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Development and pharmacokinetic evaluation of erythromycin lipidic formulations for oral administration in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this work was to enhance the bioavailability of erythromycin base when administered orally in rainbow trout (*Oncorhynchus mykiss*). Since erythromycin is normally given in the form of medicated feed, in this study three new types of feed formulation were developed. A self-emulsifying system and two types of double microemulsions (O/W/O) were prepared, characterized and adsorbed on a commercial extruded diet for fish. The emulsified systems were based on saturated polyglycolized glycerides and mono- and diglycerides of medium-chain fatty acids (as oily phase), Tween® 80 (as surfactant) and, in the case of double microemulsions, distilled water. The systems differed in percentage composition and for the amount and position of erythromycin in different phases. The three medicated feed were then administered orally by means of a gastric probe to rainbow trout and their relative bioavailability was estimated in comparison with that obtained after oral administration of feed with erythromycin powder. For each medicated feed, 80 fish were tested. Finally, plasma profiles of erythromycin after single administration of medicated feeds were used to predict profiles obtainable by administering once-daily medicated feeds for 7 consecutive days. The results proved that the feeds containing microemulsified erythromycin provided largely superior oral bioavailability and the advantage of obtaining the same efficacy against bacterial infections with a much lower dose of drug.

1. Introduction

Aquaculture production has notably increased in the last decades, mainly thanks to intensive farming. In the Mediterranean area, the average annual increment of aquaculture production in the last 20 years has raised from 4% to 13%. Yet despite the outstanding advantages of indoor recirculating aquaculture systems, to be economically feasible, they imply a very high density of fish stocking, and consequently, disease outbreaks can readily occur [1,2].

For this reason, large amounts of antibiotics are currently used to keep the fish moderately healthy. Following the discovery of the growth-promoting effect and the familiarity with antibiotics, fish farmers and livestock producers began using such drugs in animal feeds. Antibiotics effective in human medicine, including oxytetracycline, sulfamerazine in association with ormetoprim and erythromycin, are hence used for the treatment of bacterial infections in salmon, catfish, trout and other commercially raised fish [3]. Incorrect method of calculating the appropriate dose of these chemotherapeutics, drug abuse and overuse have posed a threat to human health and environmental safety. Hence, it is necessary to examine the behaviour of these antibiotics after dosing in order to obtain suitable dosing regimens [4].

Erythromycin, a macrolide bacteriostatic antibiotic, is the antibiotic of choice against gram-positive cocci, the major concern for trout farming. In particular, it is primarily used for controlling *Lactococcus garvieae*, responsible for lactococcosis, the most important risk factor for rainbow trout farming when water temperature increases over 16 °C over summer months [5–9], and against *Streptococcus iniae*, mainly responsible for infections in reared trout during colder seasons [8]. Erythromycin is also primarily used for controlling bacterial kidney disease in salmonids (*Renibacterium salmoninarum*) [10]. Despite its activity, the drug has not been approved for widespread use in fish within most countries. In the USA and in most European countries, erythromycin is commonly prescribed by veterinarians with extra-label authority [1,11].

Moreover, in aquaculture practice, erythromycin is generally delivered as medicated feed to accomplish the treatment for a vari-

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ety of systemic diseases of pre-reproductive fish [12], with a normal dose regimen of 110 mg/kg bw/day [3].

Erythromycin has low palatability for fish [10,3] and it is easily inactivated by gastric acid [3]. These two characteristics often determine an inefficient antibiotic therapy that is frequently vanished by the lack of hunger of the diseased fish and by the poor absorption of the drug. As a consequence, a huge amount of antibiotic is lost and contributes to the water pollution and to the rise of antibiotic resistance phenomena. Therefore, erythromycin is dosed in fish either as enteric-coated oral formulation or as a pro-drug, such as erythromycin ethylsuccinate ester, to obtain a taste-masking effect and to prevent the drug from inactivation [3]. However, with the administration of the pro-drug, the free drug concentration in the blood circulation depends on the rate of metabolic activation, with expected inter-species and inter-animal variations.

In this context, the aim of this work was to develop new erythromycin formulations: a self-emulsifying system (SES) and two double microemulsions (DME) to increase oral bioavailability of erythromycin base in fish. SES are mixtures of oils and surfactants, ideally isotropic, and sometimes containing co-solvents, which emulsify spontaneously to produce fine oil-in-water emulsions when introduced in gastrointestinal (GI) tract under gentle agitation [13].

