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Quantification Of Benzo[A]Pyrene-Guanine Adducts in In Vitro And in Vivo Tissue Samples By LC Tandem Mass Spectrometry

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QUANTIFICATION OF BENZO[A]PYRENE-GUANINE ADDUCTS IN
IN VITRO AND IN VIVO TISSUE SAMPLES BY
LC TANDEM MASS SPECTROMETRY

by

Po-Chang Chiang

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Chemistry

Western Michigan University
Kalamazoo, Michigan
June 2001
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Finally, I dedicate this dissertation to my soon-to-be-born child.

Po-Chang Chiang
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CHAPTER I

INTRODUCTION

The term "cancer" refers to a group of diseases in which cells grow and spread unrestrained throughout the body. It is difficult to imagine anyone who has not heard about this disease. Cancer, as an illness of man, has been described in the history of medicine since the earliest medical records were kept. For several thousand years, cancer was visible only in its outward manifestations (Health Canada, 1999).

Cancer arises from a loss of normal growth control. In normal tissues, the rates of new cell growth and old cell death are kept in balance. In cancer, this balance is disrupted. This disruption can result from uncontrolled cell growth or loss of a cell’s ability to undergo “apoptosis.” Apoptosis, or “cell suicide,” is the mechanism by which old or damaged cells normally self-destruct (National Cancer Institute, 2000).

Cancer is often perceived as a disease that strikes for no apparent reason. This is because scientists don’t know all the reasons. However, many of the causes of cancer have already been identified. Besides heredity, scientific studies point to the existence of three main categories of factors that contribute to the development of cancer: chemicals (e.g., from smoking or diet), radiation, and viruses or bacteria (Newton, Beral, Weiss, & Tooze, 1999).
Among these contributors, chemicals play a significant role in causing cancer. Since exposure to chemical carcinogens (cancer-causing agents) is responsible for triggering most human cancers, people can reduce their risk of cancer by taking steps to avoid or minimize the exposure to such agents (Harras, Edwards, Bolt, & Gloeckler Ries, 1997). Hence, a very important step in cancer prevention is to identify the behaviors or exposures to particular kinds of chemical carcinogens that represent the greatest cancer hazard.

Chemical carcinogenesis was first recognized in the human approximately two and half centuries ago by findings of Hill in 1761 who noted an increased incidence of nasal polyps and cancer after the prolonged and excessive use of tobacco snuff (Augusto, Cavalieri, Rogan, RamaKrishna, & Kolar, 1978). In 1775 Percival Pott noted the scrotal skin cancer of chimney sweeps and correlated it to the exposure of these patients for many years to large amounts of coal soot and tar (Josephy, 1997). Even in that early era, the effects of certain chemical exposures as cancer causing agents were recognized.

It is considered that for chemical carcinogens to be effective, they must interact with cellular macromolecular constituents, including proteins, lipids, and more importantly, with nucleic acids (Waalkes & Ward, 1994). One basic mechanism in chemical carcinogenesis is the formation of covalent bonds between chemical carcinogens and DNA, RNA, and protein to form adducts. These adducts are thought to interfere with replication leading to mutation or deletion of a DNA
sequence. Mutation is considered the critical step in the process of a tumor's formation (Miller, 1970).

During the past 60 years or so, following the identification of the first pure chemical carcinogen (Kennaway & Hieger, 1930) no common factors or pathways for the mechanism of action of chemical carcinogens from different chemical classes have become evident. For this very reason, each class of carcinogen is often discussed separately. Today, the potential for DNA adduct formation is recognized as a common property of most potent carcinogens and the formation of such adducts is the basis of several current strategies in molecular epidemiology and bio-monitoring (Dipple, 1995).

Despite the common aspect of mechanisms for many chemical carcinogens, the complexities of metabolic activation and of the chemistry and stereochemistry of adduct formation have tended to keep some degree of compartmentalization in research and in literature reviews of DNA adduct formation by different classes of chemical compounds (Hemminki, 1994). Typically, a single toxic chemical class, such as the polycyclic aromatic hydrocarbons (PAHs), induces many types of DNA damage. Particular adduct isomers may be dominant, however, many minor adducts are also formed. For example, even simple alkylating agents react with guanine and other bases at several sites (Augusto, Cavalieri, Rogan, RamaKrishna, & Kolar, 1990; Josephy, 1997; Netto et al., 1992). The formation of methyl guanine adducts is shown in Figure 1.
Figure 1. Multiple Sites of Guanine Alkylation.

In many cases, the pattern of DNA damage induced by an agent can serve as a distinctive "fingerprint". In general, if such a pattern is distinctive, then it may be possible to work back from an observed spectrum of DNA adducts to deduce the nature of the chemical agent responsible and to prevent or repair the damage to DNA (Josephy, 1997).

Chemical carcinogens can be classified into three major categories. They are direct acting (no metabolic activation needed), indirect acting (metabolic activation needed), and nongenotoxic or epigenetic carcinogens (Waalkes & Ward, 1994).

Most chemical carcinogens are considered to be indirect acting. They require metabolic activation to produce electrophilic intermediates in order for the tumor
formation process to take place (Josephy, 1997). For example, the hypothesized mechanism for metabolic activation of polycyclic aromatic hydrocarbons (PAH), is that activation occurs by two main routes: one-electron oxidation to yield reactive intermediate radical cations (Cavaliere & Rogan, 1984, 1985; Cavaliere & Roth, 1976; Hanson, Rogan, & Cavaliere, 1998; Miller & Miller, 1981; Rogan et al., 1988; Waalkes & Ward 1994) and monooxygenation to produce bay-region diol epoxides (Cheh et al., 1993; Conney, 1982; Sims & Grover, 1981). These activated intermediates then react with nucleotides or bases to form covalently bonded compounds and cause a “molecular lesion.” In many cases, stable DNA adducts can be found as evidence of these reactions (Blobstein, Weinstein, Grunberger, Weisgras, & Harvey, 1975; Cavaliere & Auerbach, 1974; Cavaliere et al., 1991; Chakravarti, Pelling, Cavaliere, & Rogan, 1995; Chen et al., 1997; Cheng, Hilton, Roman, & Dipple, 1989; Cremonesi, Hietbrink, Rogan, & Cavaliere, 1992; Devanesan, Cremonesi, Nunnally, Rogan, & Cavaliere, 1990; Devanesan et al., 1993; Dipple, Moschel, & Pigott, 1985; Fernando, Huang, Milliman, Shu, & LeBreton, 1996; Herreno-Saenz, Evans, Abian, & Fu, 1993; Higginbotham, RamaKrishna, Johansson, Rogan, & Cavaliere, 1993; Mulder, RamaKrishna, Cremonesi, Rogan, & Cavaliere, 1993; Mulder et al., 1996; Pachuta et al., 1988; RamaKrishna, Gao, Padmavathi, Cavaliere, & Rogan, 1992; RamaKrishna et al. 1993; Wilk & Girke, 1972).

The adducts formed by the above mechanisms can be either stable, remaining in DNA unless repaired by natural repair enzymes, or depurinated and
released from DNA by cleavage of the glycosidic bond between the purine base and deoxyribose (Josephy, 1997; Todorovic et al., 1997). Loss of adducts by depurination results in apurinic sites in the DNA, which, if not repaired, can also be mutagenic. Therefore, the importance of determination of structure of the DNA adducts formed in target tissues is a critical variable requiring extensive research.

Researchers believe that study of DNA adducts will help to yield an understanding of the mechanisms of metabolic activation and, eventually, the mechanisms of tumor initiation (Cavalieri, Rogan, Devanesan, Jankowiak, & Small, 1997). The use of DNA adducts as bio-markers for risk assessment and environmental monitoring have caught researcher’s interest (Huang et al., 1998). The DNA adduct represents direct evidence of primary damage to DNA by a chemical. Therefore, measuring DNA adducts may prove to be more precise and reliable than measuring the external exposure (Groopman & Kensler, 1993; McCull, Rindgen, Blair, & Penning, 1999).

DNA adducts are typically large molecules, and therefore structure determination represents a very challenging problem in organic analysis. Deconvolution of the chemical structures of DNA adducts is usually based on spectroscopic identification by nuclear magnetic resonance (NMR). However, NMR analysis is possible only with purification of many micrograms of pure adducts and in many cases, such quantities are hard to obtain. This is typically the case in living organisms.
The most productive approach has been to prepare authentic DNA adduct standards by the reaction of synthetic reactive intermediates with target nucleotides or bases *in vitro*. Structural analysis is performed on the synthetic standards, and DNA adducts formed in cell culture or *in vivo* are identified by Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC) co-chromatography (Josephy, 1997).

The detection and quantitation of DNA adducts by the direct methods described above is possible only when adducts are present in sufficiently large amounts (μmole/mole DNA). For samples with lower levels of DNA adducts, the radiolabeled xenobiotic method (14C or 3H) and 32P postlabeling method (two-dimensional thin-layer chromatography) are often used (Josephy, 1997). However, each method has its own limitation. The GC and HPLC methods often lack confirmation information. The radiolabeling method is expensive and is limited by the relatively low specific activities of 14C and 3H. The 32P postlabeling method also lacks confirmation information and accurate quantitative work is difficult by TLC.

Our research is focused upon the analysis of the benzo[a]pyrene-DNA/Guanine adducts at trace levels by modern instrumentation. The ultimate goal of this study is to provide a better method of detecting the "bio-markers" for cancer formation. In this work we have found upon detecting DNA adducts formed via the free-radical activation pathway. This will enable future researchers to gain a better
understanding of cancer formation, which will in turn lead to better treatments of the disease.
Benzo[a]pyrene is a five-ring polycyclic aromatic hydrocarbon (PAH), also known as 3,4-benzyrene which was first isolated from coal tar. Later, it was found to be a component of cigarette and marijuana smoke (Lee, Novotny, & Bartle, 1976). Benzo[a]pyrene belongs to the PAH family, and comprises less than five percent of the total amount of PAHs present in the atmosphere. Benzo[a]pyrene has a molecular formula of $C_{20}H_{12}$. It is a yellowish crystal with a molecular weight of 252.3. The boiling point of benzo[a]pyrene is greater than 360°C at 760 mm Hg and the melting point is approximately 179°C. The vapor pressure of benzo[a]pyrene at 25°C is $8.4 \times 10^{-7}$ Pa, enthalpy of sublimation at 25°C is $118.3 \pm 2.2$ KJ / mol, entropy of sublimation at 25°C is $183.6$ J / mol K, enthalpy of fusion is $16.6 \pm 0.3$ KJ / mol, $\log P$ (octanol/water) is 6.04, and the Henry Constant is $2.7 \times 10^{-7}$ Atm-m$^3$ / mol (Bjørseth, 1983; Ebert, 1986; Futoma, Smith, Smith, & Tanaka, 1981). It is soluble in benzene, xylene, and toluene. It is sparingly soluble in ethanol, methanol, and insoluble in water. The structure and numbering of benzo[a]pyrene are displayed in Figure 2.
Figure 2. Structure of Benzo[a]pyrene.

Benzo[a]pyrene, after metabolic activation, is known as one of the most potent PAHs to cause carcinogenic effects (Fourth Annual Report on Carcinogens, 1985). It was first determined to be a carcinogen in the early 1950s by Elizabeth Miller who found that a metabolite of benzo[a]pyrene reacts with proteins in mouse skin (Miller, 1951). The principal sources of benzo[a]pyrene and other PAHs in the atmosphere are combustion of fossil fuels in heat and power generation, refuse burning, vehicle emission and coke ovens, together contributing over 50% of the total nationwide emissions (Bjørseth & Ramdahl, 1985). Emissions from these sources are major contributors to urban environmental contamination, particularly in areas adjacent to highways, industries, and airports. Although there are other natural sources of PAHs (e.g., volcanic activity and forest fires), the anthropogenic sources are still considered to be the most significant sources of benzo[a]pyrene and other PAHs in air pollution. According to recent estimates, the annual emission of benzo[a]pyrene into the atmosphere of the United States is over 1300 tons (Harvey, 1991).
The United States Environmental Protection Agency (U.S. EPA) classified benzo[a]pyrene as a “possible human carcinogen” (Group B2) and the International Agency for Research on Cancer (IARC) considers benzo[a]pyrene a known animal carcinogen and a probable human carcinogen (Group 2A). The OEHHA (The Office of Environmental Health Hazard-Assessment) staff agrees with the IARC classification of benzo[a]pyrene (IARC Group 2A) as a probable human carcinogen based on sufficient evidence for carcinogenicity in animals and limited evidence in humans. Benzo[a]pyrene has the ability through its metabolites to arylate DNA, cause gene mutations in both prokaryotic and eukaryotic cells, induce sister chromatid exchanges in mammalian cells, and produce unscheduled DNA synthesis in mammalian cells (Environmental Protection Agency, 1998).

Several types of malignant tumors have been induced in rodents by benzo[a]pyrene. Epidemiological evidence for human cancer from exposure to benzo[a]pyrene is found in studies of roofers, tar distillers, patent-fuel workers, and creosote-exposed brickmakers. Certain complex mixtures such as soots, tars and oils are in the IARC Group 1 (sufficient evidence for carcinogenicity in humans) based on epidemiological studies. The federal government has developed standards and guidelines to protect individuals from the potential health effects of PAHs, including benzo[a]pyrene, in drinking water. The U.S. Environmental Protection Agency (EPA) has provided estimates of levels of total cancer-causing PAHs in lakes and streams associated with various risks of developing cancer in people. The EPA has
also determined that any release of PAHs of more than 1 pound should be reported to the National Response Center (Environmental Protection Agency, 1998).

