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DNA Adducts as Molecular Biomarkers of Exposure to Benzo(a)pyrene

by

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B. S., Biology
Spelman College, 1989

Submitted to the Division of Toxicology in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in Toxicology
at the

Massachusetts Institute of Technology

September 1997

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Division of Toxicology
June 30, 1997

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Abstract

It is believed that the formation of DNA adducts is an essential initial step in carcinogenesis. To better understand this initial step and its relationship to cancer, this study primarily focuses on benzo(a)pyrene (BaP), a highly carcinogenic model polycyclic aromatic hydrocarbon (PAH) and an ubiquitous environmental contaminant. The objective of this dissertation was to identify and quantify carcinogen-DNA adducts as biomarkers of exposure to BaP.

The BaP-DNA adduct levels were measured by Adduct Detection by Acylation with Methionine (ADAM), a new postlabelling method that was recently developed by our group. Nucleoside adducts of fluoranthene, 4-aminobiphenyl and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were acylated with ^{35}S-methionine and detected in femtomolar quantities. This method was adapted for analysis of BaP-DNA adducts and optimized with ^{3}H-BaP using unlabeled methionine. Acylated products of benzo(a)pyrene-deoxyguanosine (BaP-dGuo) from different sources, including ^{3}H-BaP-dGuo isolated from calf thymus DNA modified enzymatically *in vitro* or by reaction with BPDE, have shown identical RP-HPLC retention times. The ^{35}S content of acylated products was linearly correlated ($r = 0.860$) with the nucleoside adduct concentration over the range from 20-1000 femtomoles.

BaP-DNA adduct levels in random human lung samples were found to range from 240-51,120 adducts per cell. Similarly, paraffin-embedded breast tissues contained BaP-DNA adduct levels from 35-1764 adducts in 10^8 normal nucleotides (0.05-14.7 femtomole BaP/μg DNA) with an average of 44,000 adducts/cell.

Adult CD-1 mice were injected i.p. with [^{3}H]-benzo(a)pyrene ([^{3}H]-BaP) (2mg, 117 mCi/mmol) and sacrificed 24 hr later. Liver DNA was isolated, enzymatically hydrolyzed to nucleosides, purified by immunoaffinity chromatography and analyzed with the ADAM procedure for BaP-DNA adducts. BaP-DNA adduct levels were quantified by both radioactivity (^{3}H in BaP) and acylation with ^{35}S-methionine with the ADAM procedure. An excellent correlation was found between the adduct levels determined independently by ^{3}H and ^{35}S. A newborn mouse lung adenoma assay was used to generate information about BaP-DNA adducts formed *in vivo*. Newborn CD-1 mice were injected i.p. with a tumorigenic dose of 210 μg of BaP (0.83mmole) three times over a two week dosing regimen starting at birth. Control mice were given DMSO as *placebo*. The mice were sacrificed by decapitation at specific time points of 24 hr, 72 hr, 1 wk and

5.5 months. Both target (lung) and nontarget (liver, kidney) tissues were collected. Tumors were induced by this regimen in the male mice. DNA was isolated, enzymatically hydrolyzed to nucleosides, purified and analyzed with the ADAM procedure for BaP-DNA adducts. The RP-HPLC profiles of treated mice were compared with their prospective controls. Relationships of adduct levels to tumorigenesis were summarized.

Successful acylation conditions were used to analyze benzo(a)pyrene adducts in DNA extracted from samples of tissues of CD-1 mice injected with benzo(a)pyrene and samples of human lung and embedded breast tissues. RP-HPLC profiles of the acylation products of adducts isolated from mouse tissues and human lung samples eluted at the same retention time as that of an authentic BaP-dGuo standard.

The postlabelling method combined with a subsequent RP-HPLC profile allows characterization of the carcinogen-DNA adducts present in the tissues. A data base will be generated to analyze DNA as a dosimeter of exposure. This data base will be used to correlate molecular biomarkers, specifically BaP-DNA adducts with tumor frequency. A direct relationship will verify the use for carcinogen-DNA adducts as dosimeters of exposure to genotoxic chemicals and will be imperative in understanding the role carcinogens play in initiating cancer.

Thesis Supervisor: Dr. Gerald N. Wogan

Title: Professor of Toxicology

Dedication

To the members of my family.....
Let me take a few minutes to brag about important family members in my life

Mom

Andrea W. Lawrence, Ph.D., 1993 Georgia Institute of Technology

Grandad

Emory Williams, World traveler, Retired Co-op Program Director Northwestern University

Allegra/Muffin

Allegra J. Lawrence, J.D. 1996, Yale Law School

Valerie, "The Baby"

Valerie Lawrence, B.A. expected 1998, Atlanta Metropolitan College
mother of niece Alexandra "Tigger"

Dad

President & CEO, Inchworm Industries, Inc.

