid JWH-073 found in the zebrafish tank water were the same as those described for humans. The reaction leading to monohydroxy metabolites on the indole group were not observed, which could be explained by the absence of CYP2C9 orthologs in zebrafish as this enzyme was responsible for the indol hydroxylations in humans.

Diao and Huestis already summarized the advantages and disadvantages of the zebrafish model (Diao & Huestis, 2019). They found that it may in general provide human-like phase I metabolites but species differences still limit the use of the zebrafish model. Maintaining zebrafish might be challenging for typical forensic and clinical laboratories, which usually do not have the necessary facilities and/or experience.

2.2 | Metabolism studies using zebrafish larvae

Although not considered as animals, the zebrafish larvae can provide all benefits of intact organisms including absorption, distribution, and excretion processes. Using larvae instead of adult fish for studying the metabolism of synthetic cannabinoids was exemplarily done by Richter et al. (2019). They exposed the larvae to 5F-MDMB-P7AICA either via the culture medium or via microinjection into the yolk sac. Metabolites were then identified not only in the medium but also by analyzing the freeze-dried larvae. They found only a small number of metabolites after using microinjection but the exposure via medium and subsequent analysis of larvae was the most promising approach. Park et al. detected a high number of 5F-MDMB-P7AICA metabolites in zebrafish larvae after microinjection into the caudal vein, heart ventricle, or hindbrain (Park et al., 2020). Wagmann et al. also found zebrafish larvae to produce a high number of phase I and also phase II metabolites and a good agreement with metabolites detected in humans when investigating the metabolism of 3,4-DMA-NBOMe, ephylone, 4F-PHP, 1-propionyl-LSD, 4F-MDMB-BINACA (Wagmann et al., 2020). Using different in vitro systems, Gampfer et al. identified several phase I and phase II metabolites of the fentanyl homologs cyclopropanoyl-1-benzyl-4′-fluoro-4-anilinopiperidine and furanoyl-1-benzyl-4-anilinopiperidine, with the majority detected in zebrafish larvae (Gampfer et al., 2020).

Considering these findings implicates that zebrafish larvae may be a suitable method for studying the metabolism of NPS but that the route of application is crucial for the number of formed metabolites and that the best route of administration may also be different from compound to compound depending on their physicochemical properties. Data published after investigating the butyrfentanyl biotransformation in zebrafish larvae and comparison to patterns in human blood samples and a postmortem case demonstrated similarities (Kirla et al., 2021). Larvae (5 days post fertilization) were exposed to butyrfentanyl by using one larva per well in a 48-well plate. Afterwards, larvae were pooled and frozen at different time points (16 larvae per time point). This study also showed that the uptake of butyrfentanyl was driven by passive diffusion processes and should be somehow predictable by the pH corrected octanol–water partition coefficient.

Wagmann et al. also found the zebrafish and zebrafish larvae to be a promising and emerging topic in metabolism studies of NPS but also concluded that establishing and maintaining a zebrafish culture platform is expensive and experienced personnel are required (Wagmann et al., 2021). However, no matter which model—adult zebrafish or larvae—will be used, there is still a need for standardization of the model and to extent the knowledge of similarity with human metabolism (de Souza Anselmo et al., 2018).

2.3 | Further aspects

Using zebrafish (larvae) for toxicokinetic studies can be challenging in terms of experimental design. As already stated in Section 2.2, one important aspect is the route of administration as this can impact the experimental outcome dramatically. In summary, the microinjection of compounds into zebrafish embryos at an early developmental stage is a well-established technique and this issue was addressed in the study by Park et al. (2020). They investigated the metabolism of the synthetic cannabinoid 5F-MDMB-P7AICA after microinjection into different compartments of the larvae and additionally compared the outcome to human urine data and data from HepaRG cell line. They found that the route of administration to zebrafish larvae had a strong impact and that microinjection into the caudal vein, heart ventricle, or hindbrain produced highest number of metabolites in comparison to the yolk sac and exposure via water. What they also investigated was the spatial distribution of the parent compound and metabolites by using mass spectrometry imaging. This led to the conclusion that drug and metabolite distribution differed depending on the region of

