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A novel approach for long-term oral drug administration in animal research

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Abstract

In the field of pharmacological research, the oral consumption of anastrozole, an aromatase inhibitor, when added to an animal's drinking water is hindered by poor drug palatability and environmental loss of drug solution. To overcome these caveats, we developed a novel approach for the oral delivery of anastrozole mixed in a solid hydration gel matrix that functions as a replacement for water. Heated hydration gel was mixed with anastrozole and distributed into a gel delivery device consisting of a 50 mL plastic conical tube containing four stacked 200 μ L pipette tips to allow for air pressure induced gel disbursement. Transgenic female 3xTgAD mice were randomized to receive either anastrozole-treated or untreated hydration gel at 3 months of age. Body weights were recorded weekly, and gel consumption was measured every 1–3 days. Six months post treatment mice were killed and serum anastrozole levels were determined using liquid chromatography-mass spectrometry (LC-MS). Anastrozole-treated mice gained significantly more weight despite consuming significantly less hydration gel compared to vehicle treated mice. LC-MS analysis, using a low serum volume (10 μ L), revealed average anastrozole serum levels of 2.91 ng/mL. Anastrozole-treated ovarian tissue displayed ovarian cysts, massive edema-like stroma, and also lacked corpora lutea compared to control mice. These findings demonstrate that hydration gel delivered using the newly developed oral delivery method is a viable approach for pharmacological research involving compounds with poor palatability, low water solubility, and cost prohibitive compounds where environmental loss needs to be minimized.

Keywords

anastrozole; hydration gel; liquid chromatography-mass spectrometry; oral administration; ovary; pharmacology

Introduction

Long-term oral drug administration in rodents has several methodological challenges including minimizing stress to the animal, drug palatability, and in the case of liquids,

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reducing the amount of drug lost due to water bottles that leaked, and bedding contamination. The use of anastrozole, an aromatase inhibitor, which interrupts a critical step in the body's synthesis of estrogen (Plourde et al., 1994) and is used orally in breast cancer treatment for post-menopausal women (Howell et al., 2005), suffers from many of these methodological caveats. Anastrozole has sparse water solubility (Sarkar and Yang, 2008) and displays poor palatability (Gary Nunn, AstraZeneca, personal communication). In studies where rodents were given water bottles containing anastrozole for three to twelve months (Beaton and deCatanzaro, 2005; Turner et al., 2000), leakage made dose calculations difficult (Beaton and deCatanzaro, 2005). While dosing animals using a gavage syringe provides a specific drug dose and overcomes palatability issues and drug loss, oral gavage is stressful particularly when studies require daily administration over weeks to months (Balcombe et al., 2004). Therefore, investigators will often administer poorly tolerated drugs using alternate administration routes such as subcutaneous to avoid the stressful nature of gavage dosing, or intracerebrally to ensure presence in the brain. However, these *in vivo* dosing methods often differ from the route of administration used for humans thereby limiting the applicability of these findings to the human condition. Here we describe the development of a novel oral drug delivery device that combines anastrozole with a thermo-reversible hydration gel housed in a gel delivery device, which overcomes these technical problems.

Thermo-reversible hydration gel contains > 92% water and when heated transforms into a semi-liquid. When cooled to room temperature, the gel reverts back to its original solid state. Although hydration gels are available in various flavors to mask the taste of compounds with poor palatability, delivering medicated hydration gel in a manner that prevents rodents from playing with, or otherwise contaminating the gel, remains a challenge. To overcome these issues, we developed a novel gel delivery device that provides sufficient amounts of gel to hydrate/treat a cage of five mice for up to five days, protects against gel evaporation and bedding contamination, while allowing recording of gel consumption and reduces the time spent by investigators dosing mice.