Pure benzo[a]pyrene is produced in the United States only as a laboratory chemical. However, benzo[a]pyrene is a PAH, and PAHs are found in coal tar and in the creosote oils and pitches formed from the production of coal tar. The government’s goal has been to protect workers involved with the production of coal tar products. These regulations are for exposure to benzo[a]pyrene in workplace air. Although government standards are not for benzo[a]pyrene alone, they are useful in controlling exposure to total PAHs.

The National Institute for Occupational Safety and Health (NIOSH) has determined that workplace exposure to coal products can increase the risk of lung and skin cancer in workers. Based on that, NIOSH has suggests that the workplace exposure limit for coal tar products is 0.1 milligram of PAHs per cubic meter of air (0.1 mg/m³) for a 10-hour workday and 40-hour workweek. NIOSH has not suggested a specific workplace limit for benzo[a]pyrene. However, the Occupational Safety and Health Administration (OSHA) has set a legal limit of 0.2 milligrams of all PAHs per cubic meter of air (0.2 mg/m³) (California Air Resources Board and Office of Environmental Health Hazard, 1994).

Benzo[a]pyrene/PAHs are widely distributed throughout the waters of the earth, entering the food chain by being taken up by fish and plankton (Harvey, 1991). Since some PAHs including benzo[a]pyrene are only slowly degraded, they represent a potential health hazard to humans through drinking water and other
sources. For example, the average level of benzo[a]pyrene in drinking water is found to be around 0.01μg/L which is of a similar level to that from breathing reasonably clean air (Harvey, 1991).

The most common way benzo[a]pyrene enters the body is through the lungs when a person breathes in air or smoke containing it. It also enters the body through the digestive system when substances containing it are swallowed. Although benzo[a]pyrene does not normally enter the body through the skin, small amounts could enter if contact occurs with soil that contains high levels of benzo[a]pyrene (for example, near a hazardous waste site) or if contact is made with heavy oils containing it (Agency for Toxic Substances and Disease Registry, 1990).

Benzo[a]pyrene is potent, because it has two main features: (1) a relatively low ionization potential of 7.23eV (IP), which allows metabolic removal of one electron thus forming a cation (if IP < 7.35eV) (Cavalieri & Rogan, 1985; Hemminki, 1994); and (2) appreciable charge localization in the radical cation, which allows this intermediate to be especially and efficiently reactive with nucleophiles (Dipple, 1995; Hemminki, 1994; Josephy, 1997). This is demonstrated by adduct formation of benzo[a]pyrene with DNA and has been widely studied and published by various researchers (Cavalieri et al., 1990; Cavalieri et al., 1997; Todorovic et al., 1997).
Benzo[a]pyrene-DNA Adducts

Several benzo[a]pyrene-DNA adducts have been synthesized by either \textit{in vitro} or \textit{in vivo} methods and identified by modern instrumentation. Those adducts are used as standards for further studies of benzo[a]pyrene carcinogenesis effects (Rogan et al., 1988).

A large body of research exists on benzo[a]pyrene and PAHs. Among those studies, guanine nucleosides were chosen most often because this base is most frequently modified by chemical carcinogens in biological systems. It has been reported that benzo[a]pyrene forms two major depurination adducts with guanine (nucleoside) when reacted with DNA \textit{in vitro} and \textit{in vivo}. Several groups of researchers have attempted similar studies. PAHs and other aromatic compounds form covalent bonds with DNA nucleosides that provide valuable information on the binding mechanism (Casale et al., 1997; Cavalieri, Devanesan, Mulder, RamaKrishna, & Rogan, 1994; Cavalieri et al., 1990; Chen et al., 1996; Devanesan, Todorovic, Rogan, & Cavalieri, 1994; Devanesan et al., 1996; Hoffman, Lesko, & Ts’o, 1970; Humphreys, Kadlubar, & Guengerich, 1992; Jeffery, Blobstein, Weinstein, & Harvey, 1976; Li, RamaKrishna, Padmavathi, Rogan, & Cavalieri, 1994; Li et al., 1995; McCull et al., 1999; Miller, 1951; RamaKrishna et al., 1992; RamaKrishna et al., 1993; Rogan, Higginbotham, Devanesan, & Cavalieri, 1994; Rogan et al., 1990; Ross et al., 1991; Sage & Haseltine, 1984; Slaga et al., 1977; Stack, Cremonesi, Hanson, Cavalieri, & Rogan, 1995; Todorovic, Devanesan, Rogan, & Cavalieri, 1993).
According to these studies, it is believed that benzo[a]pyrene is activated by two main routes: one-electron oxidation to yield reactive intermediate radical cations (Cavalieri & Rogan, 1984, 1985; Cavalieri & Roth, 1976; Hanson et al, 1998; Miller & Miller, 1981; Rogan et al., 1988; Waalkes & Ward 1994) and monooxygenation to produce bay-region diol epoxides (Cheh et al., 1993; Conney, 1982; Sims & Grover, 1981). These activated intermediates then react with nucleotides or bases to form covalently bonded compounds and cause a "molecular lesion."

For the monooxygenation route, the major identified benzo[a]pyrene diol epoxide adduct arises from formation of a covalent bond between C-10 of benzo[a]pyrene diol epoxide and two amino of guanine moieties on DNA (Rogan et al., 1990; Weinstein et al., 1976). Figure 3 displays the scheme of formation of benzo[a]pyrene diol epoxide and its adducts by "monooxygenation" of benzo[a]pyrene and reacted with DNA.

For the one-electron oxidation route, the benzo[a]pyrene was first activated by one-electron oxidation to its radical cation. It then binds the C-6 of benzo[a]pyrene to either the C-8 or N-7 of guanine on DNA and forms adducts (Rogan et al., 1990). They are 8-(benzo[a]pyrene-6-yl)guanine and 7-(benzo[a]pyrene-6-yl)guanine (Rogan et al., 1988) (hereafter referred to as BaP-C8Gua and BaP-N7Gua). Figure 4 displays the scheme of formation of BaP-C8Gua and BaP-N7Gua adducts by one electron oxidation of benzo[a]pyrene reacted with DNA.
Figure 3. Formation of BaP-DNA Adducts by Monooxygenation.

In some of the benzo[a]pyrene/DNA research that has been done, BaP-C8Gua and BaP-N7Gua were made and used as external standards in HPLC/UV detection assays (typical detection limit was at μg/mL) for quantitative work (Rogan et al., 1988). However, the analytical work that has been done on bio-samples was lacking confirmation information due to the complexity of sample matrix, especially when tissue samples were used for analysis.

Research Goal and Instrumentation

Our research goal is to utilize high performance liquid chromatography/tandem mass spectrometry to solve the analytical problems associated with
benzo[a]pyrene-DNA adduct isomer detection and provide accurate data for the quantitative, qualitative work, and kinetic studies of benzo[a]pyrene/DNA (BaP-C8Gua and BaP-N7Gua) adduct formation in *in vitro* and *in vivo* samples.

Mass spectrometry has very unique selectivity. When coupled with GC or HPLC it becomes a very powerful tool in analytical chemistry. It has been widely used in industry and academia for years for different research. In mass spectrometry, molecules are converted to ions and the ions are separated according to their mass-to-charge ratio (m/e). The prototype mass spectrometer was first made by J. J. Thompson and the first mass spectrometer was developed by the British physicist
Francis William Aston in 1919. Since then, it has been quickly adopted by analytical chemists as one of the most important research tools.

Despite the differences in mass spectrometry ion detection techniques, all mass spectrometers have four features in common: (1) a device for sample introduction, (2) a device for ionizing the sample, (3) an analyzer/mass filter that directs the ions into the measuring device, and (4) a system for measuring the constituent ions and recording the mass spectrum.

Typically, GC, HPLC, CE, ICP, and solid probe are commonly used inlet devices. Electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmosphere pressure chemical ionization (APCI), fast atom/ion bombardment (FAB), and matrix-assisted laser desorption/ionization (MALDI) are common ionization techniques (Johnstone & Rose, 1996; Burlingame & Carr, 1996). For the analyzer/mass filter, quadrupole/triple-quadrupoles, magnetic sector, ion trap, and time of flight (TOF) are widely used. Detection and recording are often achieved by coupling an electron multiplier with a high-speed computer (Siuzdak, 1996).

The biggest advantage in utilizing mass spectrometry as an analytical tool is that it provides confirmation information on the analytes. Unlike UV or other spectrophotometer detectors, mass spectrometers do not require analytes to have any chromophores or special properties. Any ionizable chemical can be detected by the mass spectrometer. Another advantage of mass spectrometry is that it provides more than just “detection.” A good mass spectrum often provides important information such as molecular weight and special characteristic fragments of the analytes. If the
appropriate mass spectrometer and separation technique are used, the molecular structure of unknowns can be determined and confirmed. Because of the unique advantages of mass spectrometry, it has become a very useful tool for chemical analysis, especially when complicated sample matrices are encountered (e.g., the biological and environmental samples) (Siuzdak, 1996).

In general, HPLC, GC, and CE are often coupled with the mass spectrometer as inlets and to provide separation prior to mass spectrometer analysis. HPLC is by far the most favored inlet for bio-analysis. It has the capability of handling large molecules, provides better separation for special compounds, larger sample capacity, and most importantly, it does not cause thermal degradation of the analytes. Because of those advantages, liquid chromatography/mass spectrometry (hereafter referred to as LC/MS) has become increasingly popular among analytical chemists. Today, the application of LC/MS has become widely used. Application ranges from trouble-shooting, research work, and even routine quality control duties.

The most advanced LC/MS is the LC/tandem mass spectrometer (LC/MS/MS) with atmospheric pressure ionization (ESI and APCI). The electrospray ionization (ESI) model transforms ions in solution into ions in the gas phase. In ESI, ions are produced and analyzed as follows:

1. The sample solution enters the ESI capillary, to which a high voltage is applied.
2. The ESI capillary sprays the sample solution into a fine mist of droplets (aerosol) that are electrically charged at their surface. The electrical charge density at the surface of the droplets increases as solvent evaporates from the droplets.

3. The charge density at the surface of droplets increases to a critical point (Rayleigh stability limit) and then becomes divided into smaller droplets because the electrostatic repulsion is greater than the surface tension. The process is repeated many times to form very small droplets.

4. Charged sample ions are ejected into the gas phase by electrostatic repulsion and enter the mass spectrometer.

In atmospheric pressure ionization (APCI), ions are produced and analyzed as follows:

1. The APCI sprays the sample solution into a fine mist of droplets and is vaporized in a high temperature tube.

2. A high voltage is applied to a needle located near the exit end of the tube and creates a corona discharge that forms reagent ions through a series of chemical reactions with solvent molecules and nitrogen nebulizing gas.

3. The reagent ions react with sample molecules to form ions and enter the mass spectrometer.

A typical example of the mass spectrometer is the quadrupole mass spectrometer. Quadrupole mass analyzers have been used in conjunction with electron ionization sources since the 1950s (Chapman, 1993). Electron ionization coupled with quadrupole mass analyzers is employed in the most common mass
spectrometers in existence today. Quadrupole mass analyzers have found new utility in their capacity to interface with electrospray ionization. This interface has three primary advantages. First, quadrupoles are tolerant of relatively low vacuum (~5×10^{-5} Torr), which is very suitable to electrospray ionization since the ions are produced under atmospheric pressure conditions. Second, quadrupoles are now capable of analyzing compounds with m/z ratios (3000-4000), which is very useful for bio-analysis. Finally, the cost of a quadrupole analyzer instrument is lower compared with other analyzers.

Considering these mutually beneficial features of quadrupole analyzers, it is not surprising that quadrupole instruments are widely used for LC/MS applications, especially LC/MS/MS. There are other advantages of utilizing quadrupole instruments in chemical analysis. For example, the quadrupole analyzer is independent of ion velocity, therefore, the peak broadening on fragmentation seen with sector instruments is absent when a quadrupole is used. A triple quadrupole is able to provide unit mass resolution for both precursor and fragment ions and the spectra are generally free from artifacts.

The most advanced quadrupole mass spectrometer is the triple quadrupole tandem mass spectrometer. It is constructed by joining three quadrupole analyzers/filters together. The first and third quadrupole serve as analyzer/filter and the second quadrupole (radio frequency only) is designed to be the collision cell to further break down the ions (usually by introducing argon or helium as collision gases). This design offers advantages in resolution and sensitivity (signal to noise...
enhancement) for the study of ion fragmentation as well as freedom from artifact peaks (Chapman, 1993). As a result, independent mass analyzer/filters are available which can select precursor and fragment ions for a fragmentation taking place in the second quadrupole. The further fragment ions are called “daughter” ions (MS/MS). Daughter ions provide valuable information for structure determination and because of the signal to noise enhancement it also serves as a very powerful tool for trace sample or bio-sample analysis.

Analytical Approach

Our goal is to utilize LC/tandem mass spectrometry to develop a method to use DNA adducts BaP-C8Gua and BaP-N7Gua formation as bio-markers of exposure to carcinogens. A stable isotope internal standard method was developed for our analysis. The use of stable isotope analogues as internal standards for quantitative LC/MS work has been adopted by researchers because of the following major advantages (Johnstone & Rose, 1996).

1. The stable isotope internal standard has similar/same chemical/physical properties of the compound to be quantified; therefore, it compensates for any losses during work-up by behaving identically towards extraction or purification.