Uncle Ronald

Ron M. Lawrence, Attorney at Law, J.D., LL.M

Julia Marie, my baby baby sister

Acknowledgements

I would like to sincerely thank the members of my thesis committee: Dr. Peter C. Dedon, chairman, for his pragmatic outlook, Dr. Steven R. Tannenbaum for his contagious enthusiasm about science and Dr. William G. Thilly for his constant encouragement and support. I especially appreciate my thesis advisor, Professor Gerald N. Wogan for his continuous guidance and confidence in my ability to succeed.

I owe my life to GOD and my major support systems:

My parents for loving me unconditionally. You set the example of leadership as you strive for greatness. Allegra for constantly doing my editing over the phone and for wanting us to be friends in addition to being sisters. Valerie for making sure people remember that I'm human. To Uncle Ronald, my favorite Uncle who is my godfather that has his own law firm and lives in Atlanta...ha ha ha! Thank you for making me laugh when things got rough. Grandad Emory for always coming to the rescue. Samuel M. Clemons, Jr. my soul-mate, fellow movie buff and future partner for life. Thank you for spoiling me.

I owe my success to the help of Laura Trudel—for the large quantities of antibody and necessary extra pair of hands; Faye Sayedi—for the tremendous amount of work on the standard and TBM synthesis; Fayad Sheabar, William Boadi and Sarah Moody Robinson—for the development and application of the ADAM method and the animal studies. Wang—for showing me not to be afraid to repair an HPLC myself. Now I know how to take them apart and put them back together...no problem, Xia— for her animal expertise. Your work is a piece of art. The Headquarters Staff of Division of Toxicology have looked out for me well. Thank you Debra, Roni, Jack, Blair, Terri, Janet, Mary, Alice and Carrie. Thank you, Denise for all of the computer work/desktop publishing.

Other MIT checkpoints: Office of Graduate Education Dean Isaac (Ike) Colbert and Margaret (Margot) Daniels Tyler. Thank you for your faith in me. My second moms at MIT: Mary Ni and Carla Kirmani—you helped me to love myself and not take life so seriously. My allies: Tony Gray for much needed ice cream breaks; Lynn Roberson—you saved my life. Thank you for helping me ward off the energy vampires; Jennifer Thomas—you make the world's best brownies...don't tell Don. Our giggling sessions are addictive. I fully appreciate the helpful hands and encouraging words of Paul Chodak, Karl Reid, Robert Selders, Jeff Thomas, the other members of "The Crew": Carsie Hall, Charles Clarke, BGSA and Color Creations —my family away from home.

Special thanks to the people who sent in reinforcements when I became battle weary.

My thesis group Cathryn Shaw and Araba Lamouse'-Smith —you got me to the other side. Cathryn: I am inspired by your bright smile, perserverance and courage. Araba: I would not have been able to make 94-95 as BGSA chair a successful year without you. Uncle Johnny---got me interested in this whole research thing beginning with UMARC. Dawn Hilliard (Williams)—MY HUMAN DIARY. I feel like we are connected beyond words. I have enjoyed our deep discussions. JOSH—my partner "in crime" (rollerblading, movies, etc) who taught me how to get

things at a "bargain price." Thank you for the gospel music and Mikki Howard. Robbin—you've shown me how to ask for what I want and say no to the things I don't. Don Brunson—When are we going to start our catering business? Thank you for everything. You have helped me more than you know. Carla Ferguson taught me how to put aside my pride and accept help from others: "Dee, if you don't let people do for you...you are stopping them from being blessed." Cassandra Clay for helping me find my inner voice. Ken and Alan—I cannot believe I let you get away with petnames like deedree, diadora, kikuyu, lulu, kooloo...thanks for the getaways to Roxbury, Dorchester & New Hampshire.

I owe a huge amount of gratitude to my friends "back home" who have stuck by me despite my inability to return calls. Lateefah Cabey, Zabrina Furlow, Debbie Hinkson, Kim Johnson, and Kirkpatrick Terry...or should I say "captain kirk"?

TO THE PEOPLE THAT I HAVE LOST DURING MY TIME AT MIT—Grandpa Ed, Grandma Julia, Uncle Maurice and Uncle Gillis. I miss you dearly. 'Til we meet again". Please say Hello to my Gramma Jeanne.

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List of Abbreviations

ADAM	Adduct Detection by Acylation with Methionine
AP	alkaline phosphatase
BaP	Benzo(a)pyrene
BaP-dGuo	Benzo(a)pyrene-deoxyguanosine adduct
BPDE	anti-benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±)
BPDE-dGuo	BaP-dGuo adduct made using anti-benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±) and 2'-deoxyguanosine
Dnase I	deoxyribonuclease I
Glucose-6-P	glucose-6-phosphate
^{3}H-BaP	^{3}H-Benzo(a)pyrene
NP_1	Nuclease P_1
SPD	spleen phosphodiesterase
SVPD	snake venom phosphodiesterase
TBM-NHS	t-butoxycarbonyl-L-methionine,N-hydroxysuccinimidyl ester
TEA	Trielthylamine
THF	tetrahydrafuran
DIC	N',N'-diisopropylcarbodiimide
DTBDC	Di-tert-butoxy-dicarbonate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
^{35}S-TBM-NHS	^{35}S-tert-butoxycarbonyl-L-methionine, N-hydroxysuccinimidyl ester

