Identification and Quantification of S-(2-(N(7)-Guanyl)ethyl)glutathione DNA Adduct in Channel Catfish (Ictalurus Punctatus) After Exposure to 1,2-Dichloroethane.

Ahmedin Muktar Jemal
Louisiana State University and Agricultural & Mechanical College

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IDENTIFICATION AND QUANTIFICATION OF S-[2-(N' GUANYL)ETHYL]GLUTATHIONE DNA ADDUCT IN CHANNEL CATFISH (ICTALURUS PUNCTATUS) AFTER EXPOSURE TO 1,2-DICHLOROETHANE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Interdepartmental Program in Veterinary Medical Sciences Through the Department of Epidemiology and Community Health

by

Ahmedin Muktar Jemal
D.V.M., Addis Ababa University, 1986
M.S., Louisiana State University, 1993
December 1997
DEDICATION

To Peter and Suzan Moorhouse for your constant support
ACKNOWLEDGEMENTS

I am very fortunate to have been advised by Dr. Martin E. Hugh-Jones. He has helped me develop my writing skill. I will always cherish his wisdom and kindness. I thank Dr. Jay C. Means, my co-major professor, for his advice and guidance of the research project. He has helped me understand the core aspects of toxicology. I am indebted to thank the other dissertation committee members: Drs. Michael G. Groves, for his advice, motivations, and humor but most importantly for the financial support and for initiating the contact to do my dissertation research in Dr. Means project; Dr Steven A. Barker, for guiding the analytical method development and for his help in understanding the fundamentals of analytical techniques; and Dr. Daniel T. Scholl for his advice in study design and analysis of results and for his help to develop my quantitative skill. I would also like to extend my sincere gratitude to Dr. David Swenson, a former committee member, for initiating the research project and for teaching me the basics of DNA adducts.

Not enough "Thank you" for Dr. Huan Huang for the synthesis of the DNA adduct standards and analysis of the samples in LC/MS. I would also like to thank Connie David for the help in the LC/MS analysis and Michael T. Kearney for the advice in statistical analysis. The help of Marry Boudreau and Lance Lumbord during exposure of experimental animals is greatly appreciated. Also the technical assistance I received from the faculty/staff members of ECH (Blaine Elbourne, Kathleen Harrington, James Roberts) and VPT (Bruce Toth, Mona Busby, Jeannie Tyler, Shiela Rugby, Neil Magee) departments is most appreciated. I would like to thank Michael
Beck of the Louisiana Department of Environmental Quality and Mark McElroy of the Louisiana Wildlife and Fisheries for their material support.

I am indebted to thank my friends Peter and Suzan Moorhouse who made this rewarding training program possible by supporting me financially and morally. I thank Dr. Alexander Thompson for his material support and friendship. I would also like to thank my other friends Dr. Amha Lisan, Aster Geresu, Dr. Zeleke Negatu, Lulseged Ayalew, Asfaw Bekele, and Dr. Abraham Armidie for their support in many ways.

Finally, I thank my parents, Jemal and Wosila, and my brothers, Hassen, Aman, and Abdu for constant support and encouragement.
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ABSTRACT

The main goal of the study was to provide the basic technical framework for the use of native fish species as environmental sentinels for potential human exposure to genotoxic agents in general and to 1,2-dichloroethane (ethylene dichloride) in particular. First, LC/MS/ESI/ID and LC/MS/MS/ESI/ID methods for detection and quantification of S-[2-(N\(^7\)-guanyl)ethyl]glutathione DNA adduct from biological samples was developed. The methods were tested by exposing groups of rats with three dose levels of 1,2-dibromoethane. They allowed detection of ~1 adduct in 10\(^7\) bases. Second, the formation and persistence of DNA adduct in channel catfish (*Ictalurus punctatus*) from 1,2-dichloroethane exposure was studied under controlled laboratory conditions. DNA adducts formed rapidly, within two hours of exposure. The peak of the DNA adduct (265 pmol/mg DNA) formation was 4 hours post-exposure with a half-life of 2-5 days. However, the DNA adduct was detectable three weeks after end of exposure. Third, a dose-response relationship, with the dose in ppm and response as DNA adducts’ frequency per mg DNA, was established. The biological response was linear up to 200 ppm and appeared to level off thereafter. Finally, the effect of depletion of glutathione on DNA adduct formation was studied. DNA adducts were not detected in fish pre-treated with diethylmaleate (DEM) (detection limit, ~10 fmol on column).

The results from this study provide the first evidence for the potential use of channel catfish as sentinel animals for 1,2-dichloroethane environmental contamination. Furthermore, the hepatic DNA adduct in channel catfish may serve as a dosimeter of exposure and thus may be used to differentiate areas of high pollution.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

1,2-Dichloroethane is extensively used in the U.S. (6.2 x 10^9 kg/year), predominantly for the synthesis of vinyl chloride (Guengerich et al., 1994). It is an environmentally important compound in Louisiana because of the volume of production and the historical levels of contamination in the environment. About half of 1,2-dichloroethane producing capacity of chemical companies in U.S. are located in Louisiana. For example, in 1993, there were 16 facilities that produced 1,2-dichloroethane in the U.S., of which 8 were in Louisiana, accounting for about 42% (4.5 x 10^9/10.8 x 10^9 kg) of the total production (SRI International, 1993). From these production facilities and others that use 1,2-dichloroethane as a synthetic intermediate, 1,2-dichloroethane is released to the environment through different routes such as permitted discharges into water and air and accidental spills.

The Louisiana Department of Environmental Quality (LDEQ) estimates that over 7 million kg of 1,2-dichloroethane were released into the air and water of Louisiana between 1987-93 (LDEQ, 1995). Two historical spills, among many, abstracted from LDEQ public domain files are: (1) in Feb., 1993, about 7000 kg of 1,2-dichloroethane leaked from a storage tank at Pittsburgh Plate Glass in Lake Charles; (2) in March, 1994, at the Conoco Marine Dock facility an underground pipeline failed and 750,000 kg of 1,2-dichloroethane escaped.
As a result of these permitted discharges and spills, 1,2-dichloroethane is the most frequently detected volatile organic compound present above the permissible limit in the streams and in hazardous waste sites in the state. Contamination levels as high as 3.5 mg/kg were reported at a Calcasieu Parish hazardous waste site (Hanor, 1995). In 1988, 1,2-dichloroethane was detected in Monte Sano Bayou (located in East Baton Rouge Parish) down stream of some synthetic industries at a level of 114 ppb. Further investigation to locate the source of contamination of the bayou revealed levels as high as 4800 ppm of 1,2-dichloroethane in ground water monitoring wells of Formosa Plastic and Ethyl Corporation (LDEQ, 1994, Water Quality Inventory). Though contamination of the environment with 1,2-dichloroethane in other states may not be as prevalent as in Louisiana, 1,2-dichloroethane environmental contamination is ubiquitous in the U.S. The compound has been detected in approximately 35 % (484/1350) of the EPA national priority list of hazardous waste sites (HAZDAT, 1992; U.S. DHHS, 1994). Furthermore, it is among the 189 air-toxic compounds listed in The Clean Air Act Amendments of 1990 (Olden & Guthrie, 1996).

1,2-Dichloroethane is mutagenic in Drosophila melanogaster (Ballering et al., 1994) and can produce tumors in rodents (NCI, 1978b). Therefore, it is classified as a possible carcinogen in man. The compound has been demonstrated to form DNA adducts in rats (Inskeep et al., 1986; Guengerich et al., 1994). Adducts produced in rodents upon exposure to 1,2-dibromoethane (an analogue of 1,2-dichloroethane) have been characterized by Guengerich and his colleagues. The S-[2-(N'-guanyl)ethyl]glutathione adduct was the major adduct formed in liver tissue.
accounting for 95% of the total DNA adduct burden. Unlike 1,2-dibromoethane, the characteristics of adducts produced by 1,2-dichloroethane have not been well documented in the literature. Nevertheless, it has been shown experimentally that both 1,2-dichloroethane and 1,2-dibromoethane produce the same major adduct in rat liver although the extent of production is about four fold higher with the latter than with the former compound (Inskeep et al., 1986).

Besides their importance in the initiation of chemical carcinogenesis, DNA adducts can be used as markers of exposure (Perera, 1988). The high level of contamination of the environment with 1,2-dichloroethane and the carcinogenic potential of the compound may be exploited in the development of a surveillance strategy, in the long-term, through screening for levels of adduct formation in channel catfish (Ictalurus punctatus) from 1,2-dichloroethane exposure in the environment. In order to realize this goal, the following considerations must be made. First, neither of the available analytical methods (radioactivity and fluorescence intensity) are suitable for detection and quantitation of the DNA adducts from whole animal tissue. The former method requires radiolabeled compound be used in the exposure and is applicable only for experimental purposes; the latter technology has inadequate sensitivity. Hence, there is a need for development of alternative analytical methods for detection of the DNA adduct in environmental biological samples. Second, there is currently no information documenting whether native fish metabolize 1,2-dichloroethane to form the specific hepatic DNA adduct associated
with mutagenesis and carcinogenesis. To address these points, the following hypotheses were formulated along with the specific objectives.

Null Hypotheses

1. Channel catfish (*Ictalurus punctatus*) will not metabolize 1,2-dichloroethane to form S-[2-(N'-guanyl)ethyl]glutathione adduct in liver tissue.

2. There is no relationship between the concentration of 1,2-dichloroethane fish are exposed to and the levels of hepatic DNA adducts formed.

3. Chemical-depletion of glutathione will not affect DNA adduct formation in channel catfish.

Specific Objectives

1. To develop alternative methods for detection and quantification of DNA adducts in biological samples from animals exposed to 1,2-dihaloalkanes.

2. To test if channel catfish produce hepatic DNA adducts within the detection limit of the analytical system used.

3. To outline the persistence of the hepatic adduct formed in channel catfish exposed to 1,2-dichloroethane. This will provide the biological half-life of the DNA adduct.

4. To establish a dose-response relationship between the levels of exposure of 1,2-dichloroethane and formation of hepatic DNA adducts in channel catfish under controlled laboratory conditions.
5. To provide insight for future research work in interpretation of negative field results resulting merely from glutathione depleting compounds that occur concomitantly with 1,2-dichloroethane.

**Literature Review**

**Cancer and Louisiana**

Louisiana is one of the states in U.S. with highest cancer prevalence rates. A draft report, in 1974, by the Environmental Defense Fund on the implications of cancer-causing substances in Mississippi River water raised concerns of the contamination of drinking water with chlorinated hydrocarbons. Subsequently, Page *et al.* (1976) studied the relationship between Louisiana parishes that receive drinking water from the Mississippi River and 20-year cancer mortality rates for these parishes. Multivariate regression analysis indicated a statistically significant (p<0.05) relationship between cancer mortality rates (total cancer, cancer of the urinary organs and cancer of the gastrointestinal tract) in Louisiana and drinking water obtained from the Mississippi River. Furthermore, Dowty *et al.* (1975) detected volatile halogenated hydrocarbons from New Orleans drinking water and plasma of local residents. Using a case-control mortality study in which the residence and water supply of each decedent was individually linked in south Louisiana, Gottlieb and Carr (1982) studied the relation of chlorinated surface water with cancer type. But, unlike the preceding report by Page *et al.* (1976) in which chlorinated surface water was associated with different cancer types, they found the association of chlorinated surface water with cancer type only for rectal cancer.
Recently, the Lower Mississippi River Interagency Cancer Study (LMRICS) program has been initiated by the Louisiana Department of Health and Hospitals (LDHH) and the Louisiana State University Medical Center, Stanley S. Scott Cancer Center. The program was developed to generate data on human cancer in relation to factors such as environmental pollution, person, and location of chemical factories. Based on preliminary results of a 30-year cancer trend analysis, it was concluded that 1960-1993 age-adjusted average annual lung cancer mortality rates were higher in the rural study area compared to the study area as a whole (LSU Medical Center, 1996).

