Development of a vehicle for non-invasive oral administration to facilitate behavioral experiments in fish

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Abstract

Pharmacological approaches are increasingly used in fish ethological studies to investigate the proximate mechanisms underlying behavior. However, an injection-based administration can induce stress or injury, potentially interfering with natural behavioral patterns. Here, we present a non-invasive vehicle for oral administration. To demonstrate its utility, the oxytocin receptor antagonist atosiban was administered orally using this vehicle and subsequently detected in blood plasma via high-performance liquid chromatography. Our method offers an easy-to-use and minimally disruptive tool for fish behavioral experiments.

Keywords

Fish, Oral administration, Atosiban, Non-invasive administration, High-performance liquid chromatography

Introduction

In recent years, ethological studies have increasingly adopted integrative approaches that consider not only the ultimate factors of behavior but also its proximate mechanisms (Davies et al. 2012; Rubenstein and Alcock 2019). This broader perspective has enabled researchers to gain a more comprehensive understanding of the evolution of behavior and to identify conserved patterns across taxa. It has also led to novel insights that are difficult to obtain through observational studies alone. For example, Matsumoto et al. (2018) investigated filial cannibalism in male blenniid fish, *Rhabdoblennius nitidus*, and successfully resolved a theoretical paradox in behavioral ecology by incorporating an endocrinological approach. Similarly, Nowicki et al. (2020) compared the expression patterns of nonapeptides, dopamine, and opioid receptor genes in the forebrains of pair-bonded and solitary butterflyfish species and found that these systems modulate sociality as is also observed in other vertebrates. These studies illustrate that approaches bridging proximate and ultimate factors hold great potential to open new directions for research.

An accessible approach to bridge these levels is to use pharmacological manipulation within traditional behavioral experiments, in which candidate regulatory systems (e.g., neural, endocrine, or physiological bases) are enhanced or inhibited to

assess effects on behavior. This approach has been widely used; for example, in studies of territorial behavior (Santangelo and Bass 2006), paternal care (O'Connell et al. 2012), shoaling (Ataei Mehr et al. 2020), and reproductive behavior (Almeida et al. 2023). In most cases, pharmacological manipulations involve injecting compounds into subjects via intraperitoneal, intracerebroventricular, or intramuscular routes. However, because such injection-based methods are inherently invasive, they are sometimes unsuitable for behavioral experiments depending on the experimental design. For example, when experiments must be conducted over extended periods, treatments are required to be administered repeatedly, which can induce substantial stress or physical injury. These consequences may alter the physiological state and confound behavior (e.g., Sneddon et al. 2014). To avoid these problems, non-invasive compound delivery methods are needed.

One non-invasive method is oral administration, which has been used in long-term treatments (e.g., Madrid et al. 2021; Lu and Patton 2022; Ochocki and Kenney 2023). However, oral administration of a considerable number of compounds remains challenging compared with injection-based methods due to various physiological and biochemical barriers, including low permeability and degradation in gastrointestinal fluids (Song et al. 2004; Lou et al. 2023). In fish, oral administration faces similar limitations, and efforts have been made to improve its efficacy (e.g., Amezawa et al. 2018). A practical and accessible strategy is to optimize the composition of the vehicle mixed with the compounds to enhance delivery efficiency, as the vehicle's composition is known to influence oral delivery outcomes (e.g., Tønsberga 2010; Serdoz et al. 2010). Establishing an effective and easy-to-use vehicle system could provide a valuable tool for behavioral experiments in ethological studies of fish.

