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Pharmacokinetics, efficacy prediction indexes, and residue depletion of ribavirin in Atlantic salmon's (*Salmo salar*) muscle after oral administration in feed

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Ribavirin is an antiviral used in human medicine, but it has not been authorized for use in veterinary medicine although it is effective against infectious salmon anemia (ISA) virus, between others. In this study, we present a pharmacokinetic profile of ribavirin in Atlantic salmon (*Salmo salar*), efficacy prediction indexes, and the measure of its withdrawal time. To determine the pharmacokinetic profile, fishes were orally administered with a single ribavirin dose of 1.6 mg/kg bw, and then, plasma concentrations were measured at different times. From the time-vs.-concentration curve, Cmax = 413.57 ng/mL, T_{max} = 6.96 h, AUC = 21394.01 μg·h/mL, $t_{1/2}$ = 81.61 h, and K_{10} = 0.0421/h were obtained. Ribavirin reached adequate concentrations during the pharmacokinetic study, with prediction indexes of C_{max}/IC_{50} = 20.7, AUC/IC_{50} = 1069.7, and $T>IC_{50}$ = 71 h, where IC is the inhibitory concentration 50%. For ribavirin depletion study, fishes were orally administered with a dairy dose of 1.6 mg/kg bw during 10 days. Concentrations were measured on edible tissue on different days post-treatment. A linear regression of the time vs. concentration was conducted, obtaining a withdrawal time of 1966 °C days. Results obtained reveal that the dose of 1.6 mg/kg bw orally administered is effective for ISA virus, originating a reasonable withdrawal period within the productive schedules of Atlantic salmon.

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INTRODUCTION

Due its successful and accelerated growth, the salmon industry in Chile has become an important economic activity, currently ranking fourth within the export sector in the country. Currently, Chile is one of the major producers and exporters of salmon worldwide; it is the second largest global salmon producer, accounting for 27% of worldwide production, surpassed only by Norway, which accounts for 52% of worldwide production (Barton & Fløisand, 2010; Salmonchile, 2013). During 2007, the exponential growth of Atlantic salmon production suffered a severe downturn, due to the first reported 'infectious salmon anemia' (ISA) outbreak in the region of Los Lagos, Chile. As a result, there was a marked decrease in salmon aquaculture production in Chile, from 386 000 tons in 2006 to 98 000 tons in 2010. In the following years, the virus spread quickly, causing a health crisis in the sector with a financial loss valued between US$34 and 64 million in 2008, and over US$ 2 billion between 2009 and 2011 (Asche *et al.*, 2009; Barton & Fløisand, 2010; Mardones *et al.*, 2011; Bustos-Galllardo, 2013). According to the Chilean National Fisheries Service, the last known ISA outbreak occurred at a farming center in Chiloé, Region X, in January 2014 (Sernapesca, 2014).

The ISA virus belongs to the *Orthomyxoviridae* family, and is a pathogen that primarily affects Atlantic salmon grown in

captivity, causing multisystemic disorders. ISA outbreaks occur predominantly during the saltwater stage in ocean sites, and virus transmission has been described as vertical as well as horizontal. Outbreaks vary in terms of the clinical manifestation and mortality rates, with mortality reaching 100% in the most aggressive cases. These characteristics have made ISA one of the most dangerous infectious diseases in salmon farming, as measured by economic impact (Mardones *et al.*, 2011; Rivas-Aravena *et al.*, 2011; Vike *et al.*, 2014).

Currently, ISA is controlled through several biosecurity measures during both production and transportation. These measures are focused on controlling the different pathways of horizontal transmission, the dominant dissemination pathway of the virus (Scheel *et al.*, 2007; Lyngstad *et al.*, 2008). Among various biosecurity measures, physicochemical disinfectants have been proven effecting in reducing the virus's infectivity (e.g. exposure to temperatures over 50 °C, pH 4, UV radiation, ionophores, chloramine-T, and sodium hypochlorite, among others). However, commercially available vaccines have had mixed results, with outbreaks reported in vaccinated salmon populations (Torgersen, 1998; Smail *et al.*, 2004; Gómez-Casado *et al.*, 2011). To date, no antiviral treatment has been proved effective at controlling the disease (García *et al.*, 2013).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Fig. 1) is a guanosine analogue that inhibits replication of several RNA and DNA viruses, from the families *Herpesviridae, Poxviridae, Paramyxoviridae, Ortomixoviridae, Flaviviridae, Caliciviridae, Coronaviridae, Rhabdoviridae, Birnaviridae, Reoviridae, Arenaviridae*, and *Bunyaviridae* (Sidwell *et al.*, 1972; McCormick *et al.*, 1986; Hudson *et al.*, 1988; Shigeta *et al.*, 1992; Jashés *et al.*, 1996; Merck, 1999; Lanford *et al.*, 2001; Cooper *et al.*, 2003; Severson *et al.*, 2003; Day *et al.*, 2005; Chang & George, 2007; DeWitte-Orr & Bols, 2007; Marroquí *et al.*, 2007; Rockx *et al.*, 2010; Kim & Lee, 2013). The exact mechanism of action remains unknown, but evidence indicates that it inhibits messenger RNA synthesis (Merck, 1999). The *in vivo* effectiveness of the drug has been proven in rats and mice (Colombo *et al.*, 2011; Dong *et al.*, 2013; Noshy *et al.*, 2013); however, ribavirin has not been authorized for use in any non-human animal species. Whereas, in human medicine, ribavirin has been approved for use in the treatment of respiratory syncytial virus, influenza A and B, and herpes simplex virus types 1 and 2, among others (Merck, 1999).