DME are multiple microemulsions whose dispersing phase can contain droplets of another phase, leading to water/oil/water or oil/water/oil systems [14]. These fine microemulsions result in small droplets of the dispersed phase in the gastrointestinal fluids. Small droplets provide a large interfacial area and thereby promote an enhanced release of the drug. Additionally, the surfactant used for this kind of formulations potentially further improves the bioavailability by various mechanisms, including the improved solubility in gastrointestinal fluids and enhancement of intestinal epithelial permeability [15].

Improved bioavailability is favourable from a therapeutic point of view, since it ensures adequate systemic concentrations during the treatment with drug administration at a lower dose rate.

Hence with microemulsions, several aims could be hypothetically achieved at the same time: to mask the drug bad taste, to protect the drug from the inactivation, to enhance the bioavailability and to decrease its inter-animal variation and finally to reduce the dose and hence the water pollution.

In particular, in this study, a self-emulsifying system and two types of double microemulsions (O/W/O) were prepared using saturated polyglycolized glycerides and mono- and diglycerides of medium-chain fatty acids (as oily phase), distilled water and Tween® 80 (as surfactant). Erythromycin was chosen as a model drug for the above-mentioned reasons. Additionally, we aimed to formulate erythromycin inside lipidic vehicles of the internal phases, in order to protect the active ingredient from the interaction with GI fluids, and to enable a large surface area when in contact with intestinal membranes. The drug loading in the formulations was carried out taking into account the final aim of designing a formulation for trout. Once optimized, the three different formulations were characterized and then adsorbed to a commercial extruded diet for fish. Pharmacokinetics of three different feeds obtained were evaluated by administering them orally to rainbow trout. The relative bioavailability was estimated in comparison with that obtained after oral administration of feed with erythromycin powder.

2. Materials and methods

2.1. Materials

Erythromycin E.P. was purchased from Galeno S.r.l. (Prato, Italy). Mono-di-triglycerides mixtures and mono-diesters of polyethylene glycol with medium-chain fatty acids (saturated polyglycolized glycerides) [compound A] and diethylenglycole monoethyl ether [compound B] were purchased from Gattefossé S.a.s. (Saint Priest, France), whereas mono- and diglycerides of medium-chain fatty acids [compound C] were purchased from Huwell Chemicals S.r.l. (Milano, Italy). Polysorbate 80 (Tween® 80) was used as a surfactant, whereas tricainemethansulfonate (MS-222) was used as an anaesthetic; both supplied by Sigma Aldrich, Milano, Italy. Commercial non-medicated feed composed of fish meal, wheat gluten, sunflower meal, wheat and wheat by-products, fish oil, soybean oil and seed oil was kindly donated by Hendrix S.p.a. (Verona, Italy). Monohydrate lactose (Granulac® 230) was purchased by Prodotti Gianni (Milano, Italy) and cod-liver oil was purchased by Marco Viti Farmaceutici S.p.a (Como, Italy). The solvents used were of HPLC grade, and all other chemicals were of reagent grade. Double-distilled water was used throughout the study.

2.2. Determination of erythromycin solubility

To select the components of formulations, the solubility of erythromycin in various oils (see Table 1) was evaluated by adding an excess amount of drug to 5 ml of each of the selected oil or oily mixture placed in a cap vial.

After sealing, mixtures of drug and vehicles were then shaken with a shaker at 25 °C for 48 h. After reaching equilibrium, each vial was centrifuged at 3000 rpm for 5 min and the undissolved drug was eliminated by filtration using a membrane filter (RC 0.45 μm, Sartorius AG, Germany). The concentration of erythromycin was quantified by HPLC using the method reported in Section 2.7.2.

2.3. Optimization of formulations and preparation techniques

2.3.1. Construction of a ternary phase diagram and optimization of primary microemulsion

A primary O/W microemulsion (PE, O/W) composed of [B]:[C] = 1:1 (w:w) as oily phase, polysorbate 80 as surfactant and distilled water was optimized. A ternary phase diagram was constructed to determine the zone of existence of microemulsion at different ratios of the components. The phase diagram was built for the primary microemulsion according to Mackay [16], using three components: oily mixture, surfactant and water phase, according to the literature data [17]. The phase diagram was obtained by weighing appropriate amounts of each component (double-distilled water, the oily phase and polysorbate 80) into glass tubes and investigating the phase state after incubation for three hours at ambient temperature. The phase state was classified by visual observation into three, that is, clear one-phase liquid, clear one-phase gel and multiple phase.