Chapter 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are released into the environment by the burning of organic materials such as coal, fuel oils, petrol, wood, refuse, and various plant materials. The main sources of exposure for human populations include coke plants, which account for 30% of airborne PAHs, coal-fired power plants and domestic coal- and wood-burning stoves. Other contributions to air pollution are industrial activity , automobile traffic and the burning of coal for domestic use. Higher concentrations of atmospheric PAHs are found in urban areas in western countries (up to 10 ng/m^3 of benzo(a)pyrene (BaP)). These carcinogens are metabolized to reactive intermediates which covalently bind to cellular molecules, resulting in DNA adducts and protein adducts. Traditionally, measurements of contaminants in the environment (e.g. air, soil, water, food) have been used for assessment of exposure. These measurements make it difficult to assess human exposure to polycyclic aromatic hydrocarbons, since exposure to PAHs occurs mainly through inhalation, ingestion and skin absorption (Oesch, 1983). Accurate measurements of individual exposure, such as DNA damage as a result of exposure to environmental carcinogens may be used to appropriately assess human health risk factors for cancer.

It is believed that the formation of DNA adducts is an essential initial step in carcinogenesis. Concentrating investigation efforts towards DNA-adducts in the target cells will help to decipher the relationship between dose and exposure in human populations. The

objective is to identify and quantify carcinogen-DNA adducts as biomarkers of exposure to genotoxic chemicals. The need for validated sensitive methods to determine carcinogen exposure in an individual is imperative. The BaP-DNA adduct is a form of genetic damage and may lead to mutations. The appearance of a stable adduct may give way to the loss of a base or the insertion of an incorrect base during DNA repair. A mistake in the coding could lead to errors in transcription to mRNA or in translation to protein. The resulting faulty protein may could be a regulatory protein that is normally responsible for growth or the halting of growth. The error may result in over-expression or under-expression of a regulatory growth protein, thereby resulting in tumor formation.

To better understand how DNA adducts are related to cancer, this research focuses on benzo(a)pyrene (BaP) since it is a highly carcinogenic PAH and a ubiquitous environmental contaminant (Zaleski, 1991). Chapter 2 reviews the literature discussing the carcinogenicity of BaP demonstrated in animals and the advantages and disadvantages of techniques that measure BaP in humans. The major objective of this body of work was to further develop the ADAM method created in the Wogan laboratory and apply it to the identification and quantification of carcinogen-DNA adducts. The ability to measure BaP-DNA adducts will make it possible to examine the relationship between BaP-DNA adducts and exposure to BaP. The synthesis of an internal benzo(a)pyrene-nucleoside adduct is described in Chapter 3. Chapter 4 outlines the optimal digestion and purification conditions obtained through the use of *in vitro*-modified DNA. Chapter 5 describes the application of the method to quantify DNA adduct levels in animals treated with BaP. The method was applied to mouse tissues to determine the level of BaP-DNA adducts formed *in vivo*. This includes the newborn mouse

lung adenoma assay in which newborn CD-1 mice were injected i.p. with a tumorigenic dose of 210 μg BaP/35 μl DMSO three times over a two week period. Both target (lungs, livers) and nontarget (kidneys) tissues were collected and analyzed via the ADAM procedure for BaP-DNA adducts. The postlabelling method combined with a subsequent HPLC profile allowed the characterization of the carcinogen-DNA adducts present in the tissues.

The final goal is to analyze BaP-DNA adducts in human tissues. Chapter 6 describes the successful application of the ADAM method to analyze BaP-DNA adducts in human lungs and embedded breast tissues. The conclusions are summarized in Chapter 7 which also outlines ideas for future work. A data base will be generated to analyze DNA as a dosimeter of exposure. This data base will be used to correlate molecular biomarkers, specifically BaP-DNA adducts with tumor frequency. A direct relationship will verify the use for carcinogen-DNA adducts as dosimeters of exposure and will be imperative in understanding the role carcinogens play in initiating cancer.

Chapter 2 Literature Survey

2.1 Polycyclic aromatic hydrocarbons

2.1.1 Source and composition

Polycyclic aromatic hydrocarbons (PAHs) are released into the environment by the burning of organic materials such as coal, fuel oils, petrol, wood, refuse, and various plant materials. The main sources of exposure for human populations include coke plants, accounting for 30% of airborne PAHs (Harris, 1989), coal-fired power plants and domestic coal-burning and wood-burning stoves. Other contributions to air pollution are industrial activity, automobile traffic and the burning of coal for domestic use. Higher concentrations of atmospheric PAHs are found in urban areas in western countries (up to 10ng/m^3 of benzo(a)pyrene) (Hemminki, 1990).

BaP is a highly carcinogenic PAH and an ubiquitous environmental contaminant. Human exposure to BaP can be as high as 100 ng/m^3 in heavily polluted air, 23 ng/m^3 in drinking water, and 100 μg/kg in smoked foods. Exposures are particularly high for coke oven workers, gas works operators and asphalters, whose occupations have been associated with increased cancer risk. It is important to better understand the relationship between an increase exposure to BaP and an increase in cancer risk.