**1,2-Dichloroethane**

**Chemical and physical properties**

1,2-Dichloroethane is a colorless, volatile, and flammable liquid with a sweet taste. It has a molecular weight of 96.95 amu, boiling point of 83°C, and vapor pressure of 8.5 kPa; and its density (1.26 at 24°C) is slightly heavier than water. It is readily soluble in organic solvents (ether, alcohol, benzene) but only moderately soluble in water (only 6.3 g/L) (U.S. DHHS, 1994).

**Production and uses**

1,2-Dichloroethane does not occur naturally in the environment. It is synthesized by chlorination of ethylene, and is the first chlorinated hydrocarbon to be manufactured in 1795. It is extensively produced in the U.S. The annual production for 1991 was 6.2 billion kg, up over by a million from 1980 (5.04 million tons) (IPCS, 1995). As of June, 1993, there were 11 manufacturers with 16 production
facilities in the U.S. located predominantly in the southern regions, including Texas and Louisiana (SRI, 1993).

1,2-Dichloroethane is predominantly used as an intermediate for the synthesis of vinyl chloride as well as other organic solvents such as tetrachloroethylene and 1,1,1-trichloroethane. Over 88% of the production in the U.S., 90% in Canada and 99% in Britain is used for this purpose (IPCS, 1995). It has also been used (1) as an extraction solvent for decaffeinated coffee, dyes and perfumes (U.S. DHHS, 1994); (2) as an antiknock agent in gasoline; and (3) as a soil fumigant in highly productive agricultural fields to combat plant parasites and as a seed fumigant in storage. However, after the compound was established to have been carcinogenic in experimental animals in the early 1980’s, its agricultural use has been banned in many countries, including the U.S., U.K., and Canada. Also its use as an antiknock additive has been declined for the same reasons.

Sources of environmental contamination and fate

Contamination of the environment by 1,2-dichloroethane can occur (1) through direct release to the air and discharge of effluent to streams from facilities that produce 1,2-dichloroethane or use 1,2-dichloroethane as an intermediate compound for synthesis of other products, (2) by leaching into ground water or streams from landfills or hazardous waste sites, and (3) through leaks from valves and failure of underground pipelines (IPCS, 1995; U.S. DHHS, 1994).

The atmosphere is the primary sink for 1,2-dichloroethane. In the atmosphere, 1,2-dichloroethane undergoes photo-oxidation and photolysis to yield HCl, formyl
chloride, CO₂ and free chlorine radicals. The half-life of the compound in the atmosphere is estimated to be between 43 and 111 days (IPCS, 1995). In lakes and rivers, 1,2-dichloroethane vaporizes readily, and its half-life is about one day, but in the case of large spills into lakes it may stay for months (HSDB, 1993). Loss to the atmosphere by volatilization was the main fate following a chemical spill in the Rhine River in Germany (Brueggemann et al., 1991). In ground water, 1,2-dichloroethane degrades very slowly by hydrolysis and its half-life is estimated at 23 years. Due to its moderate water solubility, 1,2-dichloroethane has low sorption coefficients and hence is not well sorbed to particulate matter (Wilson et al., 1981; IPCS, 1995). A large portion of 1,2-dichloroethane applied to soil ultimately leaches into ground water. For example, contamination of ground water in Seminole County, Georgia, occurred as a result of 1,2-dichloroethane application to soils to combat crop parasites. Environmental surveys conducted by EPA have detected 1,2-dichloroethane in groundwater sources in the vicinity of contaminated waste sites (EPA, 1984).

1,2-Dichloroethane has a relatively low potential for bioaccumulation. The bioaccumulation factor was 2, with a half-life tissue clearance of two days in fresh water bluegill (Lepomis macrochirus) exposed to 96.5 μg/L for 14 days (Barrows et al., 1980).

Environmental levels

Levels of 1,2-dichloroethane have been estimated in several environmental matrixes. In ambient air, the level is typically very low. In samples gathered from 23
cities in Canada between 1988-1990, the average level of 1,2-dichloroethane was 0.13 μg/m³ (Dann, 1992). In Japan, it is found in a range of 0.004-3.8 μg/m³ and for the U.S. it is usually < 0.8 μg/m³. Of the 1350 hazardous waste sites in the National Priority List (NPL), 1,2-dichloroethane was recorded in 484 of them, and the levels in air ranged from trace to 27 μg/m³ (U.S. DHHS, 1994).

In Canada, 15 out of the 2000 drinking water samples tested were positive for 1,2-dichloroethane, and levels as high as 0.85 μg/L were recorded (IPCS, 1995). In the U.S., 1,2-dichloroethane was recorded in 24 out of 1973 ground water samples with concentration levels up to 19 μg/L (Letkiewicz et al., 1982). Of the 204 water samples collected from 14 river basins in the U.S., 1,2-dichloroethane was detected in 53% of the samples (EPA, 1977). Concentrations in surface water used as a source of drinking water ranged from trace to 4.8 μg/L. The compound was recorded in 89% of indoor air samples (770 ppt) and 100% of outdoor air samples (440 ppt) collected in Baton Rouge, Louisiana, in the winter of 1981 (Pellizarri et al., 1986). 1,2-dichloroethane was also detected in drinking water samples across the U.S., including New Orleans, Miami, Cincinnati, and Philadelphia (Clark et al., 1986).

**Kinetics and metabolism**

Studies on experimental animals revealed that 1,2-dichloroethane is readily absorbed after ingestion, inhalation, and application on the skin (Nouchi et al., 1984; Spreafico et al., 1980; Reitz et al., 1982). Peak blood levels (16 mg/L) of 1,2-dichloroethane in rats occurred within 15 minutes following single oral administration at doses between 25-150 mg/kg of body weight (Reiz et al., 1982).
Similarly, levels of 1,2-dichloroethane peaked in blood within 1-2 hours of continuous inhalation of 600 mg/m$^3$ (150 ppm) for 6 hours.

Following exposure, 1,2-dichloroethane is widely distributed throughout the body: spleen, lung, liver, brain and adipose tissue. It accumulates most rapidly in liver. However, the concentration is higher in adipose tissue (Spreafico et al., 1980). It was detected in fetal tissue of rats following maternal exposure to airborne concentrations ranging from 612-7966 mg/m$^3$ (153-1999 ppm) on day 17 of gestation. The concentration detected in fetal tissues was proportional to the level of maternal exposure (IPCS, 1995).

Metabolism of 1,2-dichloroethane essentially follows one of two pathways: a microsomal mediated p450 oxidative pathway and a cytosolic glutathione conjugation pathway. In the first pathway, p450 enzymes catalyze an oxidative transformation of 1,2-dichloroethane to form 2-chloroacetaldehyde and 2-chloroethanol. These intermediates react enzymatically or nonenzymatically with glutathione and are excreted in the urine (Guengerich et al., 1980). The second pathway involves conjugation of 1,2-dichloroethane with glutathione to form S-(2-chloroethyl)glutathione [half-mustard]. The latter non-enzymatically converts to episulfonium ion that then alkylates proteins, RNA or DNA (Fig. 1). Although the p450 pathway may induce DNA damage, the glutathione pathway is considered the most important pathway as the major route for DNA damage (Guengerich et al., 1994; Inskeep et al., 1986).
Fig. 1.1: Metabolic activation of 1,2-dihaloalkane and formation of S-[2-(N\textsuperscript{7}-guanyl)ethyl]glutathione DNA adduct
The major metabolites of 1,2-dichloroethane described are largely excreted in the urine, and the parent compound is eliminated through expired air (IPCS, 1995). 1,2-Dichloroethane has been administered to rats by inhalation (600 mg/m³) for 6 hours or by gavage (150 mg/kg of body weight). Forty-eight hours following exposure, more than 84% of total metabolites were eliminated through urine, 8% as carbon dioxide in expired air, 2% parent compound was detected in the feces and the balance (4%) was found in the carcass (Reitz, et al., 1982). The compound is unlikely to bioaccumulate in significant amounts. Recovery of metabolites in expired air and excreta was complete for young rats exposed to 1,2-dichloroethane at a dose of 150 mg/kg of body weight by gavage (Cheever et al., 1990).

**Toxicological effects**

The literature is replete with the toxicological effects of 1,2-dichloroethane, and effects vary depending upon duration and route of exposure and the presence or absence of other compounds. For the purpose of this brief review, the toxicological effects of 1,2-dichloroethane are divided into the acute toxicity, chronic exposure and carcinogenicity, and mutagenicity and related end points.

**Acute toxicity, mammals:** The LD₅₀ value of 1,2-dichloroethane ranges from 680 to 850 mg/kg of body weight in rats and from 413 to 489 mg/kg of body weight in mice (IPCS, 1995; U.S. DHHS, 1994). Daniel et al. (1995) studied the 10 and 90-day toxicity of 1,2-dichloroethane by exposing rats with 300 mg/kg of body weight and 150 mg/kg of body weight *per os*, respectively. Whereas all the animals in the 90-day study group survived, only 2/20 rats survived in the 10-day study group.
Data on toxicity of 1,2-dichloroethane on man has been accrued primarily through occupational and accidental exposures. The lethal oral doses of 1,2-dichloroethane is estimated at 20-50 ml per os (IPCS, 1995; US DHHS, 1994). For instance, a 51-year-old-man inhaled 1,2-dichloroethane for 30 minutes and died 4 days after exposure; a 63-year-old-man died 22 hours after ingesting 60 ml of 1,2-dichloroethane; a 14-year-old-boy died 6 days after ingesting 15 ml of 1,2-dichloroethane. Non-lethal acute toxicity of 1,2-dichloroethane in man includes depression of the central nervous system, cardiovascular insufficiency, gastroenteritis, and liver and kidney damage.

**Acute toxicity, aquatic species:** The acute toxicity of 1,2-dichloroethane has also been studied in aquatic organisms and ranging from microorganisms such as *Nitrosomonas* (bacteria) to vertebrates such as the guppy and fathead minnows. Guppies are reported to be the most sensitive fresh water fish species to 1,2-dichloroethane. The LC$_{50}$ value under static renewal test condition for 7-day exposure was 106 mg/L. In comparison, rainbow trout appear to be more resistant with an LC$_{50}$ value of 336 mg/L after 96 hour of static test exposure (Konemann, 1981).

**Chronic exposure and carcinogenicity:** Route of exposure substantially affects the effect of 1,2-dichloroethane. Groups of female and male rats were exposed by gavage with 1,2-dichloroethane at a dose of 95 mg/kg of body weight for 78 weeks followed by 32 weeks of observation. The incidence of squamous cell carcinoma of the stomach was significantly higher (p=0.01) in the exposed groups (9/50) than matched controls (0/20). Similarly there were significant differences in the incidence...
of adenocarcinoma (18/50, exposed groups versus 0/20, matched control) and fibroadenoma (8/59 versus 0/20) of the mammary gland (NCI, 1978b). However, exposure of 1,2-dichloroethane to groups of rodents by inhalation failed to show any significant difference in the development of tumors (Maltoni et al., 1980) or liver genotoxicity (Storer et al., 1984) compared to controls. However, the compound becomes tumorogenic at dose levels as low as 50 ppm (U.S. occupational standard) when given with 0.05% disulfiram in the diet for 2 years (Cheever et al., 1990; Igwe et al., 1986).