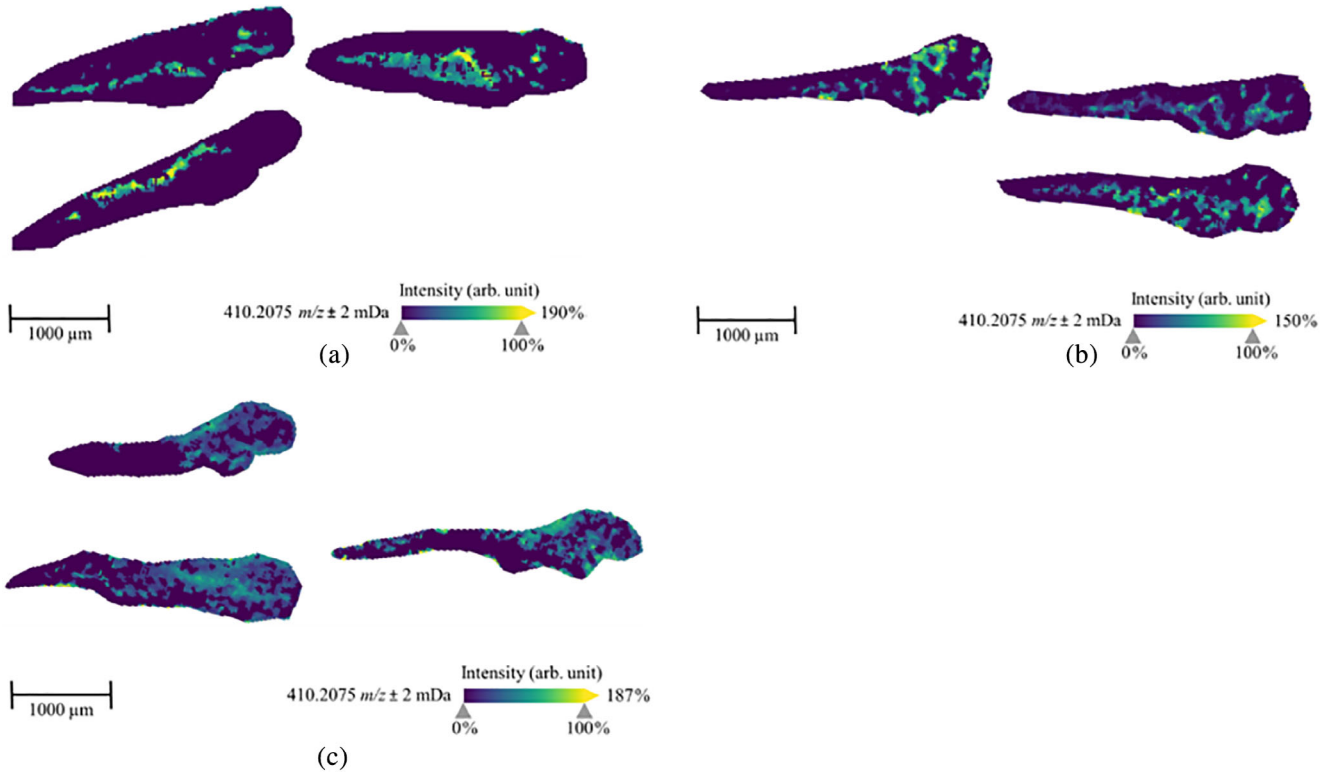

FIGURE 1 MALDI-MS images of four isomeric 5F-MDMB-P7AICA metabolites (*m/z* 410.2075) in zebrafish larvae, which were exposed via microinjection into caudal vein (a), heart ventricle (b), and hindbrain (c). These isomeric metabolites were the third most abundant in zebrafish larvae except zebrafish larvae treated through the yolk sac compartment. The presented sections originate from one representative larva per condition. The images were generated by preparing a colormap from blue (no detection) to yellow (high local concentration) and images were further processed by weak denoising in 96 dpi resolution with 24-bit color *Source*: Figure taken from Park et al. (2020)

application and that it is important to choose the right administration route when studying metabolism in zebrafish larvae. In the study by Park et al., microinjection into vital organs resulted in fast distribution and metabolism of the NPS. Figure 1 illustrates the metabolite distribution in larvae, which were exposed to 7′N-5F-ADB via microinjection into caudal vein, heart ventricle, or hindbrain.

