Short Communication

Live-Food Mediated Drug Delivery as a Tool for Disease Treatment in Larviculture. The Enrichment of Therapeutics in Rotifers and Artemia Nauplii

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(Received 27 March 1991; revised version received 8 April 1991; accepted 17 January 1992)

ABSTRACT

The live food enrichment technique using emulsions for correcting nutritional deficiencies in rotifers and Artemia was further investigated as a tool for transferring therapeutics through the food chain. Artemia nauplii and the rotifer Brachionus plicatilis were enriched with a self-emulsifying concentrate in which the therapeutic mixture trimethoprim: sulfamethoxazole was incorporated. An analytical method and protocol for the quantification of trimethoprim and sulfamethoxazole in a heterogeneous biological matrix, such as Artemia, was developed using High Performance Liquid Chromatography. When applying a 24-h enrichment period, the accumulation of both drugs in Artemia nauplii appeared to be linearly related to the concentration of the drugs in the enrichment product. Values of approximately 790 mg/kg of total drugs (protein basis; or 290 mg/kg on dry weight basis) could be obtained. In rotifers, the level of total therapeutics reached a value of about 116 mg/kg (protein basis) after 6 h of enrichment. The ratio of the two drugs detected in rotifers was different from the one found in Artemia, suggesting different accumulation kinetics.

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INTRODUCTION

The incidence of microbial diseases has increased dramatically along with the degree of intensification in the larval production of aquaculture species (Sorgeloos & Léger, 1992). Treating microbial infections in fish and shrimp larvae is most often done by dissolving relatively high doses of broad spectrum antibiotics in the culture water (Brown, 1989). A disadvantage of this method is the large amounts of expensive drugs which are used and discharged in the environment, putting the animal and human health at risk. A direct treatment through the food chain using much smaller quantities may prove more effective and safer for the environment.

Live food organisms, especially Artemia and rotifers, are extensively used as the main food sources for the larviculture of marine fish and shrimp. Both food organisms are filter feeders and have successfully been applied as biological carriers for transferring essential nutrients to predator larvae (Léger et al., 1986).

This paper reports on the determination of the accumulation rate of particular drugs in Artemia and rotifers. The sulfonamides, trimethoprim and sulfamethoxazole, were selected for this study because of their pronounced effect against Gram-negative bacteria (e.g. Aeromonas spp., Vibrio spp., Yersinia spp.), their low degree of toxicity at therapeutic doses, their low degree of resistance induction (Muir & Roberts, 1988) and because of technical reasons, i.e. the two drugs are hydrophobic enough to permit their incorporation in existing enrichment products.

MATERIALS AND METHODS

Artemia and Brachionus plicatilis samples

Artemia nauplii were hatched out of Great Salt Lake cysts at 28°C ± 1°C in filtered (1 µm) natural sea-water (35‰) under continuous aeration and illumination (2000 lx). After 24 h. Instar I nauplii were harvested, separated from hatching debris and thoroughly rinsed. The nauplii were incubated during 24 h at 28°C ± 1°C in sea-water (35‰) to which two rations of 0.3 g/litre of enrichment medium were added at $t_0$ and $t_0 + 16$ h with $t_0$ the moment of incubation (Léger et al., 1987). The enrichment medium consisted of a commercial live food enrichment diet (SELCO; Artemia Systems S.A., Baasrode, Belgium) into which various levels (1%, 5% and 10%) of a mixture trimethoprin (TMP):sulfamethoxazole (SMX) (1:5) were incorporated.
Rotifers, *Brachionus plicatilis*, previously grown on algae (*Chlorella* spp.) and fresh baker’s yeast, were incubated during 6 h in diluted seawater (25%o) at 25°C to which two rations of 50 mg/litre of the above medicated enrichment diet was added at $t_0$ and $t_0 + 3$ h. Samples were taken at $t_0$, $t_0 + 2$ h, $t_0 + 4$ h and $t_0 + 6$ h with $t_0$ the moment of the first enrichment.

After treatment the *Artemia* and rotifers were thoroughly rinsed and stored at −28°C till further analysis.