2.3.2. Preparation of formulations

We prepared 3 different erythromycin formulations, SES and two DME, type I with drug loaded just in the internal oily phase and type II with drug loaded in the internal and external oily phase.

Table 1
Solubility of erythromycin in different oils and oily mixtures at 25 °C (mean ± SD; $n = 3$).

Oils and oily mixtures	Erythromycin solubility (mg/ml)
[B]–[A] (1:1, w/w)	105 ± 1.2
[C]	190 ± 0.9
[C]–[A] (1:1, w/w)	166 ± 1.3
[C]–[B] (1:1, w/w)	138 ± 1.6
[B]	120 ± 0.7
[A]	166 ± 0.9

To prepare SES, erythromycin was incorporated in the selected oily phase, and the resulting solution was mixed with the surfactant (Tween® 80) in appropriate proportions, under gentle stirring with a magnetic stirrer for 30 min.

Double microemulsions were prepared using a two-step process [18]. In the first step, a primary microemulsion (PE) was formed, composed of appropriate amounts of [C]–[A] mixture (1:1 = w:w) as internal oily phase, distilled water as external phase and Tween® 80 as surfactant. In the second step, the double microemulsion was obtained by dispersing the primary microemulsion with [C]–[A] mixture (1:1 = w:w) (representing in this case the external oily phase) and once again Tween® 80 as surfactant. Both steps were performed at ambient temperature. Three batches of each microemulsion were independently prepared and characterized.

In particular, for the preparation of DME type I (O/W/O type I), O/W microemulsion was prepared incorporating appropriate amounts of erythromycin (166 mg/ml) in the internal oily phase which was then spontaneously emulsified with double-distilled water and surfactant, under magnetic stirring. Then, O/W microemulsion was re-dispersed in the external oily phase by stirring at 300 rpm for 2 h at room temperature, thus resulting in a double O/W/O microemulsion. DME type II (O/W/O type II) was analogously prepared, with the exception of incorporating erythromycin (83 mg/ml) in both internal and external oily phases. Percentage weight compositions of SES and of two types of DME are reported in Table 2.

2.4. Stability study

The optimized formulations were subjected to accelerated stability studies in order to assess their physical stability under the following conditions: stored at 4 °C for 48 h and then at 40 °C for 48 h. The heating/cooling cycle was repeated six consecutive times. Then, the formulations were subjected to centrifugation for 30 min at 3500 rpm and evaluated for appearance.

2.5. Preparation of medicated feeds

The classical non-medicated feed for trout was mixed with different formulations in a bench-top mixer with a 2-l bowl (Kenwood Chef., Kenwood Appliances, Dublin, Ireland) to obtain different types of feed in 0.5-kg batches. In particular, feed I was mixed with 30.12% of DME type I (% w/w), feed II was mixed with 26% of DME type II, and feed III was mixed with 11.88% of SES.

Finally, reference feed (feed IV) was prepared for comparison purpose by first mixing erythromycin with lactose (used as inert support) and then dissolving in an equal amount of cod-liver oil to obtain a final mixture of erythromycin (5%), lactose (45%) and cod-liver oil (50%). This mixture was adsorbed on the surface of non-medicated extruded diet (15.88% w/w). The drug concentration was selected according to rainbow trout feed rate and therapeutic doses suggested (75 mg kg^{-1} trout bw day^{-1}).

Table 2
Percentage weight composition of optimized SES and DME (O/W/O types I and II).

Components	SES (% w/w)	Double microemulsions O/W/O (% w/w)	
		Type I	Type II
Erythromycin	7.11	1.66	3.59
Oily internal phase	–	8.35	9.18
Water	–	10.01	10.01
Tween®80	57.14	46.65	46.65
Oily external phase	–	33.33	30.57
Oily phase	35.75	–	–

2.6. Droplet size measurement

The size of the droplets and their polydispersity index in an electric field were determined by photocorrelation spectroscopy (Nano ZS Malvern Instruments, United Kingdom). Light scattering was monitored at 25 °C at a 90° angle. This technique was used for the determination of the droplets size formed by SES and DME types I and II, when dispersed in two different buffers simulating gastric and intestinal pH values of fed fish (pH 4.5 and pH 7.4 phosphate buffer, PE 5th Ed.), and for the determination of the droplets size released from different types of medicated feeds in the same buffers.

