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Liquid Chromatographic Determination of Efficacy of Incorporation of Trimethoprim and Sulfamethoxazole in Brine Shrimp (*Artemia* spp.) Used for Prophylactic Chemotherapy of Fish

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The brine shrimp *Artemia*, an excellent live food source in aquaculture, has been studied as a carrier to deliver selected chemotherapeutic agents to fish for prophylactic treatment of infectious diseases. To monitor the efficiency of incorporation of trimethoprim and sulfamethoxazole in *Artemia* franciscana, a sensitive and specific analytical method was developed. It is based on homogenization of *Artemia* nauplii in methanol, extraction of lipids with hexane, solid-phase cleanup on C_{18} cartridges, and reversed-phase liquid chromatography with detection at 210 nm. The method is sensitive (detection limit, on the order of 3 μg/g with a sample quantity of 30 mg [dry weight]) and reproducible (coefficients of variation, 2.2 and 1.8% for trimethoprim and sulfamethoxazole at levels of 79.6 and 257 μg/g of body weight, respectively). Preliminary quantitative data indicated excellent uptake and persistence of both therapeutic agents in A. franciscana, with levels of 115 μg/g for trimethoprim and 277 μg/g for sulfamethoxazole.

The world's supply of fish and other aquatic organisms is becoming increasingly dependent on aquaculture (17). However, the success of large-scale fish and shrimp farming is commonly jeopardized by the occurrence of infectious diseases, often leading to high mortalities and, hence, serious economic losses (1, 2, 17). Several recent reviews were concerned with the pathogenesis of these diseases and the nature of the causative agents (11, 17). Typical fish pathogens are mostly gram negative and include various species belonging to the genera *Aeromonas*, *Vibrio*, and *Pseudomonas* (11, 17).

Prophylactic treatment of fish currently relies on the addition of chemotherapeutic agents to the feed (1, 19, 20). However, this approach is poorly effective and uneconomical, and it harbors risks for the environment and public health (2). In addition, formulated feeds are not readily accepted by fish in the youngest fry stages, and the drugs may leach out of the feed and/or decompose. A promising new approach to the successful prevention of infectious diseases in fish and crustaceans consists of using live food supplemented with therapeutic agents by a technique of bioencapsulation. The preferred live food for larval fish and shrimp are the nauplii of the brine shrimp *Artemia* spp. (6). The advantage of using this crustacean as a vehicle for therapeutic agents would be a controlled delivery to the fish with no risk for leaching or decomposition of the drugs into the environment.

For the first experimental trial, trimethoprim and sulfamethoxazole were selected as the therapeutic agents because this combination has already been proven to be effective against typical fish pathogens (1). Because, until recently (8, 10), it was not technically feasible to supplement *Artemia franciscana* with these or other drugs, no analytical methods are available to monitor the incorporation efficiency. Nor do high-pressure liquid chromatographic (HPLC) methods exist for the simultaneous determination of trimethoprim and sulfamethoxazole in fish, as opposed to that of the analogous drugs ormetoprim and sulfadimethoxine (19, 20). The latter two compounds have recently been determined in *A. franciscana* by a crude microbiological assay (10). The method described in this paper for *A. franciscana* uses solid-phase extraction with reversed-phase liquid chromatography and detection at 210 nm.

MATERIALS AND METHODS

Chemicals and reagents. Trimethoprim, sulfamethoxazole, and sulfisoxazole were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ormetoprim and tetroxoprim were gifts from Hoffmann-La Roche (Basel, Switzerland). Bond Elut C_{18} extraction columns (500 mg, 2.8 ml) were from Analytichem (Harbor City, Calif.). Acetonitrile, HPLC grade, was from ROMIL (Loughborough, United Kingdom). All other chemicals and reagents were analytical grade and came from E. Merck (Darmstadt, Germany) or Janssen Chimica (Beerse, Belgium).

Animals. *A. franciscana* nauplii (EG grade Great Salt Lake strain; batch 07.32603), which were produced at the Artemia Reference Center by standard procedures (15), were bioencapsulated with trimethoprim and sulfamethoxazole by incubating them for 6 h in the presence of an experimental formulation (medicated Selco; Artemia Systems NV, Ghent, Belgium) containing both therapeutic agents (7, 8).