Two epidemiologic studies have attempted to address the impact of chronic exposure of 1,2-dichloroethane on man. Men working in chlorohydrin production plants from 1940-67 were followed up to 1988. Excess death due to pancreatic cancer was higher in men working in chlorohydrin production plants when compared to US national rates (observed : expected = 8:1.6) (Benson and Teta, 1993; IPCS, 1995). The study was, however, confounded by simultaneous exposure of workers with ethylene oxide and ethylene chlorohydrin. In the second epidemiological study, the relationship between the level of 1,2-dichloroethane in drinking water and cancer incidence between 1969 to 1981 in men >55-years old was studied. The incidence of colon (222.8/100,00 persons) and rectal cancer (126.5/100,00 persons) for men whose drinking water contained > 0.1 μg/L 1,2-dichloroethane were significantly higher than for those men (colon cancer, 170.3/100,000; rectal cancer, 92.9/100,000) whose drinking water contained < 0.1 μg/L 1,2-dichloroethane/L (p < 0.05) (Isacson et al., 1985). The study was confounded by the presence of the chlorinated organic
solvents in the drinking water. Because no account was made for the confounding factors in the analysis of the data, the results may be questionable.

Mutagenicity and related end points: 1,2-Dichloroethane is found to be mutagenic and genotoxic in several biological tests including human cell lines, bacteria, insects, and mammals; a few of the reports are given in Table 1.1.

Table 1.1: Summary of selected mutagenicity and genotoxicity studies

<table>
<thead>
<tr>
<th>Species (Test System)</th>
<th>End Points</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em></td>
<td>Gene mutation</td>
<td>Milaman et al., 1988</td>
</tr>
<tr>
<td>Human periph. lymphocyte</td>
<td>Unscheduled DNA synthesis</td>
<td>Perocco et al., 1981</td>
</tr>
<tr>
<td>Mouse bone marrow</td>
<td>Sister-chromatid exchange</td>
<td>Gri &amp; Hee, 1988</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Gene mutation</td>
<td>Ballering et al., 1993</td>
</tr>
<tr>
<td>Sprague-Dawely rats</td>
<td>DNA adducts</td>
<td>Inskeep et al., 1986</td>
</tr>
</tbody>
</table>

DNA Adducts

DNA adducts are the complex products of reactive chemicals and DNA that contain one or more covalent bonds between the two moieties (Miller and Miller, 1981; Dipple, 1995). The sources of chemicals can be endogenous such as hormones or exogenous such as chemicals from the environment or drugs taken as medication (Goldring & Lucier, 1990).

Leonard (1984) suggested that oxidation of 1,2-dichloroethane by the p450 pathway gives rise to 2-haloacetaldehydes and the haloacetaldehydes may react with DNA to form DNA adducts. However, many researchers (Rannug et al., 1978, Guengerich et al., 1980; Ozawa & Guengerich, 1983; Inskeep & Guengerich, 1984) have argued that the route for generation of reactive derivatives of 1,2-dichloroethane...
involves GSH conjugation to form S(2-chloroethyl)glutathione and subsequent nucleophilic attack via an episulfonium ion intermediate (Fig. 1). Guengerich and his colleague (1983, 1986) characterized the DNA adducts formed \textit{in vivo} and \textit{in vitro} from 1,2-dibromoethane (an analogue of 1,2-dichloroethane) exposure. S-[2-(N\textsuperscript{7}-guanyl)ethyl]glutathione is the major adduct in liver tissue accounting for over 95% of the total DNA adducts formed (Inskeep \textit{et al.}, 1986; Guengerich \textit{et al.}, 1994). Other hepatic DNA adducts characterized are S-[2-(N\textsuperscript{1}-adenyl)ethyl]glutathione (2%), S-[2-(N\textsuperscript{2}-guanyl)ethyl]glutathione (\(-0.1\%\)), and S-[2-(O\textsuperscript{6}-guanyl)ethyl]glutathione (0.2%) (Koga \textit{et al.}, 1986; Cmarik \textit{et al.}, 1992, Guengerich \textit{et al.}, 1994).

When Sprague-Dawley rats were administered a single intraperitoneal (i.p.) injection of 37 mg of radiolabeled 1,2-dibromoethane per kg of body weight, adduct levels in liver tissue in the range of 0.26 nmol/mg DNA (Kim & Guengerich, 1990) to 1.3 nmol/mg of DNA (Inskeep \textit{et al.}, 1986) were reported. Levels of hepatic DNA adduct formation from 1,2-dichloroethane exposure (150 mg per kg of body weight by i.p. injection) in the Sprague Dawley rats was 0.26 nmol/mg of DNA. The major adduct was also observed to form in the kidney, lung and stomach, but to a lower degree than in liver. The levels of formation of the adduct in rats kidney and liver were linear with respect to dose of 1,2-dibromoethane administered (Inskeep & Guengerich, 1989). The stability of the major adduct both \textit{in vivo} (in rat) and \textit{in vitro} (in calf thymus DNA incubated at 37°C) was studied. The biological half-life was estimated at 70-100 hours \textit{in vivo} and 150 hours \textit{in vitro} (Inskeep \textit{et al.}, 1986).
Significance of DNA adducts

DNA adducts are important in the initiation of chemical carcinogenesis (Swenson, 1983; Van Zeeland, 1996; Denissenko et al., 1996), as markers of exposure (Ehrenberg et al., 1996; Mumford et al., 1993; Perera et al., 1994), and for risk assessment (Bartsch et al., 1983; Bodell et al., 1996; Perera, 1988).

Adducts in chemical carcinogenesis: A large body of evidence exists to support the role of adducts in the first stage of chemical carcinogenesis. Recently, an elegant piece of evidence was obtained by mapping the distribution of DNA adducts along the p53 gene using a modification of the ligation mediated polymerase chain reaction (LPCR) in benzopyrene diepoxide (BPDE)-treated HeLa cells as well as bronchial epithelial cells (Denissenko et al., 1996). The adduct hot spots and mutational hot spots for lung cancer (Codon number 157, 248, and 273) coincided, suggesting that BPDE adducts are involved in the transformation of human lung tissue. Carcinogen-DNA adducts and somatic gene mutation at the hypoxanthine guanine phosphoribosyltransferase (HPRT) locus have also been evaluated in leukocytes of workers in a foundry exposed to benzo[a]pyrene and other polycyclic aromatic hydrocarbons (PAH). A significant correlation coefficient (r=0.65; p=0.0005) was reported between PAH-DNA adducts and mutation at the HPRT locus during the two year study period (Perera et al., 1994).

More convincing evidence for the role of DNA-adducts in chemical carcinogenesis was obtained from a nested case-control study that associated adduct formation to development of cancer. The study was initiated in 1986 in Shanghai to
establish the relationship between markers for aflatoxin and hepatitis B virus and the development of cancer. About 18,000 urine samples were collected from healthy male adults, and in the subsequent 7 monitoring years, 50 men developed liver cancer. After cases were matched with controls for age and residence, a highly significant odds ratio was observed for detectable urinary aflatoxin. The presence of aflatoxin B1 adduct (AFB-N'-Gua) in urine was associated with a 2-3 fold elevation in odds of developing cancer (Ross et al., 1992; Quian et al., 1994).

Except for the case of aflatoxin, 7-guanyl-DNA adducts do not appear to be mutagenic or involved in initiation of tumors. The role of S-[2-(N'-guanyl)ethyl]glutathione in mutation was studied in Salmonella typhimurium TA 100 (Humphreys et al. 1990), in oligonucleotide d(ATGCA) and d(CATGCCT) with S[2-(N'-guanyl)ethyl]glutathione substituted for guanine (Kim and Guengerich, 1993). Neither of the studies provided a definitive answer regarding the role of the adduct in mutagenesis although a number of studies strongly suggested that GSH dependent activation of dihaloalkanes is important in the initiation of mutation (Guengerich et al., 1994).

**Adducts as biomarkers:** Exposure mis-classification is one of the major problems in environmental epidemiology. Comparison of concomitant exposure classification by air monitoring and biologic (urine) monitoring has revealed extensive disagreement (Droz et al., 1991). Unlike air monitoring, biologic monitoring measures the biological effective dose of carcinogens and allows for absorption through all routes and accounts for individual variation in absorption and metabolic transformation rates.
of a compound. Thus, DNA adducts offer a measure of the dose to which an intracellular target has been exposed. Few studies have attempted to use DNA adducts in exposure monitoring, and almost all of these are validation or pilot studies.

One outstanding example is a study carried out on the development, evaluation, and application of a bio-monitoring procedure (that involved DNA adducts in addition to other toxicological end points) for populations exposed to environmental genotoxic pollutants (PAH) in Europe. Populations exposed through occupational sources to relatively large amounts of genotoxic compounds including PAH were used as positive control whereas populations in rural areas were negative controls. Garage workers and bus mechanics who are potentially exposed to PAH through diesel exhaust and/or lubricating oils had higher adduct levels (8.3 adducts/10^8 nucleotide) compared to adduct levels in matched controls (2.5/10^8 nucleotide, p=0.0004) (Farmer et al., 1996).

Adducts in risk assessment: As discussed elsewhere, adducts may be repaired with varying efficiency depending on size and chemical complexity, and the probability of a DNA adduct becoming fixed as a genetic change is dependent on the relative rates of DNA repair and cell division. It follows therefore that for chemicals with metabolites that interact covalently with DNA, it may be more meaningful to relate tumor response to the concentration of DNA adducts in target organ rather than to the applied dose (Hoel et al., 1983). Thus, DNA adducts can be used for risk assessment. However, the research area is in its infancy.
Dose-response relationship

The dose-response relationship, dose as the chemical carcinogen and response as adducts per mg of DNA, depends upon a number of biological variables including absorption efficiency, distribution and excretion rates, and whether the compound requires metabolic activation or detoxification (reviewed by Swenberg et al., 1995; Lutz, 1990). In particular, the balance between the activation and detoxication rates determines the amount of carcinogen available to cause heritable damage (Swenberg et al., 1995). When detoxification pathways are overwhelmed or when DNA repair capacity is saturated, greater numbers of DNA adducts accumulate per unit of exposure. A classical example of this formation is the O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-MG) adduct following a single exposure to dimethylnitrosamine covering five orders of magnitude in dose. As the dose increases, the slope of the dose response for adduct formation increases because of the saturation of DNA repair while the capacity for activation of dimethylnitrosamine is not exceeded.

If critical steps in the process are not saturated, the dose-response relationship is linear. If a metabolic activation pathway is saturated, the response is supra-linear, i.e., adducts per unit dose are much less in higher dose than lower dose. On the other hand, saturation of detoxication or DNA repair results in a sub-linear dose-response curve, i.e., the amount of DNA adducts per dose is higher at higher doses than at lower doses. Nonetheless, like many pharmacokinetics products, DNA adducts attain steady-state concentration during chronic exposures (Swenberg et al., 1995). The amount formed in each unit of time equals the amount lost or repaired in the unit
period of time. For glutathione adducts, the dose response is expected to be linear until the glutathione pathway and/or the enzymatic DNA repair pathway is saturated, when the response becomes supra-linear. For instance, Kim & Guengerich (1989) found a linear response of adduct formation when rats were exposed to 1,2-dibromoethane in the range of 1-37 mg per kg of body weight. The linear range was not exceeded in this study.

**DNA adducts in fish**

DNA adduct formation in fish from exposure to chemical carcinogens has been reported both in the laboratory (Bailey *et al.*, 1988; Vranasi *et al.*, 1989a; Sikka *et al.*, 1991; Law *et al.*, 1996) and in the field (Malins *et al.*, 1990; Varanasi *et al.*, 1989b; Dunn *et al.*, 1987) (reviewed by Maccubbin in 1994). In most cases, the major adducts formed from specific carcinogenic exposures in fish were the same as those reported for mammalian model systems suggesting that fish and mammals follow similar processes of chemical bio-transformation (Maccubbin, 1994). For example, Sikka *et al.* (1991) reported that bullhead (*Ictalurus nebulosus*) liver metabolizes benzo pyrene (BP) to (+)-anti-BPDE, which forms adducts with deoxyguanosine (dG). The same adduct is prominent in mouse skin which is susceptible to BP-induced tumorogenesis.