One hundred micrograms of SES or DME was dispersed in 10 ml of buffers. The samples were bathsonicated for 30 s to disperse the samples before sizing. For the determination of the size of the droplets released from medicated feeds, 2 g of feeds was dispersed in 20 ml of buffers. Samples of supernatant were collected after 20 min and filtered using a membrane filter (RC 0.45 μm, Sartorius AG, Germany) before droplets size measurement.

2.7. Pharmacokinetic study

2.7.1. In vivo absorption experiments

Three hundred and eighty rainbow trout (mean weight 268.6 g ± 10.9) with a known disease-free status were randomly divided in four raceway tanks (12 °C, 80 fish per tank) and allowed to adapt for one week. The experiments were carried out at "Rivoli trote", a salmonid fish farm in Osoppo (Udine, Italy). Housing conditions were suitably representative of the real situation of fish farming sites. Procedures for trout care and management complied with those required by Italian laws (D.L.vo 116/92), and they adhered to ethical standards for humane treatment of experimental animals established by the ethical committee of University of Trieste.

This was a parallel design study in which each of the four tanks was used to test one of the prepared medicated feeds. Prior to the administration, fish were starved for 2 days. A specific injector (Lameplast S.p.a., Rovereto, MO, Italy) was used for feed administration at an erythromycin dose of 12.90 mg/kg for feed I, 20.62 mg/kg for feed II, 18.51 mg/kg for feed III and 20.96 mg/kg for feed IV (control group). The fish that regurgitated the feed were excluded from the trial.

At each of the scheduled times: 0 (before administration), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h, five fish were randomly selected from each tank and plasma samples (3 ml) were withdrawn from caudal vein. Five plasma samples per treatment group and time point were pooled and stored at −20 °C until analysis.

2.7.2. Assay of erythromycin

2.7.2.1. Erythromycin determination in oily mixtures, formulations and feeds. The amount of erythromycin in oily mixtures, in formulations (O/W/O type I, O/W/O type II and SES) as well as in feed pellets and in plasma samples (after extraction procedure) was evaluated by liquid chromatography–electrospray ionization tandem–mass spectrometry (LC–ESI-MS–MS) analysis. This assay was carried out by using a validated method, previously developed by Lucchetti et al. [19]. In the present study, the only modification of the method was the choice of the alternative internal standard (roxythromycin).

2.7.2.2. Erythromycin determination in trout plasma samples. Plasma fractions were separated from blood samples by centrifugation at the speed rate of 4000 rpm, for 15 min. Before analysis, extraction procedure using methanol was carried out. Ten microlitres of internal standard solution (roxythromycin in methanol 10 μg/ml),

100 μl of plasma sample and 300 μl of methanol were added to an Eppendorf vial, vortexed for 3 min and centrifuged for 10 min at 13,400 g. The supernatant was collected and filtered using 0.45-μl syringe filters. Samples were then placed in amber vial for auto sampling, which, once closed, were stored in freezer at −80 °C until analysis.

2.7.2.3. Curve calibration matrix for plasma. A methanolic erythromycin solution (1000 μg/ml) was prepared and stored at −20 °C. The calibration curve for erythromycin in plasma (range 0–4 μg/ml) was prepared by adding to blank plasma samples roxythromycin internal standard and solutions with increasing concentrations of erythromycin. Peak area ratios so obtained and corresponding nominal concentrations were used to calculate the equation of the calibration curve ($y = 0.6136x + 0.0093$; $R^2 = 0.9996$). The internal standard method was used for quantification. The limit of quantification was 0.44 μg/ml, while the limit of detection was 0.13 μg/ml.

Recovery test was performed by comparing values of area ratio obtained adding internal standard before and after the extraction procedure. Values ranged between 84% and 92%.

To calculate intra-day variation coefficient of the analytical method, six independent samples were analysed on the same day, while in order to calculate inter-day variation coefficient, six independent samples were analysed three times on three different days. The relative standard deviation ranged from 2.4% to 9.4% for the intra-day precision and from 7.5% to 10.9% for the inter-day precision.

