Health hazard nitrofuran metabolites in shrimp and its source: Bangladesh aspect

Md. Nurul Huda Bhuiyan[1]*, Matiur Rahim[1], Mohammad Tariqul Hassan[1], Mamtaz Dawlatana[1]

[1]Institute of Food Science & Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Qudrat-I-Khuda Road, Dhanmondi, Dhaka-1205, Bangladesh.

* Corresponding author: Md. Nurul Huda Bhuiyan, E-mail: nhbmb@yahoo.com

Four nitrofuran metabolites: 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2- oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD) were investigated by LC-MS-MS in shrimps and feed (both animal protein rich and without animal protein) of Bangladesh to elucidate the status and to identify the source of nitrofuran metabolites. 250 of shrimps and 150 feed samples (100 animal protein rich and 50 without animal protein) were analyzed during January 2008 to July 2010 which was collected from two main shrimp production areas (Khulna and Cox's bazaar) of the country. No nitrofuran metabolites were detected in the feed samples of without animal protein. AHD and SEM were detected in both shrimp and animal protein rich feed samples. 8 %, 11% and 4% with AHD and 13%, 18% and 3% with SEM were contaminated among the shrimp samples of 2008, 2009 and 2010 respectively. In animal protein rich feed, 12 %, 18% and 7% with AHD and 14%, 23% and 9% with SEM were contaminated among the samples of 2008, 2009 and 2010 respectively. Of the samples analyzed AHD and SEM in shrimp were detected from 0.6 μgkg^{-1} to 48.5 μgkg^{-1} and 0.7 μgkg^{-1} to 5.2 μgkg^{-1} respectively and in feed the ranges were from 0.8 μgkg^{-1} to 9.2 μgkg^{-1} and 0.9 μgkg^{-1} to 75.3 μgkg^{-1} respectively. AOZ also found in 5% and 4% of feed samples of 2008 and 2009 respectively, (range from 1.3 μgkg^{-1} to 3.2 μgkg^{-1}). AMOZ was not found in any of the samples. It was found that animal protein rich feed sample was one of the sources of nitrofuran metabolites found in shrimp and incidence of nitrofuran metabolites in shrimp showed a decreasing trend in 2010.

Keywords: Nitrofuran, metabolites, AHD, SEM, AOZ, AMOZ

Introduction:

Aquaculture is playing an important role in the economy of developing countries like Bangladesh - contributing, in particular, to reducing malnutrition and poverty, but also to economic growth through export and in providing employment opportunities. Though the aquaculture has expanded to the entire world now, it actually started in Asia (Gandini et al, 2009). Food safety regarding the growing occurrence of human and veterinary pharmaceuticals in the environment is causing increasing concern, and improving their ecological and human risk assessment constitutes a challenge for the scientific community (Dorne et al, 2007). The nitrofuran compounds (furazolidone, furaltadone, nitrofurantoin and nitrofurazone) are veterinary drugs and have been used as antibiotics for the treatment of gastrointestinal infection in cattle and poultry. They are also used in aquaculture for control of bacterial diseases and in the formulated feed (Cañada-Cañada et al, 1998). Due to the potential carcinogenicity and mutagenicity of nitrofurans (VanKoten-Vermeulenetal.1993) and their metabolites since mid 1990s using of any nitrofuran in any food-producing animal within the EU, or in any animal destined for export in to the EU has been banned (Commission Regulation, 1995). EU Member States are required to ensure compliance with this ban through their annual residue monitoring plans (Council Directive, 1996). The nitrofuran antibiotics are characterized by quick metabolism, half-life time in vivo is a few hours (McCracken et al., 1995). Therefore determination of the parent compounds is unsuitable for monitoring purposes and persistent tissue bound metabolites are formed (Vroomen et al., 1986) which may be released by mild acid hydrolysis and used as marker residues (Hoogenboom et al.1991b; Samsonova et al., 2008). The marker residues identified are: 3-Amino-oxazolid-2-one (AOZ) for furazolidone, 3-amino-5-morpholino methyl-1, 3-oxazolid-2-one (AMOZ) for furaltadone, 1 amino-hydantoin (AHD) for nitrofurantoin and semicarbazide (SCA) for nitrofurazone. It was found that high concentrations of nitrofuran metabolites accumulate in the retina of pigs, poultry egg, albumen, shell (Cooper and Kennedy, 2005; McCracken and kennedy, 2007) and that parent nitrofurazone accumulates in avian eyes (Cooper et al., 2005a, 2008). AOZ in tissues of pigs fed furazolidone has been shown to be bio