Preparation of standards. For standardization, stock solutions of trimethoprim and sulfamethoxazole were prepared in methanol. Approximate concentrations were 20 μg/ml for trimethoprim and 107 μg/ml for sulfamethoxazole. The exact concentrations were determined spectrophotometrically as follows. One milliliter of the trimethoprim stock solution was diluted to 10 ml with 1.5 ml of ethanol and 7.5 ml of 0.4% (wt/vol) sodium hydroxide. The absorption was measured at 287 nm ($\varepsilon = 7.4 \times 10^3\ M^{-1}\ cm^{-1}$) (9). For sulfamethoxazole, 1 ml of the stock solution was diluted with 9 ml of 0.1 M phosphate buffer (pH 7.5). The wavelength was 257 nm, and

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the molar absorption coefficient was $1.72 \times 10^4\ M^{-1}\ cm^{-1}$ (13). The concentrations of the respective internal standards (tetroxoprim and sulfisoxazole) were 37 and 81 μg/ml, respectively.

Sample pretreatment. About 300 mg (wet weight) of nauplii was homogenized in 1.5 ml of methanol by using a Potter-Elvehjem apparatus (all glass). After centrifugation at 1,500 × *g*, the supernatant was isolated. The extraction was repeated twice and all supernatants were combined in a 20-ml screw-cap tube. The solid residue was saved for protein determination. The two internal standards (50 μl of tetroxoprim and 100 μl of sulfisoxazole dissolved in methanol) were added together with 1 ml of 0.1 M sodium phosphate buffer (pH 7). Lipids and pigments were extracted twice with 5 ml of hexane, and the hexane layer was discarded. The methanol was removed from the semiaqueous lower layer by applying vacuum (water aspiration) with continuous vortexing of the tubes (Rotary Evapo-Mix; Büchler Instruments, Fort Lee, N.J.). To the aqueous residue was added 0.075 ml of 1 M phosphoric acid and 0.5 ml of 0.5 M sodium phosphate buffer (pH 4.85). The resulting solution was applied on top of a Bond Elut C_{18} extraction cartridge, preconditioned successively with methanol, water, and 0.15 M ammonium phosphate, containing 0.12% triethylamine (pH 4.85). After sample application, the column was washed with 1 ml of 0.15 M ammonium phosphate containing 0.12% triethylamine and was eluted with 3 ml of acetonitrile–0.15 M ammonium phosphate (35:65; vol/vol) containing 0.12% triethylamine (pH 4.85). The acetonitrile was removed under vacuum (Evapo-Mix), and a 50- to 100-μl aliquot was injected onto the liquid chromatographic column.

Chromatography. The liquid chromatograph consisted of an LKB 2150 isocratic pump (LKB, Bromma, Sweden), an *N60 valve injector* (Valco, Houston, Tex.) fitted with a 100-μl loop, an LC 95 variable-wavelength detector set at 210 nm (Perkin-Elmer, Norwalk, Conn.), and an SP 4100 integrator (Spectra-Physics, San Jose, Calif.). The column (15 by 0.46 cm) was packed with 5 μm Hypersil ODS (Shandon, Runcorn, United Kingdom) and was eluted with acetonitrile–0.15 M ammonium phosphate (15:85; vol/vol) containing 0.1% triethylamine (the final pH was adjusted to 4.85 by using 0.15 M phosphoric acid). The flow rate was 1 ml/min, and the temperature was ambient. The absorption spectra of chromatographic peaks were recorded with the aid of an HP 1040 photodiode array detector equipped with an HP 85 computer, an HP 82901M flexible disk drive, and an HP 7470A graphics plotter (all from Hewlett Packard, Palo Alto, Calif.).

Determination of dry weight and proteins. The water content of the *A. franciscana* nauplii was determined by weighing three to five 300-mg samples before and after drying (60°C, 24 h). These samples were taken simultaneously with the ones to be analyzed by HPLC, to prevent differences in water content in the two sets. Alternatively, proteins were determined in the solid residue that remained after extraction of the therapeutic agents by a modified Lowry procedure (12). The solid residue was solubilized with 10 ml of 5% sodium dodecyl sulfate in 0.5 M sodium hydroxide (3 h of incubation at 30°C). The solution was quantitatively transferred to a 100-ml volumetric flask and brought to volume with distilled water. After centrifugation of the mixture at 1,500 × *g*, a clear 0.5-ml sample (adjusted to 1 ml with 0.5% sodium dodecyl sulfate in 0.05 M NaOH) was used for protein determination by the method of Peterson (12). Standardization was carried out with bovine serum albumin.