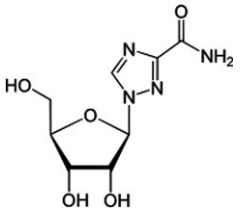

Fig. 1. Ribavirin chemical structure (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide).

In the case of viral fish pathogens, ribavirin would drastically affect the infective cycle of some viruses, such as the causative organism of viral hemorrhagic septicemia, the infectious pancreatic necrosis virus, and the chum salmon virus (Hudson *et al.*, 1988; Jashés *et al.*, 1996; DeWitte-Orr & Bols, 2007; Marroquí *et al.*, 2007). Studies carried out on Atlantic salmon have proven that ribavirin inhibits the ISA virus *in vitro* as well as *in vivo*, perceptibly reducing mortality in infected salmon. These studies have also determined that the effective concentration 50% (EC_{50}), the inhibitory concentration 50% (IC_{50}) and IC_{90} are lower than previously reported for other viruses and antivirals (Leyssen *et al.*, 2005; Rivas-Aravena *et al.*, 2011). These data indicate that ribavirin is an ideal candidate for use as palliative treatment for infectious salmon anemia caused by the ISA virus.

Regarding the ribavirin pharmacokinetics, previous studies carried out in humans have shown that the drug is quickly absorbed after oral administration and it presents a bioavailability of 50%, due to the fact that it is processed through enterohepatic circulation. Nevertheless, its absorption increases when administered along with high-fat foods. The volume of distribution is approximately 28.6 L/kg, and it tends to accumulate in blood cells and tissues, although it does not bond to plasmatic proteins. There are two pathways of ribavirin metabolism: (i) reversible phosphorylation and (ii), a degradation pathway through deribosylation and hydrolysis of the amide. Ribavirin and it metabolites are excreted by the kidneys, with an elimination half-life of 298 h (Glue, 1999).

To predict the efficacy of ribavirin as an anti-infective agent, and in order to optimize therapeutic treatment, the pharmacokinetic/pharmacodynamic (PK/PD) properties of the drug must be considered and the optimum level of drug exposure should be associated with the susceptibility of the agent (Preston *et al.*, 2003; Canut *et al.*, 2015). For this reason, the minimum inhibitory concentration (MIC) should be compared for every agent with systemic activity after its administration. Currently, the most frequently used parameters to express the PK/PD relationship are C_{max}/MIC (maximum serum concentration/MIC), %T > MIC (fraction of time in which concentration exceeds MIC) and AUC/MIC (area under the inhibitory concentration–time curve/MIC) (EMA, 2013). Nevertheless, unlike for antibiotics, there is no standard pharmacodynamic parameter to measure the susceptibility of an antiviral, such as MIC. Therefore, the Food and Drug Administration (FDA, 2006) recommends that to establish the efficacy of an antiviral, the parameters IC_{50} or EC_{50}, determined *in vitro*, should be used (Schmidt *et al.*, 2008).

Based on the aforementioned, this is the first study evaluating the pharmacokinetics of a single ribavirin dose in *Salmo salar* after oral administration by pellets in feed. The results allow us to determine: (i) whether these concentrations are higher than previously established IC_{50} and IC_{90}, values, and (ii) the therapeutic efficacy of the antiviral based on the PK/PD index. In addition, we determined the withdrawal time (WDT) for muscle + skin necessary for ribavirin through a residue depletion study.

MATERIALS AND METHODS

This study was carried out at the Veterinary Pharmacology Laboratory, part of the Department of Livestock and Veterinary Sciences at the University of Chile, accredited under ISO 17025 (INN-CHILE, 2006).