2.7.3. Pharmacokinetic analysis

Following initial graphical exploration of the data on the semi-logarithmic plot, erythromycin pharmacokinetics were assessed by fitting a one-compartment model with first-order absorption and elimination to concentration–time data using ADAPT 5 (Build 5.0.35, http://bmsr.usc.edu) software [20]. Maximum-likelihood method with a proportional variance model was used for the estimation of absorption rate constant (k_a), elimination rate constant (k_e) and apparent volume of distribution (V_d/F). Goodness of fit was estimated by visual inspection of the fitted curve. The one-compartment model was selected based on favourable Akaike Information Criterion (AIC) compared with the alternative two-compartment model. Secondary parameters, including absorption and elimination half-lives ($t_{1/2ka}$ and $t_{1/2ke}$), area under the concentration versus time curve (AUC), apparent oral clearance (Cl/F), maximum concentration (C_{max}) and time to reach maximum concentration (t_{max}), were calculated by conventional algorithms.

Relative oral bioavailability (F_r) was calculated by the following equation:

$$F_r = \frac{\mathrm{AUC}\,\mathrm{Dose}_{\mathrm{REF}}}{\mathrm{AUC}_{\mathrm{REF}}\,\mathrm{Dose}} \times 100$$

where $\mathrm{AUC}_{\mathrm{REF}}$ and $\mathrm{Dose}_{\mathrm{REF}}$ are the area under the concentration–time curve and dose, respectively, of the reference feed with erythromycin powder (feed IV). AUC and Dose are the corresponding parameters of the feed tested (feed I or feed III).

3. Results and discussion

To develop a microemulsion system for oral delivery, suitable oils and surfactant need to be selected. Erythromycin showed a discrete solubility at 25 °C in numerous oils and oily mixtures (Table 1). Among the tested solvents, the mixture [A]–[C] (1:1 = w:w), corresponding to an erythromycin solubility of 166 mg/ml, was chosen. Solvent [C], though it guarantees the highest erythromycin solubility, was not selected because it was not able to form a microemulsion.

Once the components are selected, the formulation for O/W primary microemulsion was optimized on the basis of studies performed in a previous paper where the same components were used to develop a formulation to deliver oxytetracycline hydrochloride [21,22]. As reported in that paper, the percentage composition of O/W primary microemulsion was chosen and developed with the help of a ternary phase diagram, laser light scattering and DSC analysis and on the basis of maximum drug loading and minimum toxicity criteria. In particular, the thermal behaviour at the DSC analysis was employed to distinguish between an oil-rich phase and a water-rich phase, as in our previous work [21].

Since the aim of this research was to improve erythromycin oral bioavailability, to mask its bad taste and to protect the drug from degradation in aqueous medium, the formulation was selected inside ternary diagram in the area of O/W microemulsion formation.

The following composition was hence chosen for the formulation of the microemulsion: 30% oily mixture, 30% water and 40% Tween® 80. Removing water percentage from primary microemulsion composition, the composition of SES was obtained and it is reported in Table 1.

Once optimized the formulation for primary microemulsion, two different types of DME were prepared as reported in Section 2.3. The two types of DME (types I and II) differed in the amount and position of erythromycin in different phases (see Table 2).

These types of formulations, containing the lipophilic antibiotic encircled by an interphasal layer of surfactant, were expected to avoid the bad taste of the drug and to avoid the inactivation phenomena by protecting the drug from its contact with the gastrointestinal fluids. The potential of these formulations in improving oral bioavailability and protecting from inactivation has been previously demonstrated for oxytetracycline hydrochloride [21,22].

All formulations were subjected to stability studies, and no change in the physical parameters such as homogeneity and clarity was observed.

3.1. Droplet size measurement

As previously mentioned, the droplet size distribution is the most important characteristic of an emulsion, including a microemulsion, in evaluating its stability *in vivo*.

In this case, dispersing SES and two types of DME in both simulated gastric and intestinal fluids, values of mean particle size perfectly compatible with microemulsions (in the order of 200 nm) were obtained. These values were very promising in terms of thermodynamic stability [23].

It was also interesting to check the ability of the solid systems, loaded with SES or DME, to re-form a microemulsion system when in contact with the GI fluids. From Table 3, it can be noted that the size of the droplets formed when feeds I–III were dispersed in different solutions remained in the order of 200 nm, practically equivalent to those of the droplets examined before loading on the

Table 3
Size of the droplets released after dispersion in different media. [mean (PI); $n = 3$].

