

The Occurrence of Residues of Furazolidone Metabolite, 3-Amino-2-Oxazolidone, in Eggs Distributed in Mazandaran Province, Iran

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Background: Furazolidone, as an antibiotic in nitrofurans class, is used for prevention and treatment of poultry diseases and increasing egg production. However, its use is prohibited in Iran because of toxic, carcinogenic, and mutagenic effects and antibiotic resistance in humans.

Objectives: This study was conducted as a first survey to determine occurrence of 3-amino-2-oxazolidone residue (as a metabolite of furazolidone) in eggs distributed in Mazandaran (a northern province of Iran) in 2011.

Materials and Methods: In this study, 84 samples, containing 42 branded eggs from seven companies and 42 non-branded eggs were collected by quota sampling techniques from stores of Mazandaran province cities. Samples were assessed by competitive enzyme-linked immunosorbent assay (ELISA) technique. Results were analyzed using SPSS and One-way Analysis of Variance (ANOVA), Mann-Whitney U Test and Kruskal-Wallis test were used.

Results: The percentage of the eggs polluted with 3-amino-2-Oxazolidone (AOZ) in branded, non-branded and total eggs was 71, 62 and 66.7, respectively. The mean concentration of AOZ in the samples of branded, non-branded and total eggs was 252.9, 283.7 and 268.25 ng/kg, respectively and there was no significant difference in the 3-amino-2-oxazolidone level between the two groups ($P < 0.05$). There was only one company with no contamination in any of the six samples.

Conclusions: The majority of eggs in the cities of Mazandaran contained 3-amino-2-oxazolidone, which confirmed the use of furazolidone. All detected concentrations were below the minimum required performance limit (MRPL) set at 1 ng/kg by the UE. It was also concluded that the eggs produced by certified companies are not healthier than miscellaneous eggs, in terms of 3-amino-2-oxazolidone concentration.

Keywords: Furazolidone; 3-amino-2-oxazolidone; Eggs; Enzyme-Linked Immunosorbent Assay; Iran

1. Background

A wide variety of antibiotics are used to enhance the growth rate and treat bacteria and parasitic infections in poultry industries. Despite these benefits, when antibiotic residues exceed maximum residue limit (MRL) in the meat and egg endangers consumers' health through side effects including carcinogenesis, development of drug resistance in humans and changing the intestinal flora (1-3). Nitrofurans, as a broad-spectrum group of antibiotics, include some derivatives such as furazolidone, nitrofurantoin and nitrofurazone, which their toxic, carcinogenic and mutagenic effects have been demonstrated (4, 5). Furazolidone, with N-(5-nitro-2-Furfurilidun)-3-amino-2-Oxazolidone formula, is one of the most widely used nitrofurans, which has been used in the treatment of infections caused by *Escherichia coli*, *Salmonella* spp. and *Shigella* spp. in pigs, poultry and fishes. Furazolidone could control a variety of digestive tract infections and treatment of bac-

terial enteritis, dysentery, giardiasis and poultry typhoid. Furazolidone also may increase egg production (6-8). A part of the furazolidone residue in the form of a protein-bound side chain metabolite called 3-amino-2-Oxazolidone (AOZ), which shows higher stability and survival in the body tissues and releases under acidic conditions (9-12). Detection of furazolidone residues in food is carried out by various methods including different types of chromatography like HPLC-UV, LC-MS, and LC-MS/MS. However, these are very costly procedures. Enzyme-linked immunosorbent assay (ELISA) is known as a rapid, high capacity and cheap method. This method has been included in official collection of test procedures by the German Federal Board of Health. This immunological test is based on the measurement of the antigen-antibody colored complex (2, 13-15). Utilization of furazolidone is prohibited in food animals in US and EU countries due to adverse ef-

fects on health. But some developing countries are still using it routinely. Europe Union in 2002 banned imports of poultry products from Thailand and Brazil, because of nitrofurans occurrence (8, 16, 17). Previous studies in the world show better control of public health and less use of vet drugs in developed countries in comparison with developing ones. For example, Bock et al. (2006) in Germany report that furazolidone in consumed egg of Berlin is negligible (18). While Amjad et al. (2006) in Pakistan confirmed that the remains of antibiotics in chicken and egg exceed standard levels (19). Hussein and Khalil (2013) in Egypt detected considerable levels of antibiotic residues in chicken fillet samples (2). In Iran, despite of prohibition of nitrofurans usage, these medicines are widely used. Prior studies in associated with antibiotic residues in food products have confirmed this issue. For example, studies of Peyghan et al. in 2012 about furazolidone in the muscles of Carp fish and Vahedi et al. in 2011 about antibiotic residues including furazolidone in industrial poultry carcasses have been published (20, 21).