2. The isotope internal standard is often co-eluted with the compound to be quantified via chromatography; hence, it compensates for any variation of the instrumentation. For example, a slight variation in pressure in the ion source will have the same degree of effect on both analytes and internal standards. Therefore,
the effect of the magnitude for both signals is the same. Since only the ratio of analyte and internal standard is used, the result will not change.

3. The difference in mass between standard and analyte can be easily distinguished by mass spectrometry. It is especially useful for analyzing samples with a complicated sample matrix. A carefully designed LC/MS/MS experiment usually taking cares of all the problems.

For our study, authentic standards (BaP-C8Gua and BaP-N7Gua) and stable isotope internal standards (d_{11}-BaP-C8Gua and d_{11}-BaP-N7Gua) were synthesized by the electrochemical method and purified by liquid chromatography. In vitro and in vivo methods were used to generate the bio-samples and the levels of BaP-C8Gua and BaP-N7Gua in each sample were determined by our method.
CHAPTER III

MATERIALS AND METHODS

Materials

Benzo[a]pyrene, calf thymus DNA, and horseradish peroxidase type II were purchased from Sigma Chemical Company (St. Louis, MO) and the N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from United State Biochemical Corporation (Cleveland, OH). Benzo[a]pyrene-d$_{12}$ was purchased from Cambridge Isotope Laboratories (Andover, MA) and deoxyguanosine monohydrate was purchased from Fluka Chemical Company (St. Louis, MO). All solvents used for LC/MS and LC/MS/MS analysis were HPLC grade and purchased from Fisher Scientific (Itasca, IL). C-18 Sep-Pak cartridges were purchased from Millipore (Bedford, MA).

Instruments

The electrochemical synthesis device was assembled in our laboratory. It consisted of a potentiostat (A.R.F. Product) (Boulder, CO), a calomel reference electrode, and two platinum electrodes. A 100mL Pyrex beaker was used as the synthesis cell and a magnetic stir bar was used for sample stirring. Nuclear magnetic resonance spectra (NMR) were determined on a JEOL Eclipse 400 FT NMR.
spectrometer (Peabody, MA). The LC/tandem mass spectrometer system used was a Micromass Quattro II triple-quadrupole mass spectrometer (Wythenshawe, UK) coupled with a Waters 2696 multisolvend delivery system, Waters 2487 dual wavelength UV detector, and an auto injector (Milford, MA). The HPLC system used for sample purification was a Waters HPLC system equipped with Waters 600-MS multisolvend delivery system and manual injector (Milford, MA) coupled with a Hewlett Packard 1040A Photodiode array detector (Palo Alto, CA). The HPLC system used for evaluating the homogeneity and potency of benzo[a]pyrene in dosed feed was a Hewlett Packard 1090 HPLC system coupled with a Photodiode array detector (Palo Alto, CA). The rotatory shaker used for feed preparation was from Lortone inc. (Seattle, WA). The LC/tandem mass spectrometer system used for APCI (confirmation) was a Finnigan LCQ mass spectrometer (San Jose, CA) coupled with a Hewlett Packard 1090 HPLC system (Palo Alto, CA).

Synthesis of BaP-C8Gua and BaP-N7Gua

The Rogan and Cavialier electrochemical synthesis method (Rogan et al., 1988) was used for preparing BaP-C8Gua and BaP-N7Gua. The electrochemical synthesis device was assembled in our laboratory.

In a typical preparation, DMF (20mL, dried by sodium sulfate and degassed) containing 0.5 M KClO4 as electrolyte was pre-electrolyzed at +1.45V verses the standard calomel reference electrode for 30 minutes with argon purging. The pre-dried (80 °C under vacuum for 24 hours) deoxyguanosine monohydrate (1 mmol)
and benzo[a]pyrene (0.1 mmol) were added to the solution and the resulting solution was stirred until all materials dissolved. The bubbling of argon was stopped and the electrode potential was gradually raised from zero to 1.1 V verses the standard calomel reference electrode (current is usually around 20-30 mA). The reaction was stopped when the current dropped to around 1 mA (usually after 2 hours). The dark brown, crude solution was then transferred to a separatory funnel along with 100 mL 0.05% formic acid in Milli-Q water. The solution was extracted three times with 150 mL of CHCl₃. The combined extract was dried on a rotatory evaporator and re-dissolved in 5 mL DMSO. A C18 Sep-Pak cartridge was conditioned by first eluting with 6 mL of acetonitrile and then followed by 6 mL of water. Approximately 0.25 mL of the DMSO solution was then loaded on a pre-conditioned C-18 Sep-Pak cartridge and washed with 10 mL of water followed by 5 mL of 10% methanol in water to remove unreacted deoxyguanosine. Adducts were eluted with 15 mL of 50/50 (% v/v) acetone: methanol (both containing 0.1% formic acid) and collected. The procedure was repeated for the remaining DMSO solution. All adduct fractions were combined and dried on a rotatory evaporator and then re-dissolved in 5 mL of DMSO for further HPLC purification.

HPLC purification of BaP-C8Gua and BaP-N7Gua was conducted using a Waters HPLC system equipped with a Waters 600-MS multisolvent delivery system and manual injector, and a Hewlett Packard 1040A DAD detector set to monitor the absorbance at 300 nm. Solvent A was 0.1% formic acid in acetonitrile and solvent B was 0.1% formic acid in Milli-Q water. Two HPLC steps were used for purifying...
the DNA adducts. First purification step was performed on a semi-preparative reversed-phase C-18 column (250 x 9.4 mm, 5 μm) (Phenomenex, Terrance, CA). In general, 250 μL of the DMSO (post Sep-packs) solution was injected onto the column and eluted with the following linear gradient program at a flow rate of 2.0 mL/min: 0 minute, 20% A; hold for 5 minutes; 75 minute, 100% A; hold for 10 minute; 85.1 minute, 20% A. The BaP-C8Gua eluted at 37 minutes and BaP-N7Gua eluted at 42 minutes. BaP-C8Gua and BaP-N7Gua were collected individually and dried under vacuum. The procedure was repeated for the remaining samples. Finally, all BaP-C8Gua fractions and all BaP-N7Gua fractions were combined respectively and dried under vacuum. Approximately 0.5 mL of DMSO was added to each sample to dissolve adducts for the second HPLC purification.

For the second purification step, a Zorbax SB-CN column (250 x 4.6mm, 5μm) (Hewlett Packard, CA) and a linear gradient were used for both BP-C8Gua and BaP-N7Gua samples. Typically, 20 μL of either DMSO (BaP-C8Gua or BaP-N7Gua first purification step) solution was injected onto the column and eluted with the following linear gradient program at a flow rate of 1.0 mL/min: 0 minute, 30% A; hold for 5 minutes; 55 minute, 100% A; hold for 10 minutes; 65.1 minute, 30% A (hereafter referred to as Gradient 20 HPLC method). The BaP-C8Gua eluted at 15 minutes and BaP-N7Gua eluted at 19 minutes. Each adduct was collected and dried under vacuum and the procedure was repeated for the remaining samples.

A total of eight preparations of crude mixture were made and purified by following the above procedure. The identity of BaP-C8Gua and BaP-N7Gua were
confirmed by NMR, LC/MS (ESI), LC/MS/MS (ESI), and LC/MS (APCI). Typical LC chromatograms are shown in Figure 5. The UV profile from DAD is shown in Figure 6.

![LC Chromatograms](image-url)

**Figure 5.** LC Chromatograms. ([From top to bottom] Reaction Mixture; LC chromatogram of BaP-C8Gua; LC chromatogram of BaP-N7Gua. The Gradient 20 HPLC Method was used to run all samples [Zorbax SB-CN column 250 x 4.6 mm, 5 μm]).

**Synthesis of BaP-C8Gua-d<sub>11</sub> and BaP-N7Gua-d<sub>11</sub>**

The synthesis and purification procedures used here were the same as preparing BaP-C8Gua and BaP-N7Gua. The only difference was to use of
benzo[a]pyrene-d$_{12}$ (twelve deuterated) in place of benzo[a]pyrene in the synthesis and run on a smaller scale each time.

In a typical preparation, DMF (10 mL, dried by sodium sulfate and degassed) containing 0.5 M KClO$_4$ as electrolyte was pre-electrolyzed at +1.45 V versus the standard calomel reference electrode for 30 minutes with argon bubbling. The pre-dried (80 °C under vacuum for 24 hours) deoxyguanosine monohydrate (0.5 mmol) and benzo[a]pyrene-d$_{12}$ (0.05 mmol) were added to the solution and the resulting solution was stirred until all materials dissolved. The bubbling of argon was stopped and the electrode potential was raised from 0 to 1.1V verses the standard calomel reference electrode (current was approximately 20-30 mA). The reaction was stopped when the current dropped to approximately 1mA (usually around 2 hours). The dark brown, crude solution was then transferred to a separatory funnel along with 50 mL 0.05% formic acid in Milli-Q water. The solution was extracted three times 25 mL of CHCl$_3$. The combined extract was dried.

Figure 6. (From left to right) BaP-C8Gua UV Profile From DAD; BaP-N7Gua UV Profile From DAD.
on a rotatory evaporator and re-dissolved in 5 mL DMSO. A C18 Sep-Pak cartridge was conditioned by first eluting with 6 mL of acetonitrile and then followed by 6 mL of water. Approximately 0.25 mL of the DMSO solution was loaded on a pre-conditioned C-18 Sep-Pak cartridge and washed with 10mL of water followed by 5 mL of 10% methanol in water to remove unreacted deoxyguanosine. Adducts were eluted with 15 mL of 50/50 (% v/v) acetone: methanol (both with 0.1% formic acid) and collected. The procedure was repeated for remaining DMSO solution. All adduct fractions were combined and dried on a rotatory evaporator and then redissolved in 5 mL of DMSO for further HPLC purification.

HPLC purification of BaP-C8Gua-d11 and BaP-N7Gua-d11 was accomplished using the same Waters HPLC system. It is Waters HPLC system equipped with a Waters 600-MS multisolvent delivery system and manual injector, and a Hewlett Packard 1040A DAD detector set to monitor the absorbance at 300 nm. Solvent A was 0.1% formic acid in acetonitrile and solvent B was 0.1% formic acid in Milli-Q water. Two HPLC methods were used for purifying the DNA adducts. First purification step was done on a semi-preparative reverse-phase C-18 column (250 x 9.4 mm, 5 μm) (Phenomenex, Terrance, CA). In general, 250 μL of the DMSO (post Sep-packs) solution was injected onto the column and eluted with the following linear gradient program at a flow rate of 2.0 mL/min: 0 minute, 20% A; hold for 5 minutes; 75 minute, 100% A; hold for 10 minutes; 85.1 minute, 20% A. The BaP-C8Gua-d11 eluted at 37 minutes and BP-N7Gua-d11 eluted at
42 minutes, BaP-C8Gua-d11 and BaP-N7Gua-d11 were collected individually and dried under vacuum. The procedure was repeated for the remaining samples. Finally, all BaP-C8Gua-d11 fractions and all BaP-N7Gua-d11 fractions were combined respectively and dried under vacuum. Approximately 0.5 mL of DMSO was added to each sample to dissolve adducts for the second HPLC purification.

For the second purification step, a Zorbax SB-CN column (250 x 4.6 mm, 5 \( \mu m \)) (Hewlett Packard, CA) and a linear gradient were used for both BaP-C8Gua-d11 and BaP-N7Gua-d11 samples. Typically, 20 \( \mu L \) of either DMSO (BaP-C8Gua-d11 or BaP-N7Gua-d11 first purification) solution was injected onto the column and eluted with the Gradient 20 HPLC method. The BaP-C8Gua-d11 eluted at 15 minutes and BaP-N7Gua-d11 eluted at 19 minutes. The retention times of BaP-C8Gua-d11 and BaP-N7Gua-d11 on both HPLC assays are almost identical with BaP-C8Gua and BaP-N7Gua. Each adduct was collected and dried under vacuum and the procedure was repeated for the remaining samples.

A total of three batches were made and purified subsequently and 0.3 mg BaP-C8Gua-d11 and 0.9 mg of BaP-N7Gua-d11 were obtained.

MS/MS/MRM (Multiple Reaction Monitoring) Optimization

Quantitative analysis of BaP-C8Gua and BaP-N7Gua was performed in the LC/MS/MS/MRM mode. The mass spectrometer was operated in the positive ion
ESI mode. Tuning of the ESI source and optimizing the MS/MS parameters were carried out using synthetic BaP-C8Gua and BaP-N7Gua standards. Standards were first dissolved in DMSO and then diluted with 45/45/10 (% v/v/v) acetonitrile: methanol: 2% formic acid in water and then induced by syringe pump at a flow rate of 3 to 5 μL/min. ESI interface and mass spectrometer were optimized to obtain maximum sensitivity. The electrospray current was optimized by monitoring the ion intensity of MH\(^+\) (m/z 402) from 2.0 kV to 5.0 kV for both adducts. The collision energy was optimized by monitoring the intensity of m/z 277 (major fragment) for both adducts. For the collision energy optimization, the first quadrupole of the instrument was set only to pass the MH\(^+\) at m/z 402 for both adducts. The product ions (daughters) were separated in the third quadrupole. Argon was used as the collision gas, with a gas cell pressure of \(~2.5\times10^{-3}\) mbar. The collision energy was studied from 0 V to 200 V.