Pharmaceutical formulation

A commercial ribavirin formula (Virotop®, Diagnotec Laboratory, Santiago, Chile) was used, administered by pellets. This formulation includes the active ingredient of the ribavirin, developed as oral powder to be mixed with pellets through an oil impregnation process, with the purpose of facilitating oral administration and thereby improving its bioavailability. Prior to the study, the content of ribavirin was verified in the solutions. For this purpose, standard calibration curves of certified standards of these drugs were performed.

Animals

One hundred and fifty smoltified *Salmo salar* fish were used, with an average weight of 70 g. Fish were kept in 2000-L salt water pools at 10.4 °C, with 25 ppt in recirculation with a flux of 120 L/min. Animals were fed every 24 h with a feed ration corresponding to the 0.5% of their body weight, with a diet of approximately 50% protein, 21% lipid, 11% carbohydrates, 10% ash, 8% humidity, 0.7% fiber, and 22 MJ/kg of gross energy. The salmon were maintained and euthanized, through concussion, according to animal welfare conditions as recommended by the European Agency for the Evaluation of Medicinal Products (EMA, 2009) and the Directive 2010/63/EU (European Union, 2010).

Fish were divided in two groups, for the two different phases of the study: 80 specimens were used to determine ribavirin's pharmacokinetic profile and 70 were used in the ribavirin residue depletion study. Both groups were kept in the same recirculation units, but for at different times, with an interval of time in between to clean the systems. Salmon from the pharmacokinetic study were separated into two groups: the treated group, where fish received a single dose of 5% ribavirin medicated feed of 3.5 g/fish, equivalent to a 1.6 mg/kg of body weight (bw); and the control group, where fish received pellets without ribavirin.

The fish consumed the food within 10 min, and which point measurement began. Sampling was carried out at 10 different time points: 1, 3, 6, 8, 12, 18, 24, 36, 48 and 72 h after medicated pellet ingestion. At each sampling time-point, six fish from the treated group and two from the control group were tested. Blood plasma samples were extracted from each animal, and stored in freezers −20 °C until analysis.

For the ribavirin residue depletion study, 70 salmon were used under the same characteristics of the pharmacokinetic study fish. Fish were divided into two groups: the treated group, where fish received a daily dose of 5 g of ribavirin/100 g pellet, equivalent to a 1.6 mg/kg bw, for 10 days; and the control group, where fish received fodder without ribavirin. Sampling was carried out at five time points: 1, 39, 50, 64 and 85 days after the initiation of treatment. During each sampling, 12 fish were tested from group A, and two from group B. Muscle tissue + skin samples were extracted from each fish in natural proportions, according to EMA (2009) recommendations, and were stored in freezers at −20 °C until analysis.

Chemical reactants and standards

The ribavirin 'purity standard' GmbH® (Augsburg, Germany) was used for the study (provided by Dr. Ehrenstorfer). As an internal standard (I.S), we used ribavirin [^{13}C](1-β-D-(1′-^{13}C, 3′-^{13}C, 4′-^{13}C, 5′-^{13}C)-ribofuranosyl-1,2,4-triazole-3-carboxamide, synthesized by Moravek Biochemicals Inc. (Brea, CA, USA). Methanol HPLC grade, analytical grade ammoniac, analytical grade ammonium acetate, analytical grade perchloric acid, and analytical grade acetic acid were purchased from Merck (Darmstadt, Germany). Acid phosphatase was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Standards and working solutions

Standard ribavirin solution and I.S. were prepared using HPLC-grade water, using a proportion of 1 mg/mL and 1 μg/mL, respectively. They were both stored in darkness, refrigerated at 4 ± 2 °C, over a period of less than 3 months. Calibration curves were calibrated to the matrices using a standard solution prior to extraction.