Samples	Droplet size, nm (polydispersity index)	
Medium	pH 4.5 buffer	pH 7.4 buffer
SES	117.3 (0.644)	257 (0.516)
O/W/O type I	244.3 (0.455)	221.8 (0.438)
O/W/O type II	236 (0.467)	167.9 (0.522)
Feed I	129 (0.471)	148.2 (0.261)
Feed II	148 (0.471)	179.1 (0.396)
Feed III (SES)	203 (0.968)	254 (0.426)

carrier. These droplet size values were very promising in terms of possible *in vivo* performance of the formulations. In fact, so small oil droplets provided a large interfacial area for pancreatic lipase to hydrolyse triglycerides and thereby promote the release of the drug and/or formation of mixed micelles of the bile salts containing the drug. Moreover, it may lead to a greater and more uniform distribution of the drug in the gastrointestinal tract, minimizing the irritation due to the contact between the drug and gut wall [15]. On the other hand, polydispersity was fairly small in all cases, suggesting uniformity in the droplet size of the formulations. As previously mentioned, similar droplet sizes showed the potential of these systems in terms of stability: In fact, it is well known that small droplet size of the oily phase provides stable microemulsions.

3.2. In vivo trial with medicated feeds

Plasma concentration profiles and fits of the one-compartment model are presented for each feed formulation in Fig. 1. Pharmacokinetic parameters are summarized in Table 4. For comparison purposes, pharmacokinetic parameters were normalized to 20 mg kg^{-1} dose, when applicable. As indicated in Fig. 1, erythromycin plasma concentration profile is notably affected by the drug formulation. Comparing the values of dose-normalized AUC reported in Table 4, it can be seen that feed medicated with microemulsified erythromycin resulted in a higher drug bioavailability with respect to the reference feed (feed IV) containing the drug in a solid state. This favourable performance was probably due to the *in vivo* properties of DME and SES feed formulations, ensuring the formation of drops of dispersed phase surrounded by surfactant molecules. This fact presumably prevented the drug from degradation and promoted, through an increase in surface area and permeability, an increased absorption of erythromycin.

To enter into further detail, the observed plasma concentration profiles considerably differed in the absorption rate. The concentration–time profile of feed III was characterized by the very rapid absorption phase within 2 h and a typical elimination phase from 2 h to 24 h. Compared to the reference (feed IV), there was a sevenfold difference in the absorption half-life, while there was no evident difference in the elimination rate. This difference was also manifested in the AUC truncated at 24 h (AUC_{0-24}) and the peak plasma concentration, which was approximately three times higher than that of the reference feed.

On the other hand, with the DME formulations, the terminal slope of the plasma concentration profile (parameter k_e) was smaller than with the reference feed, especially with DME I. The most likely reason for this difference was delayed slow absorption of erythromycin resulting from the diffusion of drug molecules from the inner droplets or the mass transfer due to coalescence of the inner droplets [14].

With DME I compared to the reference formulation (feed IV), approximately 2-fold improvement in the extent of erythromycin absorption was obtained, similar to SES (feed III). However, DME I and SES markedly differed in the absorption rate. Absorption of erythromycin following administration of DME I was characterized with initial rapid absorption phase until approximately 3 h after