2. Objectives

Eggs' health and safety is of great importance as one of the most widely consumed food. This study was aimed to determine furazolidone usage induced AOZ in eggs distributed in Mazandaran, Iran.

3. Materials and Methods

3.1. Preparation of Samples and Equipment

In this descriptive cross-sectional study, 84 egg samples were collected by quota sampling techniques from numerous stores in Mazandaran province of Iran in 2011. Branded eggs ($n = 42$) from seven different companies (each company: 6 samples) were coded with A, B, C, D, E, F, G and non-branded samples ($n = 42$) were coded as N. Branded eggs contain manufacturer's name, date of production and expiry on egg shells but non-branded eggs had no such info. Samples were kept at 4 °C until analysis. After cleaning and cracking the egg shell, samples were poured in clean and dried dishes, and were homogenized by a mixer machine. ELISA was chosen to determine AOZ using Ridscreen kits manufactured by R-Biopharm, Germany (detection limit: 100 ppt, recovery rate ranges 90-100%). Solvents and most chemical materials were purchased from Merck Company, Germany.

3.2. Extraction

Extraction was performed in the following steps: first, one gram of the homogenized egg with 4 mL of distilled water, 0.5 mL of hydrochloric acid 1 M and 200 mL of 2-Nitro benzoic aldehyde 10 mM (in dimethyl sulfoxide) were mixed and placed in the shaker device. The mixture was incubated at 37 °C for 16 hours. 5 mL of 0.1 M K₂PO₄, 0.4 mL of 1 M NaOH and 10 mL ethyl acetate were then

added and the tubes were shaken vigorously for 30 sec and then centrifuged at 3000 g for 10 minutes at room temperature (20-25 °C). Then, 5 mL of 0.01 M K₂PO₄, 0.4 mL of 1 M NaOH and 5 mL of ethyl acetate were added and the mixture was shaken for 30 seconds. The sample was centrifuged at 3000 g for 10 minutes at room temperature (20 to 25°C). After that, 2.5 mL of ethyl acetate (in the upper case) was passed into a new vial and evaporated. The remaining mixture resolved in 1 mL of n-hexane or n-heptane and then mixed well with 1 mL of sample buffer. Finally, the samples were centrifuged at 3000 g for 10 minutes and then 50 ml of the lower aqueous layer was passed into the well (10, 22).

3.3. Enzyme-Linked Immunosorbent Assay (ELISA)

After extraction, the samples were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the following procedure: 50 µL of each of the 84 samples and six standard solutions in duplicate were added to wells in the kit. A new sampler tip was used for everyone. Then, 50 µL of enzyme conjugate and 50 µL AOZ antibody were added and mixed gently by hand shaking for one hour, and maintained at room temperature (20-25°C). After removing the liquid of the pores, they were washed three times with 250 mL washing buffer solution embedded in the kit. Then, 100 µL of chromogenic substrate was added to create color and mixed, followed by incubation at room temperature in the dark for 15 minutes. One hundred µL of the stop solution was then added to each well and mixed to stop the enzymatic reaction. After 30 minutes, absorbance values were measured at 450 nm. Finally, concentrations of AOZ in ng/kg (ppt) were calculated by a standard curve and dilution factor of two (10, 15, 22).

3.4. Statistical Analysis

Results were statistically analyzed by using SPSS version 17. Continuous variables are presented as the Mean ± Standard Deviation (SD), median and inter Quartile Range (IQR), whereas categorical data are presented as frequency and percentages. A Kolmogorov-Smirnov test is used to find out if the recorded data are normally distributed. Independent t test, One-way Analysis of Variance (ANOVA) with Duncan post-hoc test, Mann-Whitney U Test and Kruskal-Wallis were used for continuous variables. In this study, a probability less than 0.05 ($P < 0.05$) was considered significant.

