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Toxicokinetics of N-nitrosamine in Red Swamp Crayfish (*Procambarus clarkii*): Absorption, Metabolism and Human Health Risk Implications

Jennifer L.O. Patterson, S. Ogutu, S. Willis and L.T. Walker and M. Verghese
Department of Food and Animal Sciences, Alabama A and M University, P.O. Box 1628, Normal, AL 35762, USA

Corresponding Author: M. Verghese, Department of Food and Animal Science, Alabama A and M University, P.O. Box 1682, Normal, AL 35762, USA Tel: (256) 372-4175 Fax: (256) 372-5932

ABSTRACT

N-nitrosamines are human carcinogens that may form in the environment by both natural and anthropogenic actions. Edible tissues of aquatic organisms are susceptible to contamination due to exposure from water, sediment and food processing methods; therefore, possible health risk may exist. The purpose of this study was to examine absorption and metabolism patterns of seven N-nitrosamine compounds in various compartments of commercially-available Red Swamp crayfish. Crayfish samples were purchased from a wholesale dealer and exposed to N-nitrosamines at varying concentrations (0 (control),10, 20, 40, 80 μg mL^{-1}). Samples were washed in tap water and boiled for 7-10 min at 100°C. Crayfish were dissected into shell, hepatopancreas and tail meat. N-nitrosamines were extracted using standard solid-phase extraction protocol with Extrelut and Florisil cartridges. N-nitrosamines were identified and quantified using GC-FID. N-nitrosamines were not detected in control samples. Absorption of the various N-nitrosamines depended on treatment concentration, duration and compartment. At higher concentrations, NDBA (N-nitrosodibutylamine) concentrations could be used as an indicator of NPIP, NPYR, NDPA and NMEA levels. NPIP could be used to predict the levels of NDMA, NDPA, NPYR and NMEA when crayfish are exposed to N-nitrosamines for 6 and 24 h periods. NPYR had the highest rate of metabolism while NDMA had the slowest. Understanding absorption and metabolism patterns can help determine possible health risk due to consumption of crayfish.

Key words: Nitrosamines, cray fish, metabolism, environment, carcinogens

INTRODUCTION

The expositive and continual growth of the fishery industry is of major concern as products may be potentially contaminated. The Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) have been forced to evaluate the safety of imported fish products into the U.S., to determine levels of heavy metals and carcinogens within the food.

Major carcinogens of concern include a class of compounds called N-nitrosamines. N-nitrosamines are formed endogenously or during food processing (Magee *et al*., 1976). They are a part of a larger group of compounds called N-nitroso compounds. N-nitrosamine structure consists of R_1R_2-N-N-O, where R_1 and R_2 are alkyl or acyl groups (Lijinsky, 1992; Rostkowska *et al*., 1998). N-nitrosamines have been shown to be carcinogenic to several animal species including rats (Luinsky *et al*., 1988), murines (Cardesa *et al*., 1973), hamsters (Margison *et al*., 1980; Nakagawa *et al*., 1996) and dogs (Mysliwy *et al*., 1974). The National Center for Toxicological

Research (NCTR) and the International Agency for Research on Cancer classifies several N-nitrosamine as posing a cancer risk to humans based on the carcinogenic activity in animal models (Abnet, 2007).

N-nitrosamines may form in the environment by both natural and anthropogenic actions. Two reactants are needed for this reaction to occur- nitrates/nitrites (NO_3^-/NO_2^-) and a nitrosating agent (Rostkowska *et al*., 1998) which include proteins, pesticides, organic nitrogen and nitrogen oxides (NO_x). In seafood, N-nitrosamines can be formed as a result of the nitrosation reaction between secondary amines (dimethylamine, diethylamine and trimethylamine oxide) and nitrate food additives or other environmental sources (Song and Hu, 1988; Yurchenko and Molder, 2006). In an effort to reduce possible N-nitrosamine formation in foods, FDA in 2009 has set limits on levels of nitrates and nitrites used in food processing. However, toxicology studies used to determine these limits do not take into account the concentration of nitrate, nitrite and nitrosamines that may already be present in food. Therefore, data collected from the studies cannot be used to estimate current exposure risk to these substances.

