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Solid-Phase Extraction of Sulfamerazine from Shrimp Residue and Determination by Reversed Phase High Performance Liquid Chromatography

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ABSTRACT : *The focus of this study was to investigate the suitable analytical methods for measurement of sulfamerazine and its metabolite in shrimp hepatopancreas and tail tissue, in addition to the methods for the optimization of solid-phase extraction cartridge conditions and the elucidation of sulfamerazine concentrations in aqueous buffer using HPLC with UV and EC detectors. Compared with UV detector the EC detector appears to be 10 times more sensitive than that of the UV detector. After the shrimp was exposed to 10 ppm sulfamerazine, the accumulation levels of sulfamerazine and its metabolite in tail tissue, which is edible portion, were considerably lower than 0.1 ppm. The data indicate that sulfamerazine continues to be a candidate for use at levels of sulfamerazine concentration used in aquaculture of shrimp.*

Key Words : *HPLC, Sulfamerazine, Solid-Phase Extraction, Penaeus Shrimp, Aquaculture, Accumulation, Metabolism*

I. INTRODUCTION

There is considerable interest in the aquaculture of penaeus shrimp (Overstreet, 1984). Due to several reasons including population, high temperature, suboptimal water quality, and inadequate diet during intensive culture, the commercial cultivation of penaeus shrimp may lead to the development of microbial infections (Cough, 1978). These infections must be treated if commercial goals are to be met. Once an animal population which is destined for human consumption is treated with a pharmacological agent, however, concern arises as to the presence of the drugs and their metabolites in the tissue to be marketed (Bell, 1977; Cook and Lofton 1973).

Considerable information is necessary to predict the time required after administration of a drug before the animal is safe for human consumption (Corliss, 1979). This study is designed to allow assesment of the pharmacokinetics of sulfamerazine (Fig. 1) in penaeus shrimp to be made. Data which this study will generate are particularly critical because of the dearth of knowledge concerning aquatic toxicology in general and of the behavior of xenobiotic substances in crustacean species in particular. We have chosen the brown shrimp (Penaeus aztecus) as an organism to begin the acqusition of knowledge required for making rational decisions concerning the safety of use of selected drugs for treatment of infections in shrimp. The sulfamerazine was chosen because it is widely employed to combat bacterial and fungal infections in both hatchery and grow-out phases of penaeus culture in regions of the world where crustacean aquaculture is practiced (Schnick *et al.*, 1986). Despite the widespread use of the such chemical agents as sulfamerazine little is known of the safety in shrimp (James *et al.*, 1988; Barron *et al.*, 1988; Parasrampuria *et al.*, 1986). Before studies on metabolism and pharmacokinetics of sulfadrugs can be undertaken, reliable methods for analysis of substrates and their metabolites in the medium of interest must be developed (Nielson, 1993). A literature survey conducted at the beginning of this study indicated that no completely suitable analyt-

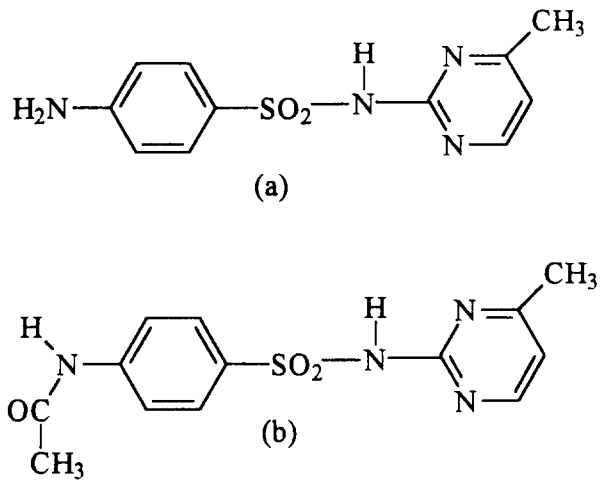

Fig. 1. Structures of sulfamerazine (a) and N^4-acylate sulfamerazine (b).

ical methods for measurement of sulfa drugs and their metabolites in shrimp tissue and biological fluids were avaliable (Astbury *et al.*, 1987; Wolley *et al.*, 1979; Doerr, *et al.*, 1980).

The specific aims of this study are to develope a solid-phase extraction method of sulfamerazine in shrimp residue and to investigate the accumulation, disposition, and metabolism in brown shrimp.