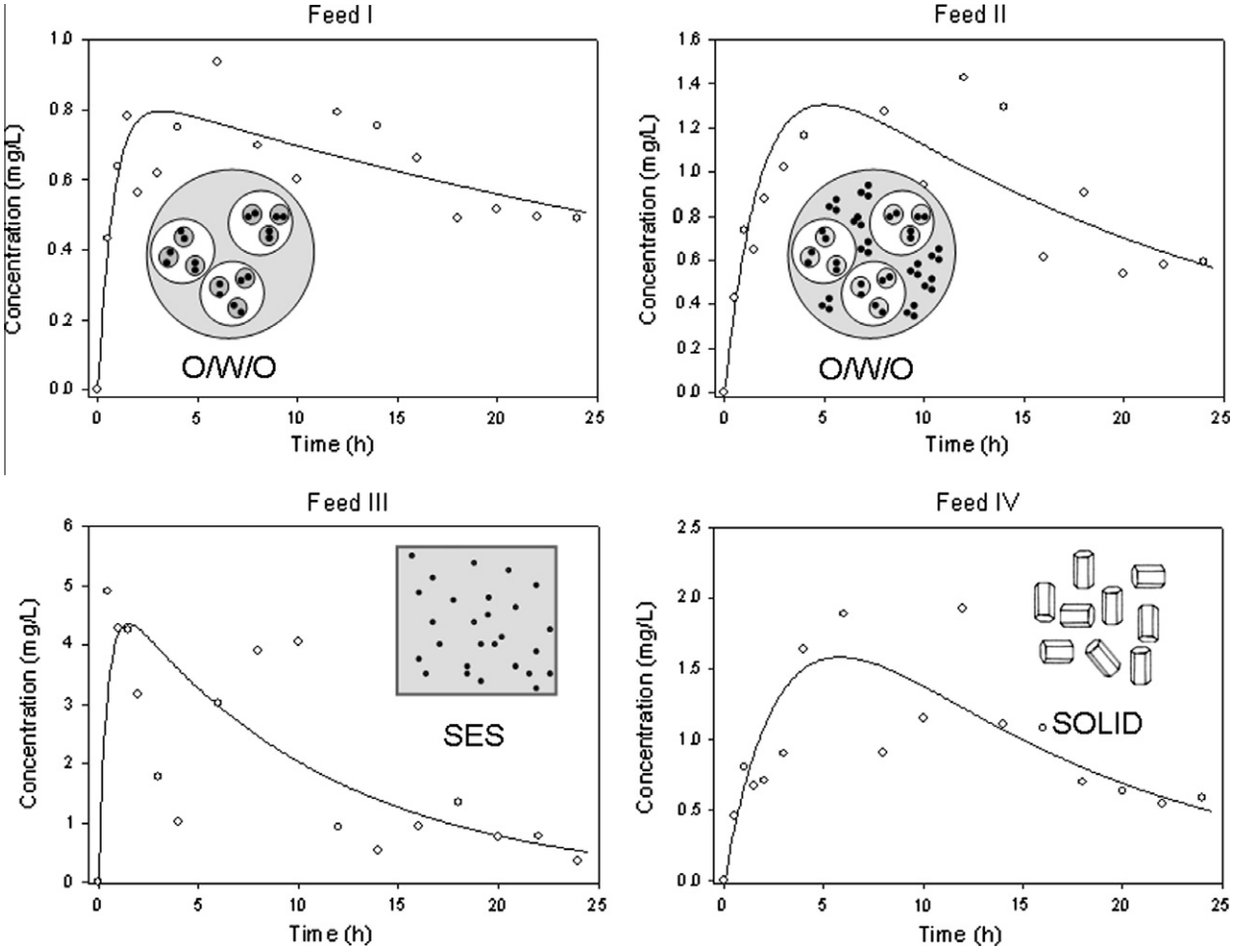

Fig. 1. Fitting of the one-compartment model (solid line) to the experimental drug concentration–time data (circles) following oral administration of four medicated feeds in rainbow trout. Each experimental point is a pooled sample from five animals.

Table 4
Parameter estimates (CV%) of erythromycin pharmacokinetics in rainbow trout following oral administration of four medicated feeds.

Parameter	Feed I	Feed II	Feed III	Feed IV
V_d/F (l kg^{-1})	15.1(9.0)	12.4(17.5)	3.7(24.0)	8.5(28.0)
k_a (h^{-1})	1.297(24.1)	0.526(29.6)	2.147(95.3)	0.325(39.4)
k_e (h^{-1})	0.0220(30.2)	0.0478(29.0)	0.0953(20.5)	0.0772(34.0)
Cl/F (l h^{-1} kg^{-1})	0.333(22.8)	0.595(14.5)	0.351(13.0)	0.653(10.6)
$t_{1/2ka}$ (h)	0.53(24.1)	1.32(29.6)	0.32(95.2)	2.14(39.4)
$t_{1/2ke}$ (h)	31.4(30.2)	14.5(29.0)	7.3(20.5)	9.0(34.0)
t_{max} (h)	3.20(15.2)	5.01(13.8)	1.52(66.3)	5.81(13.5)
C_{max}[a] (mg l^{-1})	1.23(6.7)	1.27(9.5)	4.70(19.2)	1.51(10.5)
AUC[a] (mg l^{-1} h)	60.0(22.8)	33.6(14.5)	57.0(13.0)	30.6(10.6)
F_r (%)	196(25.1)	110(18.0)	186(16.8)	100(15.0)

[a] Normalized to the dose 20 mg kg^{-1}.

dosing, followed by prolonged slow absorption. The resulting plasma concentration profile had short t_{max}, compatible with the rapid onset of therapeutic effect, followed by slow decline in systemic drug concentration ensuring prolonged action.

DME II (feed II) exhibited similar characteristics as DME I with respect to the absorption rate. Terminal half-life was longer than following the administration of the reference feed (feed IV) demonstrating prolonged erythromycin absorption. However, there was little improvement in the extent of erythromycin absorption. It appears that erythromycin loaded in the inner phase ensured prolonged release, while the portion of erythromycin loaded in the outer oily phase was more readily absorbed and presumably subjected to degradation.