In this study, we examined whether an oligopeptide compound could enter systemic circulation following oral administration in combination with a vehicle modified with reference to pharmaceutical research. As a model fish, we used the convict cichlid (*Amatitlania nigrofasciata*), a species employed in various behavioral studies, including those on reproductive behavior (e.g., Santangelo 2005), proactive prosociality (Satoh et al. 2021), and affective state (Laubu et al. 2019). As a test case, we selected atosiban, a receptor antagonist of the neuropeptide oxytocin, which has been widely studied in the context of social behavior. In some fish, atosiban introduced into systemic circulation via

intramuscular or intraperitoneal injection has been shown to affect behavior, with its effects expected to result from function within the central nervous system (e.g., Cardoso et al. 2015; Ataei Mehr et al. 2020). However, to our knowledge, it has not yet been used in behavioral experiments via oral administration in teleost fish. Therefore, we used high-performance liquid chromatography (HPLC) to determine whether orally administered atosiban, given with the modified vehicle, transfers into the systemic circulation. Based on these results, we propose a novel vehicle and oral administration method suitable for use in ethological studies of fish.

Materials and methods

Six male convict cichlids (58–99 mm SL) were collected from Okinawa-jima Island, Japan. Fish were housed in a 50 L tank at Kitasato University, where the water temperature was maintained at 26 °C under a 14 h light/10 h dark photoperiod, and fed a commercial fish food (Hikari Carnivore, Kyorin CO., LTD.), hereafter "food", twice daily.

To prepare the medicated food for oral administration, atosiban (T5148, TargetMol) was first dissolved in dimethyl sulfoxide (DMSO; 045-24511, Fujifilm Wako Pure Chemical Co.) and then mixed with either a modified vehicle or a control solution. Following Kim et al. (2017), the vehicle composition was modified. The vehicle composition was modified following Kim et al. (2017) and consisted of atosiban, 50 μg/μL; DMSO, 5% (v/v); polyethylene glycol 400 (PEG400; N0443, Tokyo Chemical Industry Co., Ltd.), 40% (v/v); Tween 80 (35703-62, Nacalai Tesque, Inc.), 5% (v/v); and reverse osmosis water. The control solution contained 50 μg/μL atosiban and 5% (v/v) DMSO in reverse osmosis water to control for the absence of other excipients. Food pellets were cut into pieces approximately 3 mm in diameter and 4 mm in length, each infused with 10 μL of either the atosiban-containing vehicle or control solution, and air-dried at room temperature for one hour. All medicated food was prepared immediately before oral administration.

The procedures for oral administration and sample collection were as follows. After a three-day fasting period, fish were divided into two groups. Three individuals were each fed three pieces of vehicle-treated food, and the remaining three were each fed three pieces of control-treated food. After 55 min, each fish was deeply anesthetized with benzocaine, and blood was collected from the caudal vessel. Blood samples were

immediately centrifuged at 10,000 × g for 15 min at 4 °C to obtain serum. The serums from three fish that had been fed the same treated food were pooled. Following the addition of 50 µl of 1 M perchloric acid (162-00695, Fujifilm Wako Pure Chemical Co.) to the pooled sample, it was centrifuged again at 15,000 × g for 20 minutes at 4°C, and the supernatant was collected. The supernatant was then passed through a 0.22 µm hydrophilic nylon filter (SLNY1322N, Hawach Scientific), and concentrated to approximately 50 µl using a centrifugal concentrator. The concentrated samples were then injected into the HPLC system. Standard solutions of atosiban (0.5 and 5 µg/ml dissolved in ultra-pure water) were used to detect atosiban in samples.

The chromatographic analysis was performed with reference to Raju et al. (2014) with a JASCO (Tokyo, Japan) HPLC system consisting of a PU-4180 quaternary gradient pump with a degasser, a CO-4060 column oven, an AS-4050 autosampler with a cooling system, an UV-4075 UV/VIS detector, and a ChromNAV data processor. The analytical column was a reversed-phase ODS-120H (4.6 × 250 mm) (Tosoh, 0023415). Elution was performed with a mixture of solvent A (30 mM potassium phosphate buffer at pH 3.2) and solvent B (acetonitrile:milli-Q water = 80:20) at 40 °C; the flow rate was set at 1.0 $mL \cdot min^{-1}$ and detention wavelength was set at 220 nm. The elution gradient was set as follows: 0–5 min, 20–20% solvent B in solvent A; 5–12 min, 20-70% solvent B in solvent A.