II. MATERIALS AND METHODS

1. Reagents

All drugs were purchased from Sigma Chemical Co., and they are analytical grade and were used as received. Distilled water was used to prepare aqueous solutions.

2. Mobile Phase

20% methanol and 0.05M $NH_4H_2PO_4$ adjusted to pH 2.5 with phosphoric acid. The mobile phase was filtered using 0.45 μm nylon filter element.

3. Bonded Phase Extraction Columns

Commercial octadecylsilyl derivatized bonded phase extraction cartridges (volume 1 ml), were used for all sulfa drug extractions. These were purchased from J. T. Baker Co., Phillipsburg, NJ., listed as Baker 10 SPE C-18 isolation cartridges.

4. Apparatus and operating conditions

1) Optimization of column capacity factor (k')

An elution volume V_R ($V_R = t_R$ x flow rate) of a zone liquid chromatography is given by $V_R = V_m + KVs$, where V_m = mobile phase initial volume (void volume) = t_x flow rate. V_s = stationary phase volume (partition). The equation shows that the retention volume of a solute is equal to the retention volume of a solute is equal to the retention volume of the unretained component V_m plus the additional volume of mobile phase required to elute the component (KV_s). Large values of K mean high affinity for the stationary phase and strong retention of the solute. Distribution coefficients are quite temperature-sensitive. Equation shows that zone separation is also changed by varying the ratio of V_m to V_s (K held constant). This ratio is expressed as the capacity factor(k') and is given by $K' = KV_s/V_m = (V_R - V_m)/V_m$. Smaller values of k' indicate that the components are little retained by the column and elute close to the unretained peak. This leads to poor separations. Large values of k' improve separation but lead to long analysis times and wide peaks difficult to detect. Therefore, the optimization of k' value is necessary. To optimize the k' value a various range of % MeOH mobile phase (between 5 and 30) in 0.05M $NH_4H_2PO_4$ was used. An optimized k' values obtained by each of UV and EC detection.

2) HPLC with ultraviolet(UV)/electrochemical(EC) detection

A high performance liquid chromatograph equipped with a constant flow pump was used (LDC). A 254 nm fixed wavelength UV detector (LDC/Milton Roy) was connected in series with an electrochemical detector and cell. HPLC separations were performed on 150×4.6 mm (i.d.) columns packed with octadecylsilyl silica gel (5 μm Nucleosil) in this study. Chromatographic elution was performed using 20% methanol/0.05M $NH_4H_2PO_4$ at a flow rate of 1 ml/min(3.45 MPa). The column effluent was monitored at 254 nm (UV) and 0.85V(EC) simultaneously.

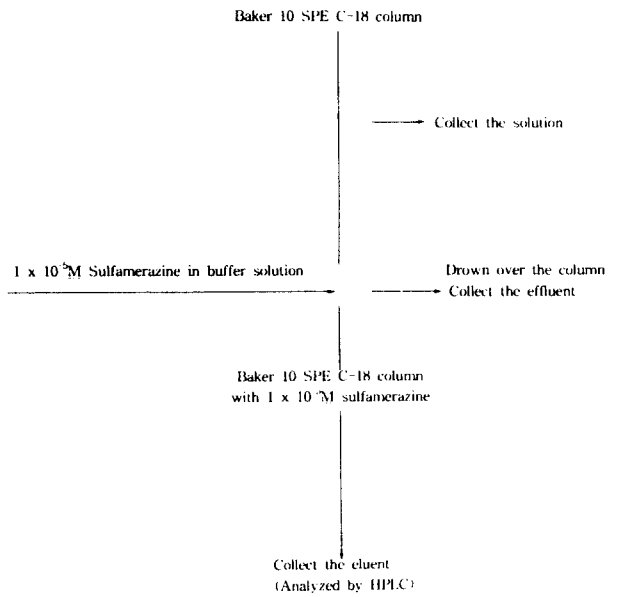

Fig. 2. Extraction of sulfamerazine from McIlvaine buffer (or shrimp tissues).