Uptake kinetics of meta-chlorophenylpiperazine into zebrafish larvae were evaluated by Kirla et al. and suggested the existence of active transport processes (Kirla et al., 2018). Further studies on the distribution showed an accumulation in the eye, which was discussed to be due to melanin serving as binding site for drugs. This assumption was confirmed by lower meta-chlorophenylpiperazine accumulation in hypo-pigmented larvae. However, it has to be considered that one important difference between human and larvae are the exposure routes, which was underlined by the findings using cocaine as test compound (Kirla et al., 2016). Human intake is usually oral or via inhalation whereas larvae are exposed to the drug via injection or surrounding water. This may result in different toxicokinetic data of the investigated NPS (Kirla et al., 2016, 2018, 2021; Park et al., 2020).

2.4 | Final comments and some take-home messages

Some points will be discussed in the following that may help scientist in working with zebrafish larvae. One may be interested in the question whether one should analyze the zebrafish (larvae) and/or the surrounding medium. Whether to analyze only the zebrafish (larvae) or additionally the surrounding medium, which can be the tank water or the culture medium, cannot be answered in general. This depends on the ability of the organism to excrete the formed metabolites. As this is usually unknown for metabolites of NPS, it might be recommended to analyze both to catch as many metabolites as possible.

A further point may be to find the best extraction method of the larvae. There are different approaches available, which might be used depending on the available equipment. Larvae should be washed before extraction to remove the compounds present in medium and can then be freeze-dried. Afterwards, an extraction using methanol and ultrasonification, followed by centrifugation, evaporation and reconstitution can be applied. As a much easier alternative, larvae might be extracted directly using methanol supported by treatment of stainless-steel balls. However, extraction will always depend on the compound.

For analyzing real world samples, the relative abundances of the metabolites found using zebrafish (larvae) in contrast to humans may be of relevance. There are examples available where the most abundant zebrafish (larvae) metabolite was also the most abundant human metabolite but there are also many examples available where this was not the case. One should not expect that the quantitative pattern between human and zebrafish are identical. The abundance of individual metabolites in the model organism is always depended on several experimental factors. This includes factors such as the way of administration (e.g., tank water or injection).

Finally, the suitability of the zebrafish for a forensic and clinical toxicological setting should be briefly discussed. The model is suitable for providing qualitative information on the metabolism of new drugs including NPS, given that the laboratory can manage a zebrafish facility. Besides metabolism, further parameters, which are of interest in a forensic and clinical toxicological setting, can additionally be monitored. Tests on the maximum-tolerated concentration in zebrafish larvae can reveal malformations and changes in the behavior of larvae after exposure to the compounds.

3 | CONCLUSION

Studies of toxicokinetics using zebrafish (larvae), particularly metabolism studies, were increasing within the last years. It was demonstrated in different previous publications and it can be concluded from them that the NPS metabolism was similar to humans and to in vitro systems such as pooled human liver microsomes. Moreover, when using larvae until 120 h postfertilization an ethical approval is usually not necessary although the larvae offer many advantages of intact organisms such as distribution and excretion processes. One critical point when using the aforementioned model is the route of application. NPS application can be done via the tank water (usually in case when using adult zebrafish) but also via microinjection into for example, the caudal vein, heart ventricle, or the yolk sac when using the larvae. Depending on the route of administration, experimental outcome can differ. The choice of the right route might also depend on the physicochemical properties of the investigated NPS. Furthermore, future tutorial articles about the extraction methods of the larvae could be interesting for the reader since larvae is not a very common matrix in forensics.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Lea Wagmann: Conceptualization (supporting); data curation (supporting); project administration (supporting); writing – review and editing (supporting). **Markus R. Meyer:** Conceptualization (lead); data curation (lead); project administration (lead); writing – original draft (lead); writing – review and editing (lead).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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FURTHER READING

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