Results

HPLC analysis detected a peak corresponding to orally administered atosiban with the same retention time as the atosiban standard only in the group that ingested atosiban with the modified vehicle (Fig. 1). These results demonstrate that oral administration using the proposed vehicle delivered atosiban into systemic circulation.

Discussion

Ethological studies by adaptationist thinking have long contributed to understanding the evolutionary background (ultimate factor) of social behaviors across vertebrates, and some theoretical and empirical frameworks of ethological studies are broadly applicable to adjacent research fields (Davies et al. 2012; Rubenstein and Alcock 2019). One of the key emerging questions is whether behaviors that evolved under similar ultimate factors

have entirely different phylogenetic origins and mechanisms, or whether they are, at least in part, underpinned by evolutionarily homologous mechanisms. Indeed, commonalities across vertebrates have been identified not only in the phenotype but also in some aspects of the underlying regulatory mechanisms for some social behaviors, such as territoriality (Oldfield et al. 2015), maternal and paternal care behaviors (Dulac et al. 2014), and monogamous mating system (Young et al. 2019). These examples illustrate how an integrative approach can reveal that some of the core mechanisms regulating these social behaviors are conserved across vertebrates. In recent years, behavioral studies focusing on psychological/ cognitive aspects in fish, such as prosociality (Satoh et al. 2021), self-recognition (Kohda et al. 2019, 2022; Kobayashi et al. 2024), and affective states (Laubu et al. 2019), have gained increasing attention. Notably, similarities have been found between fish and mammals in mechanisms underlying some cognitive abilities (Calvo and Schluessel 2021) and fear contagion (Akinrinade et al. 2023). As such findings accumulate, the importance of integrative research is likely to become even more pronounced. The method proposed in this study offers a practical tool for incorporating non-invasive pharmacological manipulation into ethological experiments without the need for complex procedures or costly equipment. PEG400 and Tween 80 are excipients used for oral administration, which are generally regarded as nontoxic and nonirritant in mammals (e.g., Rowe et al. 2009; Gullapalli and Mazzitelli 2015; Ma et al. 2017). They have also been shown to enhance the permeability of the administered compound (Tønsberga 2010), and their application has also been extended to fish (e.g., Kim et al. 2017). We expect the vehicle proposed here will be particularly useful for long-term behavioral studies and for experiments involving sensitive species or delicate behaviors, where minimally disruptive conditions are essential. We expect the vehicle proposed here will be particularly useful for long-term behavioral studies and for experiments involving sensitive species or delicate behaviors, where minimally disruptive conditions.

On the other hand, we acknowledge that several considerations should be noted in applying our method. First, although we used atosiban as a model compound, the extent to which this vehicle improves oral delivery efficiency for other substances should be validated individually. For example, while atosiban is an oligopeptide and hydrophilic, enhancements in permeability with PEG400 and Tween 80 are often reported for hydrophobic compounds (e.g., Tønsberga 2010). Second, if a greater transfer into

systemic circulation via the oral route is required, alternative strategies may be necessary. In pharmacology, several sophisticated approaches for improving oral bioavailability have been developed (Brayden et al. 2020; Cui et al. 2022; Lou et al. 2023). As our primary aim was to minimize procedural complexity, it would be valuable to consider incorporating such methods.

Therefore, the present findings should be regarded as a preliminary proof-of-concept. Elucidating the underlying mechanisms was beyond the scope of this study and will require future research. Nevertheless, we hope that our results will encourage the future development of non-invasive pharmacological methods for application in ethological experiments.

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Author contributions

Conceptualization, Methodology: SM, TY, SS, YY, KF; Investigation: SM, TY, KF; Writing - original draft preparation: SM, KF; Writing - review and editing: TY, SS, YY, KF; Funding acquisition: KF; Resources: SM, SS, KF; Supervision: TY, SS, KF

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Data availability

The datasets are available from the corresponding author on reasonable request.

Declarations

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

At Kitasato University, there are currently no institutional ethical regulations for fish.