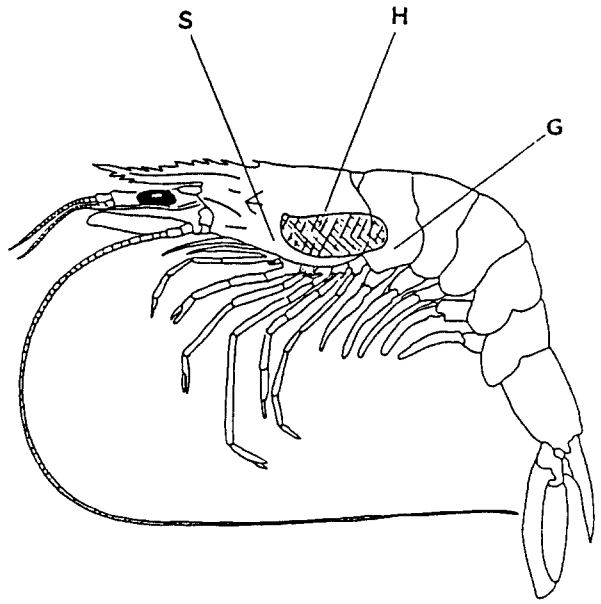

Fig. 3. The Organs of penaeus shrimp (S: stomach, H: hepatopancreas, and G: gut).

5. Extraction

1) Optimization of solid phase extraction cartridge conditions

10 m*l* aliquots of a 1×10^{-5} M solution of sulfamerazine in 0.05 M pH 7.4 McIlvaine buffer (mixing 0.2 M $NH_4H_2PO_4$ and 0.1 M citric acid) were placed on C-18 adsorption cartridge. Concentration of sulfamerazine was varied between 1×10^{-5} M and 1×10^{-6} M and 5 m*l* aliquots of methanol were used to elute the bound sulfamerazine. The volume of methanol required for optimizing drug recovery was determined by placing 10 m*l* aliquots of the sulfamerazine standard solution on the cartridge columns. The columns were then eluted with different volumes of methanol. The sulfamerazine extracted by this procedure (Fig. 2) was quantitated by HPLC with UV/EC detections.

2) Extraction of sulfamerazine from spiked buffers

A standard solution of 1×10^{-5} M sulfamerazine in McIlvaine buffer was prepared. 10 m*l* aliquots of the standard solution were placed on a C-18 bonded phase extraction cartridge, and the bound sulfamerazine eluted with 5 m*l* of methanol. The concentration of sulfamerazine extracted from solution was quantitated by HPLC. Percent recovery was calculated by comparing the detector response for 10 μ*l* aliquots of standard sulfamerazine solution injected directly onto the chromatograph. The average percent recovery was calculated using data from triplet extraction repetitions.

3) Extraction of sulfamerazine from spiked shrimp tissue

A homogenate of shrimp hepatopancreas or tail tissue(Fig. 3) in tris buffer 1 g/10 m*l* was prepared. This was spiked with sulfamerazine to give a final concentration of 10 ppm drug in the tissue homogenate. The spiked homogenate was centrifuged at 9,000×g for 15 minutes and supernatant centrifuged at 10,000×g for 10 minutes. The cartridge was washed with 2 m*l* of methanol and 2 m*l* of distilled water and the bound sulfamerazine eluted with 5 m*l* methanol. The concentration of sulfamerazine was quantitated by HPLC and the percent recovery calculated as above.

4) Exposure of shrimp to sulfamerazine

The shrimp were placed in a 5 liter capacity glass tank, containing 4 liters of artificial seawater. It was found that a population density of 1 shrimp/liter of water was acceptable, provided the water

was aerated. No food was given during the experiment, to minimize tank fouling. Several holding tanks were set up under these conditions, and the shrimp exposed to 10ppm sulfamerazine in the tank. Shrimps were sacrificed after known periods of exposure and specific tissues isolated (hepatopancreas or tail tissue). The tissue under investigation was then processed and the sulfamerazine was extracted and quantitated as described previously.

5) Extraction of sulfamerazine from shrimp tissue after in vivo drug exposure

Shrimps that had been exposed to 10 ppm sulfamerazine were sacrificed and time of exposure recorded. The hepatopancreas was removed and weighed, and a homogenate of the hepatopancreas tissue in tris buffer(final concentration 1 g/10 m*l*) was prepared. To precipitate shrimp fats and proteins diluted acetic acid was added to the homogenate and the results mixed with celite (ca. 1 m*l*). The mixtures were placed on cartridge columns. The columns were then eluted with 2 m*l* of methanol. The liquid was drawn through the cartridge under the gentle vacuum, and the cartridges washed with buffer and aspirated to dryness. Finally the eluent was evaporated with prepurified N_2 gas. The residues were reconstituted in 200 μ*l* methanol, and analyzed by HPLC with UV/EC detection.

III. RESULTS AND DISCUSSION

1. Optimization of column capacity factor (k')

After k' vs. % MeOH in 0.05 M $NH_4H_2PO_4$ was plotted, optimum values of k' are achieved between 1.5 and 4.