1. Material and methods

1.1. Animals

Three-month old female 3xTgAD mice, which over expresses the familial Alzheimer's disease (FAD) genes APP_{SWE}, PS1_{M146V}, and Tau_{P301L} (Oddo et al., 2003), were generated from breeding pairs provided by Dr. Frank LaFerla from the University of California Irvine. Mice were housed four to five per plastic cage and maintained under constant room temperature and humidity on a 12:12-hour light:dark cycle (lights on at 6 am). Littermates were randomized to receive either anastrozole-treated or untreated hydration gel (N = 8–9/group) as well as food pellets (2020X; Teklad, Indianapolis, IN), *ad libitum*. Cages were changed twice a week. Body weights were recorded weekly, and gel consumption was collected every 1–3 days. Vaginal cytology was used to identify the proestrus phase during the last week of treatment (Goldman et al., 2007). Mice in proestrus were deeply anesthetized using a mixture of ketamine (95 mg) and xylazine (5 mg) (per kg body weight, respectively) and cardiac blood was collected prior to transcardial perfusion with ice-cold saline. Dissected ovaries were fixed in 10% buffered formalin and processed for paraffin embedding. Serum was separated from whole blood by centrifuge (10 min, 2,000 rcf), transferred to a new 1.5 mL tube and stored at –80°C. All animal care and procedures were conducted with approved institutional animal care protocols and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

1.2. Drug preparation and administration

Anastrozole powder (45 mg, AstraZeneca, UK) was mixed with red food coloring (40 μ L, McCormick; Sparks, MD) and propylene glycol (1 mL, Fisher Scientific; Pittsburgh, PA) on a glass slide forming a slurry, which was then transferred to a heated bag of no-sugar-added banana-flavored, hydration gel consisting of purified water, sucralose, fruit and natural flavoring, hydrocolloids, potassium sorbate, sodium benzoate, phosphoric acid (8 oz LabGel; ~236 mL; ClearH2O; Portland, ME). Pilot data demonstrated that animals consumed approximately 2.5 mL of gel/day, which equates to an anastrozole dose of approximately 0.5 mg/animal/day. The bag was closed and vigorously shaken until the red-dyed anastrozole was homogeneously dispersed throughout the bag. The gel was immediately transferred to 50 mL conical plastic tubes. Following cooling, the gel delivery device was assembled by placing four stacked pipette tips (200 μ L) or one serological pipette (2 mL) between the wall of the plastic tube and the gel. The device was inverted and tapped to ensure airflow (Fig. 1). The gel delivery device was placed with the gel-side down upon the grate of the cage lid. Food pellets were located in a restricted area on the other side of the lid. As the mice consumed the gel, the air coming into the gel delivery device forced the gel to move down the tube while the grate provided sufficient resistance to prevent the gel from being extruded (see Fig. 1). Cages were undocked from the automatic watering system, and mice acclimated to the hydration gel within 24–30 h of initial exposure. Vehicle gel was prepared in the same manner as the anastrozole-containing gel with the exception of the drug and half the amount of food dye so that the investigator could distinguish anastrozole-containing gel from vehicle gel.

1.3. Histology

Paraffin embedded mouse ovaries were sectioned at 4 μ m and stained with a 10% hematoxylin and eosin (H&E) solution. Slides were cover slipped using DPX and representative photomicrographs of ovarian structure were acquired with the aid of a Zeiss Axioplan 2 microscope. Images were adjusted for contrast and size using Adobe Photoshop (version 7).

1.4. Anastrozole quantification

1.4.1. Serum extraction—Serum anastrozole levels were quantified from a small volume of serum (10 μ L) with the aid of liquid chromatography-mass spectrometry (LC-MS) using a modification of a previously described method (Mendes et al., 2007). LC-MS was performed in duplicate runs per sample. Solvent-resistant pipette tips with charcoal filters (Molecular BioProducts, San Diego CA) were used for all procedures involving solvents. Briefly, duplicate serum samples were spiked with the 25 ng/mL internal standard (10 μ L) and 10X PBS (180 μ L, 1.37 M NaCl, 27 mM KCl, and 119 mM diphosphates; pH 8.0 adjusted with NaOH). Samples were vortexed for 40 s and extracted with diethyl ether and dichloromethane (100 μ L, 70:30, respectively), vortexed for 40 s, and centrifuged (3 min, 7,000 rcf). The supernatant fraction was then transferred to deactivated vials (Waters; Milford, MA) containing HPLC- grade acetonitrile:water (50 μ L, 50:50 mix). This extraction process was repeated two more times (300 μ L total extraction solvent). Samples were dried under a hood until only acetonitrile:water remained, transferred to deactivated vial inserts (Waters; Milford, MA) and quantified by LC-MS (see below).