**LC/MS/MS/MRM (Multiple Reaction Monitoring) Method**

For the quantitative study of adducts, a LC/MS/MS/MRM (multiple reaction monitoring) method was developed and used. For the mass spectrometer, the first quadrupole of the instrument was set only to pass the MH\(^+\) at m/z 402 for both adducts and m/z 413 for both deuterated internal standards. The collision induced fragmentation that occurred at the second quadrupole. The product ions (daughters) were separated in the third quadrupole. Argon was used as the collision gas, with a gas cell pressure of \(~2.5\times10^{-3}\) mbar. The collision energy was set at 40 V. The
quantitative analysis of adducts was done by MS/MS/MRM mode by monitoring their daughter ions. For the authentic adducts, (BaP-C8Gua and BaP-N7Gua) m/z 402 → 277 and m/z 402 → 360 transitions were monitored. For the internal standards (BaP-C8Gua-d11 and BaP-N7Gua-d11) m/z 413 → 287 and m/z 413 → 371 transitions were monitored.

For the HPLC, a Zorbax SB-CN column (250 x 4.6 mm, 5 μm) fitted with a Zorbax SB-CN guard column was used for all the sample analysis. The flow rate was 0.7 mL/min. Formic acid, 0.2% in water, was used as solvent A and 0.2% formic acid in acetonitrile was used as solvent B. A linear gradient was run as follows: 0 minutes, 48% B; 5 minutes, 48% B; 20 minutes, 100% B; 25 minutes, 100% B; 25.1 minutes, 48% B; 30 minutes, 48% B. The BaP-C8Gua and BaP-C8Gua-d11 were co-eluted at approximately 7 minutes, and the BaP-N7Gua and BaP-N7Gua-d11 were co-eluted at approximately 10 minutes under the above conditions.

For a typical analysis, 10 μL of sample was injected. A standard curve was constructed by replicate analysis of solutions containing BaP-C8Gua and BaP-N7Gua standards from 1 to 32 ng per injection and the internal standards BaP-C8Gua-d11 and BaP-N7Gua-d11 at 2 ng per injection in all of the solutions.
Adducts Stability Evaluation and Method Suitability

Several spike and recovery studies were performed by addition of the authentic standards at various sample preparation stages to assess the method validity, adduct stability, and procedure efficiency. Different types of bio-samples were used to verify the method’s ruggedness and robustness.

The adduct stability and the Sep-Pack procedure efficiency were demonstrated by treating adducts at extreme conditions, spiking the internal standards into the post Sep-Pack fraction, and assaying for recovery. Duplicate samples were prepared for each level of the study. The samples were prepared by spiking 15, 75, 150, 300, and 450 ng of authentic adducts into separate test tubes with 2 mL of 0.02 N HCl. The spiked concentrations were equal to 1, 5, 10, 20, and 30 ng on column based on a final volume of 150 μL and 10 μL injection. The solutions were hydrolyzed at 75 °C for 1.5 hours. After cooling, each sample was neutralized to pH 7.0 by addition of NaOH. The C18 Sep-Pak cartridges were activated by first rinsing with 6 mL of acetonitrile followed by 6 mL of purified water. Each sample was then loaded onto a separate cartridge and washed with 5 mL of purified water. Adducts were eluted with 3 mL of methanol. The methanol fraction for each sample was collected separately and spiked with 30 ng of the deuterated internal standards (both BaP-C8Gua-d11 and BaP-N7Gua-d11). The final methanol fraction was dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μL of DMSO for LC/MS/MS/MRM analysis.
The method validation was demonstrated by measuring adduct levels in rat liver DNA samples spiked with a known amount of authentic standards. Duplicate samples were prepared for each spiked concentration level. Rat liver DNA was obtained as described in the protocol provided by Life Technologies Co. For each spiked sample, 1.0 gram of liver was minced and homogenized in 30 ml of dnazol reagent using a hand held glass homogenizer for approximately 30 seconds. The solution was then centrifuged at 10,000 × g for 10 minutes to remove insoluble tissue fragments, RNA, and excess polysaccharides. The top layer was transferred to a new tube and the DNA was precipitated by the addition of a half volume of absolute ethanol. DNA was recovered by centrifugation at 1700 × g for 2 minutes and washed three times with 70% ethanol. The DNA pellet was air dried briefly, dissolved in 8 mm sodium hydroxide solution and the ph was then adjusted to 7.0 with HEPES and centrifuged at 10,000 × g for 2 minutes to remove any impurities. The DNA was then re-precipitated by addition of ethanol and recovered by centrifugation at 1700 × g for 3 minutes. The DNA pellet was then briefly air dried, and then redissolved in 8 mm sodium hydroxide solution and the ph was adjusted to 7.0 with HEPES. The quantity of DNA was estimated by reading the absorbance (1AU = 50 µg DNA/ml) at 260 nm. The purity of the DNA was estimated by scanning the UV spectrum from 200 to 400 nm and calculating the 260/280 ratios. Typical yield of each preparation was approximately 3.7 to 4.1 mg of DNA per 1000 mg of liver tissue (3.7 ~ 4.1 µg/mg).
For each spike, 15, 75, 150, 300, and 450 ng of adducts were added separately into individual DNA solutions (approximately 4 mg equal to prepare from 1 g of liver tissue). The spiked levels were equal to 1, 5, 10, 20, and 30 ng on column based on a final sample volume of 150 μL and 10 μL injection per sample. The pH of each spiked solution was adjusted to approximately 2.5 with HCl and hydrolyzed at 75 °C for 45 minutes (Citti, Gervasi, Turchi, Bellucci, & Bianchini, 1984; Dong & Jeffrey, 1991; Josephy, 1997; McCull et al., 1999). After cooling, each sample was neutralized to a pH of 7.0 by the addition of sodium hydroxide solution and then spiked with 30 ng of the deuterated internal standards (both bap-C8Gua-d11 and bap-N7Gua-d11). The C18 Sep-Pak cartridges were activated by first rinsing with 6 ml of acetonitrile and followed by 6 ml of purified water. Approximately 1/3 of the sample was then loaded on a cartridge and washed with 5 ml of purified water. Adducts were eluted with 3 ml of methanol. The procedure was repeated for each sample and methanol fractions were then combined and dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μL of DMSO for LC/MS/MS/MRM analysis.

Blank rat liver DNA samples were prepared by following the procedure described above. Neither authentic nor internal standards were added for the blank preparations in order to assess the possible bias.

The method suitability for different bio-samples was demonstrated by measuring adduct levels in rat urine and feces samples spiked with a known amount of authentic standards. For the rat urine experiment, duplicate samples were
prepared for each spiked concentration. The spiked samples were prepared by the addition of 15, 75, 150, 300, and 450 ng of adducts respectively onto separate 5 ml aliquots of blank rat urine and then prepared for analysis by the following procedure.

Briefly, the urine sample was extracted with five 5 ml aliquots of chloroform. The protein precipitated from the extraction was transferred into a separate tube and extracted with five 5 ml aliquots of acetonitrile. The acetonitrile and chloroform extracts were combined. The combined organic extract was spiked with 30 ng of the deuterated internal standards (both bap-C8Gua-d11 and bap-N7Gua-d11) and then dried under ultrapure nitrogen. The residue was redissolved in 150 μL DMSO and the recovery was evaluated by LC/MS/MS/MRM. The spiked levels were equal to 1, 5, 10, 20, and 30 ng on column based on a final sample volume of 150 μL and 10 μL injection per sample. Blank urine samples were prepared by following the procedure described above. Neither authentic nor internal standards were added for the blank preparations in order to assess the possible bias.

For the rat feces experiment, duplicate samples were prepared for each spiked concentration. Rat feces was first dried at 80 °C with an in-house vacuum for 24 hours and then pulverized by mortar and pestle. The spiked samples were prepared by addition of 15, 75, 150, 300, and 450 ng of adducts respectively onto separate 1.0 g of dried feces powder and prepared for analysis by the following procedure. Briefly, each spiked feces sample was dried at 80 °C with an in-house vacuum for 24 hours. The dried sample was extracted with three 5 ml aliquots of
chloroform (5 hours shaking on a shaker for each fraction) followed by three 5 ml aliquots of acetone (one hour shaking for each fraction). The chloroform and acetone extracts were combined. The combined organic extract was spiked with 30 ng of the deuterated internal standards (both bap-C8Gua-d11 and bap-N7Gua-d11) and dried under ultrapure nitrogen. The residue was redissolved in 1.0 ml of 50/50 (% v/v) DMSO: acetone. The C18 Sep-Pak cartridges were activated by first rinsing with 6ml of acetonitrile followed by 6 ml of purified water. Approximately 1/3 of the sample was then loaded on a cartridge and washed with 5 ml of purified water. Adducts were eluted with 3 ml of methanol. This procedure was repeated for the rest of the sample and methanol fractions were combined and dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μL of DMSO for LC/MS/MS/MRM analysis.

The spiked levels were equal to 1, 5, 10, 20, and 30 ng on column based on a final sample volume of 150 μl and 10 μl injection per sample. Blank feces samples were prepared by following the procedure described above. Neither authentic nor internal standards were added for the blank preparations in order to assess the possible bias.

In Vitro Sample Preparation

The in vitro experiment was performed by following a slightly revised procedure of Rogan and Cavalieri (Rogan et al., 1988). Rat liver DNA (isolated and
purified in our lab) and calf thymus DNA (purchased from Sigma-Aldrich Co.) were used for our in vitro experiment.

Rat liver DNA was obtained as described in the protocol provided by Life Technologies Co. Briefly, 1.0 gram of liver was minced and homogenized in 30 ml of dnazol reagent using a hand held glass homogenizer for approximately 30 seconds (about 10 strokes). The solution was centrifuged at 10,000 × g for 10 minutes to remove insoluble tissue fragments, RNA, and excess polysaccharides. The top layer was transferred to a new tube and the DNA was precipitated by the addition of a half volume of absolute ethanol. DNA was recovered by centrifugation at 1700 × g for 2 minutes and washed three times with 70% ethanol. The DNA pellet was air dried briefly, dissolved in 8 mm sodium hydroxide solution and the pH was adjusted to 7.0 with HEPES and centrifuged at 10,000 × g for 2 minutes to remove any impurities. The DNA was re-precipitated by addition of ethanol and recovered by centrifugation at 1700 × g for 3 minutes. The DNA pellet was briefly air dried, and redissolved in 8 mm sodium hydroxide solution and the pH was adjusted to 7.0 with HEPES. The quantity of DNA was estimated by reading the absorbance (1AU=50 μg DNA/ml) at 260 nm. The purity of the DNA was estimated by scanning the UV spectrum from 200 to 400 nm and calculating the 260/280 ratios. Typical yield of each preparation was approximately 3.7 to 4.1 mg of DNA per 1000 mg of liver tissue (3.7 ~ 4.1 μg/mg). A total of 150 mg of rat liver DNA was prepared by following the above procedure.
For each of the *in vitro* experiment, 50 mg of DNA was dissolved in 100 ml of 60 mm sodium hydroxide solution and the pH was adjusted to 7.0 with phosphoric acid. A solution of 0.1 mg/ml Horseradish peroxidase (HRP) (typeii, Sigma-Aldrich) containing 0.5 mm of hydrogen peroxide was added. Benzo[a]pyrene was added at 10 mm by spiking with a 2.5 mg/ml stock DMSO solution. The above solution was purged with Argon, sealed, and incubated at 37 °C for a period of 30 minutes. The solution was cooled to room temperature and the procedure was repeated once with same amount of HRP, H₂O₂, and benzo[a]pyrene and incubated at 37 °C for another 30 minutes. At the end of the reaction, DNA was precipitated and collected by the addition of 2 volumes of ethanol followed by centrifugation at 1700 × g for 2 minutes. For the depurinating adducts, the supernatant was used and the DNA pellet was extracted with three 5 ml aliquots of chloroform. The supernatant and chloroform extracts were pooled and evaporated under vacuum and the soluble residue was redissolved in 1 ml of 50/50 (% v/v) DMSO: meoh, to which 30 ng of deuterated internal standards were then added. The C18 Sep-Pak cartridges were activated by first rinsing with 6ml of acetonitrile followed by 6ml of purified water.

Approximately one quarter of the above sample (0.25 ml) was load on a cartridge and washed with 5 ml of purified water. Adducts were eluted with 3 ml of methanol. This procedure was repeated for the remaining sample and methanol fractions were combined and dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 µl of DMSO for LC/MS/MS/MRM analysis.
The stable DNA adducts were released by thermal/acid hydrolysis (Citti et al., 1984; Dong & Jeffrey, 1991; Josephy, 1997; McCull et al., 1999). The DNA was dissolved in 25 mm NaOH. The pH of the solution adjusted to approximately 2.5 with HCl and hydrolyzed at 75 °C for 45 minutes. After cooling, the sample was neutralized to pH of 7.0 by addition of NaOH and spiked with 30 ng of the deuterated internal standards. The C18 Sep-Pak cartridges were activated by first rinsing with 6 ml of acetonitrile followed by 6 ml of purified water. Approximately 1/3 of the above sample was loaded on a cartridge and washed with 5 ml of purified water. Adducts were eluted with 3 ml of methanol. This procedure was repeated for the rest of the sample and methanol fractions were combined and then dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μl of DMSO for LC/MS/MS/MSR analysis.

In order to assess the possible interference and bias, blank samples were prepared. Both calf thymus DNA and rat liver blanks were prepared by following the procedure described above, but without the addition of benzo[a]pyrene for the reaction. Internal standards were not added in final sample preparations.