Therefore, all procedures adhered to the ASAB/ABS guidelines for the treatment of animals in behavioral research.

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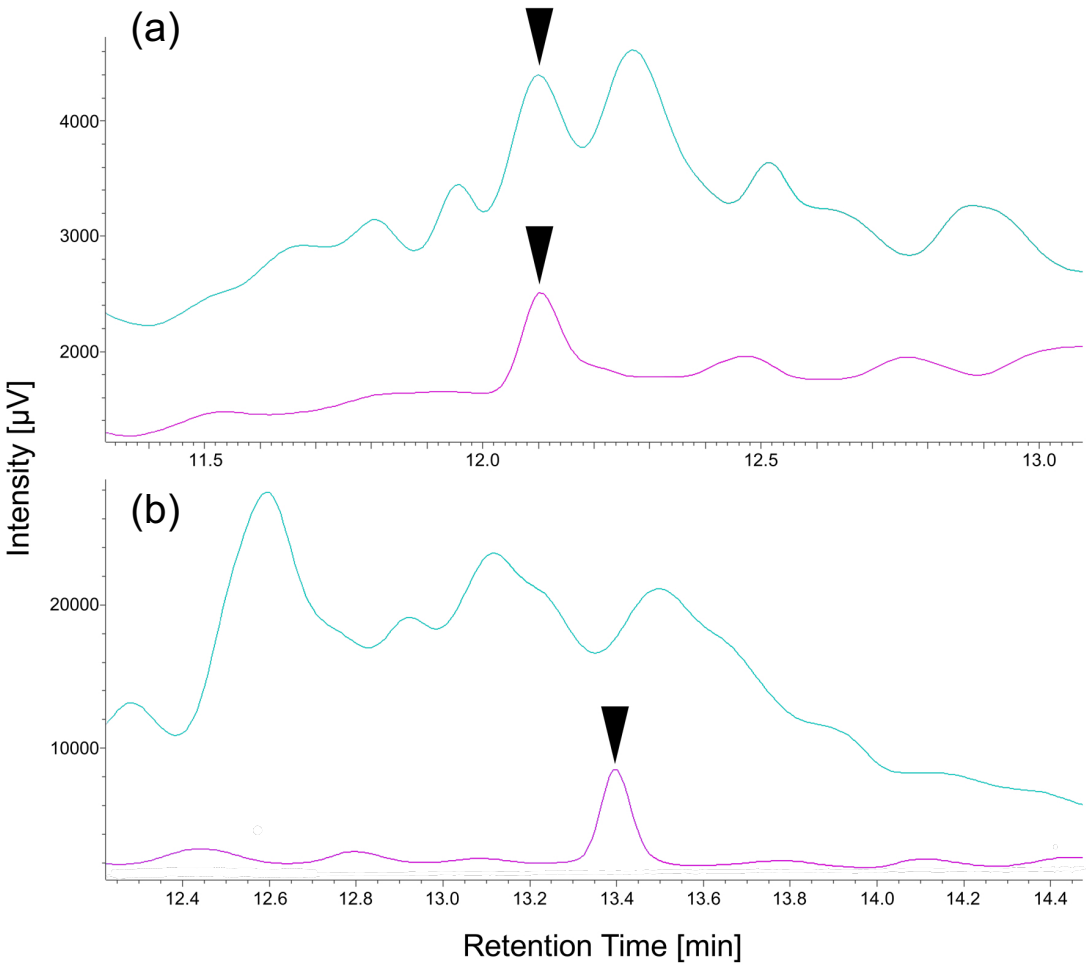

Fig1. Chromatograms of (**a**) a blood extract from fish that ingested atosiban with the modified vehicle solution, and of the 0.5 µg/mL atosiban standard, and (**b**) a blood extract from fish that ingested atosiban with the control solution, and of the 5 µg/mL atosiban standard. Black arrowheads indicate the peak of atosiban. Cyan and magenta lines represent the chromatogram of the blood extract and standard, respectivery.