2.1.2 Metabolism and biological effects of BaP

PAHs are metabolized to reactive intermediates which covalently bind to cellular molecules, resulting in DNA adducts and protein adducts. The metabolism of BaP takes place in two phases. Phase I involves the conversion, by oxidative enzymes and epoxide hydrolases attached to the microsomal membrane, to oxidized derivatives (epoxides, phenols, quinones) is performed. The oxygenase is also know as "aryl hydrocarbon hydroxylase' (AHH). In phase 2, these polar metabolites are conjugated with glutathionine by enzymes mainly present in the cytosol which make the substances soluble enough to be excreted (Osborne and Crosby, 1987).

Figure 2.1 illustrates the microsomal activation of BaP which covalently binds deoxyguanosine. The biologically active important metabolites of benzo(a)pyrene are the diol epoxides 7,8-dihydroxy-9,10-epoxybenzo(a)pyrene (BPDE) in which the epoxide group is situated in the bay region of the molecule (C^{10}-C^{11}). The most abundant reaction products of the (+) and (-) *anti*-BPDE enatiomers with native DNA are adducts with a covalent linkage between the C^{10} position of BPDE and the exocyclic amino group of deoxyguanosine residues (Geacintov *et al.*, 1997). There are two diasterereomeirc forms of each diol epoxide that are distinguished from one another because the most distant hydroxyl group can either be *anti* or *syn* relative to the orientation of the epoxide group. In the case of the bay region BaP diol epoxides, each of the two diasteromers, 7*r*, 8*t*-dihydroxy-*t*9,10-epoxy and 7*r*,8*t*-dihydroxy-*c*9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (called *anti*- and *syn*-BPDE, respectively), can be resolved into two optically active (+)- and (-)-enantiomers. For *anti*-BPDE, the absolute configurations of the two enantiomers are (+)-7(*R*),8(*S*),9(*S*),10(*R*) and (-)-7(*S*),8(*R*),9(*R*),10(*S*) ((+)- and (-)*anti*-BPDE

respectively). The 4 adduct conformations for the DNA lesion are stereoisomers but chemically identical. They are (+)-*trans*-, (-) *trans*-, (+)-*cis*-, and (-)-*cis-anti*-BP-N2-dG. The major adduct formed by the metabolism of BaP arose from the *trans* addition of 7R-anti-BPDE to the amino group of deoxyguanosine (Osborne and Crosby, 1987) . The newly formed compound, BaP-DNA adduct, is a form of genetic damage and may lead to mutations. It is believed that DNA adducts are an essential initial step in carcinogenesis since these mutations could activate an oncogene or inactivate a tumor suppressor gene resulting in uncontrollable growth.

In experiments where the binding of BaP to DNA was examined (Meehan *et al.*, 1977), two adducts were obtained for both deoxyguanosine and deoxyadenosine. The formation of the two adducts could have resulted from the reaction with both stereoisomers of (±) BaP diol epoxide or alternatively, a single isomer forming *cis* and *trans* addition products. High-resolution mass spectroscopy has quantitatively determined that the exocyclic amine of guanine is the preferred binding site. The excision repair of adenine carcinogen adducts in a number of cases is known to occur at a greater rate than those of deoxyguanosine (Dipple, 1977). The N2-exocyclic amine adduct of deoxyguanosine may therefore have a relatively long biological half life (Meehan, 1977).

The exocyclic amino groups of purine residues in native DNA are the primary targets of the covalent binding reactions of PAH diol epoxides. The attachment of PAH diol epoxides to the N^2 groups of guanine or N^6 of adenine residues can occur by either *trans* or *cis* addition. Thus each PAH diol epoxide entiomer can give rise to two adducts with different absolute configurations about the PAH-exocyclic amino group linkage site. The spectrum of adducts fromed when (+)- or (-)-*anti*-BPDE reacts with nucleic acids in an aqueous environment depends

on the nucleic acid composition, secondary structure of the DNA, solution composition, etc. Cheng et al., (1989) reported typical results on the binding of these two entionmers to native DNA. In the case of the (+)*anti*-BPDE enantiomer, the dominant adduct (>90%) is the (+)-*trans-anti*-BPDE-N^2-dG. Whereas in the case of of (-) *anti*-BPDE, a smaller proportion of (-)-trans-adducts (63%) but greater proportions of (-)-*cis-anti*-BPDE-N^2-dG (22%) and (-)-*trans-anti*-BPDE-N^6-dA adducts (15%) were found.

There are several things known about the properties of DNA containing BPDE adducts. The bound BPDE can be detected by fluorescence and the spectrum is similar to that of BPDE (Osborne and Crosby, 1987). The temperature at which the double stranded structure breaks up ("melting point") is lowered by treatment with BPDE. This implies that the modified bases do not properly fit into the normal DNA structure. When *anti*-BPDE or *syn*-BPDE was incubated at neutral pH, apurinic sites developed which gave rise to single strand breaks in alkaline conditions (Shooter *et al.*, 1977). These sites resulted mainly from the loss of BPDE-7-guanine adduct from the DNA (Osborne and Merrifield, 1985).