available to rat (Mc Cracken and Kennedy 1997). The metabolites of nitrofurans are almost stable during storage and cooking (Cooper & Kennedy, 2007). Due to their higher stability and ability to accumulate in animal tissues the monitoring of food stuffs is carried out by determination of these tissue-bound metabolites. All current screening (ELISA) or confirmatory methods (LC–MS/MS) are based on this principle (Cooper et al., 2004 a, b, 2005b; Leitner et al.,2001).

The acidic conditions in the human stomach would also liberate these potentially carcinogenic metabolite side-chains and pose a threat to human health (Cooper & Kennedy, 2007). There is a 'zero-tolerance' attitude towards nitrofurans in food-producing animals with in the EU. The development of methods and legislation with regard to the monitoring of nitrofuran residues has recently been reviewed (Kennedy et al. 2003). Legislators and consumers may also wish to know if nitrofuran residues in food are destroyed when that food is cooked. Between 1995 and1999, Rose and co-workers in the Central Science Laboratory, Norwich, demonstrated that residues of a range of veterinary drugs have varying degrees of stability during cooking and, therefore, that cooking influences the level of risk posed by such residues (Rose et al. 1999).

The aim of this study was to elucidate the status of health hazard nitrofuran and to identify the source of nitrofuran metabolites found in shrimps. When some consignment from EU has been rejected and rapid alert shown to the member states of EU (European Commission RASFF n.d.) and findings of antibiotics in shrimp of exporting countries has become big concern for Bangladesh as a shrimp exporting country. Apart from that, no data or publication is available relating to these types of antibiotics in shrimp or in any food of Bangladesh.

Materials and Methods:

Sampling:
250 of shrimps and 150 feed samples (100 animal protein rich and 50 without animal protein) were collected during January 2008 to July 2010 from two main shrimp production areas (Khulna and Cox's bazaar) of the country.

The control samples of shrimp, collected from deep sea, were considered as blank.

Apparatus and Accessories:
Vortex Mixer, Ultra-Turrax, Cabinet Drier (Gellencamp), Centrifuge (Universal 320R, Hittich), pH Meter (35xx, Jenway), Shaker, Water Bath, Heating Block, Top loading Balance (exact to 0.01 g), Precision Balance (exact to 0.01 mg), Pipette with disposable tip (10-100 uL, 20-200 uL, 100-1000uL, 0.5-5mL), Volumetric Pipette 25 ml, Disposable glass pipette, Measuring Cylinder (500mL, 250mL, 100mL, 50 mL, 10mL), Volumetric flask (500mL, 100mL, 50mL, 25mL, 10mL), Brown glass bottle (100ml), PE-Centrifugation tube 50 mL, PE – Centrifuge tube 15 mL, Disposable Syringe Filter (13mm, 0.45 µm, Nylon), Disposable Syringe (3mL with 2.5" long needle), HPLC-Vials 1.5mL and Hybrid Ion Trap based Triple Quadrupole MS/MS system , Applied Biosystems 3200 Q TRAP, Software: Analyst version 1.5 coupled with High Performance Liquid Chromatography (HPLC) system (Auto Sampler: SIL20A : Shimadzu Prominence Series AD, Injection loop on injection port: 50µL, Syringe in the auto sampler: 250µL, Sample vial tray: 105positions of 1.5mL, Binary Pumps:20AD, Shimadzu Prominence UFLC, Column Oven, System controller: CBM20 Alite, LC Column-Phenomenex Luna 3u C18(2) 150mm X 2mm).