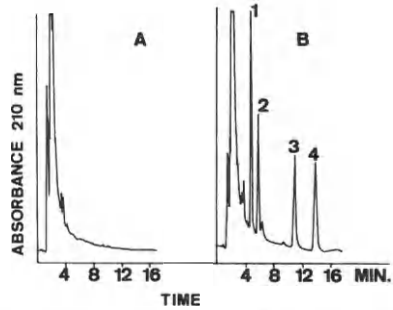

FIG. 1. Chromatograms of extracts of *Artemia* nauplii (EG grade; batch 07.32603). (A) Blank *A. franciscana*; (B) *A. franciscana* supplemented with trimethoprim and sulfamethoxazole. Peak identities were as follows: 1, trimethoprim; 2, tetroxoprim (internal standard for trimethoprim); 3, sulfisoxazole (internal standard for sulfamethoxazole); 4, sulfamethoxazole.

Quantitation. For standardization, 300-mg samples of blank nauplii were homogenized as the unknowns. The homogenates (4.5 ml) were supplemented with known amounts of trimethoprim (1 to 8 μg) and sulfamethoxazole (2.7 to 21.4 μg). After the addition of the internal standards, they were analyzed as the unknowns. Standard curves were constructed by plotting peak height ratios (trimethoprim/tetroxoprim and sulfamethoxazole/sulfisoxazole) versus the respective amounts of trimethoprim and sulfamethoxazole in each sample. The amounts of both therapeutic agents in the unknown samples were calculated by extrapolation from the standard curves. Results were expressed as micrograms of trimethoprim or sulfamethoxazole per gram (dry weight) or per milligram of protein. In the latter case, the concentrations could be extrapolated to dry weight by taking into account the average protein content of dry (lyophilized) *A. franciscana* (EG grade; batch 07.32603) (89.8 ± 0.6%; $n = 3$). However, this value differs from strain to strain.

RESULTS AND DISCUSSION

Representative chromatograms of blank *A. franciscana* and *A. franciscana* supplemented with trimethoprim and sulfamethoxazole through the enrichment procedure are shown in Fig. 1. Trimethoprim, sulfamethoxazole, as well as both internal standards tetroxoprim and sulfisoxazole all eluted in blank positions. Their retention times were 4.9, 6.0, 11.1, and 14.0 min, respectively. Ormetoprim, which was initially used as an internal standard but was later abandoned in favor of tetroxoprim, eluted after 6.2 min. Figure 2 presents the structural formulae of the five compounds. Identification of the trimethoprim and sulfamethoxazole peaks in extracts of *A. franciscana* enriched in both therapeutic agents was based on a comparison of their retention times and absorption spectra with those of authentic standards. Retention times were in close agreement, and the absorption spectra (unknown in relation to the corresponding standard) coincided completely. The experimentally determined absorption maxima were 271 nm for trimethoprim and 269 nm for sulfamethoxazole.

Methanol was used for the initial homogenization of the *Artemia* nauplii because it was clearly superior to aqueous

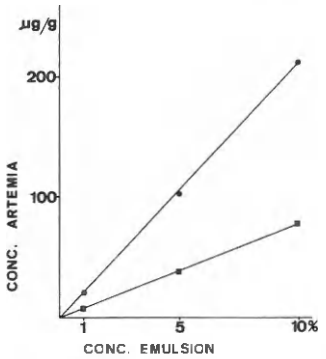

FIG. 3. Relationship between levels of trimethoprim (■) and sulfamethoxazole (●) in *Artemia* nauplii and dosage (concentrations) of trimethoprim and sulfamethoxazole in the enrichment formulation.

method to the analysis of fish larvae fed on *A. franciscana* loaded with trimethoprim and sulfamethoxazole.

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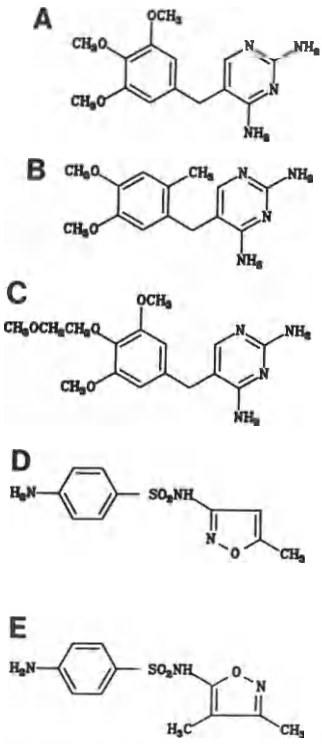

FIG. 2. Chemical structures of trimethoprim (A), ormetoprim (B), tetroxoprim (C), sulfamethoxazole (D), and sulfisoxazole (E).