The freshwater crayfish species (*Procambarus clarkii*) are harvested and consumed seasonally in high quantities in the southeastern region of the United States. The demand and consumption of foods such as crayfish is a growing trend, with approximately 25% of Americans indicating that they consume Cajun cooking. According to the USDA Continuing Survey of Food Intakes by Individuals, the mean consumption of crayfish is 0.012 g/person/day (EPA., 1980). However, this data does not consider the seasonal aspect of crayfish consumption. Though consumption styles vary with individuals, most individuals prefer the abdominal tail meat, while others consume the contents of the head, foregut and midgut.

Edible tissues of aquatic organisms are very susceptible to contamination because exposure can occur from water, sediment, endogenous formation and formation during food processing (Escartin and Porte, 1996). Although seafood is highly recommended as a substitute for meats (Zhang, 2008), these factors suggest that populations that ingest large quantities of seafood may be at risk of adverse health effects as a result of N-nitrosamine exposure. Freshwater crayfish species have been used as a biological indicator of clean water, monitoring levels of heavy metals, PCBs, organochlorine pesticides and other toxic substances and yet, they are also highly consumed in certain regions of the U.S. In general, crustaceans accumulate heavy metals when there is an increase in the bioavailability of these metals in the water. In polluted water systems, crayfish are able to bioaccumulate large amounts of these metals in their tissues as reported by Alikhan *et al*. (1990), Anderson *et al*. (1997) and Bollinger *et al*. (1997). Likewise, N-nitrosamines have the potential to bioaccumulate in various edible and non-edible tissues of crayfish. However, they can also be formed endogenously when the crayfish are exposed to nitrogenous compounds in the water system and during processing (Alibaud *et al*., 1985; Escartin and Porte, 1996).

Though much research on nitrosamines exist, toxicity data for nitrosamines accumulated in specific tissues of seafood are outdated and therefore cannot be used to estimate the potential risk to target populations due to changes in industrialization and pollutants in water bodies. Recent studies on nitrosamines in seafood focus on compounds being formed during various processing methods without consideration of the nitrosamines already present in the fresh fish. Determining the N-nitrosamine content in specific compartments is necessary to estimate hazardous risk due to differences in consumption styles. In addition, toxicokinetic studies may help predict the accumulation of N-nitrosamines in specific compartments of the crayfish. Therefore, the objective of this study is to determine the toxicokinetics for N-nitrosamines in compartments of red swamp crayfish by studying absorption and metabolism.

MATERIALS AND METHODS

Sampling: Samples of live Red Swamp crayfish were purchased from commercial wholesalers in Louisiana (LaBlanc Crawfish Farm, Inc.) in June 2010.

Chemicals: A standard N-nitrosamine mixture containing N-nitrosomethylethylamine (NMEA), N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodibutylamine (NDBA), N-nitrosodipropylamine (NDPA), N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP) and individual nitrosamines were purchased from Sigma-Aldrich, St. Louis, MO.

Toxicokinetic experiment: Live crayfish were exposed to N-nitrosamines at varying concentrations. Crayfish species (*P. clarkii*) were placed in (2000 mL) glass beakers to simulate the natural fresh water habitat of the crayfish. N-nitrosamines were dissolved in water and approximately 175 mL of the stock solution, diluted to appropriate concentrations (0,10, 20, 40, 80 and 100 mM) and added to the beaker. Crayfish were exposed for a period of 6, 12 and 24 h. Three samples were assigned to each concentration and exposure time. Crayfish were processed, dissected and analyzed. Water samples were collected and prepared for analysis using nitrosamine extraction methods described. Mortality rate (if any) was also noted.