DNA adduct formation in feral fish obtained from a PAH contaminated aquatic environment were studied and some of the studies revealed that there was a correlation between hepatic DNA adduct levels and the degree of contamination in the aquatic environment. For instance, van Schooten *et al.* (1995) studied DNA
dosimetry in eel (*Anguilla anguilla*) living on PAH-contaminated soil and sediments. They reported a significant correlation \( r^2 = 0.59, p<0.001 \) between the total aromatic DNA adducts level in the livers of eel and the level of PAH in the sediment. Also Dunn *et al.* (1987) reported formation of DNA adducts by \(^{32}\)P-postlabelling system in Brown bullheads (*Ictalurus nebulosus*) sampled from sites in Buffalo and the Detroit River with high levels of sediments containing bound PAH. Vsaranasi *et al.* (1989b) also reported elevated DNA adduct levels in livers of English sole (*Parophrys vetulus*) sampled from contaminated areas of Puget Sound where they were exposed to high concentrations of sediment-associated chemical contaminants. These fish also exhibited an elevated prevalence of hepatic neoplasms. The levels of total DNA adducts (assessed by \(^{32}\)P-postlabelling) reported for two sites in the Puget sound area were on an average of 17 to 26 nmol adducts/mg of nucleotide. Interestingly, English sole, treated with extracts of the contaminated sediments, had similar adduct profiles to those wild populations of English sole captured from the contaminated area. In comparison, no similar adducts were detected from DNA digest of English sole from a reference site.

There are differences among fish species in binding of chemicals to DNA post exposure. Aflatoxin B1 (AFB1) binding was 7-56 times greater in rainbow trout than in salmon liver. Among other factors, the species difference in AFB1-DNA binding was related to the lower efficiency of cytochrome p-450 metabolic activation of aflatoxin to the reactive 8,9-epoxide in salmon as compared to trout (Bailey *et al.*, 1988).
DNA repair following chemical exposure is generally characterized as low in fish as compared to mammals. A modest amount of DNA repair capacity was reported in fish in both *in vitro* (Walton *et al.*, 1983; Walton *et al.*, 1984a, 1984b) and *in vivo* (Ishikawa *et al.*, 1978; Nakatsuru *et al.*, 1987) following exposure to a variety of chemicals. Also fish are deficient or low in DNA excision repair activity except for UV dimers. Nakatusuru *et al.* (1987) studied the O<sup>6</sup>-methylguanine DNA methyl transferase (O<sup>6</sup>-MT) activity in livers from eight fish species. They found that the activity of O<sup>6</sup>-MT was lower in all fish species when compared to mammals except for the Japanese medeka (*Oryzias latipes*) which showed a level of O<sup>6</sup>-MT activity comparable to that measured in the mouse.

As a result of the deficient DNA repair in fish, the biological half-life of DNA adducts in fish, formed following exposure to chemicals, is expected to be significantly longer than the half-life of similar adducts in mammalian systems. For example, the major DNA adducts formed by AFB1 has a half-life of 3-4 weeks in rainbow trout (Goeger *et al.*, 1986) compared to 7-8 hours in the rat (Croy & Wogan, 1981). This comparatively long biological half-life of DNA adducts in fish suggests fish may be better sentinel animals for monitoring of exposures to environmental carcinogens through biological markers than traditional laboratory mammals.

**The Study Animal (Channel Catfish)**

Channel catfish (*Ictalurus punctatus*) is widely distributed in the southeastern U.S. (Becker, 1983). It is abundant in streams and lakes of Louisiana (Douglas, 1974)
with an abundance range of 6 - 840 fish/acre (McElroy, 1997). Channel catfish is also one of the most commercially important freshwater fish in the state. Two and half million kg of channel catfish were harvested in Louisiana in 1985 with a gross value of $2.67 million (McElroy et al., 1990).

Perry et al. (1985) have studied the migration of channel catfish in Louisiana by capturing, tagging, and releasing in the Salvador wildlife management area from 1979 through 1984. About 2% of the tagged catfish were recovered. The maximum migration recorded was for a 14" fish which traveled 48 km in 60 days from the point of release. A similar migration study of channel catfish in the upper Mississippi River showed a maximum downstream migration of 345 km in 14-16 months and upstream migration of 345 km in 33 months. Movement as fast as 5 km/day (179 km in 36 days) was recorded. In contrast, about 89% of catfish tagged in Trempealeau Bay (Trempealeau County) were recaptured within 21 km.

Unlike brown bullheads (Ictalurus nebulosus), channel catfish have rarely shown pollutant-mediated tumor formation in field studies (Harshbarger 1979, 1981; Hasspieller, 1994). This was attributed in part to the glutathione-dependent defense because they have higher constitutive levels of glutathione compared to other fish (Hasspieller, 1994). No documented report was available on the toxicity of 1,2-dichloroethane in channel catfish.

In summary, this chapter presented important characteristics of 1,2-dichloroethane: its carcinogenic potential and its ability to form DNA adducts. It is envisaged that these characteristics of the compound along with its availability in the
environment at high level may be exploited in the development of a surveillance
system, in the long run, through screening of DNA adducts in feral population of
channel catfish. Therefore, the subsequent chapters in this manuscript provide basic
information towards achieving this goal. Chapter 2 addresses the development of
analytical methods for detection and quantification of S-[2-(N'-
guanyl)ethyl]glutathione from biological samples. Chapter 3 deals with the formation
and persistence of the DNA adduct in channel catfish (Ictalurus punctatus) following
exposure to the compound. Chapter 4 focuses on dose-response relationships of the
DNA adduct and the effect of depletion of glutathione on adduct formation. The last
chapter summarizes over all results and provides recommendations on future studies.
CHAPTER 2

LIQUID CHROMATOGRAPHY (LC)/ MASS SPECTROMETRY (MS)/ AND TANDEM MASS SPECTROMETRY (MS/MS)/ ELECTROSPRAY IONIZATION (ESI)/ ISOTOPE DILUTION (ID) METHODS FOR DETECTION AND QUANTIFICATION OF S-[2-(N\textsuperscript{7}-GUANYL)ETHYL]GLUTATHIONE DNA ADDUCT

Introduction

DNA adducts can be used as markers of genotoxic exposure (Wogan & Gorelick, 1985; Wogan, 1992; Perera et al., 1996; Mumford et al., 1993; Dunn et al., 1987) in addition to their importance in the initiation of chemical carcinogenesis (Miller & Miller, 1981; Swenson, 1983; Grollman & Shibutani, 1994; Mass et al., 1996). S-[2-(N\textsuperscript{7}-guanyl)ethyl]glutathione is the major hepatic DNA adduct formed in rats from 1,2-dibromoethane exposure, accounting for >95% of the total adduct burden (Inskeep et al., 1986). This specific adduct also forms from 1,2-dichloroethane exposure, though to a lesser degree. Both 1,2-dibromoethane and 1,2-dichloroethane have been reported to be carcinogenic in experimental animals (NCI, 1978a; 1978b).

Common methods in use for detection and quantification of S-[2-(N\textsuperscript{7}-guanyl)ethyl]glutathione are fluorescence intensity and radioactivity. The former method lacks the sensitivity to detect adducts at a toxicologically relevant level. The latter method, though very sensitive, is useful only for experimental purposes and is undesirable for use in environmental matrices; it requires labeled compound upon exposure and is also inconvenient due to the health risks associated with handling of radioactive material. Moreover, neither method provides structural information. These
underlying problems are overcome by using Mass Spectrometry (MS) coupled with Liquid Chromatography (LC)/Electrospray Ionization (ESI).

Examples of the use of LC/MS for detection and quantification of DNA adducts from biological samples have emerged in the last few years (Chaudhary et al., 1996; Rindgen et al., 1995; Yen et al., 1996; Wolf & Vouros, 1994; Vanhoutte et al., 1995). Yen et al. (1996) used the LC/MS/ESI method for analysis of N², 3-ethenoguanine with a detection limit of 5 fmol on column and 50 fmol in biological sample using a 150 mm x 0.3 mm ID capillary column. Rindgen et al. (1995) used capillary LC/tandem mass spectrometry with ESI for the detection of 2-aminol-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) adducts of DNA with a detection limit of approximately 40 pg (80 fmol) on column.

This chapter addresses the use of LC/MS/MS/ESI/ID using Multiple Reaction Monitoring (MRM) for qualitative and LC/MS/ESI/ID using Selected Ion Monitoring (SIM) for quantitative analysis of S-[2-(N⁷-guanyl)ethyl]glutathione hepatic DNA adducts. The application of the methods for quantitation of DNA adduct from whole animal tissue was demonstrated by exposing groups of rats to different levels of 1,2-dibromoethane.

**Materials and Methods**

**Chemicals**

1,2-dibromoethane (purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO; Milwaukee, WI), and Proteinase-K was obtained from Amresco (Oslo, OH). All other reagents used were of the highest quality commercially available.
Synthesis of S-[2-(N⁷-guanyl)ethyl]glutathione Standard

DNA adduct standards were synthesized following the method of Peterson et al. (1988). Briefly, 1,2-dibromoethane (2.5 ml, 28.4 mmol) was added to guanosine monohydrate (0.5 g, 1.7 mmol) dissolved in DMSO (5 ml). The mixture was stirred at room temperature in the dark for three days and then added slowly to rapidly stirred ice-cold ethyl acetate. The white precipitate, crude N⁷-(2-bromoethyl)-guanosine, was collected by vacuum filtration and washed with ethyl acetate. The crude material was dissolved in DMSO (10 ml) and used for the next step without further purification.

Sodium metal (58 mg, 2.5 mmol) was added to glutathione (154 mg, 0.5 mmol) suspended in dry methanol (10 ml). After sodium and glutathione were dissolved, the crude N⁷-(2-bromoethyl)guanosine solution was added drop wise and the mixture was stirred at room temperature for two hours. The reaction was stopped by addition of acetic acid (1 ml). The mixture was added to 200 ml ethyl acetate. The white precipitate was collected by filtration, and heated in distilled water (20 ml) at 100°C for 75 minutes to depurinate all 7-substituted products. The solution was cooled to room temperature and left overnight for guanosine to precipitate. The product, S-[2-(N⁷-guanyl)ethyl]glutathione in the supernatant, was then purified by semi-preparative HPLC. The peak corresponding to S-[2-(N⁷-guanyl)ethyl]glutathione was collected and lyophilized. The synthesis of the deuterated standard followed the same procedure except 1,2-dibromoethane-²H₄ was used instead of 1,2-dibromoethane.
**MS/LC/ESI Condition**

A Quatro II mass spectrometer (Micro Mass Inc.) equipped with Hewlett-Packard Series II 1090 HPLC system was used to record the mass spectra in the positive-ion (+) ES mode. The HPLC system consisted of a C18 5μ Microbore HPLC column (250 x 1 mm I.D., 5μm adsorphosphere packing) (Alltech, Deerfield, IL) and mobile phases of 25 mmol formic acid (solvent A) and acetonitrile (solvent B) with a flow rate of 100 μl per minute. The solvent program was as follows: solvent B, 3% from 0-2 minutes, 40% from 5-15 minutes, 100% from 17-27 minutes, and 3% at 30 minutes. The sample (10 μl) was injected by autosampler into the LC/MS system for analysis. The system was allowed to equilibrate for 10 minutes before the next injection. Reserpine and the standards for adduct analysis were used to tune the ESI source. Instrumental voltage was set at 3.5 kV for the capillary, 0.5 V for HV lens, 40 V for the cone, and 850 V for the multiplier. For the LC/MS/MS/ESI/ID, the collision gas was argon and the cell pressure was 2-3 mBar. The dwell time was 0.1 second.

**Experimental Animal**

Twelve two-month-old male Fischer 344 rats were obtained from the Louisiana State University (LSU) School of Veterinary Medicine (SVM) breeding colony and maintained on chow diet and water *ad lib*. They were assigned randomly into four experimental groups: three treatment and one control group with three rats per group. 1,2-Dibromoethane dissolved in 0.4 ml of dimethyl sulfoxide (DMSO) was administered by intraperitoneal (i.p.) injection at a single dose of 2, 20, or 40 mg/kg to
each animal in treatment groups 1, 2, and 3, respectively. Rats in the control group received the vehicle only (0.4 ml of DMSO) by the same route of administration.