In Vivo Rat Experiment

For the in vivo rat study, a total of 36 1-month post weaning male Fisher 344 rats were obtained (Charles River, MI) for this study. The rats were put on an in-house basal diet and water ad lib for 2 weeks prior to use. They were assigned randomly into one control group and three treatment groups, with 9 rats in each
group. Three exposure levels for benzo[a]pyrene were chosen and chronic dietary (feed) dosing was used for the study. Benzo[a]pyrene was added in the rat feed (pellet) for the dosing groups at three different concentrations; 1 mg/g (1000 ppm), 0.1 mg/g (100 ppm), and 0.01 mg/g (10 ppm). For the feed preparation, a general procedure was developed and followed. Briefly, a 20 mg/ml benzo[a]pyrene stock solution was made by dissolving benzo[a]pyrene in toluene. In a 4000 ml polypropylene bottle, 2000 g of pellet feed, 300 ml of 95% ethanol, and aliquot of stock solution were added and sealed. The mixture was then put on a rotating shaker and rotated for about 4 hours until all liquid soaked into the feed. The pellet was then dried in the vacuum oven at 80 °C with in-house vacuum for 48 hours to evaporate the solvent. The same process was used for preparing the feed for the control group except that no benzo[a]pyrene was added.

A HPLC assay was run on each feed sample to ensure the homogeneity and potency of benzo[a]pyrene in feed before it was used. For the HPLC assay, a Hewlett Packard 1090 HPLC system equipped with a DAD detector and a Zorbax SB-C8 column (150 × 4.6 mm, 5 μm) (Hewlett Packard, CA) were used for all the sample analysis. The detector was set to monitor the absorbance at 300 nm for benzo[a]pyrene. The flow rate was 1.0 ml/min and the injection volume was 10 μL. Formic acid (0.2%) in water was used as solvent A and 0.2% formic acid in acetonitrile was used as solvent B. A linear gradient was run as follows: 0 min, 60% A, hold for 1 min; 7 min, 100% A, hold for 3 min; 10.1 min, 60% A.

Benzo[a]pyrene eluted at 7.1 minutes. For the HPLC sample preparation, briefly, at
each dose level, 2 to 5 grams of dosed feed was soaked in diluent containing 70/20/10 (% v/v/v) ACN: meoh: acetone with constant shaking for 24 hours and then filtered through a 0.22 μm filter. A calibration curve was made by analyzing 4 levels of benzo[a]pyrene standards in triplicates via HPLC.

For each rat, weekly body weight and daily feed consumption were recorded for the entire dosing period in order to calculate the actual dosage on animal. At the end of each dosing period (2, 4, and 8 weeks), 3 rats from each group were euthanatized and the livers were excised for the dose response analysis. Excised livers were immediately frozen in liquid nitrogen and stored at −70 °C until analysis.

For the in vivo rat liver DNA adducts analysis, the protocol provided by Life Technologies Co. was used. Briefly, 1.0 gram of liver was minced and homogenized in 30 ml of dnazol reagent using a hand held glass homogenizer for approximately 30 seconds (about 10 strokes). The solution was then centrifuged at 10,000 × g for 10 minutes to remove insoluble tissue fragments, RNA, and excess polysaccharides. The top layer was transferred to a new tube and the DNA was precipitated by the addition of a half volume of absolute ethanol. DNA was recovered by centrifugation at 1700 × g for 2 minutes and washed three times with 70% ethanol. The DNA pellet was air dried briefly, dissolved in 8 mm sodium hydroxide solution and the ph was then adjusted to 7.0 with HEPES and centrifuged at 10,000 × g for 2 minutes to remove any impurities. The DNA was re-precipitated by addition of ethanol and recovered by centrifugation at 1700 × g for 3 minutes. The DNA pellet was briefly air dried, and then redissolved in 8 mm sodium hydroxide solution and the ph was
adjusted to 7.0 with HEPES. The quantity of DNA was estimated by reading the absorbance (1AU = 50 μg DNA/ml) at 260 nm. The purity of the DNA was estimated by scanning the UV spectrum from 200 to 400 nm and calculating the 260/280 ratios. Typical yield of each preparation was approximately 3.7 to 4.1 mg of DNA per 1000 mg of liver tissue (3.7 ~ 4.1 μg/mg).

The stable DNA adducts were released by thermal/acid hydrolysis (Citti et al., 1984; Dong & Jeffrey, 1991; Josephy, 1997; McCull et al., 1999). The pH of the above DNA solution was adjusted to approximately 2.5 with HCl and hydrolyzed at 75 °C for 45 minutes. After cooling, the sample was neutralized to pH of 7.0 by addition of NaOH and spiked with 30 ng of the deuterated internal standards. The C18 Sep-Pak cartridges were activated by first rinsing with 6 ml of acetonitrile followed by 6 ml of purified water. Approximately 1/3 of the above sample was loaded on a cartridge and washed with 5 ml of purified water. Adducts were eluted with 3 ml of methanol. This procedure was repeated for the rest of the sample and methanol fractions were combined and then dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μL of DMSO for LC/MS/MS/MRM analysis.

**In Vivo Fish Experiment**

For the *in vivo* fish study, a total of 53 4 to 6 inches bluegill fish were obtained (Minnesota Muskies Farm, Brandon, MN) for this study. They were assigned randomly into one control group and three treatment groups, with 13 to 14 fish in each group. For each group, fish were put in a 40 gallon tank with oxygen-
saturated and dechlorinated water at room temperature. A flow-through system was used to ensure the freshness of water. The fish were kept on an in-house diet 2 weeks prior to use. Three exposure levels for benzo[a]pyrene were chosen and chronic dietary (feed) dosing was used for the study. Benzo[a]pyrene was added to the feed (pellet) for the dosing groups at three different concentrations; 1 mg/g (1000 ppm), 0.1 mg/g (100 ppm), and 0.01 mg/g (10 ppm). For the feed preparation, a general procedure was developed and followed. Briefly, a 20 mg/ml benzo[a]pyrene stock solution was made by dissolving benzo[a]pyrene in toluene. In a 1000 ml polypropylene bottle, 200 g of pellet feed, 30 ml of 95% ethanol, and aliquot of stock solution were added and sealed. The mixture was then put on a rotating shaker and rotated for about 4 hours until all liquid soaked into the feed. The pellet was then dried in the vacuum oven at 80 °C with in-house vacuum for 48 hours to evaporate the solvent. The same process was used for preparing the feed for the control group except that no benzo[a]pyrene was added.

A HPLC assay was run on each feed sample to ensure the homogeneity and potency of benzo[a]pyrene in feed before it was used. For the HPLC assay, a Hewlett Packard 1090 HPLC system equipped with a DAD detector and a Zorbax SB-C8 column (150 × 4.6 mm, 5 μm) (Hewlett Packard, CA) were used for all the sample analysis. The detector was set to monitor the absorbance at 300 nm for benzo[a]pyrene. The flow rate was 1.0 ml/min and the injection volume was 10 μL. Formic acid (0.2%) in water was used as solvent A and 0.2% formic acid in acetonitrile was used as solvent B. A linear gradient was run as follows: 0 min, 60%
A, hold for 1 min; 7 min, 100% A, hold for 3 min; 10.1 min, 60% A. Benzo[a]pyrene eluted at 7.1 minutes. For the HPLC sample preparation, briefly, at each dose level, 2 to 5 grams of dosed feed was soaked in diluent containing 70/20/10 (% v/v/v) ACN: meoh: acetone with constant shaking for 24 hours and then filtered through a 0.22 μm filter. A calibration plot was made by analyzing 4 levels of benzo[a]pyrene standards in triplicates via HPLC.

For the exposure, certain amounts of feed were given to each group every day for a period of four weeks. At the end of dosing period, all fish were euthanatized and the livers were excised for the dose response analysis. Excised livers were immediately frozen in liquid nitrogen and stored at −70 °C until analysis.

For the in vivo fish liver DNA adducts analysis, the protocol provided by Life Technologies Co. was used. Briefly, 1.0 gram of pool fish liver was minced and homogenized in 30 ml of dnazol reagent using a hand held glass homogenizer for approximately 30 seconds (about 10 strokes). The solution was then centrifuged at 10,000 × g for 10 minutes to remove insoluble tissue fragments, RNA, and excess polysaccharides. The top layer was transferred to a new tube and the DNA was precipitated by the addition of a half volume of absolute ethanol. DNA was recovered by centrifugation at 1700 × g for 2 minutes and washed three times with 70% ethanol. The DNA pellet was air dried briefly, dissolved in 8mm sodium hydroxide solution and the ph was then adjusted to 7.0 with HEPES and centrifuged at 10,000 × g for 2 minutes to remove any impurities. The DNA was then re-precipitated by addition of ethanol and recovered by centrifugation at 1700 × g for 3
minutes. The DNA pellet was then briefly air dried, and then redissolved in 8 mm sodium hydroxide solution and the ph was adjusted to 7.0 with phosphoric acid. The quantity of DNA was estimated by reading the absorbance (1AU=50 μg DNA/ml) at 260 nm. The purity of the DNA was estimated by scanning the UV spectrum from 200 to 400 nm and calculating the 260/280 ratios. Typical yield of each preparation was approximately 3.7 to 4.1 mg of DNA per 1000 mg of liver tissue (3.7 ~ 4.1 μg/mg).

The stable DNA adducts were released by thermal/acid hydrolysis (Citti et al., 1984; Dong & Jeffrey, 1991; Josephy, 1997; McCull et al., 1999). The ph of the above DNA solution was adjusted to approximately 2.0 with hcl and hydrolyzed at 75 °C for 45 minutes. After cooling, the sample was neutralized to ph of 7.0 by addition of naoh and spiked with 30 ng of the deuterated internal standards. The C18 Sep-Pack cartridges were activated by first rinsing with 6 ml of acetonitrile followed by 6 ml of purified water. Approximately 1/3 of the above sample was loaded on a cartridge and washed with 5 ml of purified water. Adducts were eluted with 2.5 ml of methanol. This procedure was repeated for the rest of the sample and methanol fractions were combined and then dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μl of DMSO for LC/MS/MS/MRM analysis.
CHAPTER IV

RESULTS AND DISCUSSION

Synthesis of BaP-C8Gua and BaP-N7Gua

Electrochemical synthesis of BaP-C8Gua and BaP-N7Gua was very successful. The structures of the synthesized adducts were elucidated by a combination of analytical instrumentation which includes NMR, MS (ESI and APCI), and MS/MS. They perfectly matched the literature values (Rogan et al., 1988).

The BaP-C8Gua NMR spectrum shown excellent correlation with the literature value. The absence of both the C-6 proton in the benzo[a]pyrene moiety and C-8 proton in the guanine moiety indicates that it is BaP-C8Gua. Figure 7 shows the structure and numbering of BaP-C8Gua.

The obtained NMR values were: 6.42(s, 2H, 2-NH2 [Gua]), 7.76-7.78(d, 1H, 5-H), 7.85 (m, 1H, 8-H), 7.93-8.01(m, 2H, 7-H, 9-H), 8.07-8.15(m, 2H, 2-H, 4-H), 8.26(d, 1H, 3-H), 8.46(d, 1H, 1-H), 8.58(d, 1H, 12-H), 9.33-9.37(m, 2H, 10-H, 11-H), 10.67(s, 1H, 1-H NH [Gua]), and 12.9(s, 1H, 7-H NH [Gua]).

The mass spectrum of BaP-C8Gua by electrospray showed a rational match with literature values (Rogan et al., 1988). Mass spectrum of BaP-C8Gua by
electrospray showed an \((\text{M+H})^+\) ion of \(m/z\) 402. The mass spectrum of BaP-C8Gua by ESI is shown in Figure 8.

![Figure 7. Structure and Numbering of BaP-C8Gua.](image)

![Figure 8. Mass Spectrum of BaP-C8Gua by ESI.](image)
The molecular weight of BaP-C8Gua ((M+H)^+ ion of m/z 402) was confirmed by APCI. The vaporization temperature was set at 450 °C and 5 μA discharge current was used for APCI operation. The mass spectrum of BaP-C8Gua by APCI is shown in Figure 9.

Figure 9. Mass Spectrum of BaP-C8Gua by APCI.

Electrospray (ESI) was used for all sample analysis instead of APCI. The high vaporization temperatures used for APCI cause a bio-sample's matrix to decompose, therefore, a very frequent ion source and compartment clean up would have been needed if APCI was used.
The MS/MS (ESI) spectrum of BaP-C8Gua was obtained by selectively passing the m/z 402 ion through the first quadrupole, bombarding the ion by inducing argon gas in the second quadrupole and then analyzing with the third quadrupole. Upon collision (collision energy 35V), the 402 ion decomposes to produce the most abundant ion at m/z 277 which is likely to be BaP-CN⁺. The greater abundance of the m/z 277 in the spectrum of BaP-C8Gua as compared with BaP-N7Gua is attributed to the greater stability of BaP-CN⁺ versus BaP-NC⁺ (Rogan et al., 1988). The MS/MS spectrum of BaP-C8Gua by ESI is shown in Figure 10:

![Figure 10: Daughter Mass Spectrum (MS/MS) of BaP-C8Gua by ESI. Collision energy set at 35V.](Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.)
The greater abundance of the m/z 277 in the spectrum of BaP-C8Gua compared with BaP-N7Gua is very unique. Thus, it provides a rapid distinguishing characteristic between BaP-C8Gua and BaP-N7Gua. In addition to the above, the fragment ion of m/z 385 is assigned as \((\text{M+H-NH}_3)^+\) and m/z 360 is assigned as \((\text{M+H-NH}_2\text{CN})^+\).

The BaP-N7Gua NMR spectrum correlates very well with literature values. In comparison with the standard spectrums in the literature (Rogan et al., 1988), the absence of the C-6 proton in the benzo[a]pyrene moiety and sharp singlet C-8 proton in the guanine moiety indicate that it is BP-N7Gua. Figure 11 shows the structure and numbering of BaP-N7Gua.