4. Results

The results are shown in tables 1 and 2. Table 1 shows mean concentrations of the AOZ metabolite in the eggs of producing companies and non-branded eggs. Comparison of the AOZ mean concentrations showed that highest and lowest values were seen in eggs produced in company A (460 ng/kg) and company C (not detectable AOZ levels), respectively. Statistical analysis showed that company C had a significant difference in AOZ concentrations with

Table 1. The Mean Concentration of 3-Amino-2-Oxazolidone (AOZ) in Samples of Different Companies (A, B, C, D, E, F, G) and Non-Branded Eggs (N)^a

Name	Samples, No.	AOZ Concentrations, ng/kg			
		Mean ± SD	Minimum	Maximum	Median ± IQR
A	6	460 ± 127.72 ^b	240	628	478 ± 88
B	6	273.17 ± 189.42 ^b	- ^c	588	271.00 ± 97
C	6	-	-	-	-
D	6	201.67 ± 128.47 ^b	-	346	255 ± 330
E	6	212.67 ± 134.33 ^b	-	390	271 ± 342
F	6	304.67 ± 168.25 ^b	-	476	335 ± 142
G	6	318 ± 62.71 ^b	220	404	318 ± 72
N	42	283.62 ± 219.68 ^b	-	790	296 ± 492

^a Abbreviations: AOZ, 3-Amino-2-Oxazolidone; IQR, Inter Quartile Range.^b Similar letters are not significantly different ($P < 0.05$).^c Limit of detection of ELISA for AOZ: 100 ng/kg.**Table 2.** Concentration of 3-Amino-2-Oxazolidone (AOZ) in Branded Eggs (M) and Non-Branded Eggs (N)^a

Type of Eggs	Number of Samples	AOZ concentrations, ng/kg	
		Mean ± SD	Median ± IQR
M	42	252.88 ± 166.53 ^b	287 ± 384
N	42	283.62 ± 219.68 ^b	296 ± 492

^a Abbreviations: AOZ, 3-Amino-2-Oxazolidone; IQR, Inter Quartile Range; M, Branded Eggs; N, Non-Branded Eggs.^b Similar letters are not significantly different from each other.

other groups ($P < 0.05$). There was no significant difference between AOZ levels of groups of A, B, D, E, F, G and N ($P < 0.05$). AOZ concentration for all analyzed samples were below the minimum required performance limit (MRPL) set at 1 µg/kg by the EU in 2003 (23). Table 2 shows the comparison between the AOZ mean concentration of branded eggs (M: including groups of A, B, C, D, E, F and G) and non-branded eggs (N). Our studies revealed that the AOZ mean concentration of branded samples was lower than non-branded samples, but there was no significant difference between the groups of M and N, based on statistical analysis ($P < 0.05$). Mean concentration of total eggs including branded and non-branded eggs was 268.25 ng/kg. Our results demonstrated that out of 84 samples collected from Mazandaran stores, 56 samples (66.7%) were AOZ positive. The frequency of polluted samples in two categories of branded eggs and non-branded eggs were 71% and 62%, respectively. Limit of detection as a minimum limit was 100 ng/kg and AOZ was not detected in none of the six samples of company C. Meanwhile, AOZ was present in all six samples of A and G. Due to the lack of AOZ occurrence or its undetectable levels in some samples, the zero in data has caused large standard deviations towards means.

5. Discussion

Results revealed among seven surveyed companies, only one company (C) - probably - did not use furazolidone. The results also indicated that furazolidone was

seen in all the eggs in two companies (A and G) and in most of the eggs in four companies (B, D, E and F). Another finding of this study was insignificant difference in AOZ concentrations of branded eggs (M) compared to non-branded eggs (N). Unexpectedly, the percentage of contaminated samples in branded eggs was 71.43% which was 9.5% higher than non-branded eggs, while it was supposed that branded eggs have a significant better quality than non-branded samples because of more accurate monitoring by relevant organizations, higher production quality, and professional staff. According to nitrofurans prohibition for food animals in Iran, all positive eggs in this study (66.7%) could be unacceptable. There is no research about furazolidone residues in eggs in Iran to be compared. However, bacterial contamination of eggs from retails markets of Iran was determined by Ghasemian Safaei et al. in 2011. They founded *E. coli* is known to contaminate the surface of egg while *Salmonella* spp. and *C. jejuni* does not make up a serious health hazard (24). Also, in similar studies, Vahedi et al. (2011) determined antibiotic residues in poultry industry carcasses in Mazandaran and reported that of 815 carcasses, which were examined, antibiotic residues were detected in 533 (65.4%) similar to our study, indicating that poultry products are contaminated with antibiotic residues and can threaten the health of consumers. Furazolidone mean concentration in this study was 128 µg/g that is much higher than the level obtained in our study (21).