**DNA Extraction**

After 8 hours of exposure, rats were sacrificed to remove livers. Excised livers were immediately frozen in liquid nitrogen and stored at -70°C until analysis. DNA was extracted using a standard method (Strauss, 1994). Briefly, a gram of liver tissue was minced, suspended in 12 ml of proteinase-K buffer, and homogenized in a glass homogenizer tube for less than a minute. The solution was incubated overnight in a shaking water bath maintained at 50°C. A method blank (reagents without tissue) was prepared simultaneously and subjected to a similar sampling process. DNA was extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and was precipitated with a 1/2 volume of ammonium acetate and one times the original volume of isopropanol. DNA was recovered by centrifugation at 1700 x g for two minutes and washed twice with 70% alcohol to remove organic solvents and salts. The DNA pellet was air-dried briefly and dissolved in 3 ml of Saline Sodium Citrate (SSC) buffer in Teflon®-sealed and mininert-capped, conical bottom, glass vials. DNA level was estimated by reading absorbance (1 Au = 50 μg DNA/ml) at 260 nm λ.

**DNA Adduct Isolation**

DNA adducts were released from total DNA by neutral thermal hydrolysis (Beranek et al., 1980; Kim and Guengerich, 1990). Briefly, DNA samples dissolved in the SSC buffer in the conical glass vials were evacuated and hydrolyzed at 100°C for 30 minutes. After cooling, the partially apurinic acid was precipitated in 0.1N HCl at 0°C
and removed by centrifugation at 1700 x g for 7 minutes. The resulting supernatant were transferred to a 4 ml glass tubes and spiked with 30 ng deuterated internal standard for the analyte, S-[2-(N\textsuperscript{2}-guanyl)ethyl]glutathione adduct.

**Desalting**

First, the sample was adjusted to pH 6.5 by addition of either ammonium hydroxide or formic acid. A C\textsubscript{18} Sep-Pak\textsuperscript{*} cartridge (Millipore, Waters) was conditioned with 6 ml of acetonitrile and subsequently flushed with an equal volume of HPLC water. Then, the sample was loaded onto the cartridges and the salts were eluted with 2 ml of HPLC water. The DNA adducts were eluted with 2 ml of acetonitrile into a 2 ml Eppendorf tube and concentrated under a stream of ultrapure nitrogen. Finally, the precipitate was resuspended in 150 µl of HPLC grade water and filtered through a 0.2 µm membrane filter, 4-mm syringe (Nalge Company), into 250 µl glass insert (Alltech, Deerfield, IL) for the LC/MS analysis.

**Results and Discussion**

**Mass Spectral Characterization of the Standards**

The full mass spectra of the non-deuterated standard (\textsuperscript{1}H\textsubscript{4}[M + H]) and deuterated internal standard (\textsuperscript{2}H\textsubscript{4}[M+H]), as obtained by LC/MS/ESI/ID are depicted in Figs. 2.1 & 2.2. The molecular ions at mass/charge (m/z, Da/e) 485 and 489 for the non-deuterated and the deuterated internal standards, respectively, were the most intense peaks and were used as the monitoring ions under SIM for quantitative purposes. Confirmation of the analyte was achieved by scanning the daughter ions of each standards' molecular ion (Figs. 2.3 & 2.4) using the LC/MS/MS/ESI/ID method,
Fig. 2.1: Mass spectrum of S-[2-(N⁷-guanyl)ethyl(¹H₄)]glutathione as obtained by LC/MS/ESI/ID

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Fig. 2.2: Mass spectrum of S-[2-(N7-guanyl)ethyl{2H4}]glutathione as obtained by LC/MS/ESI/ID

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Fig. 2.3: Daughter ions of S-[2-(N²-guanyl)ethyl\{¹H₄\}]glutathione as obtained by LC/MS/MS/ESI/ID

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Fig. 2.4: Daughter ions of S-[2-(N\(^7\)-guanyl)ethyl\(\{\text{H}_4\}\)]glutathione as obtained by LC/MS/MS/ESI/ID

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a more selective procedure. The daughter ions (m/z 188 and 356) of the non-deuterated standard and the corresponding daughter ions (m/z 192 and 360) of the deuterated standard were selectively monitored using MRM (Fig. 2.5). The intensity ratio of daughter ions’ responses of the non-deuterated standard (I188/I356) to the ratio of daughter ions’ responses of the deuterated standard (I192/I360) was close to unity.

The reconstructed ion chromatogram profile in Fig. 2.5 shows that the retention times of the deuterated standard (13.40 minutes) and the non-deuterated standard (13.42 or 13.44 minutes) are virtually the same. The resolution of the deuterated standard by 0.02 - 0.05 minutes is a well known isotope effect on elution characteristics of $^2$H containing compounds (Dizdaroglu, 1993).

The detection limit of the analyte was evaluated by monitoring the molecular ion of non-deuterated standard (m/z 485) under SIM conditions. It was estimated at 200 fmol on column and 400 fmol in biological samples, which were subjected to sample processing stages, with a signal-to-noise ratio (S/N) of 3:1 or greater in both cases.

**Standard Calibration Curve**

A standard calibration curve was generated to quantitate the analyte from unknown samples, using the isotope dilution technique. The curve relates the ion-response ratios of the standards (I485/I489) as a function of the ratio of molar amounts (non-deuterated/deuterated) on column. The amount of the non-deuterated standard used varied from 1 ng (~2 pmol) to 30 ng (~60 pmol) while the internal standard was fixed at 2 ng (~4 pmol) in all samples. The molecular ion of each standard was monitored by LC/MS/ESI/ID using the SIM mode, and the ratio of these molecular ion
Fig. 2.5: Selected ion chromatogram at m/z 192 & 360 representing S-[2-(N²-guanyl)ethyl[²H₄]]glutathione and at m/z 188 & 356 representing S-[2-(N²-guanyl)ethyl[¹H₄]]glutathione from analysis of synthesized material by LC/MS/MS/ESI/ID.

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responses (peak heights) as measured in duplicate injections were regressed using the least square method against amounts of ratios (Fig. 2.6). An $r^2$ of 0.999 was obtained. Hence, the ion abundance ratios measured in the unknown samples, spiked with a known amount of internal standard, can be used with the standard curve equation to predict the quantity of analyte present in unknown samples. The raw data used in the standard curve are presented in Table 2.1.

Table 2.1: The raw data used in the standard curve

<table>
<thead>
<tr>
<th>Standard amount (ng)</th>
<th>Response (peak height)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDST$^a$</td>
<td>DST$^b$</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$NDST and $^b$DST stand for non-deuterated and deuterated standards, respectively. $^c$Ratio of amount of non-deuterated standard to deuterated internal standard. $^d$RF (response factor) was calculated by taking the ratio of signals of molecular ions of the non-deuterated internal to the deuterated internal standard.

Detection of DNA Adducts in Biological Samples

To demonstrate the suitability of the method for quantification of DNA adducts from whole animal tissue, groups of rats exposed to three dose levels of 1,2-
Fig. 2.6: The standard curve used for estimating DNA adduct levels in unknown samples
dibromoethane plus controls were used. After hydrolysis and before sample cleanup, all samples except samples from control animals were spiked with 30 ng (~60 pmol) of the internal standard. The molecular ions for the analyte (m/z 485) and deuterated internal standard (m/z 489) were selectively monitored by the LC/MS/ESI/ID method using SIM. The ratio of the peak heights (I485/I489) in conjunction with the standard curve were used to estimate the concentration of the analyte in the unknown sample.

Characteristic structural information for the analyte was obtained by monitoring the daughter ions of the analyte (m/z 188 and 356) and the corresponding daughter ions of the internal standard (m/z 192 and 360) by LC/MS/MS/ESI method using MRM (Fig. 2.7). A qualitative ratio [(I188/I356) / (I192/I360)] close to one was obtained for each sample analyzed.

DNA adducts were detected in all treatment groups but not in the control group. The level of DNA adduct formation ranged from 13.1 pmol/mg of DNA, in the lowest dose group (2 mg/kg of body weight), to 240 pmol/mg of DNA, in the highest dose group (40 mg/kg of body weight) (Table 2.2). Response (DNA adduct frequencies) was linear with respect to dose, \( r^2 = 0.976 \) (Fig. 2.8).

The level of adduct formation in the highest dose group (40 mg/kg of body weight i.p.) of this study was compared to a previous report by Kim and Guengerich (1990) in which Fischer 344 rats were exposed to 1,2-dibromoethane at a dose of 37 mg/kg of body weight by i.p. injection. They reported a mean response of 284±84 pmol/mg of DNA adduct formation, slightly higher than that was recorded in this study (211.8 ± 23.9 pmol/mg of DNA). The relatively lower yield in our study could be
Fig. 2.7: Analysis of liver sample from 1,2-dibromoethane exposed rat after spiking with the internal standard. The reconstructed ion chromatogram at m/z 192 & 360 represent S-[2-(N'-guanyl)ethyl\{\text{^{2}H}_{6}\}]glutathione and at m/z 188 & 356 represent S-[2-(N'-guanyl)ethyl\{\text{^{1}H}_{6}\}]glutathione.

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Fig. 2.8: Dose response relationship of S-[2-(N\textsuperscript{7}-guanyl)ethyl]glutathione formation as estimated by LC/MS/ESI/ID in rats exposed to 1,2-dibromoethane.
attributed to, among other factors, difference in laboratory techniques, animal colony conditions, glutathione status, or stability of the adduct during long term storage of samples (about 4 months at -70°C) before analysis. The apparent difference in coefficient of variation between the two studies (~ 10% in our study versus ~ 30% in Kim & Guengerich) could be ascribed to the different type of analytical techniques employed in the analysis (the isotope dilution technique versus fluorescence intensity/radioactivity). It is worth noting, however, that adduct persisted for at least six months in liver tissues stored at -70°C. This is important from a potential molecular epidemiologic point of view in that it allows screening of samples collected for other reasons and stored in tissue banks.

Table 2.2: Levels of S-[2-(N\(^7\)-guanyl)ethyl]glutathione hepatic DNA adduct formation in Fischer 344 rats as estimated by LC/MS/ESI following exposure to different doses of 1,2-dibromoethane.

<table>
<thead>
<tr>
<th>Dose (1,2-dibromoethane)(^a)</th>
<th>No. of rats</th>
<th>DNA adduct (pmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>Not detected</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>15.40 ± 2.38(^b)</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>106.32 ± 7.22</td>
</tr>
<tr>
<td>40</td>
<td>3</td>
<td>211.90 ± 22.72</td>
</tr>
</tbody>
</table>

* Dose was in mg per kg of body weight and was administered by i.p. injection
* Value represents mean ± SD

In conclusion, the utility of LC/MS/ESI/ID and LC/MS/ESI/ID methods for detection and quantification of dihaloalkane-DNA adducts in biological samples has been demonstrated in this study. Moreover, it is shown that the methods can be used to investigate dose-response relationships in animals at levels above their detection limit. The level of detection of the LC/MS/ESI/ID method (~ 200 fmol) is lower than the

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detection limit of fluorescence intensity (80 pmol) but not that of the radioactivity (3 fmol). However, a detection limit of ~200 fmol allows for detection of DNA adducts at physiologically relevant levels (~1 adduct in $10^7$ bases).
CHAPTER 3

FORMATION AND PERSISTENCE OF S-[2-(N7-GUANYL)ETHYL]GLUTATHIONE DNA ADDUCT IN LIVERS OF CHANNEL CATFISH (Ictalurus punctatus) FOLLOWING 1,2-DICHLOROETHANE EXPOSURE

Introduction

1,2-Dichloroethane is carcinogenic to rats when administered per os (NCI, 1978b). Consequently, its use as a fumigant in the agricultural industry has been banned since the early 1980’s in the US. But the compound is still extensively used in the U.S. (~6.2 x 10^9 kg/year) as a synthetic precursor of vinyl chloride (Guengerich et al., 1994) and other chlorinated organic solvents. Hence, it is widely distributed in the environment and has been detected in streams (LDEQ, 1994) and hazardous waste sites in different parts of the state (U.S. DHHP, 1994). Thus methods for monitoring 1,2-dichloroethane exposure are of interest.