![Structure and Numbering of BaP-N7Gua](image)

The obtained NMR values were: 6.32 (s, 2H, 2-NH$_2$[Gua]), 7.38 (d, 1H, 5-H), 7.57 (d, 1H, 7-H), 7.85 (t, 1H, 8-H), 7.96 (t, 1H, 9-H), 8.11-8.17 (m, 2H, 4-H, 2-
H), 8.30 (d, 1H, 3-H), 8.36 (s, 1H, 8-H [Gua]), 8.50 (d, 1H, 1-H), 8.62 (d, 1H, 12-H), 9.38-9.40 (m, 2H, 10-H, 11-H), and 10.94 (s, 1H, 1H[NH Gua]).

The mass spectrum of BaP-N7Gua by electrospray shows perfect correlation with literature values as well (Rogan et al., 1988). The mass spectrum of BaP-N7Gua by electrospray showed an (M+H)+ ion of m/z 402. The mass spectrum of BaP-N7Gua by ESI is shown in Figure 12.

![Mass Spectrum of BaP-N7Gua by ESI](image)

Figure 12. Mass Spectrum of BaP-N7Gua by ESI.

The molecular weight of BaP-N7Gua ((M+H)+ ion of m/z 402) was confirmed by APCI. The vaporization temperature was set at 450°C and 5 μA.
discharge current was used for APCI operation. The mass spectrum of BaP-N7Gua by APCI is shown in Figure 13.

![Mass Spectrum of BaP-N7Gua by APCI](image)

**Figure 13. Mass Spectrum of BaP-N7Gua by APCI.**

The MS/MS spectrum (ESI) of BaP-N7Gua was obtained by selectively passing m/z 402 ion through the first quadrupole, bombarding the ion by inducing argon gas in the second quadrupole and then analyzing with the third quadrupole. Upon collision (collision energy was set at 35V for this experiment in order to retain some parent ion), the m/z 402 ion decomposes to produce abundant ions of m/z 252 and 277. The m/z 252 ion is the benzo[a]pyrene moiety resulting from facile protonation at the N7 position of BaP-N7Gua followed by a loss of the neutral
guanine moiety. The m/z 277 ion it is likely to be BaP-NC\(^+\). The MS/MS spectrum of BaP-N7Gua is shown in Figure 14.

![Image of MS/MS spectrum](image)

**Figure 14.** Daughter Mass Spectrum (MS/MS) of BaP-N7Gua by ESI. Collision energy set at 35 V.

The higher abundance of the m/z 252 daughter ions in the MS/MS spectrum of BaP-N7Gua compared with BaP-C\(^8\)Gua is very unique. Thus, it provides a rapid distinguishing characteristic between BaP-C\(^8\)Gua and BaP-N7Gua. In addition to the above, the fragment ion of m/z 385 is assigned as (M+H-NH\(_3\))\(^+\) and m/z 360 is assigned as (M+H- NH\(_2\)CN)\(^+\).
Synthesis of BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}

Electrochemical synthesis of BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} were successful. The structures of the synthesized adducts were elucidated by MS and MS/MS. The BaP-C8Gua-d_{11} mass spectrum obtained by electrospray showed excellent correlation with the spectrum of BaP-C8Gua. The (M+H)^+ ion of m/z 413 is 11 units greater than BaP-C8Gua which indicates to the 11 deuteriums on benzo[a]pyrene-d_{11} moiety. The mass spectrum of BaP-C8Gua-d_{11} by ESI is shown in Figure 15.

![Mass Spectrum of BaP-C8Gua-d_{11} by ESI](image)

Figure 15. Mass Spectrum of BaP-C8Gua-d_{11} by ESI.
The daughter (MS/MS) mass spectrum of BaP-C8Gua-d11 by ESI was obtained by selectively passing m/z 413 ion through the first quadrupole, bombarding the ion by inducing argon gas in the second quadrupole and then analyzing with the third quadrupole. Upon collision (collision energy set at 35V to monitor the parent compound), the 413 ion decomposes to produce the most abundant ion of m/z 287 and it is likely to be BaP-CN$^{+}$-d10. The formation of BaP-CN$^{+}$-d10 (m/z 287) instead of BaP-CN$^{+}$-d11 (m/z 288) indicates a possible deuterium/hydrogen exchange during the fragmentation. The daughter (MS/MS) mass spectrum of BaP-C8Gua-d11 by ESI is shown in Figure 16.

Similar to the BaP-C8Gua spectrum, the greater abundance of the m/z 287 observed in the spectrum of BaP-C8Gua-d11 as compared with BaP-N7Gua-d11 is attributed to the greater stability of BaP-CN$^{+}$-d10 versus BP-NC$^{+}$-d10. This unique difference provides a rapid distinguishing characteristic between BaP-C8Gua-d11 and BaP-N7Gua- d11. In addition to the above, the fragment ion of m/z 396 is assigned as (M+H-NH3)$^{+}$ and m/z 371 is assigned as (M+H- NH$_2$CN)$^{+}$.

The BaP-N7Gua-d11 mass spectrums obtained by electrospray showed excellent correlation with the spectrum of BaP-N7Gua. The (M+H)$^{+}$ ion of m/z 413 is eleven units higher than BaP-N7Gua which refers to the eleven deuteriums on the
Figure 16. Daughter Mass Spectrum (MS/MS) of BaP-C8Gua-d11 by ESI. Collision energy set at 35 V.

benzo[a]pyrene-d11 moiety. The mass spectrum of BaP-N7Gua-d11 by ESI is shown in Figure 17.

The daughter (MS/MS) mass spectrum of BaP-N7Gua-d11 by ESI was obtained by select passing m/z 413 ion through first quadrupole, bombarding the ion by inducing argon gas in the second quadrupole and then analyzing with the third quadrupole. Upon collision (collision energy 35V), the 413 ion decomposes to produce the most abundant ions of m/z 263 and 287. The m/z of 263 is the 11 deuterated benzo[a]pyrene moiety and m/z 287 ion is the BaP-CN²⁺-d10. The
formation of BaP-CN^-dio (m/z 287) instead of d_{11}-BaP-CN^+ (m/z 288) indicates a possible deuterium/hydrogen exchange during the fragmentation. The daughter (MS/MS) mass spectrum of BaP-N7Gua-d_{11} by ESI is shown in Figure 18.

The m/z 263 ion is the benzo[a]pyrene-d_{11} moiety resulting from facile protonation at N7 position of BaP-N7Gua-d_{11} followed by a loss of the neutral guanine moiety. The greater abundance of the m/z 263 in the daughter mass spectrum of BaP-N7Gua-d_{11} as compared with BaP-C8Gua-d_{11} is very unique. This unique difference provides a rapid distinguishing characteristic between
BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}. In addition to above, the fragment ion of m/z 396 is assigned as (M+H-NH\textsubscript{3})\textsuperscript{+} and m/z 371 is assigned as (M+H-NH\textsubscript{2}CN)\textsuperscript{+}.

MS/MS/MRM Optimization

In order to achieve the maximum sensitivity for our analysis, the electrospray current voltage and collision energy effects for both adducts were carefully studied and revised to obtain the best operating conditions.

The electrospray current for BaP-C8Gua and BaP-N7Gua which gave the most intense signal at MH\textsuperscript{+} (m/z 402) was 3 kV. The intensity of m/z 402 ion signal

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for both adducts decreased when lower ionization energy was applied. The decreased sensitivity may have been caused by incomplete sample ionization.

The intensity of m/z 402 ion signal for both adducts also decreased when a higher ionization energy was applied. The decreased sensitivity may have been caused by an arcing effect or sample break down. In addition to the above phenomenon, high background noise was observed when higher ionization energy was used. The results of the electrospray current study are shown in Figure 19.

![Graph](image-url)

Figure 19. The Electrospray Current Voltage (kV) vs. Response by Infusion Method.

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The collision energy for BaP-C8Gua and BaP-N7Gua which produced the most intense signal for their respective daughter ions m/z 277 (most abundant fragment) was 40V. The intensity of m/z 277 ion for both adducts decreased when high collision energy was applied.

The decreased sensitivity may have been caused by excessive fragmentation or further break down of the daughter ion. The optimized conditions were used for LC/MS/MS/MRM (m/z 402 $\rightarrow$ m/z 277) sample analysis in order to obtain the maximum sensitivity. The results of the collision energy study are shown in Figure 20.

**LC/MS/MS/MRM Method**

Two fragment ions were selected for the LC/MS/MS/MRM analysis for both authentic and deuterated standards. For BaP-C8Gua and BaP-N7Gua, m/z 402 $\rightarrow$ 277 (major) and m/z 402 $\rightarrow$ 360 (minor) transitions were monitored. For the internal standards (BaP-C8Gua-d11 and BaP-N7Gua-d11) m/z 413 $\rightarrow$ 287 (major) and m/z 413 $\rightarrow$ 371 (minor) transitions were monitored. The standard curves were constructed by three replicated analyses of solutions containing BaP-C8Gua and BaP-N7Gua, from 1 to 32 ng (1, 2, 4, 8, 16, and 32 ng) per injection and internal standards (BaP-C8Gua-d11 and BaP-N7Gua-d11), containing 2 ng per injection in all of the solutions. Results from LC/MS/MS/MRM are very linear.
Figure 20. The Collision Energy (V) vs. Response of m/z 277 Ion by Infusion Method.

The response ratio of BaP-C8Gua to BaP-C8Gua-d_{11} was linear and it is presented as Figure 21.

The standard plot was constructed based on peak height instead of peak area to achieve a more accurate result. The linear equation of $y = 1.0967x$ and $R^2 = 0.9998$ were obtained with accuracy from 93% to 111% (Table1). The standard deviation of three injections for all levels of standards was less than 5.6% and the highest percent relative standard deviation (%RSD) for all levels of standard injections was less than 5.5%. The limit of detection (LOD) for BP-C8Gua...
The response ratio of BaP-N7Gua to BaP-N7Gua-d11 was linear and it is presented as Figure 22. The standard plot was constructed based on peak height instead of peak area to achieve a more accurate result. The linear equation of \( y = 0.3945x \) and \( R^2 = 0.9999 \) were obtained with accuracy from 96% to 111% (Table 2). The highest
### Table 1

**BaP-C8Gua LC/MS/MS/MRM Linear Plot Accuracy Calculation**

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<th>BaP-C8Gua on column (ng)</th>
<th>BaP-C8Gua Calculated (ng)</th>
<th>Accuracy (%)</th>
<th>Average % accuracy ± standard deviation</th>
<th>%RSD</th>
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<td>30.78</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32.00</td>
<td>33.32</td>
<td>104</td>
<td>99 ± 3.4</td>
<td>3.43</td>
</tr>
<tr>
<td>32.00</td>
<td>31.37</td>
<td>98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The standard deviation of three injections for all standard injections was less than 4.1% and the %RSD for all levels of standards was less than 3.9%. The limit of detection (LOD) for bap-N7Gua standards was 96 pg (239 fmole) (S/N = 3) and limit of quantitation (LOQ) was 192 pg (477 fmole)(S/N = 6).
Figure 22. The Standard Plot of BaP-N7Gua by LC/MS/MS/MRM.

These results indicate that the method is accurate and precise. Our LC/MS/MS/MRM method demonstrated the ultimate advantage over traditional UV-VIS or FLR detection methods by having extremely low LOD and LOQ values. Both values are important for trace level analysis, especially, when analyzing bio-samples.

Chromatographic separation between BaP-C8Gua and BaP-N7Gua peaks was very good. The resolution between the two peaks was 6.36. The tailing factor for BP-C8Gua was 1.07 and 1.24 for BaP-N7Gua at 5% peak height. Both the resolution and tailing factor were calculated based on the 8 ng of analytes on
Table 2

BaP-N7Gua LC/MS/MS/MRM Linear Plot Accuracy Calculation

<table>
<thead>
<tr>
<th>BaP-N7Gua on column (ng)</th>
<th>BaP-N7Gua Calculated (ng)</th>
<th>Accuracy (%)</th>
<th>Average % accuracy ± standard deviation</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>1.07</td>
<td>107</td>
<td>106 ± 4.1</td>
<td>3.87</td>
</tr>
<tr>
<td>1.00</td>
<td>1.01</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>1.11</td>
<td>111</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>2.04</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>2.06</td>
<td>103</td>
<td>102 ± 0.5</td>
<td>0.49</td>
</tr>
<tr>
<td>2.00</td>
<td>2.05</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>4.17</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>4.18</td>
<td>105</td>
<td>104 ± 0.5</td>
<td>0.48</td>
</tr>
<tr>
<td>4.00</td>
<td>4.17</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.00</td>
<td>8.06</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.00</td>
<td>8.18</td>
<td>102</td>
<td>102 ± 0.5</td>
<td>0.49</td>
</tr>
<tr>
<td>8.00</td>
<td>8.16</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.00</td>
<td>15.61</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.00</td>
<td>16.16</td>
<td>101</td>
<td>99 ± 1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>16.00</td>
<td>15.93</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32.00</td>
<td>30.91</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32.00</td>
<td>32.42</td>
<td>101</td>
<td>100 ± 2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>32.00</td>
<td>32.60</td>
<td>102</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

column standard injection. The above result showed an outstanding improvement when compared with the literature methods (Cavaliere et al., 1990; Chen et al., 1996; Devanesan et al., 1996; Rogan et al., 1988). Our method not only provides better sensitivity, accuracy, and precision, but also provides confirmation information. The time saving by using our LC/MS/MS/MRM method was very significant. Our method was three times faster than the other methods and provided much more information than that provided by UV detection. Representative chromatograms of standard injections are illustrated in Figure 23.
Figure 23. Representative LC/MS/MS/MRM Chromatograms of the Blank and Standard Injections. (From left to right, Blank injection, Std injection [1 ng on column per adduct]. Top channel is the transition for internal standards [m/z 413→ 287 and 371] and the bottom channel is transition for adducts [m/z 402→277 and 360]. The early eluter is the BaP-C8Gua and the late eluter is BaP-N7Gua).