Processing methods: Live crayfish were processed according to Baek and Cadwallader (1996) method. Live crayfish were washed in tap water and boiled for 7-10 min at 100°C. After cooling, abdominal tail meat, hepatopancreas and shell were dissected to prepare for extraction.

N-Nitrosamine extraction: Crayfish samples were crushed using a mortar and pestle and homogenizer. Samples were prepared using a two-step solid-phase extraction using Extrelut and Florisil as described by Yurchenko and Molder (2006) with modifications. Approximately 6.0±1.0 g of sample was mixed with 6.0 mL of 0.1 N NaOH in a 100 mL beaker. A prepacked Extrelut (EMD Chemicals, MO) column was wetted with 20 mL hexane/dichloromethane 40:60 (v:v) and the sample eluted with two 20 mL portions of hexane/dichloromethane solution. The eluate was collected in a 50 mL concentrator flask and evaporated in a water bath at 60°C. In the second step, a 1 g prepacked Florisil cartridge (Phenomenex, CA) was wetted with 6 mL of dichloromethane/methanol 95:5 (v:v) and eluted with 6 mL of dichloromethane/methanol solution. The solution was evaporated to 0.5 mL using a Buchi Switzerland Rotavapor R-215 at 60°C and atmospheric pressure. The prepared solution was transferred to an amber sample vial. Extractions were performed in duplicate.

Chromatographic analysis: Gas Chromatography (GC) analysis was carried out using Hewlett Packard 5890 gas chromatograph equipped with flame ionization (GC-FID) (Agilent Tehcnologies, Palo Atlo, CA). One half microliter of the extracted solution sample was injected into a Rtx-1 Crossbond® 100% dimethylsiloxane column (30 m×0.25 mm I.D×0.1 film thickness (df)). For the gas chromatograph separation of N-nitrosamines, the oven temperature program was started at 60°C (held for 1 min), set at a rate of 5°C min^{-1} from 60-100°C and held isothermally at 100°C for 2 min. Temperature was again increased from 100-250°C at a rate of 15°C min^{-1} and held isothermally at 250°C for 2 min. The velocity of the helium carrier was 1 mL min^{-1}.

Statistical analysis: Data was analyzed in a 2×4 factorial design with factor A being processing method (boiled, raw) and factor B being body compartments (whole, tail meat, shell and head).

Statistical analysis was conducted using SAS 9.1, 2004 (SAS Institute Inc., Cary, NC) using analysis of the variance (ANOVA). The N-nitrosamines concentration were correlated by treatment concentration, compartment and exposure duration.

RESULTS

Absorption: The correlations between absorption of various N-nitrosamines are shown in Table 1-3, with data sorted by treatment concentration, compartment and exposure duration, respectively. At higher concentrations (80-100 μg mL^{-1}) a strong negative correlation exists between NDMA and other N-nitrosamines. At 40 μg mL^{-1}, the relationship between NDMA and NDEA was positive; however, it was negative with exposure to 80 μg mL^{-1}. NDBA displayed a positive relationship with NPIP, NPIP, NPYR, NDPA and NMEA at 100 μg mL^{-1}.

Table 2 shows the correlations ($p<0.05$) between N-nitrosamines in each compartment. Absorption of NDMA and NMEA displayed a positive relationship in both the hepatopancreas and the shell. Similarly, absorption of NDBA and NDPA in the shell and remaining in the water was positively correlated.

The relationship between NDPA and NPIP absorption is the same (positive) at 6 and 24 h of exposure periods. However, at 12 h exposure no relationship ($p<0.05$) exist between these two compounds. Furthermore, NDPA and NDBA had the strongest correlation after 24 h exposure to the seven N-nitrosamines.

Metabolism: The metabolized percentage for each N-nitrosamine compound was calculated using the total amounts absorbed in the various compartments (tail, hepatopancreas and shell) and the amount remaining in the water. Among the seven N-nitrosamines, NDMA had the slowest metabolism compared to all other compounds. Therefore, the metabolism percentage of NDMA is only shown in Fig. 1.