Peyghan et al. (2012) measured furazolidone residues in the muscle of 100 cultured common carp using the HPLC technique. The results of this study showed that furazolidone was detected in the muscles of 40 cultured fish (40%) that is lower than positive ratio of eggs in the present study (67%). However, the average level of furazolidone in the fishes (953 µg/kg) was much higher than our finding regarding eggs. This study revealed that furazolidone was residually retained in the muscles of the fish, when examining them after 10 days. Peyghan et al. (2012) also investigated furazolidone residue persistence in the fishes after feeding with 75 mg/L furazolidone for 10 days. After 20 days, the furazolidone concentration was 116 µg/kg and subsequently after 30 days, no furazolidone was detected (20). Antibiotic contamination in poultries is not a main challenge in developed countries, because they consider the withdrawal time of furazolidone and antibiotic side effects in humans. Bock et al. (2007) conducted a study about furazolidone residues in eggs consumed in Berlin using the LC-MS/MS method. The mean residue of AOZ metabolite of eggs in Berlin was 30 ng/kg (ppt), which is much lower than the results of the current research (268.25 ng/kg) in Mazandaran, Iran (18). Rodziejewicz in 2008 determined nitrofurans metabolites in raw milk of Poland by LC-ESI-MS-MS. Detection capability was 0.15-0.37 µg/kg and below the MRPL set at 1 µg/kg by EU (25). In addition, Verdon et al. (2007) in France surveyed five nitrofurans including furazolidone in the poultry meat through their metabolites identified by the LC-MS/MS method. The detected amounts of these metabolites were acceptable and corresponded with approved standards of the EU commission in 2002 (26). Lower pollution with furazolidone in EU countries associated with legislation related to consumers' health and adequate supervision, while in developing countries, antibiotics use is presumed to be high. Amjad et al. (2006) in Pakistan studied some antibiotic residues in eggs. The mean levels of enrofloxacin and ciprofloxacin in eggs were determined 872 and 71 µg/kg respectively, which are higher than the MRL of 30 µg/kg (19). In another study, Hussein et al. (2013) evaluated enrofloxacin and oxy-tetracycline antibiotic residues in one hundred fresh and frozen broiler fillet samples (50 of each) by HPLC method in Egypt. Their results revealed that the 34% and 8% of the fresh broiler fillet and frozen broiler fillet samples were positive for antibiotic residues, respectively. Oxy-tetracycline concentration of all positive samples and 66.7 % of positive enrofloxacin samples were above the MRL (0.1 mg/kg) (2). Cooper et al. in 2005 gave feed medicated pigs with nitrofurans drug for ten days and evaluated depletion of the parent nitrofurans and their metabolites (AOZ). Pig tissue samples collected for analysis for six weeks following withdrawal of medicated feed. The parent drugs were detectable only in pigs subjected to no withdrawal period. This confirms the instability of the major nitrofurans antibiotics in edible tissues. In contrast, the metabolites of all drugs were still readily

detectable in tissues six weeks after treatment cessation (27). The results of the present study which evaluate furazolidone metabolite (AOZ) residues in eggs distributed in Mazandaran, Iran seem concerning. Although detected AOZ concentrations were lower than MRPL (1 µg/kg) by the EU, because no safe limit for the presence of these drugs in food product for human consumption could be assigned, nitrofurans have been banned for food-producing animals by Iran. Presence of furazolidone residues in some eggs shows illegal use in the poultry. Carcinogenic and mutagenic side effects of some antibiotics like nitrofurans have been demonstrated. Efficient supervision and regular monitoring of production and notifying farmers and poulterers of antibiotic risks seem very effective to produce healthier food.

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Authors' Contributions

Study concept and design: Meskarpour Amiri and Mousavi; Acquisition of data: Meskarpour-Amiri and Hashemi; Analysis and interpretation of data: Mousavi, Meskarpour Amiri, Tavakoli and Hashemi; Drafting of the manuscript: Hashemi, Rostami and Gholian; Critical revision of the manuscript for important intellectual content: Hashemi; Statistical analysis: Gholami Fesharaki; Administrative, technical, and material support: Meskarpour Amiri, Mousavi and Tavakoli; Study supervision: Tavakoli and Mousavi

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