Adducts Stability Evaluation and Method Suitability

The efficiency of the Sep-Pack cleaning procedure and stability of both adducts were very good. For this study, the efficiency of the Sep-Pack clean up procedure was tested by adding the internal standards into the post Sep-Pack cartridge clean up fraction instead of pre-cartridge. The adduct stability was tested by treating sample at extreme conditions for an extended period.
Samples prepared at extreme conditions (0.02N HCl at 75 °C for 1.5 hours) were observed to be very stable and the Sep-pack procedure showed high efficiency. No significant adduct loss was observed throughout the study.

The recovery for BaP-C8Gua was from 95% to 105% and the average recovery was 98%. Results and additional details are presented in Table 3.

Table 3

<table>
<thead>
<tr>
<th>BaP-C8Gua on column (ng)</th>
<th>BaP-C8Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.99</td>
<td>99</td>
</tr>
<tr>
<td>1.0</td>
<td>1.05</td>
<td>105</td>
</tr>
<tr>
<td>5.0</td>
<td>4.88</td>
<td>98</td>
</tr>
<tr>
<td>5.0</td>
<td>4.90</td>
<td>98</td>
</tr>
<tr>
<td>10.0</td>
<td>10.01</td>
<td>100</td>
</tr>
<tr>
<td>10.0</td>
<td>10.08</td>
<td>101</td>
</tr>
<tr>
<td>20.0</td>
<td>19.51</td>
<td>98</td>
</tr>
<tr>
<td>20.0</td>
<td>19.81</td>
<td>99</td>
</tr>
<tr>
<td>30.0</td>
<td>28.94</td>
<td>96</td>
</tr>
<tr>
<td>30.0</td>
<td>28.48</td>
<td>95</td>
</tr>
</tbody>
</table>

The recovery for BaP-N7Gua was from 97% to 107% and the average recovery was 101%. Results and additional details are presented in Table 4.

The above data indicate that both adducts are very stable at elevated temperature, and the acid hydrolysis procedure does not cause significant breakdown of the adducts. The approximate 100% recovery for both adducts indicates no adduct loss during the Sep-Pack clean up. The internal standards added on the post-Sep-Pak column served as an indicator (fixed numerator). Therefore, any adduct lost during the Sep-Pak clean up procedure would be detected by a decreasing ratio. The
Table 4
Stability Study of BaP-N7Gua

<table>
<thead>
<tr>
<th>BaP-N7Gua on column (ng)</th>
<th>BaP-N7Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.04</td>
<td>104</td>
</tr>
<tr>
<td>1.0</td>
<td>0.98</td>
<td>98</td>
</tr>
<tr>
<td>5.0</td>
<td>5.33</td>
<td>107</td>
</tr>
<tr>
<td>5.0</td>
<td>5.21</td>
<td>104</td>
</tr>
<tr>
<td>10.0</td>
<td>9.57</td>
<td>96</td>
</tr>
<tr>
<td>10.0</td>
<td>10.00</td>
<td>100</td>
</tr>
<tr>
<td>20.0</td>
<td>19.11</td>
<td>96</td>
</tr>
<tr>
<td>20.0</td>
<td>20.51</td>
<td>103</td>
</tr>
<tr>
<td>30.0</td>
<td>31.66</td>
<td>106</td>
</tr>
<tr>
<td>30.0</td>
<td>30.53</td>
<td>102</td>
</tr>
</tbody>
</table>

excellent recovery demonstrated a high efficiency for the Sep-Pak clean up procedure. Representative chromatograms of stability samples are shown in Figure 24.

The recovery of authentic adducts spiked in rat liver DNA was good and the blank rat liver DNA preparation showed no interference peak when compared with the spike. For BaP-C8Gua, the recovery range was from 82% to 101% and the average recovery was 93%. For BaP-N7Gua, the recovery range was from 83% to 104% and the average recovery was 94%.

For the BaP-C8Gua, the limit of detection (LOD) of rat liver DNA spike was 98 pg (61 fmole/mg DNA) (S/N = 3) and limit of quantitation (LOQ) was 196 pg (122 fmole/mg DNA) (S/N = 6). For the BaP-N7Gua, the LOD was 99 pg (62 fmole/mg DNA) and LOQ was 198 pg (124 fmole/mg DNA). The LOD and LOQ for both adducts in rat liver DNA spikes were very close to the values that were
Figure 24. Representative LC/MS/MS/MRM Chromatograms of the Blank and Recovery/Stability Samples. (From left to right, blank injection, recovery sample [1 ng on column per adduct]. Top channel is the transition for internal standards [m/z 413→287 and 371] and the bottom channel is the transition for adducts [m/z 402→277 and 360]. The early eluter is BaP-C8Gua and the late eluter is BaP-N7Gua).

calculated from their pure form injections (see standard plots). The above data indicate that the method is valid, rugged, free of bias, and well suited for DNA analysis. Results and additional details are presented in Tables 5 and 6. Representative chromatograms of DNA spike are presented in Figure 25.

The recovery of authentic adducts spiked into rat urine was good and the blank rat urine extraction showed no interference peak when compared with the spike. For BaP-C8Gua, the recovery range was from 91% to 104% and the average recovery was 95%. Results and additional details are presented in Table 7.
Table 5

Recovery Study of BaP-C8Gua Spiked on Rat Liver DNA

<table>
<thead>
<tr>
<th>BaP-C8Gua on column (ng)</th>
<th>BaP-C8Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.82</td>
<td>82</td>
</tr>
<tr>
<td>1.0</td>
<td>0.99</td>
<td>99</td>
</tr>
<tr>
<td>5.0</td>
<td>4.43</td>
<td>89</td>
</tr>
<tr>
<td>5.0</td>
<td>4.62</td>
<td>92</td>
</tr>
<tr>
<td>10.0</td>
<td>9.21</td>
<td>92</td>
</tr>
<tr>
<td>10.0</td>
<td>9.23</td>
<td>92</td>
</tr>
<tr>
<td>20.0</td>
<td>20.14</td>
<td>101</td>
</tr>
<tr>
<td>20.0</td>
<td>17.36</td>
<td>87</td>
</tr>
<tr>
<td>30.0</td>
<td>28.99</td>
<td>97</td>
</tr>
<tr>
<td>30.0</td>
<td>29.41</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 6

Recovery Study of BaP-N7Gua Spiked on Rat Liver DNA

<table>
<thead>
<tr>
<th>BaP-N7Gua on column (ng)</th>
<th>BaP-N7Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.83</td>
<td>83</td>
</tr>
<tr>
<td>1.0</td>
<td>1.04</td>
<td>104</td>
</tr>
<tr>
<td>5.0</td>
<td>4.16</td>
<td>83</td>
</tr>
<tr>
<td>5.0</td>
<td>4.64</td>
<td>93</td>
</tr>
<tr>
<td>10.0</td>
<td>9.53</td>
<td>95</td>
</tr>
<tr>
<td>10.0</td>
<td>9.55</td>
<td>96</td>
</tr>
<tr>
<td>20.0</td>
<td>19.63</td>
<td>98</td>
</tr>
<tr>
<td>20.0</td>
<td>17.65</td>
<td>88</td>
</tr>
<tr>
<td>30.0</td>
<td>31.33</td>
<td>104</td>
</tr>
<tr>
<td>30.0</td>
<td>27.94</td>
<td>93</td>
</tr>
</tbody>
</table>

For BaP-N7Gua, the recovery range was from 91% to 106% and the average recovery was 99%. Results and additional details are presented in Table 8.

For the BaP-C8Gua, the limit of detection (LOD) of spike on rat urine bio-sample was 146 pg (73 fmole/mL urine) (S/N = 3) and limit of quantitation (LOQ)
Figure 25. Representative LC/MS/MS/MRM Chromatograms of the Bio-Sample Spike Studies. (From left to right, rat liver DNA blank, rat liver DNA spike [1ng on column per adduct], rat urine blank, rat urine spike [1ng on column per adduct], rat feces blank, rat feces spike [1ng on column per adduct]. Top channel is the transition for internal standards [m/z 413→287 and 371] and the bottom channel is the transition for adducts [m/z 402→277 and 360]. The early eluter is BaP-C8Gua and the late eluter is BaP-N7Gua).

was 293 pg (146 fmole/mL urine) (S/N = 6). For the BaP-N7Gua, the LOD was 125 pg (62 fmole/mL urine) and LOQ was 250 pg (124 fmole/mL urine).

The LOD and LOQ results for both adducts in bio-sample (urine) spikes were similar to the injections of their pure forms (from standard plots) indicating that the method is free from bias.

The above data indicate that the method is rugged and robust and is well suited for different types of bio-sample analysis. Representative chromatograms of rat urine spikes are presented in Figure 25.
Table 7
Recovery Study of BaP-C8Gua Spiked in Rat Urine

<table>
<thead>
<tr>
<th>BaP-C8Gua on column (ng)</th>
<th>BaP-C8Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.93</td>
<td>93</td>
</tr>
<tr>
<td>1.0</td>
<td>0.91</td>
<td>91</td>
</tr>
<tr>
<td>5.0</td>
<td>4.57</td>
<td>91</td>
</tr>
<tr>
<td>5.0</td>
<td>4.60</td>
<td>100</td>
</tr>
<tr>
<td>10.0</td>
<td>9.99</td>
<td>91</td>
</tr>
<tr>
<td>10.0</td>
<td>9.62</td>
<td>91</td>
</tr>
<tr>
<td>20.0</td>
<td>19.61</td>
<td>98</td>
</tr>
<tr>
<td>20.0</td>
<td>20.75</td>
<td>104</td>
</tr>
<tr>
<td>30.0</td>
<td>27.34</td>
<td>91</td>
</tr>
<tr>
<td>30.0</td>
<td>29.79</td>
<td>99</td>
</tr>
</tbody>
</table>

Table 8
Recovery Study of BaP-N7Gua Spiked in Rat Urine

<table>
<thead>
<tr>
<th>BaP-N7Gua on column (ng)</th>
<th>BaP-N7Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.02</td>
<td>102</td>
</tr>
<tr>
<td>1.0</td>
<td>1.01</td>
<td>101</td>
</tr>
<tr>
<td>5.0</td>
<td>5.15</td>
<td>103</td>
</tr>
<tr>
<td>5.0</td>
<td>5.29</td>
<td>106</td>
</tr>
<tr>
<td>10.0</td>
<td>9.81</td>
<td>98</td>
</tr>
<tr>
<td>10.0</td>
<td>9.69</td>
<td>97</td>
</tr>
<tr>
<td>20.0</td>
<td>19.41</td>
<td>97</td>
</tr>
<tr>
<td>20.0</td>
<td>19.91</td>
<td>100</td>
</tr>
<tr>
<td>30.0</td>
<td>29.79</td>
<td>99</td>
</tr>
<tr>
<td>30.0</td>
<td>27.35</td>
<td>91</td>
</tr>
</tbody>
</table>

The recovery of authentic adducts spiked in rat feces was also good and the blank rat feces extraction showed no interference peak when compared with the spike. For BaP-C8Gua, the recovery range was from 82% to 94% and the average recovery was 88%. Results and additional details are presented in Table 9. For
Table 9
Recovery Study of BaP-C8Gua Spiked in Rat Feces

<table>
<thead>
<tr>
<th>BaP-C8Gua on column (ng)</th>
<th>BaP-C8Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.87</td>
<td>87</td>
</tr>
<tr>
<td>1.0</td>
<td>0.88</td>
<td>88</td>
</tr>
<tr>
<td>5.0</td>
<td>4.67</td>
<td>93</td>
</tr>
<tr>
<td>5.0</td>
<td>4.36</td>
<td>87</td>
</tr>
<tr>
<td>10.0</td>
<td>8.36</td>
<td>84</td>
</tr>
<tr>
<td>10.0</td>
<td>8.16</td>
<td>82</td>
</tr>
<tr>
<td>20.0</td>
<td>17.42</td>
<td>87</td>
</tr>
<tr>
<td>20.0</td>
<td>17.78</td>
<td>89</td>
</tr>
<tr>
<td>30.0</td>
<td>27.89</td>
<td>93</td>
</tr>
<tr>
<td>30.0</td>
<td>28.19</td>
<td>94</td>
</tr>
</tbody>
</table>

BaP-N7Gua, the recovery range was from 80% to 99% and the average recovery was 87%. Results and additional details are presented in Table 10. For BaP-C8Gua, the limit of detection (LOD) of spiked rat feces was 137 pg (340 fmole/gm feces) (S/N = 3) and limit of quantitation (LOQ) was 273 pg (679 fmole/gm feces) per injection (S/N = 6). For the BaP-N7Gua, the LOD was 190 pg (472 fmole/gm feces) and the LOQ was 380 pg (946 fmole/gm feces). The slightly higher numbers observed for LOD and LOQ of spiked rat feces versus the results observed for spiked DNA and urine samples were possibly due to the complexity of the feces sample matrix. Despite this, the LOD and LOQ for both adducts in bio-sample (feces) spikes are still comparable to the LOD and LOQ of their pure forms (from standard curve data), thus proving the method is free from bias. The above data indicate that the method is very rugged and robust and it is suitable for different
types of bio-sample analysis. Representative chromatograms of spiked rat feces are presented in Figure 25.