Table 1: Correlations between N-nitrosamines absorbed in crayfish by treatment concentrations

Treatment concentration (μg mL^{-1}) and N-nitrosamine correlations	Relationship	p<R
10		
NMEA-NDPA	++	0.017
NDPA-NPIP	++	0.006
20		
NDPA-NPIP	++	0.03
40		
NDMA-NDEA	++	0.02
NMEA-NPYR	++	<0.0001
80		
NDMA-NDEA	--	0.04
NDEA-NPIP	++	0.008
NDPA-NDBA	--	0.04
100		
NMDA-NMEA	--	0.05
NMEA-NPYR	++	0.007
NMEA-NDBA	++	0.014
NDPA-NDBA	++	0.01
NPYR-NDBA	++	0.03
NPIP-NDBA	++	0.0005

Results are significant at $p \leq 0.05$. NDMA: N-nitrosodimethylamine, NDEA: N-nitrosodiethylamine, NPYR: N-nitrosopyrrolidine, NPIP: N-nitrosopiperidine, NDPA: N-nitrosodipropylamine, NDBA: N-nitrosodibutylamine, NMEA: N-nitrosomethylethylamine

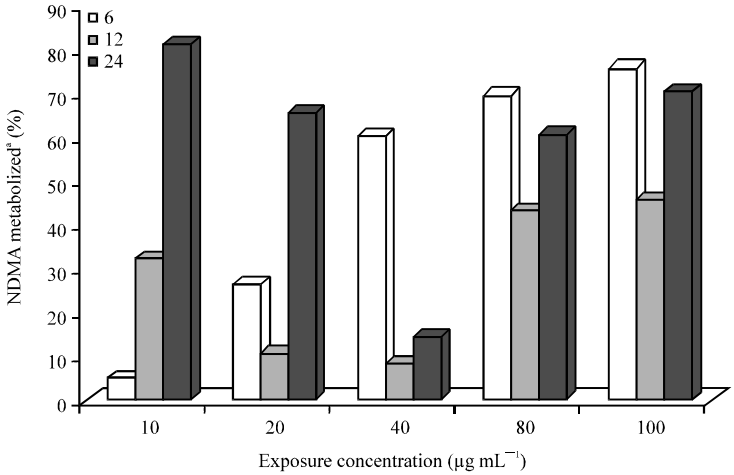

Fig. 1: Metabolism of N-nitrosodimethylamine (NDMA) by crayfish after 6, 12, 24 h exposure, [a]Percent NDMA metabolized was calculated using the following equation: $\text{Metabolized} = \frac{Ct + Ch + Cs + Cw}{100} \times 100$ where, C_t: Conc. in tail, C_h: Conc. in hepatopancreas, C_s: Conc. in shell; C_w: Conc. remaining in water

Table 2: Correlations between N-nitrosamines absorbed in crayfish by compartment

Compartment and N-nitrosamine correlations	Relationship	p<R
Hepatopancreas		
NDMA-NMEA	++	0.009
NDBA-NPIP	++	0.004
Tail		
NDPA-NDBA	++	0.019
Shell		
NDMA-NMEA	++	<0.001
NDEA-NDBA	++	0.03
NDPA-NPIP	++	0.0002
Water		
NDBA-NDPA	++	0.004

Results are significant at p≤0.05, NDMA: N-nitrosodimethylamine, NDEA: N-nitrosodiethylamine, NPYR: N-nitrosopyrrolidine, NPIP: N-nitrosopiperidine, NDPA: N-nitrosodipropylamine, NDBA: N-nitrosodibutylamine, NMEA: N-nitrosomethylethylamine

At the lowest concentration (10 µg mL^{-1}), NDMA metabolized percentage increases as exposure time increases. NDMA metabolized at 24 h was 15 and 2 fold higher than the percentage metabolized at 6 and 12 h, respectively. At 20 µg mL^{-1} of exposure, the metabolized percentage at 24 h was 2.5 and 6 times higher than NDMA metabolized of 6 and 12 h, respectively. However, at higher treatment concentrations, a higher percentage of NDMA has been metabolized at lower exposure concentrations. Furthermore, after 6 h of exposure, NDMA metabolism increased with increasing exposure dose, displaying a Michaelis-Menton-like plot.