2. Optimization of elution volume

The volume of methanol required for optimizing sulfamerazine % recovery was determined. It was found that 10 m*l* of methanol would efficiently elute sulfamerazine extracted from buffer solution.

3. Detectors

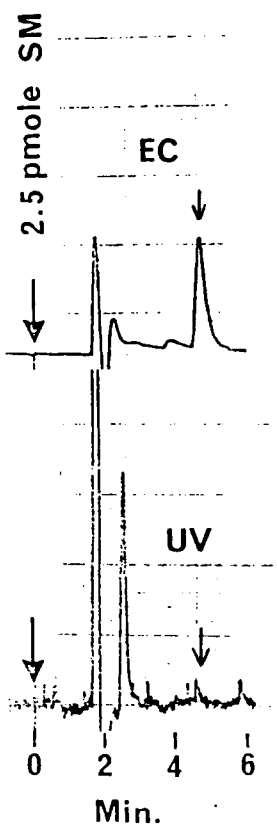

Fig. 4. Analysis of 2.5 pmole of sulfamerazine by combine UV and EC detectors. This amount of the drug is easily detected by EC detector (top trace), but not by the UV detector (bottom trace), even when operated at its most sensitive setting (0.05 AUFS).

1) UV detector

Since UV detector is general detector which responds to any substance absorbing light at the wavelength used, it was necessary to determine the wavelengths of sulfamerazine and its possible metabolite(N^4-acylated sulfamerazine). The UV detector was found to be sensitive for sulfamerazine at levels less than 0.3 - 0.5 nmoles, and exhibited linear response over the range of between 0.2 and 1.0 nmoles.

2) EC detector

The optimum applied potential for sulfamerazine detection was determined to be 0.85 V. The detector was determined to be sensitive to sulfamerazine at levels less than 3 - 5 pmoles, and exhibited a linear response over the range investigated (1.5 - 10 pmoles).

Both detectors provide linear responses over the

range studied and that the EC detector appears to be about ten times more sensitive that the UV detector. The two detectors were operated in series, with chromatographic effluent being analyzed by both detectors and 2.5 pmoles of sulfamerazine was analyzed. The resulting chromatogram (Fig. 4) shows that the analyte is easily detected by the EC detector (top trace) while the UV (bottom trace) shows no evidence of the substance. It should be emphasized , however, that the UV detector, because of its ability to respond to many types of substances, is very valuable for this study. In addition, the amount of material analyzed in Fig. 4 is extremely small; in most cases much more material is be available for analysis.

4. Extraction of sulfamerazine from McIlvaine buffer

Peak area for 100% efficiency was determined by injecting 10 μl sample of preextraction mixture onto the chromatographic column. Varying the pH of buffer, % recovery has been evaluated in Table 1.

5. Extraction of sulfamerazine from spiked shrimp tail tissues

Peak area for 100% efficiency was determined by injecting 10 μl sample of the preextraction mixture onto the chromatographic column. % Recovery has been summarized in Table 2.

6. Extraction of sulfamerazine and possible metabolite(N^4-acylated sulfamerazine) from shrimp tissues after in vivo drug exposure.

The exposure of penaeus shrimp to 10 ppm sulfamerazine has been described in the experimental section. Figures showing accumulation of sulfamerazine and its possible metabolite (N^4-acylated sulfamerazine) in hepatopancreas and tail tissues are shown Fig. 5 and Fig. 6. Fig. 5 indicates that sulfamerazine accumulates in hepatopancreas tissue, and reaches a maximum concentration.

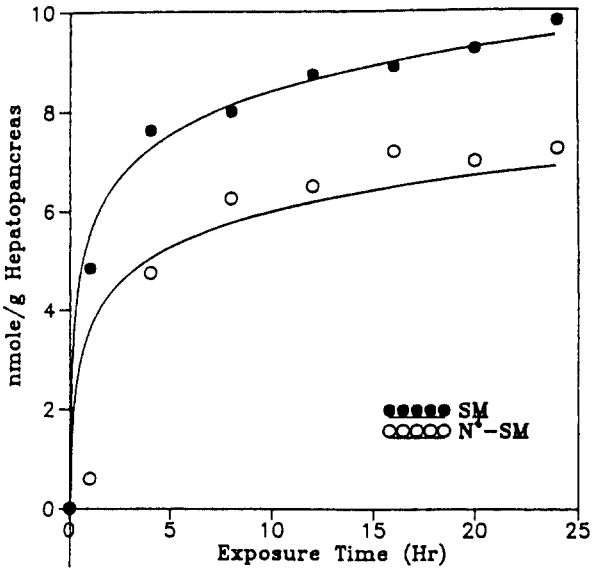

Fig. 5. Levels of sulfamerazine and its N^4-acylated metabolite in hepatopancreas of shrimp exposed to 10 ppm sulfamerazine.