Chemicals and Solvents:
Hydrochloric Acid, (HCl) (aq), Nitrobenzaldehyde, Di-methy1 sulfoxide (DMSO), Di-Potassium Hydrogen Phosphate (K2HPO4) (water free), Sodium Hydroxide, Ethy1 Acetate, n-Hexane, Methanol, Ammonium acetate, Nano Pure / HPLC grade water. Standards – AMOZ, AOZ, AHD, SCA (Sigma Aldrich), and Internal Standards - AMOZ-D5, AOZ-D4, 13C15N2 SCA (Sigma Aldrich).

Sample preparation, Extraction & Clean up:
Sample preparation, Extraction & Clean up was done according to US FDA method (US FDA, 2004) with minor modification followed by validation.

Frozen whole shrimps were thawed and rinsed under running water. Excess water drained off, then shell and head removed. Mussel chopped with knife on a clean board, collected in a beaker and kept in the fridge until analyzed.

2.0 g ± 0.1 g of chopped samples in a 50 mL PE-centrifugation tube were dervatized for 16 h in the dark at 37° C after adding 4 mL HPLC-Water, 3mL hydrochloric acid (1M), 400µLof 5 mM 2-nitrobenxaldehyde solution, 1 mL of 2 ng/mL internal standard and homogenization. pH was adjusted to 7(±0.4) of the derivatized sample by adding 2 mL of 0.1M di-potassium hydrogen phosphate solution, 2.5mL of 0.8 mol/L NaOH(aq) and 0.125 mol/L hydrochloric acid (if necessary). Sample was extracted with 12 mL ethyl acetate after centrifugation at 24º C for 8 min at 5000 rpm. The extract was dried at 45º C under the stream of Dry nitrogen. After adding 1.0 ml methanol /water 50% / 50% and 1.0 mL n-hexane the sample was again centrifuged for 5 min at 5000 rpm and the bottom (clear) part was collected in vial of 1.5 mL.

Sample analysis:
The nitrofuran metabolites were analyzed by LC-MS-MS. The HPLC condition was- Auto sampler & Injection: Shimadzu LC system Equlibration time = 0.50 min, Shimadzu LC system Injection Volume = 20.00 ul, Rinsing Volume: 200 uL, Needle Stroke: 52 mm, Rinsing Speed: 35 uL/sec, Sampling Speed: 15.0 uL/sec, Purge Time: 25.0 min, Rinse Dip Time: 0 sec, Rinse Mode: Before and after aspiration. Pump conditions: Pumping Mode: Binary Flow, Total Flow: 0.2500 mL/min, Pump B Pct: 5.0 %, B Curve: 0, Pressure Range (Pump A/B): 1.0 - 40.0 MPa, Total run time was 20 min and the running program was gradient where B pump was 95% at time 8 to 16 min. Mobile phases in pump A was 0.5 mM ammonium acetate in water and pump B was 0.5 mM ammonium acetate in Methanol. Mass Spectrometer settings was- CUR: 25.00, CAD: Medium, IS: 5500.00, TEM: 450.00, GS1: 45.00, GS2: 65.00, ihe: ON, CXP 4.00. The mass spectrometer operated in electro spray positive mode and data acquisition was in multiple reactions monitoring mode (MRM). The precursor/ product ions monitored are listed in Table I. Analyte concentrations in samples were calculated by comparing the ratio of an analyte base peak response to its appropriate internal standard response with the same ratio in calibration curve standards. Quantification of AHD utilized the AOZ-D4 internal standards.