1.4.2. Calibration standard and quality control—Stock solutions of anastrozole and dexchlorpheniramine maleate (US Pharmacopeia, Rockville, Maryland) were prepared in acetonitrile:water (50:50 v/v) at concentrations of 5 mg/mL and aliquotted (10 μ L). Calibration curves of anastrozole were prepared in duplicate by spiking naive mouse plasma (Lampire Biological Laboratories; Pipersville, PA) at concentrations of 0.78, 1.56, 3.125,

6.25, 12.5, and 25 ng/mL. Quality control samples were prepared using blank plasma at concentrations of 1 and 5 ng/mL.

1.4.3. Chromatographic conditions—A Waters Alliance 2695 Separation Module was used to resolve a 5 μ L portion of each plasma extract using a Waters Xbridge C18, 3.5 μ m analytical column (100 mm $\times$ 2.1 mm i.d., Waters, Milford, MA) at room temperature. The compounds were eluted with an isocratic mobile phase consisting of acetonitrile:methanol:water:acetone (60:20:15:5, v/v/v/v) with 10 mM ammonium acetate (pH = 5.5) and 0.1% acetic acid at a flow-rate of 0.20 mL/min. Total run time for each sample was 5 minutes.

1.4.4. Mass-spectrometric conditions—Mass spectrometry was performed using a Thermo-Finnigan TSQ Quantum mass spectrometer (West Palm Beach, FL, USA) equipped with an electrospray source operating in positive mode. Multiple reaction monitoring (MRM) was performed for transitions 294 $\rightarrow$ 225 and 275 $\rightarrow$ 230 for anastrozole and the internal standard, respectively. The source temperature was set at 336 $^{\circ}$ C and the ESI voltage set to 4.4 kV. Collision energy was set to 20 eV for the internal standard and 30 eV for anastrozole using a scan width of 2.0 atomic mass units and a scan time of 1.0 sec for each compound.

1.5. Data analysis

1.5.1. Calculation of assay results—All data points were converted to a ratio of the integrated areas of anastrozole to internal standard and a standard curve generated using a sigmoid log dose-response (variable slope) curve (GraphPad Prism 5 for Windows, San Diego, CA). The concentrations for all samples and quality controls (QCs) were then calculated based on this equation. This method is similar to those routinely employed for radioimmuno assays and recently validated for use in mass spectrometry quantification (Findlay and Dillard, 2007; Gelfanova et al., 2007). Differences between the two groups were evaluated using linear regression and Student's t-test. The level of significance was set at p -value < 0.05.

1.5.2. Method validation for anastrozole quantification—Precision Method – Assay precision was calculated as a % relative standard deviation (%RSD) from a minimum of six data points collected either on the same day (Intra-day %RSD) or across the entirety of the batches (Inter-day %RSD) and at a high (12.5 ng/mL) and low (0.78 ng/mL) concentration. Accuracy – Accuracy was calculated from high (5 ng/mL) and low (1 ng/mL) QC samples and expressed as % deviation from the true value. In addition, the lower limit of quantification (LLOQ) and lower limit of detection (LLOD) were calculated from $10\sigma_{(\text{blank})}$ and $3.29\sigma_{(\text{blank})}$, respectively, using the mean of the inter-day batches (Vial and Jardy, 1999).

2. Results

2.1. Body weight

Body weights were collected weekly and reported as mean $\pm$ SEM (Fig. 2). There was no significant difference between the anastrozole- (17.6 $\pm$ 1.4 g) and vehicle- (19.33 $\pm$ 0.726 g) treated mice at the start of the study (p > 0.05). However, during the 24-week study period anastrozole-treated mice gained significantly more weight over time (slope 95% CI 0.43 to 0.65) than the vehicle treated mice (slope 95 % CI 0.27 to 0.40).

2.2. Gel consumption

Gel consumption was measured using the milliliter gradation scale provided on the conical tube component of the gel delivery device every 1–3 days. The averaged consumption per day per animal (Fig. 3) is reported as mean $\pm$ SEM. Vehicle-treated mice (2.7 ± 0.09 mL/animal/day) consumed, significantly ($p < 0.0001$) more gel per day than anastrozole-treated mice (1.9 ± 0.1 mL/animal/day). However, no signs of dehydration were observed during the six-month study period.

2.3. Ovarian Histology

The morphology of ovarian tissue was evaluated from anastrozole and vehicle-treated mice. Ovaries from the anastrozole-treated mice contained ovarian cysts, which were often hemorrhagic, whereas vehicle-treated mice did not show such pathology (Fig. 4). The stroma in anastrozole treated mice displayed extensive edema (Fig. 4) with occasional fibroblast cells scattered throughout dense fibrous tissue. While there were follicles at various stages of progression in both groups, anastrozole-treated mouse ovaries contained many dead follicles (data not shown), and virtually no structural evidence of corpora lutea. While antral follicles were present in both treated and untreated mouse ovaries, the granulosa layer in the anastrozole-treated mice was narrow compared to the vehicle-treated mice (data not shown).