solvents in terms of its extraction efficiency for both trimethoprim and sulfamethoxazole. However, a relative disadvantage of methanol was its ability to coextract lipids, including high amounts of carotenoid pigments. A major part of these lipophilic constituents was removed by partitioning in hexane, which extracted neither trimethoprim nor sulfamethoxazole. Still, the remaining extract had to be substantially purified, particularly in view of the use of a nonselective detection wavelength. To this end, double-phase extraction, which has been used in connection with HPLC determination of trimethoprim and sulfamethoxazole in plasma (3, 4, 16, 18) and trimethoprim alone in fish (5), was initially tried. However, the impure nature of *Artemia* extracts as well as the failure of ethyl acetate to efficiently extract trimethoprim, unlike sulfamethoxazole, at a pH of <7 led to the dismissal of this approach. Alkaline conditions increased the extraction yield of trimethoprim but caused its degradation in the course of the evaporation of the ethyl acetate. This problem could theoretically be overcome by substituting ethyl acetate with dichloromethane and adding a positive counter ion to the negatively charged sulfamethoxazole. The efficacy of this approach has been demonstrated for the isolation of ormetoprim and sulfadimethoxine from tissues (20). Alternatively, solid-phase extraction on Sep Pak C_{18} from fish has recently been reported for this combination (19). By the method described here, C_{18} reversed-phase cartridges were also used for the final purification of the extracts containing trimethoprim and sulfamethoxazole. The total analytical recovery of both compounds from *A. franciscana* was 81.3 ± 1.9% ($\bar{x}$ = 65 μg/g; n = 6) and 79.3 ± 1.1% ($\bar{x}$ = 357 μg/g; n = 6), respectively.

The linearity of standard curves was investigated in the range of 1 to 8 μg for trimethoprim and 2.7 to 21.4 μg for sulfamethoxazole (total amounts per sample). This corresponds to concentrations of approximately 34 to 266 and 99 to 713 μg/g (dry weight), respectively. Equations of standard curves obtained by analyzing spiked samples were $y = 0.740x + 0.02$ (r = 0.9999) for trimethoprim and $y = 0.136x + 0.00$ (r = 0.9999) for sulfamethoxazole. The corresponding equations for the standard curves obtained by direct injection of the analytes plus the internal standards (in the chromatographic solvent) were $y = 0.704x + 0.06$ (r = 0.9998) for trimethoprim and $y = 0.138x - 0.00$ (r = 0.9999) for sulfamethoxazole. The good agreement between the slopes of the two sets of standard curves suggests a similar behavior of the analytes and the internal standards in the course of the sample pretreatment and, accordingly, also an efficient compensation by the latter for analytical variability. This result as well as the documented specificity of the method (absence of matrix interferences) support its accuracy (14). The use of two close structural analogs of the analytes as internal standards undoubtedly contributed to the excellent reproducibility of the method. Other methods either do not use an internal standard (20) or use a compound that is not structurally related to either of the therapeutic agents of interest (19). Intra-assay coefficients of variation were 2.2% ($\bar{x}$ = 79.6 μg/g; n = 8) for trimethoprim and 1.9% ($\bar{x}$ = 257 μg/g; n = 8) for sulfamethoxazole. When the HPLC determination was coupled with the protein assay, coefficients of variation were 6.3% ($\bar{x}$ = 0.28 μg/mg of protein) for trimethoprim and 6.8% ($\bar{x}$ = 0.58 μg/mg of protein) for sulfamethoxazole. The limits of detectability (absolute amounts of trimethoprim and sulfamethoxazole extracted from 27 mg of dry *A. franciscana* injected) for both compounds were about 4 ng. This corresponds to a concentration of 3 μg/g (dry weight).

When a 10% preparation was used to enrich *A. franciscana*, i.e., an emulsion theoretically containing approximately 1.67% trimethoprim and 8.33% sulfamethoxazole (ratio, 1:5), levels of up to 115 μg of trimethoprim per g and 277 μg of sulfamethoxazole per g were obtained. Concentrations experimentally found in the emulsion were 1.4 and 8.1%, respectively (ratio, 1:5.79). Remarkably, in enriched *A. franciscana* this ratio was reduced to 1:2.4. This may indicate a more efficient uptake of trimethoprim, a slower elimination and metabolism of trimethoprim compared with that of sulfamethoxazole, or more rapid leaching of sulfamethoxazole from the formulation into the alkaline water used for culturing the *A. franciscana*. On the other hand, the use of enriching formulations of different strengths (1, 5, and 10% of total therapeutic agents) led to a linear increase of trimethoprim and sulfamethoxazole levels in the animal (Fig. 3).

On the basis of the high levels of both therapeutic agents observed in *Artemia* nauplii, optimistic predictions can be made with regard to the efficacy of the bioencapsulation approach as a tool for prophylactic chemotherapy in aquaculture. Work is in progress to extend the present analytical