Analytical quality control:
At six calibration levels ranging from 0.05 to 2.5 ng/mL injected (covering the 0.05–2.5µg/kg range). The concentration of isotopically labeled ISs was fixed at 1.0 ng/mL. The stability of the calibration solutions was verified by checking the slope of the calibration curves, which was 0.9984 (should be 1.0±0.2). Calibration standards were injected before and after each analytical series, and both data sets were used to establish the calibration curves. The linearity was checked by calculating the standard deviation of the average of response factors (peak area ratios divided by the corresponding analyte concentration ratios of all standards), which should be <15% to assume a linear response. Final results were expressed as the free nitrofuran metabolites, values obtained from the calibration curves.

Nitrofuran metabolites were considered as positively identified in samples when: (a) the ratio of the retention time of the analyte to that of the corresponding IS corresponded to that of the calibration solution within a ±2.5% tolerance, and (b) the peak area ratios of the various transition reactions were within the tolerances set by the EU criteria (Commission decision, 2002) and shown in Table 1.

The estimation of measurement uncertainty was based on the results of in-house testing of spiked samples. Its significant relevance corresponds to the range over which analytical results will fall provided that the analytical system is "under control". The analytical parameters taken into account were precision (repeatability, intermediate reproducibility), trueness and calibration data (standard preparation, linear regression).

Each step involved in the sample preparation (i.e., weight of test portion, preparation and dilution of ISs, volumes, injection, etc.) was assigned to a defined uncertainty and summed as a final value (cause and effect diagrams). The final uncertainty was calculated using an expansion coefficient of 2, which represents a confidence interval of 95%. In the case of unavailability elaborate data of any elements, a rectangular distribution was used. The selectivity of this method is warranted by the use of two transitions reactions for each analyte, which count for 4 identification points (IPs), as defined by the EU criteria (Commission decision, 2002). Recoveries ranged between 85 and 122%. Decision Limit CCα and Detection capability CCβ limits were calculated following the procedure as explained in the EU guidelines (Commission decision, 2002). Table-2 has shown the ccα & ccβ of different metabolites.

Results & Discussion:

A total of 250 shrimps and 150 feed samples (100 animal protein rich and 50 without animal protein) were analyzed. The results found are shown in Table-3.

No nitrofuran metabolites were detected in the feed samples of without animal protein in any of the year of 2008, 2009 and 2010. AHD and SEM were detected in both shrimp and animal protein rich feed samples. 8 %, 11% and 4% with AHD and 13%, 18% and 3% with SEM were contaminated among the shrimp samples of 2008, 2009 and 2010 respectively. In animal protein rich feed, 12 %, 18% and 7% with AHD and 14%, 23% and 9% with SEM were contaminated among the samples of 2008, 2009 and 2010 respectively. From Table-3, it is found that the samples analyzed, AHD and SEM in shrimp were detected from 0.6 µgkg-1 to 48.5 µgkg-1 and 0.7 µgkg-1 to 5.2 µgkg-1 respectively and in feed the ranges were from 0.8 µgkg-1 to 9.2 µgkg-1 and 0.9 µgkg-1 to 75.3 µgkg-1 respectively among the years. AOZ also found in 5% and 4% of feed samples of 2008 and 2009 respectively; range from 1.3 µgkg-1 to 3.2 µgkg-1. AMOZ was not found in any of the samples.

From the result, it was found that the incidence of nitrofuran metabolites both in shrimp and feed showed a decreasing trend in 2010 (contamination free shrimp and feed are 93 % and 88 % respectively) than 2008 (contamination free shrimp and feed are 84 % and 79 % respectively) and 2009 (contamination free shrimp and feed are 76 % and 68 % respectively). It is also found that the animal protein rich feed sample was one of the sources of nitrofuran metabolites found in shrimp because no nitrofuran metabolites was detected in the feed samples of without animal protein. In our study we have looked only into feed to reveal the source of the metabolites but there may be other source of these contaminants. As residues monitoring programmes have revealed that while most of the incidences of nitrofuran metabolites in food are connected with illegal usage of these drugs, the same was not always true for nitrofurazone and its marker semicarbazide (SEM). As an example, SEM residues were discovered in foods (European Food Safety Authority, EFSA, 2003) not of animal origin and it has now been proven unequivocally that various causes can led to SEM residues accumulating in different food types (Becalski et al., 2004; Hoenicke et al., 2004; Stadler et al., 2004). In one example azodicarbonamide was shown to be responsible for SEM discovered in baby foods due to