Sample preparation

Extraction was carried out according to the methods described by Yeh *et al.* (2005). For muscle + skin, 100 mg was measured and in the case of plasma, 500 μL was put into 10-mL glass test tubes. I.S. and 1.8 mL of 5% perchloric acid in methanol was added to each sample. Then, samples were agitated with vortex formation for 5 min, sonicated for 5 min and centrifuged at 492 ***g*** for 5 min. Supernatant was transferred to a different glass tube and added to a mixture of 75% ammonium acetate 0.1 M at 4.8 pH and 25% ammonium, until pH was adjusted to 4.5. With the exception of positive and negative controls, 800 μL of acid phosphatase was added to each sample and then samples were left incubating at 37 °C for 1 h. Subsequently, samples were run through SPE Sep-Pak® Classic NH2 columns (Waters Corporation, Mildford, MA, USA), and fluids were collected in glass tubes. Fluids were dried under a mild nitrogen flux at 45 °C ± 5 and residues were reconstituted with 100 μL of mobile phase (MP). Tubes were agitated for 5 min, sonicated for 5 min, and the content was transferred to 1.5-mL Eppendorf® tubes (Sigma-Aldrich Quimica Ltda., Santiago, Chile). Eppendorf® tubes were centrifuged at 11 337 ***g***, and their content was transferred to HPLC vials. An aliquot of 50 μL was injected into the chromatography system. Residues were separated by liquid chromatography and identified by mass detection (HPLC MS/MS). The limits of detection

(LOD) were established as 0.05 μg/g for muscle + skin and 0.05 μg/mL for plasma. The limits of quantification (LOQ) were established as 0.07 μg/g for muscle + skin, and 0.07 μg/mL for plasma.

Liquid chromatography

Ribavirin was analyzed using a HPLC Agilent 1200 system, equipped with a binary pump, an autosampler, and a deaerator (Agilent, Waldbronn, Germany). Chromatographic separation was reached with a Chromolith RP-18E column (4.6 mm × 100 mm diameter; Merck) with a Chromolith RP-18E precolumn (4.6 mm × 10 mm; Merck). The mobile phase consisted of 0.1% acetic acid in water, and the chromatography was carried out at 30 °C, with a mobile phase flux of 0.6 mL/min.

Mass spectrometry

The Sciex API 4000 mass spectrometer (AB Sciex, Concord, ON, Canada) was used for detection. Ribavirin ionization was carried out using the Turbospray ion-positive mode under the following conditions: Nitrogen (N_2) gas curtain at 20 psi, ion source gases at 1 and 2, and 40 and 35 psi, respectively, source temperature at 400 °C, ion spray at 5500 V, and a collision gas pressure of 4 psi. MS data on precursor and product ions were collected in multiple reactions monitoring (MRM) mode. Product and precursor ions, declustering potential, entrance potential, collision energy, and cell exit potential are shown in Table 1.

Validation of analytical method

Previous to the determination of concentrations in plasma and muscle + skin, and to establish the WDT, a validation of the analytical method was carried out by HPLC MS/MS, according to the instructions of the European Commission Decision 2002/657/EC (2002). Precursor ions and the two product ions were identified for ribavirin. Values for essential parameters were estimated for the validation of the analytical method on both matrices: specificity, recovery, repeatability, intralaboratory reproducibility, decision limit (CCα), detection capability (CCβ), and linearity.

Quantification of experimental samples

Ribavirin concentrations in plasma and muscle + skin were calculated using a linear regression equation from the calibration curves adjusted to matrices ($r > 0.99$) of different concentrations, to avoid extrapolation. The concentration ranges were determined to be 0.05–0.4 μg/g and 0.4–1 μg/g for muscle + skin, and 0.05–0.4 μg/mL and 0.4–1 μg/mL for plasma.

Table 1. Multiple reactions monitoring (MRM) ribavirin analysis

Precursor ion	Product ions	DP	EP	CE	CXP
245.1	113.2*	86	10	13	8
	96.0†			37	6

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, cell exit potential.
*Quantification ion, †Confirmation Ion.

Data analysis

The mean values of the concentration of ribavirin in plasma, vs. time data were sequentially fitted to 1-, 2-, and multiple-compartment models, using the computer program WinNonlin (Version 6.3; Pharsight Corporation, Mountain View, CA, USA). The best-fit model was selected as that with the lowest Akaike's information criterion (AIC) value (Yamaoka *et al.*, 1978). The two-compartment model was the best fit for the plasma concentration–time curve. This model was used to establish kinetic characteristics. After a single oral administration, the plasma curve for ribavirin was fitted to the following exponential equation:

$$C = A_1 \times e^{-\alpha t} + A_2 \times e^{-\beta t} - A_3 \times e^{-Kat},$$

where C is the concentration of compounds; A_1, A_2, and A_3 are mathematical coefficients (*i.e.* A_1 and A_2 are the plasma or erythrocyte concentrations extrapolated to time zero of the first and second elimination phases of the compound and A_3 for the absorption phase); α is the hybrid rate constant for the distribution phase; β is the hybrid rate constant for the elimination terminal phase (*i.e.* α and β are the slopes of the first and second elimination phases of the compound disposition); K_a is the first-order absorption rate constant and t is time. Absorption half-life ($t½_a$), half-life of the α phase ($t½_\alpha$), half-life of the β phase ($t½_\beta$), distribution rate constants for transfer of the compound from the central to the peripheral compartment (K_{12}) and from the peripheral to the central compartment (K_{21}), and the elimination rate constant (K_{10}) were calculated by use of standard equations as described by Wagner (1975). After oral administration, the area under the concentration–time curve (AUC) was calculated as follows:

$$\mathrm{AUC} = (A_1/\alpha) + (A_2/\beta) - (A_3/K_a).$$

Maximum plasma concentration (C_{max}) after ribavirin oral administration and the time at which C_{max} was achieved (T_{max}) was determined directly from the concentration-vs.-time curves.