Table 1. Extraction of sufamerazine from McIlvaine buffer

pH Buffer	Pre-extraction peak area*	Post-extraction peak area*	% recovery
3.3	140.8±0.24	131.0±4.54	93.0±3.29
5.7	222.5±0.68	207.0±5.88	93.0±2.37
7.4	227.5±0.40	197.7±4.49	87.0±1.92
8.2	107.8±0.76	69.7±18.5	64.6±16.9

*Peak area =height x width (1/2 height), all units in mm.

Table 2. Extraction efficiency of sufamerazine from spiked shrimp tissue

Replicate Number*	Pre-extraction peak area	Post-extraction peak area	% recovery
1	227.8±0.67	207.0±0.79	90.8±0.13
2	255.6±0.42	231.5±2.72	90.5±1.20
3	288.3±0.08	273.9±3.95	95.0±1.36
4	246.7±2.77	233.7±3.96	94.7±1.34

*Each replicate value calculated from the averaged peak heights of 3 chromatograms.

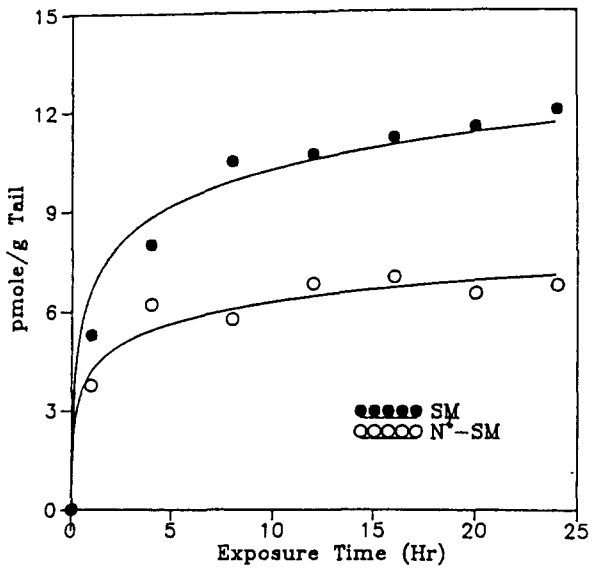

Fig. 6. Levels of sulfamerazine and its N^4-acylated metabolite in tail of shrimp exposed to 10 ppm sulfamerazine.

IV. CONCLUSION

Analysis of sulfamerazine was optimized for HPLC using EC and UV detectors. The capacity factor, k', was determined for octadecylsilyl derivatized reverse phase columns using mobile phases with varying amount of organic modifier. Capacity factors between 1.5 and 4.0 were observed, and a mobile phase consisting of 20% MeOH and 0.05 M $NH_4H_2PO_4$ was selected which yielded a k' of 2.8.

The two detectors used (UV and EC) exhibited a linear response to the sulfa drugs investigated: the EC detector having a sensitivity ten times greater than UV detector to sulfamerazine. The UV detector was found to be valuable when sulfamerazine metabolites and analogs were not electroactive.

A method for the isolation of sulfamerazine and its possible metabolite present in shrimp hepatopancreas and tail tissue was developed, in addition to a method for the elucidation of sulfamerazine concentrations in aqueous buffers.

After the shrimp were exposed to 10 ppm sulfamerazine in seawater, the accumulation of sulfamerazine and N^4-acylated sulfamerazine in hepatopancreas was determined to be 1.5 ppm and 0.61 ppm. In contrast, accumulation of sulfamerazine and N^4-acylated sulfamerazine were determined to be 3.16 ppb and 1.84 ppb. respectively, in edible tail tissue. Since the levels of sulfamerazine and its metabolite in edible portions of shrimp were considerably lower than 0.1 ppm, exposure of shrimp to 10 ppm levels of during aquaculture probably will not render the animals unfit for human consumption. Thus, sulfamerazine continues to be an attractive candidate for use at the levels of sulfamerazine concentration used in aquaculture of shrimp destined for human consumption.

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