There is a growing interest in the use of biological markers particularly of DNA adducts in environmental health for mainly two reasons. Firstly, DNA adducts represent the biological effective dose for mutagenesis and carcinogenesis at the target tissue, taking into account individual differences in rates of absorption and metabolism (Nestmann et al. 1996; McDiarmid et al., 1990). Therefore, it has been suggested that the level of the DNA adducts be used in cancer risk assessments (Groopman et al., 1995; Perera, 1988). Secondly, the half-life of some adducts is longer than that of the parent compound or its metabolites, allowing identification of exposure for longer period of time after the occurrence of exposure (Dunn et al., 1987; Varanasi et al., 1989b; van Schooten et al., 1995). Inasmuch as the field of biological indicator
development is young, present activities are centered mainly on marker identification, characterization, quantification, and validation.

Hepatic DNA adducts in rodents resulting from exposure to 1,2-dibromoethane, an analogue of 1,2-dichloroethane, have been characterized (Koga et al., 1986; Inskeep et al., 1986; Kim & Guengerich, 1990; Guengerich et al., 1994). S-[2-(N'-guanyl)ethyl]glutathione is the major adduct formed, accounting for over 95% of the total hepatic DNA adduct production. Inskeep et al. (1986) also reported that the same isomeric form is the major DNA adduct formed in rat liver upon 1,2-dichloroethane exposure. The half-life of the adduct in vivo was estimated to be 3-5 days. The formation and persistence of hepatic DNA adducts from 1,2-dichloroethane exposure in fish have not been previously reported. This chapter presents the evidence for the formation and persistence of hepatic S-[2-(N'-guanyl)ethyl]glutathione adduct in the channel catfish (Ictalurus punctatus) upon exposure to 1,2-dichloroethane. The potential of channel catfish as a sentinel organism for environmental contamination by 1,2-dichloroethane is discussed.

Materials and Methods

Chemicals

1,2-Dichloroethane (purity 99+%) was obtained from Sigma Inc. Co. (St. Louis, MO). Proteinase-K was obtained from Amresco (Oslo, OH). All reagents used were of the highest quality commercially available. The DNA adducts standard (both deuterium labelled and unlabelled) were synthesized following the method of Peterson et al. (1988) (see Chapter 2).
Experimental Animal

One year-old channel catfish, weighing between 40-100 g, were obtained from the culture stock at the School of Veterinary Medicine, Louisiana State University. Fish were kept in a 200 L stainless steel tank in recirculating, dechlorinated tap water with a 12 hour light and dark photoperiod. The fish were fed with 3/8" pelleted trout and salmon starter. The water quality of the system was: pH, 7.5; NH$_4$, 2 ppm; dissolved oxygen, 6 ppm; temperature, 24°C; alkalinity, 75 ppm; and hardness, 100 ppm.

Thirty-three fish were assigned randomly to treatment (27 animals) and control groups (6 animals). Fish in the treatment group were exposed for four hours to 200 ppm of 1,2-dichlorethane in 50 L dechlorinated aerated water in a 100 L glass tank. Fish in the control group were maintained in a 30 L tank filled with aerated dechlorinated water. Aeration was kept low during exposure to minimize evaporation of 1,2-dichloroethane from the tank. At the end of the exposure period, fish in the treatment and control groups were rinsed with dechlorinated water and transferred into four pre-labelled tanks (three treatment and one control) filled with dechlorinated water. Fish in group of three were sampled at 2, 4, 8, 24, 48, 96, 168, 360, and 504 hours after the beginning of the exposure in the treatment group, and at 2 and 504 hours in the control group. The fish were killed humanely and subsequently dissected to remove livers. Livers were snap frozen in liquid nitrogen immediately and stored at -70°C until analysis.
DNA Extraction

DNA was recovered following the method of Strauss (1994) as described in Chapter 2. Briefly, livers were thawed and homogenized in digestion buffer (12 ml/g of tissue) and incubated overnight in a water bath maintained at 50°C. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA in the resultant aqueous fraction was precipitated by adding a 1/2 volume of ammonium acetate and one original volume of cold isopropanol. The DNA pellet was recovered by centrifugation of sample at 1700 x g for 2 minutes (General Laboratory Centrifuge, Dupont Instruments). Residual salts from the pellet were removed using 70% ethanol washes.

DNA Adducts Isolation

To release the DNA adducts, total DNA was dissolved in three ml of Saline Sodium Citrate (SSC) and heated at 95-100°C for 30 minutes. The apurinic acid was precipitated in 0.1N HCl at 0°C and centrifuged at 1700 x g for 7 minutes (Beranek et al., 1980; Kim and Guengerich, 1990). The supernatant was transferred to a four ml tube, spiked with 30 ng deuterated internal standard, adjusted to pH 6.5, and desalted in C18 Sep-Pak* cartridges after conditioning (for details see desalting section in Chapter 2). The DNA adducts in the final eluent (200 μl acetonitrile) were precipitated under streams of ultrapure nitrogen and re-suspended in 150 μl of HPLC grade water. This final solution was filtered through a 0.2 μm nylon membrane 4-mm syringe filter (Nalge Company) into a 250 μl glass limited volume insert (Alltech, Deerfield, IL) ready for analysis.
DNA Adducts Analysis

DNA adducts were analyzed by LC/MS/MS/ESI/ID in a positive ion mode using Multiple Reaction Monitoring (MRM). Briefly, the parent ions at m/z 489 & 485 for the deuterated internal standard and the analyte, respectively, were selectively passed by the first mass analyzer. Subsequently these selected parent ions were dissociated, using argon as a reactant gas, into their respective daughter ions to be scanned by the second mass analyzer (for details see Chapter 2). A gradient mobile phase of 25 mmol formic acid (Solvent A) and a pesticide grade acetonitrile (Solvent B) was used. Solvent B was 3% from 0-2 minutes, 40% from 5-15 minutes, 100% from 17-27 minutes, and 3% at 30 minutes. The system was allowed to stabilize for 10 minutes before the next injection. An aliquot of 10 μl of the sample was injected by an autosampler into the capillary LC/MS-MS/ESI/ID system, and ions at m/z values of 188 and 356 for the analyte and 192 and 360 for the internal standard were monitored in MRM mode.

To estimate the level of DNA adducts in experimental groups, a calibration plot was developed using a fixed level of the deuterated standard (2 ng) and five levels of the non-deuterated standard (0.4, 1, 10, 20, and 50 ng), all within the expected range of adducts in the unknown samples. Triplicate measurements of ion responses (peak height) were taken at each data point. The standard curve was generated by regressing cumulative ion response ratios of the standards [(I188 + I 356) / (I192 + I360) on the corresponding molar (non-deuterated standard/ deuterated standard). The first order curve had an r² of 0.9976 (Fig. 3.1).
Fig. 3.1: The standard curve used for estimating DNA adduct levels in unknown samples

\( r^2 = 0.9976 \)
Statistical Analysis

The mean of DNA adduct levels for a group of fish at each sampling point, along the time course of the study, was estimated along with the standard curves.

Results and Discussion

Figure 3.2 shows the formation of the S-[2-(N'-guanyl)ethyl]glutathione DNA adduct in the liver of channel catfish using LC/MS/MS/ESI/ID in the MRM mode isotope dilution technique. The use of an HPLC/MS/MS/ESI/ID for detection and quantification of DNA adducts from fish is the first of its kind. Other methods that have been used to detect DNA adducts in fish without requiring radiolabelled material include HPLC/Flourescence intensity (Shugart et al., 1987), GC/MS/SIM (Law et al., 1996; Malins et al., 1990), $^{32}$P-postlabelling (Varansi et al., 1989; Dunn et al., 1987), and immunochemical (Nakatsuru et al., 1990) techniques. The sensitivity of the LC/MS/MS/ESI/ID method (~1 adducts in $10^7$ bases) is better than the fluorescence intensity (~1 adducts in $10^6$ bases) but less than the GC/MS/SIM (1 adduct in $10^{15}$ bases) and $^{32}$P-postlabelling (1 adducts in $10^9$ bases). Nevertheless, the method allowed detection of DNA adducts at physiologically relevant levels. Furthermore, unlike the $^{32}$P-post labelling and flourescence intensity, the method provides definitive structural characteristics of the DNA adducts.

Channel catfish (*Ictalurus punctatus*) have seldom been observed to express pollutant-mediated neoplasia in field studies (Harshbarger, 1979, 1981; Hasspieller, 1994). This could be attributed in part to the glutathione-dependent defense not only because they have higher constitutive levels of hepatic total glutathione and reduced
Fig. 3.2: LC/MS/MS/ESI/ID analysis of liver sample from 1,2-dichloroethane exposed fish (200 ppm for 4 hours) after spiking with the internal standard. The reconstructed ion chromatogram at m/z 192 & 360 represent S-[2-(N7-guanyl)ethyl]glutathione and at m/z 188 & 356 represent S-[2-(N7-guanyl)ethyl]glutathione.
glutathione but also because they possess a greater capacity for conjugation of xenobiotics (including PAH) to reduced glutathione compared to bullheads, a species that often express neoplasia in contaminated systems (Hasspieller, 1994). To our knowledge, this study is the first to demonstrate the formation of DNA adducts from chemical exposure in channel catfish. The formation of the DNA adduct proceeded, because unlike other compounds, 1,2-dihaloethanes are activated by conjugation with glutathione to produce reactive metabolites (episulfonium ions) that covalently bind to cellular macromolecules including DNA (Guengerich, 1992; Jean & Reed, 1992; Ozawa & Guengerich, 1983; Guengerich et al., 1994; Peterson et al., 1988; Goldestein & Falleto, 1993).

The formation of the DNA adduct was rapid, being observable after only two hours of exposure. Peak adduct level (264 pmol/mg DNA) was observed eight hours after the beginning of exposure (Fig. 3.3). However, adduct levels at 2, 4, 8, 24, and 48 hours after the beginning of exposure were relatively close to each other, and there appears to be a balance between formation and removal of the hepatic adduct in the first two days post exposure. By 96 hours, adduct levels had dropped by more than half (101 pmol/mg of DNA), indicating that the half-life of the adduct was between 2-5 days. This is consistent with the half-life of the adduct reported in rat (3-5 days) when rats were exposed to 1,2-dibromoethane by intraperitoneal injection (Inskeep et al., 1986). Inskeep et al. (1986) also studied the chemical half-life of the adduct in naked calf thymus DNA incubated in 50 mmol sodium phosphate buffer at 37°C. They reported a chemical half-life of 150 hours for the adduct and suggested that chemical
**Fig. 3.3**: Formation and persistence of S-[2-(N\(^7\)-guanilyl)ethyl]glutathione hepatic DNA adduct in channel catfish from 1,2-dichloroethane exposure.
depurination may play a major role in degradation. Owing to the poor DNA excision repair activity of most fish (Walton et al., 1984b; Walton et al., 1983; Nakatsu et al., 1987), the half-life of the adduct would be expected to be longer in channel catfish than in rat. For instance, the half-life of the major DNA adducts formed by aflatoxin B1 in trout is 3-4 weeks (Goeger et al., 1986) compared to only 7.5 hr in rats (Croy and Wogan, 1981). The lack of a demonstrable difference between half-lives of the hepatic DNA adduct in catfish and rats supports earlier suggestions by Inskeep et al. (1986) that depurination may play a major role in the removal of the S-[2-N\(^7\)-guanyl]ethyl]glutathione adducts. Alternatively, unlike most fish, enzymatic repair mechanism of channel catfish may be comparable to rodents as was reported for the Japanese medaka for the activity of O\(^6\)-methylguanine DNA methyltransferase (Nakatusuru et al., 1987).