DISCUSSION

Toxicokinetic studies focus on the change in concentration of a chemical in a particular tissue or blood/plasma and the biological processes that cause those changes (Creton *et al*., 2009). The

Table 3: Correlations between N-nitrosamines absorbed in crayfish by duration of exposure

Exposure duration (h) and correlations	Relationship	p<R
6		
NDMA-NPIP	-	0.03
NDPA-NPIP	++	0.05
12		
NDMA-NDPA	-	0.04
NMEA-NPYR	++	0.007
NDEA-NPIP	-	0.03
NDEA-NDBA	++	0.05
24		
NMEA-NPIP	++	0.008
NDEA-NPYR	++	0.01
NPYR-NPIP	++	0.001
NDPA-NPIP	++	0.006
NDPA-NDBA	++	0.0006

Results are significant at $p \leq 0.05$. NDMA-N-nitrosodimethylamine, NDEA-N-nitrosodiethylamine, NPYR-N-nitrosopyrrolidine, NPIP-N-nitrosopiperidine, NDPA-N-nitrosodipropylamine, NDBA-N-nitrosodibutylamine, NMEA-N-nitrosomethylethylamine

relationship between exposure dose/time and changes in absorption, metabolism and accumulation in the various organs of the Red Swamp crayfish (post processing) was examined. Such information may be used to determine possible human exposure through the consumption of crayfish.

The majority of the N-nitrosamines (only NDMA data shown) were metabolized by over 90% when exposed to the lowest dose (10 μg mL^{-1}) and shortest time (6 h). The metabolism rate of a toxin depends on the nature of the toxic challenge presented to the organism (Concon, 1988; Creton *et al*., 2009). All N-nitrosamines, except NDMA, were metabolized above 90%, at the lowest exposure dose and time. This suggests that these compounds are possibly more toxic than NDMA or that they are absorbed at a faster rate. Results showed organ specificity for N-nitrosamine absorption. Organ specificity of NDMA may to be related to dose and duration of exposure and can be clearly seen with NDMA. The levels of NDMA, in general, were higher in the hepatopancreas and tail as concentration and exposure time increased. At low doses and short exposure time, most NDMA was unabsorbed (present in the water) and very small amounts (5.2 and 26.6%) (Fig. 1) were biotransformed. However, with increasing doses, there was an increase in the percentage of NDMA being absorbed from the water; NDMA was either absorbed by the tail, hepatopancreas or biotransformed. At higher concentrations, NDMA was negatively correlated with NDEA and NMEA. This change in relationship with increasing exposure concentration is an indication that as NDMA was being absorbed, NDEA and NMEA accumulation was decreasing, due to metabolism. Furthermore, NDMA metabolism increased steadily with increasing dose and exposure time. An increase in metabolism in response to increasing dose maybe due to an induction of detoxification/biotransformation enzymes as a result of the toxic load (Liska *et al*., 2006). Concentrations exceeding the capabilities of the biotransformation enzymes resulted in accumulation in the hepatopancreas and tail. The steady increase in metabolism was only seen with NDMA, which implies that NDMA had a slower rate of absorption in the hepatopancreas (or other tissues) compared to other N-nitrosamines. NDMA has the lowest octanol/water partition coefficient at -0.64 log L kg^{-1}, which may have resulted in the slowest absorption of NDMA.