Therapeutic prediction indexes of ribavirin

The most frequently used therapeutic efficacy indexes were determined for antimicrobials as described by EMA (2013: time over IC (T>IC, where IC is the inhibitory concentration), the relationship between the maximum concentration and IC (C_{max}/IC), and the relation between the area under the curve and IC (AUC/IC). For this purpose, IC_{50} and IC_{90} values determined for the ISA virus were used (Rivas-Aravena *et al.*, 2011).

Determination of withdrawal time

Seventy salmon with the same characteristics as the pharmacokinetic study group were used and maintained under the same conditions, divided into a treatment and a control group. Samples were taken at five time points and muscle + skin tissue was sampled in natural proportions according to EMA's recommendations (2009).

EMA's (1996) recommendations were considered to determine the number of experimental specimens necessary, as well as the WDT. These parameters were calculated from linear regression analyses on the muscle + skin concentrations, transformed into logarithms and established as the time in which the tolerance limit of 95% remained below the maximum residue limit (MRL), with 95% confidence. WDTs were determined as temperature °C-days (EMA, 2011).

RESULTS

Validation parameters

The results for the validation parameters are shown in Table 2.

Quantification of plasma ribavirin levels

Plasma calibration curves were linear ($r > 0.99$), and they were used for the quantification of experimental samples. Concentrations reached in plasma are shown in Table 3. Figure 2 shows a semi-logarithmic graph, assuming an IC_{50} of 0.02 μg/mL and an IC_{90} of 0.4 μg/mL, as established by Rivas-Aravena *et al.* (2011). Bars represent the mean concentration of the six salmon analyzed during each sampling.

Pharmacokinetic parameters obtained from the concentration vs. time profiles

Pharmacokinetic parameters were calculated from the concentration average curve of plasma vs. time, using version 6.3 of the WinNonlin software (Table 4).

Table 2. Validation parameters for ribavirin on muscle + skin and plasma samples

Parameters	Values	
	Plasma (μg/mL)	Muscle + Skin (μg/g)
Recovery (%)	112	110
Repeatability (CV%)	3.4	2.2
Intralaboratory reproducibility (CV%)	10.1	9.3
CCα (decision limit)	0.05	0.05
CCβ (detection capability)	0.07	0.07
Calibration curves	0.05–0.4	0.05–0.4

Therapeutic efficacy prediction indexes of ribavirin

Table 5 shows the values of the three most frequently used indexes of therapeutic efficacy prediction, obtained from pharmacokinetic parameters and IC_{50} and IC_{90} values.

Determination of withdrawal time

Detected ribavirin concentrations on muscle + skin in salmon treated with a dose of 1.6 mg/kg bw for 10 days reached an mean value of 2.82 μg/g on the first day post-therapy, decreasing progressively until the LOD was reached around day 156 post-treatment. Linear regression analysis of muscle + skin ribavirin depletion is shown in Fig. 3. Based on EMA's guidelines (1996), WDT corresponds to 1966 °C day (equivalent to 189 days, at a temperature of 10.4 °C), considering a tolerance limit of 95% and 95% confidence (Table 6).

DISCUSSION

The ISA virus is a pathogenic agent that primarily affects Atlantic salmon grown in captivity with devastating consequences for the salmon farming industry, due to the disease's high mortality rates and its quick dissemination between indi-

Table 3. Ribavirin concentrations reached in Atlantic salmon (*Salmo salar*) plasma after oral administration of a 1.6 mg/kg bw dose in fodder. Six fish were measured per sampling time