its use in the manufacture of plastic gaskets in jar lids (Stadler et al., 2004). SEM also can occur naturally in specific foods, mainly in aquatic products (shrimps, algae) and low levels of SEM in cooked crayfish samples were possibly from natural oigin or a by product of boiling (Hoenicke et al., 2004; Saari & Peltonen, 2004). But it has been reported that nitrofuran metabolites have a considerable potential to accumulate in poultry tissues, even when the parent drug is present in their feed at concentrations equal to 0.01 % of the therapeutic dose (McCracken et. al. 2005). That is why; feed rich in animal proteins is one of the considerable sources of nitrofuran metabolites found in the shrimp. More studies are required to draw conclusion regarding the exact source of these metabolites.

In conclusion, it may be mentioned that hazardous antibiotic nitrofurans have been banned in Bangladesh. Even then, further studies are required to identify any possible source of nitrofurans in shrimp, a potential export sector of the country.

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Table-1: LC-MS-MS precursor/ product ion combinations monitored in MRM ESI positive mode for the analysis of the nitrophenyl derivatives of 4 nitrofuran metabolites.

	MRM used for		
Analyte	Quantitation	Qualification	Peak Area Ratio, ± Limit %
NP - AOZ	236.11 → 134.04	236.11 → 134.04	0.08 ± 5%
NP-AOZ D4	240.13 → 133.93		
NP-AHD	249.12 → 134.20	249.12 → 104.00	0.19 ± 4%
NP-SCA	209.13 → 166.10	209.13 → 192.2	0.88 ± 12%
NP-AMOZ	335.15 → 291.30	335.15 → 262.10	0.37 ± 18%
NP-AMOZ D5	340.21 → 269.20		
NP-SCA 13C	212.12→ 195.20		

Table-2: ccα & ccβ of different metabolites.

Analyte	ccα	ccβ	Std Dev
	µg/kg	µg/kg	
AHD	0.08	0.218	0.084
AMOZ	0.03	0.153	0.075
AOZ	0.04	0.225	0.113
SCA	0.10	0.379	0.170

Table-3: Nitrofuran metabolites in Shrimps and feed samples of 2008, 2009 & 2010

	January to December 2008									**January to December 2009**									**January to June 2010**								
	Shrimp (100 samples)			Feed (40 samples animal protein rich)			Feed (20 samples without animal protein)			Shrimp (100 samples)			Feed (40 samples animal protein rich)			Feed (20 samples without animal protein)			Shrimp (50 samples)			Feed (20 samples animal protein rich)			Feed (10 samples without animal protein)		
	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)	Number of samples detected (%)	Highest concentration (µgkg-1)	Lowest concentration (µgkg-1)
AOZ	-	-	-	9	1.8	1.3	-	-	-	-	-	-	4	3.2	1.4	-	-	-	-	-	-	-	-	-	-	-	-
AHD	8	27.8	0.9	12	7.1	1.0	-	-	-	11	48.5	1.6	18	9.2	1.2	-	-	-	4	22.1	0.6	7	5.3	0.8	-	-	-
SEM	13	7.2	1.4	14	59.0	14.9	-	-	-	18	2.9	1.1	23	60.3	0.6	-	-	-	3	2.6	0.8	9	75.3	2.8	-	-	-
AMOZ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Not Detected (%)	84			79			100			76			68			100			93			88			100		