From an epidemiological point of view, the half-life of the hepatic adduct (2-5 days) may not appear long enough for surveillance purposes. However, it is longer than the half-lives of the parent compound in surface water (1-2 days) (HSDB, 1993; IPCS, 1995) and as well as its metabolites in tissue about 1-2 days (Brarrows et al., 1980). Moreover, the adduct was detectable three weeks after exposure, and it is possible that adducts may persist for months in quantities below the detection limit of the analytical technique we used. Considering the rapidly advancing areas of analytical techniques, it may not be long before new methods are developed with detection limits low enough to detect ultratrace amounts of adducts as a result of low exposure or exposures in the distant past.
Unlike many adducts, such as PAH-DNA adducts that are non-specific and may arise from complex exposure mixtures, the S-[2-(N\(^7\)-guanyl)ethyl]glutathione adduct is much more specific and occurs only from 1,2-dihaloalkane exposure. Therefore, in an environment where there is no history of production or use of 1,2-dibromoethane as an intermediate for the synthesis of other products, identification of the specific DNA adduct in the environmental matrices unequivocally reveals the contamination of the environment with 1,2-dichloroethane.

In summary, this study provided evidence that channel catfish metabolize 1,2-dichloroethane to reactive metabolites that covalently bind to liver DNA to form the S-[2-(N\(^7\)-guanyl)ethyl]glutathione adduct. In contrast to the widely held opinion that channel catfish are a resistant organism to carcinogens, the resultant DNA adducts are suggestive of the sensitivity of the channel catfish to certain carcinogens, satisfying a requirement of sentinel animals for environmental contamination (NRC, 1991). The biological half-life of the adduct in channel catfish was not different from that of rat. The dynamics of the hepatic DNA adduct upon chronic low levels of 1,2-dichloroethane exposure is an area that requires further investigation for molecular epidemiologic purposes along with the genetic consequences of dihaloalkane exposure to this organism and others.
CHAPTER 4

DOSE-RESPONSE RELATIONSHIP FOR FORMATION OF S-2-(N'-GUANYL)ETHYLGLUTATHIONE DNA ADDUCT IN LIVERS OF CHANNEL CATFISH (ICTALURUS PUNCTATUS) EXPOSED TO 1,2-DICHLOROETHANE; EFFECT OF GLUTATHIONE DEPLETION ON ADDUCT FORMATION

Introduction

1,2-Dichloroethane is used extensively in the U.S. (6 x 10^9 kg/year) predominantly for the synthesis of vinyl chloride and other chlorinated organic solvents (Guengerich et al., 1994). As a result it is widely available in the environment (LDEQ 1995; U.S. DHHS, 1994). The compound is reported to be mutagenic in Drosophila (Ballering et al., 1994) and carcinogenic in rodents (NCI, 1978b). It is also known to form DNA adducts in experimental animals (Inskeep et al., 1986; Kim & Guengerich, 1990, Chapter 3). S-2-(N'-guanyl)ethyl]glutathione is the principal DNA adduct formed in rodents (Inskeep et al., 1986).

DNA adducts resulting from ambient exposure to several chemical groups have been detected in man, fish, and, wild animals. Therefore, DNA adducts have become useful in exposure identification (Ehrenberg et al., 1996; Mumford et al., 1993; Nestmann et al., 1996; Dunn et al., 1990) and have a significant potential in cancer prevention as an early warning system or in assessing compliance in a control program (Perera et al., 1996). It has also been reported that DNA adducts may serve as a dosimeter for cancer risk assessment in organisms living in contaminated environments (van Schooten, 1995; Varanasi et al., 1989b; Gaylor et al., 1992; Choy, 1993; Ehrenberg, 1995; Griem et al., 1995). To qualify as a valid measure for risk assessment,
the organism must activate the specific compound into DNA-reactive species and has to respond differently to varying doses received under ambient conditions.

Channel catfish (*Ictalurus punctatus*), which are widely distributed in the south-eastern U.S. and Canada, may be a potential indicator organism for 1,2-dichloroethane. It rapidly forms hepatic DNA adducts upon exposure to the compound (Chapter 3), but the dose response-relationship for DNA adduct formation has not yet been established. This chapter presents a dose-response relationship for S-[2-(N⁷-guanyl)ethyl]glutathione formation from a single administration of 1,2-dichloroethane in channel catfish. The effect of chemical-depletion of glutathione on the level of DNA adduct formation is also presented.

**Materials and Methods**

**Chemicals**

1,2-Dichloroethane (purity 99+%%) was purchased from Sigma Inc. Co. (St. Louis, MO), and proteinase-K was obtained from Amresco (Oslo, OH). All reagents used were of highest quality commercially available. The DNA adduct standards were synthesized following the method of Peterson *et al.* (1988) (see Chapter 2).

**Animals**

One-year-old channel catfish (*Ictalurus punctatus*), weighing 40-80 g, were obtained from the fish culture stock of the School of Veterinary Medicine, Louisiana State University. The catfish were maintained in a 200 L stainless steel tank with recirculated dechlorinated water at a temperature of 24-26°C and a 12 hours light and dark cycle. Fish were fed 3/8" pelleted trout and salmon starter diet daily. The average
water quality in the aquaria was pH 7.2, dissolved oxygen 6 ppm, ammonia <2 ppm, hardness 75 ppm, and alkalinity 100 ppm.

Dose-response

Twenty channel catfish were assigned randomly in groups of four fish into six experimental groups. Fish in groups 1, 2, 3, & 4 were exposed to 50, 100, 200, or 600 ppm, respectively, to 1,2-dichloroethane in 4-liters of water for 4 hours, while fish in the control group (group 6) were kept in the vehicle only (dechlorinated water). At the end of exposure, fish were rinsed with dechlorinated water and transferred to pre-labelled 10 L tanks (one tank per group) filled with clean water. After a holding period of four hours, fish were killed and the livers removed.

Effect of glutathione depletion upon DNA adduct formation

To examine the effect of glutathione depletion on the level of DNA adduct formation, twelve channel catfish were assigned in groups of four fish each to three experimental groups. Catfish in group 1 received diethylmaleate (DEM) (0.6 ml/kg in corn oil, intraperitoneal injection) while catfish in groups 2 and 3 received the vehicle only (150 μl corn oil). Three hours after DEM/corn oil administration, catfish in groups 1 and 2 were exposed to 1,2-dichloroethane (DCE) at a dose of 200 ppm in 4 liter dechlorinated water for four hours; whereas, fish in group 3 (naive group) were kept in the vehicle only (dechlorinated water). At the end of the experiment, fish were sacrificed to remove livers.
DNA Extraction

DNA was extracted from the livers following the method of Strauss (1994) as described in Chapter 2. Briefly, liver was immediately homogenized in digestion buffer (1 g tissue/12 ml of digestion buffer) after harvesting and incubated overnight in a shaking water bath maintained at 50°C. The solution was extracted twice with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1). One-half volume of ammonium acetate and one original volume of cold isopropanol were added to the aqueous portion of the solution to precipitate the DNA. The DNA pellet was recovered by centrifugation at 1700 x g for 2 minutes (General Laboratory Centrifuge, Dupont Instruments). The pellet was washed twice with 70% alcohol to remove residual salts and organic solvents.

DNA Adduct Isolation

DNA adducts were released by neutral thermal hydrolysis (Beranek et al., 1980; Kim and Guengerich, 1990). Briefly, DNA was dissolved in 3 ml of saline sodium citrate (SSC) in a 4 ml Teflon®-sealed mininert, conical bottom, glass vials. The solution was heated at 95-100°C for 30 minutes. After cooling the sample, the partially apurinic acid was precipitated in 0.1 N HCl at 0°C and removed by centrifugation at 1700 x g for 7 minutes. The supernatant was transferred to a 4 ml glass tube and spiked with 30 ng of the deuterated internal standard. Excess salt was removed in C18 Sep-Pak® cartridges (see desalting section in Chapter 2). The final eluent in ~200 µl of acetonitrile was evaporated under ultrapure nitrogen and resuspended in 150 µl of HPLC water. The resuspended solution was filtered through a 0.2µm nylon membrane,
4mm-syringe filter (Nalge Company), into a 250 μl glass insert (Alltech, Deerfield, IL). DNA level was estimated by reading absorbance (1 Au = 50 μg/ml) at 260 nm λ from an aliquot of sample taken before hydrolysis.

**Analysis of DNA Adducts**

The sample was analyzed by Liquid Chromatography/Tandem Mass Spectrometry (MS/MS)/Electro Spray Ionization (ESI)/ Isotope Dilution (ID) method using Multiple Reaction Monitoring (MRM) as described in Chapters 2 & 3. A calibration plot was developed for estimating the concentration of the DNA adduct in the unknown samples. The amount of the standard used varied between 0.4 ng (~0.8 pmol) and 50 ng (~100 pmol), while the deuterated internal standard was fixed at 2 ng (~4 pmol). Triplicate measures of molecular ion responses at each of the five concentration points were regressed by the least square method against the molar amounts. The calibration plot was linear with an r² value of 0.998 (Fig. 4.1).

**Analysis of Glutathione**

Glutathione (GSH) level was measured following the method of Lakritz et al. (1997). Briefly, 0.1 g of liver tissue was placed immediately in 2.5 ml of 200 mmol methane sulfonic acid containing 5 mmol diethylenetriaminepentaacetic acid (DTPA). The tissue was homogenized in Potter-Elvejheim homogenizer and centrifuged at 12,000 × g for 30 minutes. The supernatant was filtered through a 0.45μm nylon membrane 4mm-syringe filter and injected directly onto the HPLC column (20 μl loop). The reduced and oxidized glutathione were separated using an Alltech Adsorbosphere C₁₈ 5 micron column (Deerfield IL), and the column was eluted isocratically. The
Fig. 4.1: The standard curve used for estimating DNA adduct levels in unknown samples
mobile phase was 50 mmol NaH$_2$PO$_4$, 0.05 mmol octane sulfonic acid, and 2% acetonitrile adjusted to pH 2.7 with phosphoric acid and a flow rate of 1 ml/minute. An electrochemical detector (guard cell = 950mv, conditioning cell = 400mv, analytical cell = 880 ma) was used to measure the reduced glutathione. Peaks were quantitated by height and were compared to peak areas of known standards. Because the oxidized glutathione is the secondary form of glutathione accounting for <5% of the total hepatic glutathione in channel catfish (Hasspieler et al., 1994), its value was not measured.

**Statistical Analysis**

For the experiment on the effect of depletion of glutathione, change in levels of glutathione and DNA adducts for experimental groups were tested by one way analysis of variance. When the calculated F value was significant (P < 0.05), the difference between group means was tested by the Scheffe multiple comparison test (Dowdy & Wearden, 1985).

**Results and Discussion**

**The Dose-Response Relationship**

The dose-response relationship curve was linear at the lower doses and appeared to level off above the 200 ppm dose (Fig. 4.2). This was especially evident when the level of DNA adducts formed was normalized to dose level, i.e., DNA adducts divided by dose (nmol). For doses ≤ 200 ppm (1617 nmol), the values were approximately the same. Whereas for the highest dose group (600 ppm or 4850 nmol), the amount of DNA adduct per unit dose was lower by more than two-fold (Fig 4.3). This suggested that the glutathione activation pathway was nearly saturated at the higher dose (600 ppm).
Fig. 4.2: Dose response relationship for S-[2-(N^7-guanyI)ethyl]gluatathione DNA adduct formation in channel catfish liver from 1,2-dichloroethane exposure
Fig. 4.3: Formation of S-[2-(N\(^7\)-guanyl)ethyl]glutathione hepatic DNA adduct per nmol of 1,2-dichloroethane at different dose levels

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Alternatively, owing to the short exposure period (4 hours), the fraction of 1,2-dichloroethane absorbed may be too low to reach the desired concentration in the target organ, liver tissue.