Even though over 90% of NMEA and NDEA were biotransformed after 6, 12 and 24 h, the highest absorption was seen in the tail. At 6 h, there was an increase in absorption in the tail as

exposure dose increased for both NMEA and NDEA. NPYR was least absorbed in the tail, hepatopancreas and shell compared to other N-nitrosamines. The percentage of NPYR metabolized at all exposure concentrations and time period exceeded 90%. There was also very low concentrations retained in the various organs and low concentrations of NPYR present in the water. Therefore, NPYR was probably quickly absorbed and immediately biotransformed. It is plausible that absorption mainly took place in the hepatopancreas, since this is the organ containing high levels of CYP 450 enzymes in the crayfish and other crustaceans (James and Boyle, 1998; Snyder, 2000).

NDPA and NPIP had similar absorption and organ specificity (Table 1 and 2). At lower concentrations (10-20 μg mL^{-1}) and shorter exposure times (6 h), these two compounds were mainly absorbed in the shell. The shell, which is primarily composed of chitin, is the first line of defense for the crayfish. Some compounds pass this barrier more easily than others. However, the affinity for the tail increased as the exposure dose increased, which could be due to an increase in diffusion rate beyond the shell until equilibrium is reached. At longer exposure periods (12 and 24 h), there was an inverse relationship between dose and absorption in the hepatopancreas. This maybe an indication that detoxification occurred more rapidly as the dose increased.

At 10-20 μg mL^{-1} (6 h), the biotransformation of NPIP differed from NDPA in that over half of NDPA was biotransformed, while most of NPIP was absorbed (data not shown). Furthermore, no absorption occurred in the hepatopancreas for either compound. This difference may be explained by the significantly higher levels of NPIP in the tail at these exposure doses and time. The absorption of NDBA after 6 hours at the lowest concentration (10 μg mL^{-1}) mainly occurred in the tail. At other time durations and doses, absorption was specific in the shell.

Factors affecting absorption and metabolism of a substance may be related to the molecular size, lipid solubility and degree of ionization, among others. The biotransformation of compounds mainly depend on the chemical structure and nature of the specific compound (Concon, 1988). Overall, the N-nitrosamines with the higher molecular weights were more rapidly absorbed and biotransformed. NDMA which had the lowest molecular weight was more steadily metabolized. The factor that had the greatest influence on absorption was lipid solubility, since NDMA is more water-soluble than all other N-nitrosamines. The metabolism of NPYR occurred at the highest rate, which may be an indication of levels of specific isoforms of CYP 450s responsible for biotransformation of cyclic N-nitrosamines.

Absorption did not occur in a dose-dependent manner in this study. According to Creton *et al.* (2009), a substance is absorbed until equilibrium is reached, which is probably one explanation for why absorption was not dose-dependent. In a multi-compartment model for accumulation and distribution, the concentration of a toxin in various tissues is not proportional to the dose or exposure time (as seen in one compartment models/first-order kinetics). Assuming "non-linear" kinetics, the differences in distribution and absorption location exists between N-nitrosamine compounds because some toxicants require more time to reach blood-tissue equilibrium (Creton *et al.*, 2009). Furthermore, absorption depends on the nature of the body tissues, specifically, water, fat and protein content of the specific tissues (Concon, 1988).

Percent metabolized was calculated based on the amount of N-nitrosamines not accounted for in the tail, hepatopancreas, shell and water. However, specific metabolites were not identified in this study.

CONCLUSION

Levels of N-nitrosamines could be related to the concentration of N-nitrosamines and its precursors already present in the crayfish. However, further studies may be conducted related to

their absorption, metabolism and accumulation to consider other factors (season of harvest, farming practices, or other environmental factors) that may affect the toxicokinetics of N-nitrosamines in crayfish. The information gathered from this study can be used to construct the first three components of a human health risk assessment: hazard identification, dose-response assessment and exposure assessment. Once the risk is characterized, the assessment can be used to make and validate decisions on regulations regarding crayfish/products.

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