Sampling time (h)	Concentrations (ng/mL)							
	Salmons							
	1	2	3	4	5	6	Average	Standard deviation
1	102.8	137.0	168.1	131.9	87.4	118.2	124.2	28.3
3	194.3	156.1	280.6	197.8	255.6	218.3	217.1	45.0
6	458.4	437.8	443.4	389.7	365.2	444.5	423.2	36.9
9	520.4	461.8	493.7	722.2	940.9	572.1	618.5	182.5
12	289.6	252.9	391.4	242.3	340.7	326.3	307.2	56.7
18	177.3	173.5	137.1	224.2	189.0	222.2	187.2	32.8
24	114.6	166.8	138.8	124.8	158.4	126.0	138.2	20.6
36	117.8	127.6	123.9	109.9	111.5	107.1	116.3	8.2
48	79.2	104.3	107.7	103.1	129.5	137.2	110.2	20.8
72	81.6	83.9	70.6	73.4	70.4	72.5	75.4	5.9

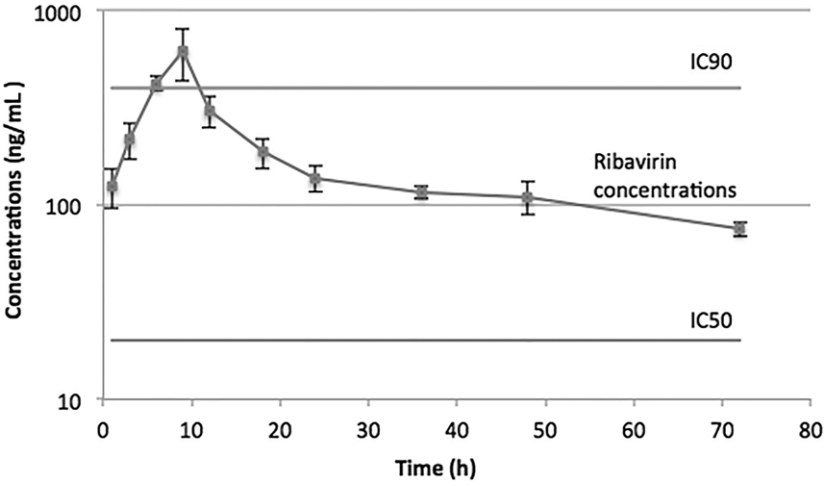

Fig. 2. Plasma ribavirin concentrations on Atlantic salmons (*Salmo salar*) after oral administration in fodder of a 1.6 mg/kg bw single dose. Horizontal lines correspond to inhibitory concentration 50% (IC_{50}) and IC_{90} values considered; vertical lines represent the standard error of each sampling time.

Table 4. Kinetic parameters for ribavirin after a single oral dose of 1.6 mg/kg bw in Atlantic salmon (*Salmo salar*)

Pharmacokinetic parameter	Value
A_1 (ng/mL)	3236.04
A_2 (ng/mL)	142.54
A_3 (ng/mL)	3378.58
α (h^{-1})	0.142
β (h^{-1})	0.0085
K_a (h^{-1})	0.186
$t_{1/2a}$ (h)	3.72
$t_{1/2\alpha}$ (h)	4.88
$t_{1/2\beta}$ (h)	81.61
K_{12} (h^{-1})	0.0798
K_{21} (h^{-1})	0.0286
K_{10} (h^{-1})	0.0421
AUC (μg·h/L)	21 394.01
C_{max} (ng/mL)	413.57
T_{max} (h)	6.96

Table 5. Therapeutic efficacy indexes calculated on salmon for a single ribavirin dose of 1.6 mg/kg orally administered in fodder, using both IC determined for ISA virus

Therapeutic efficacy index	IC_{50} value	IC_{90} value
C_{max}/IC	20.7	1.0
AUC/IC	1069.7	53.5
T > IC (h)	71 h	5.3 h

viduals and fish farms (Mardones *et al.*, 2011; Vike *et al.*, 2014). To date, there is no effective treatment for the virus, although there are studies proving the effectiveness of ribavirin as an antiviral, by effectively reducing mortality among infected salmon (Leyssen *et al.*, 2005; Rivas-Aravena *et al.*, 2011; García *et al.*, 2013). Ribavirin is the only antiviral with a proven spectrum of action against DNA and RNA viruses, and it is currently being used for treatment of influenza, pneumonia caused by respiratory syncytial virus, and chronic hepatitis C, among others (Sidwell *et al.*, 1972; Merck, 1999). Although its effectiveness has been proven against viral disease in laboratory animals, this molecule is not authorized for use in veterinary medicine. The present study is the first to show ribavirin's pharmacokinetics administered by pellet on salmon, estimating its efficacy against the ISA virus and also determining its withdrawal time in muscle + skin.