There are two types of anti-BPDE adducts that have been characterized on the basis of how the BaP molecule is aligned with the DNA. Type I adducts have the pyrene chromophore intercalated into the DNA structure. This minimizes the contact of the hydrophobic flat aromatic ring systmes with the aqueous solvent environment. This binding is noncovalent. Type II adducts describe the external binding of the BaP molecule, lying outside of the DNA bases. These react to form covalent adducts. Based on UV optical characteristics of *anti*-BPDE-polynucloetide and stereochemically defined, site specific oligonucleotide adducts, it was shown that *trans-anti*-BPDE-N^2-dG adducts have type II conformations, while the isomeric *cis*-adducts have type I

conformations (Geacintov, 1991; Geacintov, 1997).

The biological activities of racemic *anti*-BPDE have been investigated more extensively that those of the *syn* diastereomer. Racemic *anti*-BPDE is known to cause mutations in the critical codons 12 and 61 of the human c-*Ha-ras1* protoncogene (Vousden et al.,1986). Since the (+)-*anti*-BPDE is highly tumorigenic while the (-)-*anti*-BPDE is not (Buening et al., 1978, Slaga et al., 1979) and the (+)-*anti*-BPDE is more mutagenic in mammalian cell systems (Brookes 1982) , it seems that the chirality exerts a influence on the biological activities of *anti*-BPDE (Geacintov *et al.*, 1997).

Bulky PAH-DNA lesions are known to stall DNA polymerases at or near the sites of the adducts which could allow for the formation of bulged intermediates in which the modified nucleic acid residues are no longer paired with their partner bases on the complementary strands (Geacintov, et al, 1997). Such slipped, misaligned framshift intermediates are believed to give rise to deletion and point mutations (Shibutani et al., 1993).

2.1.3 Selection of benzo(a)pyrene

Benzo(a)pyrene was isolated from coal tar in 1930. Upon application of BaP synthesized by Cook *et al.* (1933) to the backs of 10 mice, tumors developed in all five of the surviving animals. Since then, many researchers have tried to understand the mechanism by which BaP causes cancer. To better understand how DNA adducts are related to cancer, this study proposes to focus on benzo(a)pyrene. Benzo(a)pyrene was chosen in this study because it is a highly carcinogenic PAH and it is ubiquitous in the environment and therefore may be relevant to human

cancer.

2.2 Dosimetry of exposure to genotoxic compounds

2.2.1 Covalent adducts as dosimeters

DNA adducts

The level of binding of BaP to DNA applied to mouse skin has been determined many times (Phillips et al., 1978; Pereira et al., 1979; Ashurst et al., 1983). When the mice of three different strains were treated with 1 μmole BaP each, DNA isolated from the skin 19 hours later contained about 29 nmole BaP, whether the strain was resistent or not. Tthis level was approximately linear with dose. The extent to which BaP binds internally is less well known. Dunn et al (1983) demonstrated that the extent of binding to DNA was proportional to the dose over a wide dose range (10^{-7} to 10^{-3} g/mouse). A linear relationship was similarly found at low dose for binding to liver, lung or stomach DNA (Adriaenssens et al., 1983)

Approaches for using DNA lesions, to elucidate dose-response relationships in carcinogen-exposed humans, have been developed through molecular dosimetry. Molecular dosimetry can measure an individual's exposure after taking into account differences in absorption, distribution, biotransformation and DNA repair. This is more accurate for dose-response relationships than determining external exposure. The challenge in measuring DNA lesions in humans is that they occur in relatively low quantities. The upper limit in humans is

about 100 fmole/mg DNA (3 DNA lesions per 10^5 normal nucleotides) (Lohman, 1992). The disadvantage is that samples of human target tissues cannot be collected routinely. However, surrogates like DNA from nucleated blood cells must be used if the level of DNA adducts in target and surrogate tissues is parallel.

Protein adducts

Adducts to serum albumin and to hemoglobin (Hb) are the protein adducts used for molecular dosimetry in humans. Protein adducts in hemoglobin and serum albumin can be analyzed by physicochemical methods. These include gas chromatography (GC)-MS or by immunoassay (Wogan, 1992). Hb adducts have been used to monitor occupational exposures. HPLC combined with synchronous scanning fluorescence spectroscopy allowed for the detectin of covalently bound BaP residues in Hb and DNA (Weston et al., 1989).

Protein adducts have several advantages. They are generally easier to collect than DNA adducts and therefore can be obtained in large quantities. Since they have a relatively low turnover rate they are useful in determining accumulated exposure.