3.3. Simulation of multiple dosing

Pharmacokinetic parameters of erythromycin estimated from the data after administration of a single dose of medicated feeds were used to predict plasma concentration profiles with multiple dosing for 7 consecutive days. Results of the simulation for feeds I, III and IV are presented in Fig. 2A. Plasma concentration profiles during a dosing interval in the steady-state are shown in Fig. 2B.

Among the many ways to optimize antimicrobial treatment, pharmacokinetic–pharmacodynamic relationships have received particular emphasis with very good results. Analyses of these relationships have led to the optimizations of dosage regimen and reduction of the selection of resistant bacteria. A large number of studies in the last 20 years have studied the pharmacokinetic–pharmacodynamic properties of the major class of antibiotics. The results of these studies suggest that antibiotics can be classified into two major groups: those that exhibit concentration-dependent effect and those that exhibit time-dependent effect. Macrolide antibiotics, including erythromycin, belong to time-dependent effect group. Therefore, serum concentrations above the minimum inhibitory concentration (MIC) for at least 50% of the dosing interval are the major determinant of bacteriologic cure rate [4,24]. In order to calculate the target exposure, multiplication of the MIC determined *in vitro* by a factor of 4 was suggested [25]. In a recent study in *Lactococcus garvieae* strains isolated from cultured rainbow trout, MIC of erythromycin ranged between 0.06 and 0.125 μg/mL [26].

Our simulation results indicate that adequate erythromycin exposure is achieved with feed I and feed III at a dose rate of 50 mg/kg bw/day. With such dosing plasma concentration was above the target concentration (four times MIC) more than half of the daily dosing interval. On the other side, when erythromycin powder is used at such dosage rate, plasma concentrations are continuously below the target concentration. However, further studies, preferably with treatment success as an endpoint, are necessary to confirm this advantage of the delivery systems developed in this study.

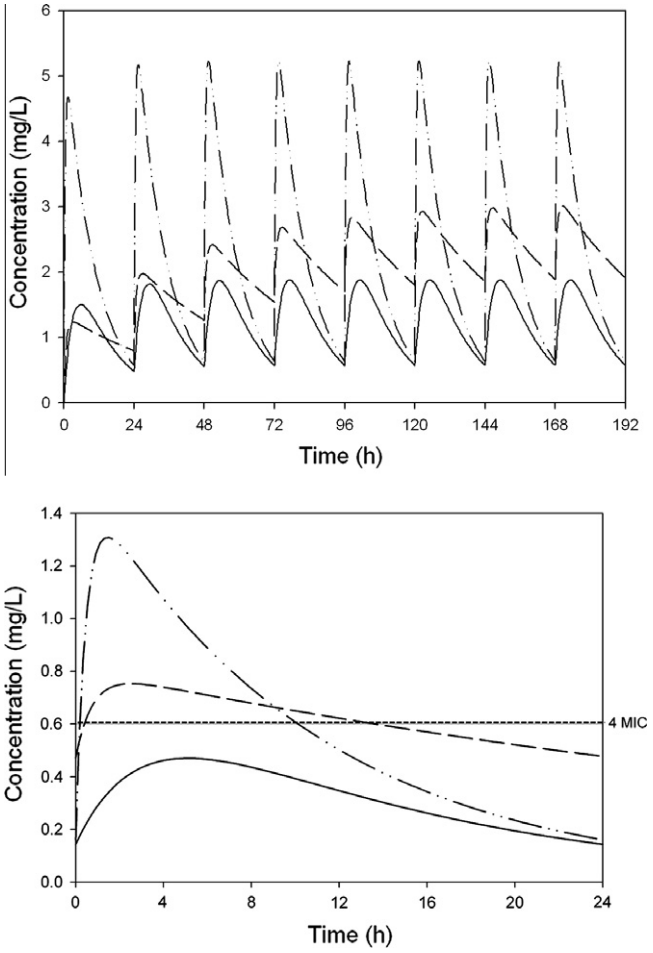

Fig. 2. Simulated plasma concentration profiles of erythromycin with multiple dosing of erythromycin 20 mg/kg/day (upper panel) formulated as feed I (dashed), feed III (dash-dot-dot) and feed IV (solid). Erythromycin concentration–time course during a dosing interval in the steady-state at a dose rate of 5 mg/kg/day (lower panel) with four times MIC as target concentration (reference line).

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