2.4. Anastrozole serum quantification using LC-MS

Assay validation - The mean intra-day and inter-day %RSD values were 13.9% and 20.54% for the low (0.78 ng/mL) concentration samples and 10.6% and 11.0% for the high (12.5 ng/mL) concentration samples. Accuracy was determined to be within a margin of 3.715% for the high QC (5 ng/mL) and within 7.95% for the low (1 ng/mL) QC samples. The LLOQ and LLOD values were calculated to be 0.61 ng/mL and 0.07 ng/mL, respectively. Representative data for samples measured in this study is provided in Figure 5. **Study Results** - Serum anastrozole concentrations were measured using LC-MS and reported as mean $\pm$ SEM. Treated mice had an average anastrozole serum concentration of 2.92 ± 0.37 ng/mL, while levels in vehicle treated mice were below the level of quantification.

4. Discussion

Thermo-reversible hydration gels have been used primarily as an alternative water source during the shipment of rodents (Fredenburg et al., 2009), as well as marketed for medication delivery in animal research. In the present study, we combined thermo-reversible hydration gel technology with a novel gel delivery device for long-term oral delivery of anastrozole. The development of the current drug delivery system was undertaken to overcome the observation derived from a pilot study showing that anastrozole mixed in water was not palatable for rodents. In this study, ad lib water intake was volumetrically measured over a 72 hours period in a cage containing 4 female 3xTgAD mice. The average intake was 4.5 mL of water/animal/24 h. The same mice were then given ad lib access to anastrozole-treated water (3.3 mg/30 mL water) for an additional 72 hours. Anastrozole-treatment reduced water consumption to 1.67 mL/animal/24 h or 37% of their normal intake. In consultation with our veterinarian staff, it was determined that continued maintenance of mice on anastrozole-treated water would result in clinical dehydration. This finding led to use of hydration gel technology to overcome the palatability issue associated with anastrozole treated water for our ongoing studies of the effects of estrogen sources upon AD pathology in a triple transgenic mouse model of this disease (Oddo et al., 2003; Oh et al., 2010; Overk et al., 2009). In line with the findings that vehicle-treated mutant mice consumed more gel than those in the anastrozole group, we demonstrated a similar response using age-matched female wild-type mice (data not shown). Therefore, while this method

offers a viable method for chronic oral delivery of anastrozole, our findings suggest that the drug's poor palatability was not completely resolved despite the use of banana-flavored gel. The use of our oral drug delivery system by collaborators at the University of Illinois, Chicago, has proved successful for the delivery of other compounds to rodents, as well (unpublished observations). Our flavored hydration gel technology increased palatability and curtailed clinical dehydration during the six-month treatment regime, further supporting the use of this novel system for drug delivery of compounds with poor palatability in research.

Although results of the present study indicate that the dose of anastrozole used induces biological alterations in 3xTgAD mice, allometric scaling between humans and animals remains challenging. In humans, a 1 mg/person dose of anastrozole resulted in a plasma concentration of 25 ng/mL (Plourde et al., 1994). In the current study, mice were treated with a higher concentration per body weight (0.38/kg), but the serum concentration was only 2.9 ng/mL, which is nearly one-tenth the human concentration. Pharmacologically, the amount of anastrozole detected in the serum was 9.7 μ M, which is more than sufficient to inhibit the aromatase enzyme, since anastrozole has been reported to have an IC_{50} of 15 nM (Plourde et al., 1994). Moreover, mice in the anastrozole group gained more weight during the course of the study than the vehicle treated animals consistent with the reported effects of anastrozole in rats (Kubatka et al., 2008; Sadlonova et al., 2009). Most strikingly, morphological analysis of ovaries from long-term anastrozole-treated mice displayed hemorrhagic cysts, a narrow granulosa layer in antral follicles, scarce corpora lutea, large numbers of atretic follicles, edema in the interstitial tissue and occasional fibroblasts scattered throughout dense fibrous tissue similar to that reported in estrogen receptor α and β knockout mice (Dupont et al., 2000). Although one report found no change in the histological appearance of ovaries from mice treated with anastrozole, these mice were superovulated and treated with anastrozole for only four days (Fatum et al., 2006). Taken together, our results suggest that long-term administration of anastrozole induces ovarian pathology similar to that seen in animals lacking estrogen receptors.