DNA adduct and protein adduct analyes have been useful in the detection and quantification of exposure through occupation, smoking and dietary contamination. The measurement of DNA and protein adducts is designed to serve as a marker of exposure and biologically effective dose. The pattern of DNA adducts formed can be altered by repair mechanisms and by different tissues and cell types. The replication of carcinogen-modified DNA can result in the fixation of a mutation which could be the initial carcinogenic step (Wogan, 1992).

2.2.2 Relevance of covalent modification of DNA to carcinogenesis

Evidence suggests that there is a relationship between adduct levels and the promotion of tumors. A linear dose-response relationship up to the dose of 1mg/kg was found when BaP was administered to rats by single injection over the dose range of 4 μg/kg to 4 mg/kg body weight (Lutz, 1978). Animals were sacrificed 50 hours later and total carcinogen binding to liver DNA determined. The dose response relationship was nonlinear at higher doses. This direct relationship between binding level and dose over a wide dose range (10^{-8} to 10^{-3}g/mouse) was found when BaP-DNA adduct levels were measured in target (stomach) and nontarget (liver) organs of mice (Dunn, 1983; Wogan and Gorelick, 1985) A significant consideration in determining the carcinogenicity of BaP is the extent to which the DNA adducts are repaired and/or persist in the target organ. For example, DNA adducts were measured in skin epidermis (Albert, et al) by ELISA method in mice given single weekly application of BaP (8 μg-64 μg). The linear increase in DNA adducts was not as steep above the 32 μg/week, yet there was a sharp rise in tumor response above the 32 μg/week dose rate. All of the tumors were initially papillomas, which converted to carcinomas after an average of 8 weeks.

^{32}P-Postlabelling analysis revealed the presence of multiple adducts in the WBC DNA of smokers with a total concentration of 1 adduct in 10^{8}-10^{10} normal nucleotides (Jahnke *et al.*, 1990).

2.2.3 Methods for detecting carcinogen-DNA adducts

To study the interaction of BaP with DNA, sensitive methods are necessary to detect DNA-bound BaP in experimental models or in exposed individuals.

Immunoassays

Using antibodies to detect BaP-DNA adducts has the advantage of having high specificity for BaP or for one particular BaP-nucleoside adduct. In the immunological approach of detecting carcinogens, monoclonal or polyclonal antibodies are raised against either carcinogen-modified DNA or carcinogen-nucleoside DNA adduct coupled to protein carriers. The antibodies are then used to quantify specific adducts in the DNA of exposed cells. The antiserum of the animal can be used in several ways (Osborne and Crosby, 1987): (1) Radioimmunoassay: Antiserum was raised in rabbits by injecting an (+)-*anti*-BPDE-DNA+methylated bovine serum albumin comples. Samples were tested by determining how much they competitively inhibited the reaction between this antiserum and tritiated (+)-*anti*-BPDE-DNA (Poirier et al., 1980). (2) Enzyme-linked immunosorbent assay (ELISA): The immunoprecipitate was detected by linking it to an enzyme, e.g. with a complex consisting of goat antibody to rabbit immunoglobin G and alkaline phosphatase, and measureing the enzyme activity. This assay was reported to detect 0.1-0.4 fmole BaP adducts in 1μg of DNA (Hsu *et al.*, 1981, Perera et al., 1982). (3) Visualization of bound antibody. The antibody bound to BaP-DNA can be observed under a microscope after labelling it with a fluorescein derivative, or individual molecules observed with an electron microscope after labelling with a ferritin complex (Slor et al., 1981). (4) Monoclonal antibodies:

Cell lines created by fusing myeloma cells with spleen cells from mice treated with a BaP-protein conjugate can be cloned to produce highly specific antibodies which will detect as little as 3 fmole BPDE (Santella et al., 1984).

Fluorescence

In 1975, fluorescent spectra like that of BaP were obtained (Kodama and Nagata, et al., 1975; Jerntrom et al.,1978). Up until then solutions of DNA from mouse skin or hamster embryo cells treated with BaP were weakly flourescent (Daudel et al., 1975; Ivanovic et al., 1976). The better fluorescence spectra have been obtained a number of ways: (1) Hydrolysis of the DNA: The free bases, released from the polymer with mild acid gave better results because the nucleosides have a higher fluorescence intensity and a clearer spectrum than the intact nucleic acid (Osborne and Crosby 1987). Good results were obtained by treating DNA containing BPDE adducts with acid at 80°C to release BaP-tetrahydro-tetrols which were seperated by HPLC and quantified by their fluorescence (Rahn, et al., 1982; Shugart, et al., 1983). (2) Synchronous fluorescence: By simultaneously scanning the excitation and emission wavelengths with a difference of 34 nm, BaP-tetrahydrotetrols liberated from BaP-DNA with acid gave a sharp peak at 379nm. This allowed the detection of adducts down to 1 in 10^7 DNA nucleotides (Vahakangas *et al.*, 1985). (3) Cooling to 77K. The fluoresence intensity is increased by a factor of 40 (Ivanovic et al., 1982). (4) Cooling to 4K and exciting with laser light at 377 nm. The emission spectrum consisted of many sharp peaks (Heisig et al., 1984; Osborne and Crosby, 1987). The fluorescence assays are sensitive but can only be used to quantitiate adducts that have fluorescent characteristics.