Table 10

Recovery Study of BaP-N7Gua Spiked in Rat Feces

<table>
<thead>
<tr>
<th>BaP-N7Gua on column (ng)</th>
<th>BaP-N7Gua Calculated (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.81</td>
<td>81</td>
</tr>
<tr>
<td>1.0</td>
<td>0.87</td>
<td>87</td>
</tr>
<tr>
<td>5.0</td>
<td>4.32</td>
<td>86</td>
</tr>
<tr>
<td>5.0</td>
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<td>89</td>
</tr>
<tr>
<td>10.0</td>
<td>8.00</td>
<td>80</td>
</tr>
<tr>
<td>10.0</td>
<td>8.24</td>
<td>82</td>
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<tr>
<td>20.0</td>
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<td>85</td>
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<td>16.46</td>
<td>82</td>
</tr>
<tr>
<td>30.0</td>
<td>29.80</td>
<td>99</td>
</tr>
<tr>
<td>30.0</td>
<td>28.92</td>
<td>96</td>
</tr>
</tbody>
</table>

The above study results have shown that our LC/MS/MS/MRM is valid. It has excellent accuracy, precision, and has been shown to be very rugged and robust. Our method has been proven far better than the traditional LC/UV or LC/FLR methods (Cavaliere et al., 1990; Chen et al., 1996; Devanesan et al., 1996; Rogan et al., 1988). Based on the above information, the advantages of using our LC/MS/MS/MRM method may be summarized as follows: (a) provides both quantitative and qualitative information; (b) provides confirmation information (m/z matching rather than UV absorbance); (c) efficient bio-sample analysis (because of the selectivity); (d) highly sensitive, selective, accurate, and precise; and (e) rugged, robust, free of bias, and time saving.
**In Vitro** Experiment Results

The *in vitro* experiment results indicate that BaP-N7Gua is the major depurinating adduct content for both calf thymus and rat liver DNA samples while BaP-C8Gua were dominated in the stable adduct analysis. Blank preparation showed no interference with our analysis and was bias free. The above result agrees with the finding of Rogan et al. (1988) and Devanesan et al. (1996). Once BaP-N7Gua is formed it will break away from DNA faster than the BaP-C8Gua. The *in vitro* results and additional details are presented in Table 11. LC/MS/MS/MRM chromatograms are presented in Figure 26.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>BaP-C8Gua (ng)/% total adduct</th>
<th>BaP-N7Gua (ng)/% total adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf Thymus DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Stable Adducts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf Thymus DNA</td>
<td>11.9 ng/26.1%</td>
<td>6.8 ng/14.9%</td>
</tr>
<tr>
<td>(Stable Adducts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf Thymus DNA</td>
<td>5.0 ng/11.0%</td>
<td>21.9 ng/48.0%</td>
</tr>
<tr>
<td>(Depurinating Adducts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat Liver DNA</td>
<td>10.9 ng/35.7%</td>
<td>3.9 ng/12.8%</td>
</tr>
<tr>
<td>(Stable Adducts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat Liver DNA</td>
<td>4.5 ng/14.8%</td>
<td>11.2 ng/36.7%</td>
</tr>
<tr>
<td>(Depurinating Adducts)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 26. Representative LC/MS/MS/MRM Chromatograms of the Horseradish Peroxidase Experiments. (From left to right, row 1-calf thymus DNA: blank, stable adducts, depurinating adducts, row 2- rat liver DNA: blank, stable adducts, depurinating adducts. Top channel is the transition for internal standards [m/z 413→287 and 371] and the bottom channel is the transition for adducts [m/z 402→277 and 360]. The early eluter is BaP-C8Gua and the late eluter is BaP-N7Gua).
In Vivo Rat Experiment Results

The benzo[a]pyrene calibration plot generated for evaluating content uniformity and potency of the feed is presented in Figure 27.

![Benzo[a]pyrene Calibration Plot](image)

Figure 27. Benzo[a]pyrene Calibration Plot.

The uniformity and potency of the processed feed were evaluated before dosing to ensure uniform exposure. The standard plot was constructed based on peak area to achieve the more accurate result. The linear equation of $y = 23953x$ and $R^2 = 0.9996$ were obtained with accuracy from 99% to 104%. The standard deviation of three injections for all levels of standards was less than 0.5% and the highest percent relative standard deviation (%RSD) for all levels of standard injections was less than 0.5%. The above data indicates that the method is accurate.
and precise and is suitable for the analysis. Representative chromatograms of feed samples are shown in Figure 28.

![Chromatograms](image)

**Figure 28. LC Chromatograms of Feed Samples.**

For the *in vivo* rat study, the average weight gain of the high dose group was significant lower than other groups throughout the study. The high dose rat appeared to be sick after 4 weeks of dosing. Abnormal fur loss was observed. They were less active than other groups and when surgery was performed we found that their internal organs were morphologically different from other groups as well. The *in vivo* rat experiment average weight gain results and corresponding dosages are presented in Table 12. The average weight gain results versus feed consumption are presented in Figures 29 and 30.
The *in vivo* rat experiment results and corresponding dosages are presented in Table 13. No adduct was detected in rat livers at all dosage levels after dosing for two weeks. Small amount of BaP-C8Gua was found in all three high-dose rat livers after four weeks of dosing. Slightly more BaP-C8Gua was found in all three high-dose rat livers after 8 weeks of dosing.
Figure 29. The *In Vivo* Rat Experiment Average Weight Gain Normalized for 100g Feed Consumption.

Figure 30. The *In Vivo* Rat Experiment Average Feed Consumption Normalized for Body Weight (per 100g).

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Table 13
In Vivo Dosage and Rat Liver Sample Result

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Dosage mg/Kg per day</th>
<th>Number of rats Euthanatized / Analyzed</th>
<th>Dosing period (Weeks)</th>
<th>BaP-C8Gua p mole/gm liver</th>
<th>BaP-N7Gua p mole/gm liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59.4</td>
<td>3</td>
<td>2</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
<tr>
<td>2</td>
<td>6.4</td>
<td>3</td>
<td>2</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>3</td>
<td>2</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>3</td>
<td>2</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
</tbody>
</table>

The above result agrees with the finding of \textit{in vivo} studies done by Rogan et al. (1990), Ross et al. (1991) and Devanesan et al. (1996). Once BaP-N7Gua is formed, it will break away from DNA faster than the BaP-C8Gua; therefore, no BaP-N7Gua was expected to be found as stable adduct in the \textit{in vivo} sample. However, based on our \textit{in vitro} study results and the \textit{in vivo} experiment done by Rogan et al. (1990) and Devanesan et al. (1996) the BaP-N7Gua is expected to be the dominant depurinated adduct. We believed that most BaP-N7Gua exists as the free adduct form and the total amount should be greater than BaP-C8Gua. Our finding indicates that benzo[a]pyrene is bio-available through the oral route; however, the finding of adduct levels were very low. This may be caused by fast
depurination of the adducts, low bioavailability, or different metabolic routes of the benzo[a]pyrene through oral exposure. Therefore, a large and chronic dose was needed in order to see the detectable adduct formation in the liver sample.

Representative chromatograms of *in vivo* rat samples are shown in Figure 31.

![Figure 31. Representative LC/MS/MS/MRM Chromatograms of the *In Vivo* Rat Experiment. (From left to right, control rat liver without internal standards, with internal standards, high dose rat liver with internal standards. Top channel is the transition for internal standards [m/z 413→287 and 371] and the bottom channel is the transition for adducts [m/z 402→277 and 360]. The early eluter is BaP-C8Gua and the late eluter is BaP-N7Gua.)](image)

*In Vivo* Fish Experiment Results

For the *in vivo* fish experiment, the uniformity and potency of the processed feed were evaluated before dosing to ensure uniform exposure. The HPLC method
and calibration plot used to evaluate the benzo[a]pyrene potency of fish feed was the same as used for rat feed. No interference was found in any sample; therefore, the determination of the benzo[a]pyrene potency in fish feed was very straightforward.

The above data indicate that our HPLC method was very rugged and bias free and our quick HPLC method is suitable for similar analysis. Representative chromatograms of feed samples are shown in Figure 32.

![Figure 32. LC Chromatograms of Fish Feed Samples.](image)

The *in vivo* fish experiment results and corresponding dosages are presented in Table 14. No adduct was detected in fish livers at all dosage levels after dosing for four weeks. We believe that the negative finding may be due to the fast
Depurination of the adducts, low bioavailability, or different metabolic routes of the benzo[a]pyrene through the oral exposure. We also believe that the negative finding was dose related. Even though the dose levels of the benzo[a]pyrene in both experiments were the same, because the feed consumption of fish was much lower than the feed consumption of rat the total dosage on fish was much lower.

Table 14

*In Vivo* Dosage and Fish Liver Sample Result

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Dosage mg/Kg per day</th>
<th>Number of Fish Euthanatized / Analyzed</th>
<th>Dosing period (Weeks)</th>
<th>BaP-C8Gua p mole/g liver</th>
<th>BaP-N7Gua p mole/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.7</td>
<td>13</td>
<td>4</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>13</td>
<td>4</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>13</td>
<td>4</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>14</td>
<td>4</td>
<td>Non detected</td>
<td>Non detected</td>
</tr>
</tbody>
</table>

In order to demonstrate that our method is bias free for fish liver samples, two spike samples were prepared by addition of both BaP-C8Gua and BaP-N7Gua at 100 pg (on column) on control fish liver DNA and carried though the sample preparation and then analyzed by our method. Both spikes show positive results which indicate that our method is suitable for fish liver analysis. Representative LC/MS/MS/MRM chromatograms of *in vivo* fish samples are shown in Figure 33.
According to our rat experiment results, we believe that a larger dose may needed in order to see the detectable adduct formation in the fish liver sample.

Figure 33. Representative LC/MS/MS MRM Chromatograms of the *In Vivo* Fish Experiment. (From left to right, control fish liver without internal standards, with internal standards, with 100 pg [on column] standards spike. Top channel is the transition for internal standards [m/z 413 → 287 and 371] and the bottom channel is the transition for adducts [m/z 402 → 277 and 360]. The early eluter is BaP-C8Gua and the late eluter is BaP-N7Gua.)

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CHAPTER V

CONCLUSION

High performance liquid chromatography coupled with tandem mass spectrometer has proven to be very powerful instrumentation in the analysis of DNA adducts in complex biological tissues. Our study was focus on quantitative study of benzo[a]pyrene DNA nucleoside adducts in *in vitro* and *in vivo* samples by utilizing this technique. The specific isotope internal standard method was used for our study in order to achieve the best result.

Both standards (BaP-C8Gua and BaP-N7Gua) and internal standards (BaP-C8Gua-d11 and BaP-N7Gua-d11) were successfully synthesized. A very rugged LC/MS/MS/MRM method was developed by carefully studied the ionization parameters. Our LC/MS/MS/MRM method is developed by setting the first quadrupole of the instrument to pass the MH+ at m/z 402 for both adducts and m/z 413 for both deuterated internal standards. The collision induced fragmentation that occurred at the second quadrupole. The product ions (daughters) were separated in the third quadrupole and two of them were monitored. Our method has proven to be very suitable for the analysis, it provides low detection limit, quick turnaround time, additional specificity, and structure confirmation.

Our LC/MS/MS/MRM method has shown excellent accuracy, precision, and has been proven to be very rugged and robust. Our method has been proven far better than the traditional LC/UV or LC/FLR methods, especially for bio-sample...
analysis such as DNA, urine, and feces. The *in vitro* samples were obtained from the horseradish peroxidase catalyst from both calf thymus and rat liver DNA with benzo[a]pyrene. The *in vivo* samples were prepared by chronic dietary dosing Fisher 344 rats and bluegill fish with feed containing benzo[a]pyrene. Both *in vitro* and *in vivo* samples were analyzed by our method and proven to be successful.

Based on the results of our experiment, the advantages of utilizing our LC/MS/MS/MRM method for sample analysis may be summarized as follows: (a) provides both quantitative and qualitative information; (b) provides confirmation information (m/z matching rather than UV absorbance); (c) efficient bio-sample analysis, despite the complexity of sample matrix; (d) highly sensitive, selective, accurate, precise, and reproducible; and (e) rugged, robust, free of bias, and time saving.

We conclude that our method was suitable for bio-sample analysis and is superior to traditional methods. Therefore, more research and application effort should be made in liquid chromatography/tandem mass spectrometry and the importance of liquid chromatography/tandem mass spectrometry in future research cannot be overemphasized.

The low findings of the stable adducts in our *in vivo* experiment may be due to a higher than expected excretion rate, low bioavailability, or different metabolic routes of the benzo[a]pyrene through oral exposure and thus, further study should be considered.
For future studies, a better formulation or dosing route should be used for animal dosing in order to achieve better bioavailability. We should also look into the formation of adducts by both one-electron oxidation and monooxygenation routes. For checking the excretion, animal feces, urine, and all the organs should be collected and analyzed after dosing. A mass balance study should be performed in order to better understand the distribution and excretion rate of the adducts.

Furthermore, different PAHs should be included in the study to assess the risk of their carcinogenesis effects. By gathering the information of all these studies, more information about cancer formation could be provided and lead to better cures of the disease.
BIBLIOGRAPHY


adenine, guanine, and cytosine bases of DNA and optically active bay-region 3,4-diol 1,2-epoxides of benz[a]anthracene. J. Org. Chem. 58, 4013–4022.


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