**Analytical procedures**

A new method was developed for the determination and quantification of the drugs in *Artemia* and *Brachionus plicatilis* (Nelis *et al.*, 1991). Weighed samples were homogenised in methanol. After centrifugation the residue was separated from the methanol fraction. This step was repeated twice on the residue. The three methanol fractions were combined. A protein determination was performed on the residue, in order to express the results on a protein basis, according to a simplified Lowry method (Peterson, 1977). The interfering lipids and carotenoids were removed from the methanol fraction by a double hexane extraction at pH 7·0. The methanol was then evaporated under vacuum. The pH was adjusted to 4·85 with 0·15 m H$_3$PO$_4$ and the mixture was applied on a solid phase C18 column (octadecyl phase). After washing the column with a 0·15 m pH 4·85 ammonium phosphate buffer, the drugs were eluted with a mixture of 35% acetonitrile and 65% 0·15 m ammonium phosphate buffer: pH 4·85. The acetonitrile was evaporated under vacuum and the extract injected on a 15 × 0·46 cm 5 μm Hypersil ODS column (C18, Shandon Southern). The other HPLC operational conditions are as follows: pump and reservoir, Varian 8500 or 5020; UV 10 Vario-Chrom Liquid Chromatograph Detector (Varian). The eluent consisted of a mixture of 17% acetonitrile (15% for rotifers) and 83% (resp. 85%) 0·15 m (NH$_4$)$_2$H$_2$PO$_4$ containing 0·12 triethylamine, adjusted to pH 4·85 with 0·15 m H$_3$PO$_4$. Flowrate: 1 ml/min. Ormetoprim and sulfisoxazole are used as internal standards for trimethoprim and sulfamethoxazole respectively.

Dry weight analyses are carried out by dessicating the biological sample during 24 h at 60°C.

**RESULTS**

Table 1 shows the results of the TMP and SMX accumulation in *Artemia* nauplii. After 24 h enrichment using an enrichment emulsion containing
10% of the therapeutics, the total concentration of TMP+SMX in Artemia exceeds 290 mg/kg on a dry weight basis or 790 mg/kg on a protein basis with a coefficient of variation below 8% \((n=8)\). With the modified Lowry method, the amount of protein in the Artemia nauplii was found to be 37% on dry weight basis (coefficient of variation 2.4% with \(n=7\)). Contrary to the 1:5 ratio (TMP:SMX) in the enrichment medium (1:5.89 as per analysis) this value amounts to 1:2.73 in medicated Artemia nauplii suggesting that TMP accumulates faster than SMX in the Artemia nauplii or is more slowly eliminated.

The accumulation rate of these drugs was further studied in an experiment using enrichment media containing different levels of the therapeutic combination, i.e. 1, 5 and 10% of the TMP:SMX (1:5) mixture. The results presented in Fig. 1 demonstrate a linear relationship between the concentration of the drug mixture in the enrichment medium and the concentration found in the Artemia enriched with this diet. The correlation coefficients for TMP and SMX were \(r=0.99996\) \(r=0.99975\) respectively. The values obtained with the 10% mixture confirm those obtained in the first experiment (see Table 1).

Six hours enrichment of the rotifers with the therapeutic mixture yields an accumulation level of about 116 mg/kg TMP+SMX in the rotifers (Fig. 2). TMP is again accumulating faster than SMX yielding a TMP:SMX ratio of 2.1:1 after 6 h against the 1:5 ratio in the enrichment medium. Contrary to what was found with Artemia, the concentration of TMP in the rotifers is higher than that of SMX.

**DISCUSSION**

In this series of experiments is has been demonstrated that when fed with a medicated enrichment emulsion Artemia nauplii and rotifers efficiently
Fig. 1. Accumulation rate (in ng/mg protein) of trimethoprim (TMP) and sulfamethoxazole (SMX) in *Artemia* nauplii enriched during 24 h with emulsions containing increasing levels of TMP and SMX (1:5).

Fig. 2. Accumulation rate (in ng/mg protein) of trimethoprim (TMP) and sulfamethoxazole (SMX) in rotifers (*Brachionus plicatilis*) fed an emulsion containing 10% TMP and SMX (1:5).

Fig. 3. Accumulation rate (in ng/mg dry weight) of chloramphenicol in the caudal tissue of *Cichlosoma nigrofasciatum* fed one ration of chloramphenicol-enriched *Artemia* nauplii.
accumulate trimethoprim and sulfametoxazole. The accumulation rate of TMP is faster than that of SMX in *Artemia* and even more so in rotifers. Under the given experimental conditions, the accumulation rate of the drug mixture in *Artemia* nauplii is linearly related to its level in the enrichment diet.

The next step of this ongoing study is the determination of the accumulation rate of these drugs into predator tissue. Preliminary results with chloramphenicol have indicated that this transfer is very fast (F. Nin, pers. comm. 1988), i.e. as can be seen in Fig. 3 chloramphenicol quickly builds up in the caudal tissue of *Cichlosoma nigrofasciatum*, reaching a maximal level already 1 h after the fish larvae were fed one single portion of drug-enriched *Artemia* nauplii (i.e. the enrichment emulsion contained 10% chloramphenicol palmitate).

A similar accumulation study is now underway with the TMP–SMX enriched life food for seabass *Dicentrarchus labrax* larvae and *Macrobrachium rosenbergii*.

As a final verification, the effects of food-chain-mediated drug delivery will be evaluated in purposely infected fish larvae.

ACKNOWLEDGEMENTS

This study has been supported by the EEC-project FAR AQ194 GR B UK and the Belgian National Science Foundation.

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