Postlabelling

Aromatic carcinogen-DNA adducts have been analyzed via ^{32}P-postlabelling techniques (Randerath, 1989). This method involves enzymatic digestion of DNA to deoxynucleoside 3'monophosphates. A T4 polynucleotide kinase-catalyzed transfer of ^{32}P from adenosine (gamma-^{32}P)triphosphate converts the 3'monophosphates to deoxynucleoside 3',5'-(5'-^{32}P)diphosphates. In previous adaptations of the approach, the DNAs were first digested with a mixture of micrococcal nuclease (MN) and spleen phosphodiesterase (SPE), yielding 3'-monophosphates of normal (Np) and adducted nucleosides (Xp). The nucleotides were subsequently converted to 5'-^{32}P-labelled nucleoside 3',5'-bisphosphates by incubation with (gamma-^{32}P)-ATP and T4 polynucleotide kinase (PNK) (Randerath, 1981). The labelled adducts are then purified and analyzed by thin-layer chromatography, autoradiography and scintillation counting. This procedure detected 1 adduct in 10^8-10^{10} nucleotides. This method was modified for fluoranthene-DNA adducts with the use HPLC-liquid scintillation counting (Gorelick, 1989). With both ^{32}P-postlabelling techniques, the sensitivity was increased with the use of higher amounts of DNA.

An alternative ^{32}P-postlabelling procedure (Randerath, 1989b) introduced the use of nuclease P1 prostastic acid phosphatase (PAP) to generate a mixture of adducted dinucleoside monophosphates (XpN) and normal nucleosides (N). The enzymatically enriched dinucleotides, not the nucleosides are labelled at the 5'termini to yield ^{32}P-labelled dinucleoside diphosphates ([^{32}P]pXpN). The dinucleotides were either directly mapped by polyethyleneimine (PEI)-cellulose TLC or cleaved with snake venom phosphodiesterase to produce 5'-monophosphates. Distinct carcinogen-specific chromatographic profiles were generated by the adduct of the pX type and

differed from those of the corresponding dinucleotide (pbpN) and nucleoside bisphosphate (pXp) adducts.

^{32}P-postlabelling is very sensitive. It is reported to be able to measure 1 bulky adduct in 10^{10} normal nucleotides. The disadvantage is the cumbersome nature of the sheilding necessary with the use of ^{32}P. With regards to the applications that use TLC, the adduct spots are sometimes not readily identifiedIn addition, there dependence on an enzymatic reaction for the postlabelling of the adduct. The use of enzymes introduces complicated substrate kinetic factors that can be difficult to account for. The use of ^{35}S removes the need to use protective sheilding, although proper procedures for the use of radioactive substances must be adhered to. Two (^{35}S)phosphorothioate postlabelling procedures for the HPLC analysis of PAH-DNA adducts have been developed using ^{35}S (Lau, 1991). BaP-modified DNA was digested to nucleotide 3'-phosphates by micrococcal nuclease and spleen phosphodiesterase and the adducted nucleosides were extracted with 1-butanol. The adducted nucleoside-3'-phosphates were 5'-thiophosphorylated by T4 polynucleotide kinase (T4PNK) and adenosine 5'-O-(3-(^{35}S)thiotriphosphate) to yield (^{35}S)BaP-nucleoside-5'-phosphorothioate-3'-phosphate adducts. (^{35}S)BaP-nucleoside-5'-phosphorothioate adducts were also prepared using a nuclease P1/prostatic acid phosphatase DNA degradation method. There were no significant differences in the HPLC profiles of the (^{35}S)phosphorothioate-postlabelled adducts obtained from these two procedures. This method sensitively measures PAH-DNA adduct but not non-PAH adducts.

In the Wogan laboratory, another postlabeling method (Sheabar, 1994) was developed which subjects nucleoside adducts to acylation with ^{35}S-methionine. This published method has detected femtomolar (10^{-12} molar) quantities of dGuo, 4-ABP-dGuo and fluoranthene adducts. A

chromatographically pure adduct is incubated in the presence of t-butoxycarbonyl-L-methionine, N-hydroxysuccinimidyl ester (TBM-NHS), tetrahydrafuran (THF) and pyridine for 2 hours at 37°C. The reaction mixture was dried, solvent extracted and analyzed via reversed phase HPLC. The chemical reaction resulted in products that are more nonpolar and therefore appear later in the chromatogram than the expected retention time of the adduct. Chromatograms generated in the presence of TBM-NHS distinctly differ from reaction mixtures in the absence of TBM-NHS. ^{35}S has a half life 88 days which is more than 6 times longer than the half life of ^{32}P (14 days) The new method takes advantage of the long half life and the high specific activity at which the product is available. The research in this thesis describes the further development of the method, its validation to measure BaP-dGuo adducts and some recent improvements in the method.