Validation of the analytical method was carried out based on the EU Commission's decision 2002/657/CE (EC, 2002), and the calibration of equipment was carried out considering the ISO/IEC guidelines 17025:2005 and ILAC (2010), where functioning of the analytical methods and result interpretation are indicated, in order to guarantee their quality. Prior to the study, the selected method to quantify ribavirin concentrations on plasma and muscle + skin in Atlantic salmon (*Salmo salar*) was validated according to the European Commission Decision guidelines 2002/657/EC (EC, 2002), and all the validation parameters met the established criteria by this norm. This analytic method has been used in a prior study to determine antiviral residues on monkey's livers (Yeh *et al.*, 2005).

Concentrations reached in plasma after an oral ribavirin dose of 1.6 mg/kg bw administered by pellet were evaluated. This dose reached a successful AUC of 21 394.01 μg·h/L and a C_{max} of 0.414 μg/mL (Table 2). The T_{max} of 6.96 h indicates an unexpectedly long absorption phase (as in humans absorption has been described as quick), which could be associated with the slow release of the antiviral (Glue, 1999). When analyzing the behavior of the semi-logarithmic curve of concentration vs. time (Fig. 2), it can be concluded that it is a two-compartment model, as a rapid distribution phase to the central compartment is observed, followed by a slow distribution phase to the peripheral compartment (DiPiro *et al.*, 2010). The elimination rate constant of 0.0421/h and the vast half-life elimination, equal to 81.61 h (Table 2), cause slow excretion of the antiviral, probably due to the fact that ribavirin does not bind to plasmatic proteins, but rather is widely distributed within cell compartments (Glue, 1999). According to Khakoo *et al.* (1998), this could explain, at least in part, the prolonged elimination phase of the drug, as it is slowly eliminated from 'deep compartments', associated with the great

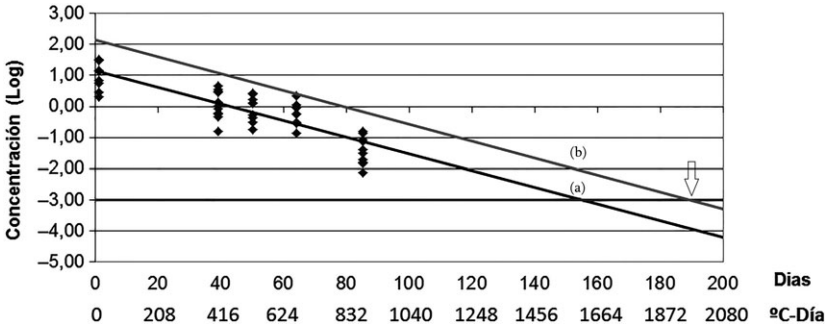

Fig. 3. Ribavirin WDT determination in *Salmo salar* muscle ($r = -0.8396$) after oral administration of 1.6 mg/kg bw during 10 days. Each point represents an individual concentration of the antiviral measured a day. (a) linear regression line; (b) tolerance limit of a 95% with a 95% confidence. Arrow indicates time in which WDT was calculated, according to LOD.

Table 6. Ribavirin concentrations in *Salmo salar* muscle after oral administration of 1.6 mg/kg bw during 10 days

	Sampling time (days)				
Salmon	1	39	50	64	85
1	3.15	1.74	1.11	0.99	0.34
2	2.10	0.71	1.11	0.58	0.12
3	4.41	0.79	0.69	0.78	0.16
4	2.32	1.59	0.60	0.97	0.16
5	3.14	1.03	0.47	0.78	0.17
6	4.52	1.61	1.25	1.39	0.18
7	1.58	1.94	1.12	0.77	0.43
8	1.35	0.81	1.51	0.60	0.22
9	ND	1.14	0.76	0.42	0.33
10	ND	0.94	1.47	1.05	0.45
11	ND	0.45	ND	0.95	0.25
Average	2.82	1.15	1.01	0.84	0.26

ND, nondeterminated.

volume of distribution described in humans, equal to approximately 28.6 L/kg (Glue, 1999). Once inside nucleated cells, ribavirin is phosphorylated, and then, a slow dephosphorylation process occurs, contributing to generate an extremely long final phase and a vast half-life, which in the case of humans reaches up to 40 days (Page & Connor, 1990; Khakoo *et al.*, 1998; Glue, 1999; De Franceschi *et al.*, 2000). Another factor promoting an extended elimination phase is the temperature at which salmons were maintained, 10.4 °C. In contrast, the recommended temperature for the species is around 12–15 °C: This lower temperature likely caused a decrease in their metabolism (EMA, 2011).