Kim and Guengerich (1989) reported a linear dose-response relationship for the DNA adduct formation in rats exposed by intraperitoneal injection to 1,2-dibromoethane over a range of 0.5-37 mg/kg of body weight, suggesting the glutathione metabolic activation pathway was not saturated at the highest dose level of the experiment. The results of the present study and those of Kim & Guengerich (1989) are not directly comparable because the dose-response relationships involve different compounds (1,2-dichloroethane versus 1,2-dibromoethane), different species (fish versus rats), and different routes of exposure (aqueous immersion versus intraperitoneal injection). Nevertheless, the level of DNA adduct formation in fish was equal to or greater than in rats when expressed DNA adduct formation per nmol of test compound. However, it is also important to note that rats have a 4- to 7-fold greater concentration of total hepatic glutathione than fish (Wallace, 1989). Therefore, under similar conditions, rats may be more competent than fish to metabolically activate 1,2-dihaloethanes through the glutathione pathway before the system is saturated or glutathione depleted.

Similar linear and nonlinear dose-response relationships for carcinogen-DNA adducts have been reported in both laboratory (Troxel et al., 1997; Buss et al., 1990; Murphy et al., 1990; Heck & Casanova, 1987; Belinsky et al., 1987) and field studies (Perera et al., 1996; van Schooten et al. 1995). For instance, zebrafish injected
intraperitoneal with 50-400 µg $[^{3}H]$afatoxin B1 (AFB1)/kg body weight showed a linear dose-response for hepatic DNA binding at 24 hour (Troxel et al., 1997). Belinskey et al. (1987) reported that O\(^{\text{6}}\)-methylguanine methyltransferase (O\(^{\text{6}}\)-MG) showed a supra-linear curve (adducts per unit dose is much less at higher doses) in rat lung following exposure to the tobacco specific carcinogen 4-($N$-nitrosomethylamino)-1-(3-pyridyl)-1-butanone. On the other hand, in a situation where the detoxication pathway or the enzyme repair system is saturated, the dose response curve may be sublinear (adducts per unit dose is much higher at a higher dose). For example, Heck & Casanova (1987) reported that there were 4-7 fold less DNA-protein cross links at low dose than at high doses because of the saturation of the detoxication pathway.

In field studies, Perera et al. (1996) reported an apparent dose-response of maternal leukocyte polycyclic aromatic hydrocarbons (PAH)-DNA adduct to increasing ambient pollution in female residents of Krakow (Poland), a city with elevated air pollution. van Schooten et al. (1995) studied DNA dosimetry in eel (Anguilla anguilla) living on PAH-contaminated soil and sediments. They found a significant correlation ($r^2 = 0.59$, $p<0.001$) between the total aromatic DNA adduct level in the livers of eel and the level of PAH in the sediment.

**Effect of Chemical-Depletion of Glutathione on the Formation of DNA Adduct**

Because the level of S[2-(N\(^{2}\)-guanyl)ethyl]glutathione hepatic DNA adduct formation in rodents is dependent on hepatic glutathione level (Ozawa & Guengerich, 1983; Guengerich et al., 1994; Kim & Guengerich, 1990), the effect of depletion of glutathione on the level of DNA adduct formation in channel catfish was examined.
Pretreatment of fish with DEM significantly reduced ($P < 0.05$) the level of glutathione (Table 4.1). The concentration of glutathione in the DEM + DCE treated group dropped to less than 5% of the naive group. This result is in agreement with a previous study in which the level of hepatic glutathione in channel catfish dropped to 15% of the control level six hours after DEM administration (Gallagher et al., 1992a). However, the excess loss of glutathione in this study over the value reported by Gallagher et al. (1992b) may be attributed to the subsequent exposure of the catfish to 1,2-dichloroethane following DEM administration.

Table 4.1: Effect of depletion of glutathione on S-[2-(N'-guanyl)ethyl]glutathione hepatic DNA adduct formation in channel catfish exposed to 1,2-dichloroethane

<table>
<thead>
<tr>
<th>Group</th>
<th>No. fish</th>
<th>GSH nmol/g liver tissue</th>
<th>DNA adducts pmol/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>4</td>
<td>1839 ±190$^a$</td>
<td>Not detected</td>
</tr>
<tr>
<td>DCE only</td>
<td>4</td>
<td>736 ± 90$^b$</td>
<td>187.20 ± 34.51</td>
</tr>
<tr>
<td>DEM + DCE</td>
<td>4</td>
<td>79 ± 9.6$^c$</td>
<td>Not detected*</td>
</tr>
</tbody>
</table>

Means ± SE with different letter are significantly different ($p < 0.05$, Scheffe test)

F value = 53.7, df = 9

*The limit of detection for DNA adduct was ~ 10 fmol on column

DCE, 1,2-dichloroethane; DEM, diethylmaleate

Though not as severe as the EDC + DEM group, the level of glutathione is also depleted in the 1,2-dichloroethane only group. The concentration of glutathione in this group was ~ 40% of the control group. The depletion is high in contrast to that reported by Kim and Guengerich (1990) in which they observed a glutathione concentration of 75% in 1,2-dibromoethane-treated rats compared to the control. The 4-7 fold higher
hepatic glutathione concentration in rats than in fish may explain the condition between results from rats and the present study in fish.

DNA adducts were undetected in the group that was exposed to 1,2-dichloroethane following treatment by DEM (Table 4.1). This was attributed to the severe depletion of glutathione by DEM (< 5% of the control level) at the time of exposure of fish to 1,2-dichloroethane. In a similar study by Kim and Guengerich (1990), the level of depletion of glutathione in rats treated with DEM 30 minutes before 1,2-dibromoethane injection was ~ 43% of the control level. Unlike the present study, DNA adducts (214 ± 26 pmol/mg) were detected in the DEM pre-treated group though it was significantly different (p < 0.05) from the 1,2-dibromoethane only group (332 ± 58 pmol/mg DNA). The mean DNA adducts formed (187 ± 34 pmol/mg DNA) for 1,2-dichloroethane only treated group was lower compared to the same dose level of the mean DNA adducts formed (249 ± 26 pmol/mg DNA) in the dose-response relationship of this study. Reasons for the apparent discrepancy may include: individual variation and conditions of animals at the time of experiment; although the relatively small Standard error of the specific DNA adduct response in each of the experiments does not suggest a substantial level of individual variation.

In conclusion, this study has established a dose-response relationship for S-[2-(N7-guanyl)ethyl]glutathione hepatic DNA adduct formation in channel catfish following exposure to varying doses of 1,2-dichloroethane. The response appears to be linear over the ≤ 200 ppm and supralinear at the highest dose (>600 ppm). Pre-exposure to DEM severely depleted the glutathione level, and the trace amount of DNA adducts

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that may have been formed were below the detection limit of the analytical method used.
CHAPTER 5

CONCLUSIONS

Summary

The study demonstrated the use of LC/MS/MS/ESI/ID and LC/MS/ESI/ID methods for detection and quantification of selected dihaloalkane-DNA adducts in biological samples. Furthermore, it has been shown that the methods can be used to investigate a dose-response relationship in both rats and fish at levels above the detection limit. The sensitivity of the method with respect to limit of detection (LOD) (~200 fmol) is better than that of fluorescence intensity (80 pmol) but lower than what can be detected by a method using radioactivity (3 fmol). However, the detection limit of ~200 fmol on column allows for detection of DNA adducts at physiologically relevant levels (1 adduct in ~ 10^7 bases) and an unambiguous confirmation of the structure of the adduct.

Channel catfish (*Ictalurus punctatus*) metabolize 1,2-dichloroethane to DNA-reactive metabolites that covalently bind to DNA to form the S-[2-(N7-guanyl)ethyl]glutathione adduct. The formation of the hepatic DNA adduct is rapid, being detectable after two hours of exposure. The peak DNA adduct level (264 pmol/mg DNA) was observed 8 hrs after the onset of exposure and had a half-life of 2-5 days. This half-life is similar to that of the hepatic DNA adduct formed in rats exposed to 1,2-dibromoethane, an analogue of 1,2-dichloroethane. The half-life of the DNA adduct may appear short for monitoring purposes. Nonetheless, the DNA adduct was detectable three weeks after a single exposure and may possibly persist for months...
at levels near the detection limit of the analytical instrument used, particularly if exposures are intermittent.

The formation of the S-[2-(N^7-guanyl)ethyl]glutathione adduct varied with respect to dose. The dose-response curve was linear up to 200 ppm and appeared to level off thereafter. Pre-exposure of catfish with diethylmaleate (DEM) severely depleted physiologic glutathione levels. The concentration of glutathione in the DEM + DCE (1,2-dichloroethane) treated group dropped to less than 5% of untreated control. As a result, no DNA adducts were detected in this group. The trace amount of DNA adducts that may have been formed were below the detection limit of the technique used (500 fmol on column and an average of 7.5 pmol in total sample) at the time of sample processing. Similarly, glutathione was depleted to 40% of the control level in the EDC only treatment group (200 ppm in dechlorinated water). Adducts were formed at a level of 187 pmol/mg DNA.

Future Research

The present study provided basic information for the long-term objectives of using channel catfish (*Ictalurus punctatus*) as an environmental sentinel for 1,2-dihaloalkane exposure. The following issues need to be addressed in order to expand upon the current results:

1. Upon low chronic carcinogenic exposure, repairable DNA adducts are expected to be removed as quickly as they are formed. But, unrepairable or slowly repaired DNA adducts may accumulate gradually. It is these accumulated DNA adducts that are likely to be detected in an epidemiological surveillance program. Therefore, the dynamics of
S-[2-(N^7-guanyl)ethyl]glutathione following chronic low level exposure to 1,2-dichloroethane needs to be studied along with repair kinetics and genetic consequences.

(2) Organisms are not typically exposed to a single compound in nature, but more frequently to a complex mixture of substances which may represent several classes of compounds. Identification of all compounds that concomitantly occur with 1,2-dichloroethane in the environment is a challenge and assessing their effects on the formation of DNA adducts is an equally difficult task. Nevertheless, understanding the dynamics of S-[2-(N^7-guanyl)ethyl]glutathione in the presence of compounds commonly found along with 1,2-dichloroethane is of paramount importance for an accurate interpretation of field results and to provide proper recommendations to decision making bodies.

(3) Before application of the current method to an epidemiological surveillance program on a wide scale, the method should be validated by trapping feral fish from areas with known 1,2-dichloroethane or 1,2-dibromoethane contamination and screening for the specific dihaloethane-DNA adduct.
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Ahmedin Jemal was born in 1962 in Butajira, Ethiopia. He received his high school diploma from Butajira High School in 1980. He graduated with Doctor of Veterinary Medicine in 1986 from Addis Ababa University. After he worked for over four years in the Ministry of Agriculture (Ethiopian Government), he came to Baton Rouge in 1991 to pursue graduate training in epidemiology. He received his Master of Science degree in epidemiology from Louisiana State University, and presently he is a candidate for a Philosophy of Doctor degree in Veterinary Medical Sciences.
Candidate: Ahmedin Muktar Jemal

Major Field: Veterinary Medical Sciences

Title of Dissertation: Identification and Quantification of S-[2-(N\(^7\)-guanyl)ethyl]glutathione DNA Adduct in Channel Catfish (Ictalurus punctatus) After Exposure to 1,2-Dichloroethane

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]
Jay Means, Co-Chairman

[Signature]

[Signature]

[Signature]

[Signature]

Date of Examination: October 27, 1997