Despite the average collected serum volume ranged between 150–250 μ L/animal, we were able to determine serum concentrations from each mouse using only 10 μ L per replicate, thereby avoiding the need to pool samples. Although very little serum was required for our LC-MS analysis, the LLOQ values were not as low as those previously reported (500 μ L aliquots) (Yu et al.) suggesting the need to further refine our LC-MS method. One refinement would be to use a capillary or nano-ESI probe with accompanying chromatographic alterations for measuring very low (5–20 μ L) quantities of serum while maintaining high sensitivity and precision.

Although disadvantages of using medicated hydration gel are few, they include caution when using heat-labile compounds and reliance on animal consumption. In addition, group housed mice inherently create a challenge in determining individual drug dosing. In the present investigation, gel consumption was recorded per cage, which was then divided among the number of mice housed per cage. Alternatively, if a more precise determination was required, mice could be individually housed animals utilizing a smaller version of the gel delivery device containing a finer gradation scale. In this study, serum concentrations confirmed that mice were dosed with similar amounts of anastrozole. Using food dye to color the anastrozole powder aids in ascertaining whether the drug is homogeneously distributed within the gel. Moreover, using less dye for the vehicle controls helped differentiate between the two gels to ensure treatments are not confused. The banana-flavored LabGel worked exceedingly well with our drug delivery device, in part due to forming a matrix that was more rigid at room temperature than the HydroGel. The banana gel matrix maintained its structure when the pipette tips were added ensuring that the gel

moved down the delivery device during consumption. Mice adapted to this alternate water source within 24–30 hours following the removal of the cage water docking station.

In summary, hydration gel combined with our gel delivery device provides a simple yet elegant method to successfully dose mice with unpalatable drugs such as anastrozole for at least six months. This was confirmed by the quantitation of anastrozole serum concentrations using LC-MS. Our drug hydration device provides a new method for drug delivery based pharmacologic research.

Research Highlights

- Hydration gel combined with a gel delivery device is a viable method for the oral delivery of anastrozole.
- Anastrozole levels were quantified from 10 μ L of serum using LC-MS.
- Anastrozole caused weight gain and changes in ovarian structure indicating that the dosing was biologically active.

Abbreviations

%RSD	% relative standard deviation
ESI	electrospray ionization
LLOD	lower limit of detection
LLOQ	lower limit of quantification
MRM	Multiple reaction monitoring
QCs	quality controls

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Fig. 1.
 (A) Photograph showing placement of the gel delivery device within the cage lid. (B) Schematic drawing of the 50 mL inverted conical tube containing 2 mL stereological pipette to create an air space between the inner wall of the plastic conical tube and the gel, which allows the gel to slide down during consumption. Milliliter gradations enable quantification of gel consumption.

Body weight change over time

Fig. 2.

Body weights were measured weekly (average $\pm$ SEM). While there was no significant difference between the anastrozole-treated and vehicle-treated groups at the beginning of the study, there was a significant difference in the rate of body weight increase over the 24 weeks between groups (slope of the linear regression $p < 0.01$).

Fig. 3. Average daily gel consumption rate. There was a significant difference in the amount of gel consumed between vehicle-treated and anastrozole-treated mice (t-test, $p < 0.0001$).

Fig. 4. Photomicrographs of H&E stained (A) control and (B) anastrozole-treated ovarian tissue. While control ovaries were histological typical and presented with follicles in all stages of progression (F and arrows), nodules of luteinized cells (N), and corpus albicans (CA), anastrozole-treated ovary morphology displayed ovarian cysts (CY), large hemorrhagic cysts (HC), and edematous stroma within the medulla (M/S; outlined with dashes). Abbreviations: A, antrum; CA, corpus albicans; CY, ovarian cyst; D, atretic (degenerating) follicle; F (follicle), arrows; primordial follicles; HC, hemorrhagic cyst; M/S, medulla/stroma; N, nodules of luteinized cells;. Scale bar = 100 μ m.

Fig. 5.
Representative mass spectral data for treated animals. (A) Trace of the total ion current; MRM and fragmentations of the (B) internal standard and (C) anastrozole.