The antiviral activity of ribavirin has not been previously described. Antimicrobial activity can be divided in three general groups: (i) those that show a time-dependent action with minimal to mild persistent effects; (ii) those with a concentration-dependent action with prolonged and persistent effects; and (iii) those that are equally dependent on concentrations over IC value and the period of time in which these concentrations exceed IC. For concentration-dependent antimicrobials, it is expected that $C_{max}/IC > 10$ or an AUC/IC relationship of 25–125, depending on the drug. Whereas for time-dependent antimicrobials the maximal effect is observed at concentrations exceeding IC value 4- to 5-fold, approximately, during at least 40–50% of the administration time interval. Nevertheless, there are situations, such as the case of amprenavir, where an efficacy index of $T/EC_{95} = 80\%$ of the interval between doses is needed. Yet, the measurement parameter for the antivirals' pharmacodynamics still remains erratic and in constant evolution. For this reason, although it is indicated that the IC_{50} value must be used for this calculation, some authors recommend the use of a more rigorous parameter, such as IC_{90} (Mueller *et al.*, 2004; Schmidt *et al.*, 2008; EMA, 2013; Canut *et al.*, 2015). Based on this observation, we used both the IC_{50} and IC_{90} established values for ribavirin against ISA virus in this study, to carry out a complete analysis of the dose under investigation. According to these criteria, when estimating the efficacy indexes for ribavirin using IC_{50}, the ribavirin dose used in this study results in a $Cmax/IC_{50}$ value close to 20, an AUC/IC_{50} value of over 1000, and a $T>IC_{50}$ of 71 h, corresponding to 100% of the measures carried out for the pharmacokinetic study (Table 3). These approximations indicate that ribavirin may be an effective treatment against ISA virus, with a dose of 1.6 mg/kg bw when orally administered by pellet. It is even possible to decrease the dosage, or to administer it every 72 h in a case of time dependency. On the other hand, if the IC_{90} value is considered when calculating the efficacy prediction indexes, this dosage results in very low values compared what is expected for the parameters C_{max}/IC_{90} and $T>IC_{90}$. It is arguable that our result of $AUC/IC_{90} = 53.5$, while it is within the range previously described for antimicrobials in the literature, is positioned among the lower range, besides the fact that it tends to be a value assigned specifically for each virus or bacteria.

It is important to consider that these therapeutic efficacy indexes do not consider PK population variability on IC values of the pathogenic agent; therefore, they are useful only to show what is possible, rather than what is likely to occur. To obtain indexes that consider these variations, studies using data based on different dosages and different IC values or using a simulation medium, such as the statistical modeling method of Monte Carlo, should be considered (Schuck & Derendorf, 2005).

After administering a 1.6 mg/kg ribavirin dose for 10 days, a WDT for muscle + skin, the edible tissue on *Salmo salar*, was determined. A WDT of 1966 °C days was established, a length consistent with the previously described long permanence of the antiviral in the organism. Even though the fact that the WDT amply exceeds the assigned values for other antimicrobials administered to fish, which generally do not surpass 600 °C day (SAG, 2014), a vast WDT should not be an obsta-

cle for the use of ribavirin in salmon farming facilities, since the 6 months of WDT for this therapeutic regime are achievable given that the productive process may last up to 24 months (FAO, 2014).

Also is important to consider the recent evidence in literature about the incidence and development of antiviral resistance in different genera of virus (Hanson & Swaminathan, 2015; Hayashi *et al.*, 2015). Some authors have demonstrated that the antiviral resistance can occur by a selection pressure that leads to therapeutic failures after repeated administrations (Eltahla *et al.*, 2015; Hayashi *et al.*, 2015). Considering this information, it is necessary to think in the possible negative effects that could happen when administering ribavirin in the food and some residues remains in the pools or in the sea, being this a potential causative of the appearance of antiviral resistance to ribavirin in salmon and other aquatic species, such us nowadays is happening with the antibacterial resistance (Huang *et al.*, 2015).

After studying the administration of the antiviral ribavirin in *Salmo salar* for the first time, it can be concluded that when orally administered in feed with a dose of 1.6 mg/kg bw, ribavirin currently becomes the best alternative for the treatment of ISA virus, achieving a prolonged permanence in salmon and appropriate plasma concentrations when considering IC_{50}, which is reflected in advantageous therapeutic indexes for this species. Furthermore, WDT should not considerably affect the slaughtering process and further study including pharmacokinetic analysis of an intravenous administration of ribavirin on salmon is needed, in order to compare it against oral administration, and to determine therapeutic efficacy indexes on dynamic models *in vivo*, based on time–kill curves. Subsequently, an in the field challenge for ribavirin on ISA virus-infected salmon could be carried out, in order to prove its effectiveness in practice.

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