2.2.4 Use of DNA adducts as molecular biomarkers of exposure

Smoking

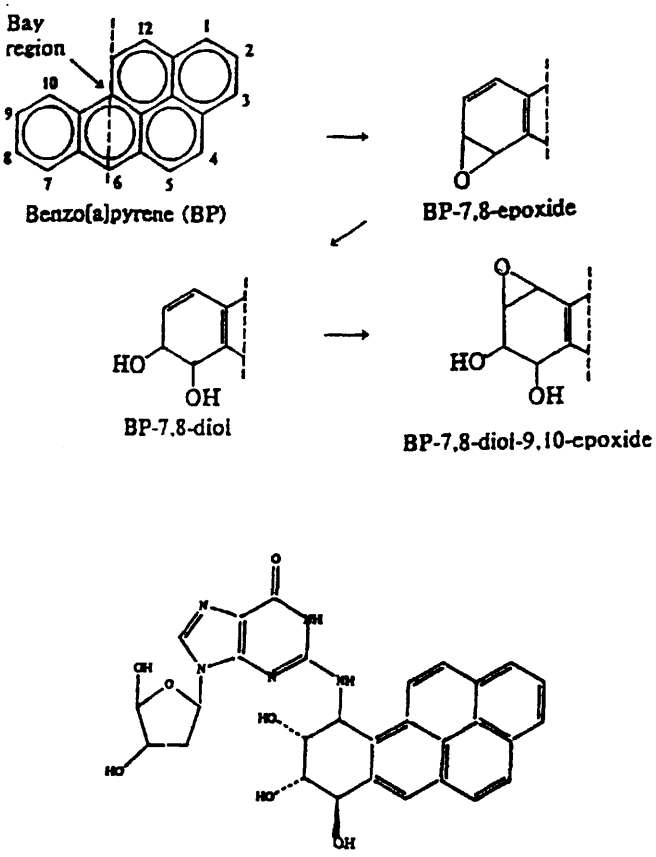
Although many carcinogens have been identified in cigarette smoke, there is still controversy about which carcinogens are predominately responsible for the development of lung cancer. Smoking is a major source of BaP exposure at 20-50 ng/cigarette (Poirier, 1980) and is considered to contribute to human lung cancer risk. The aryl hydrocarbon hydroxylase (AHH) activity is believed to reflect the cytochrome P450IA1-mediated reactions which are expected to control the rate at which aromatic carcinogen-derived electrophiles react with DNA in target cells of the lung. An HPLC/fluorescent assay allowed the measurement of low levels of BaP-DNA adducts in human lung (Alexandrov, 1992) and showed the formation of *anti*- and *syn*-BaP-DNA

adducts in lung samples of smokers. A positive correlation between the lung microsomal AHH activity and the formation of BaP-DNA adduct was shown. This finding is supported by Geneste et al (Geneste, 1991), whose laboratory compared the pulmonary DNA adducts and AHH activity. Non-tumorous lung specimens were collected from smokers and ex-smokers who were undergoing thoracic surgery for malignant lung diseases. The four ex-smokers tested had significantly lower amounts of DNA adducts in comparison to smokers. The levels ranged from 1-13 adducts /10^8 unmodified nucleotides in smokers and were of the same order of magnitude as those reported in human bronchial epithelia of smokers (Phillips, 1990). It was found that the AHH activity was significantly higher in smokers who had smoked until 1 week before surgery. In addition a positive linear correlation was observed between DNA adduct levels and AHH activity. This relationship could explain why AHH activity or inducibility appears to be a marker for lung cancer risk in smokers (Petruzzeli, 1988). These findings are supported by another case (Izzotti, 1991) where the presence of BaP-DNA adducts were investigated via synchronous fluorescence spectrophotometry in cells, mostly pulmonary alveolar macrophages, recovered by bronchoalveolar lavage. Out of the 39 individuals no adduct was detected in samples from nonsmokers or ex-smokers, whereas 84.6% of samples from current smokers exhibited typical fluorescent peaks The samples from 4 subjects that quit smoking during the last 6 months were positive. It appears that when assessing the internal dose of inhaled BaP near target cells of the respiratory tract, the detection of BaP-DNA adducts in alveolar macrophage represents a sensitive biomonitoring tool.

Occupational exposure

Emissions from coke-ovens pose a significant risk of cancer to exposed coke workers when monitoring human exposure. In a biomonitoring study (Haugen, 1986), in which the BaP-DNA adducts were analyzed in coke oven workers, analysis of the topside coke-oven samples from the work atmosphere showed that the exposure to PAH was high. Silesia is a highly industrialized region in southern Poland and is at present one of the most polluted areas in the world. Sensitive methods such as enzyme-linked immunoassays, synchronous fluorescence spectrophotometry, and ^{32}P-postlabelling assay, are used to detect carcinogen-DNA adducts in exposed individuals. Enzyme-linked immunosorbent assay (ELISA) was used to analyze peripheral while blood cells from Polish residents for the presence of PAH covalently bound to DNA (Perera, 1992) in order to evaluate the biologic dose and effects of ambient pollution. The ^{32}P-postlabelling method and sister chromatid exchange (SCE) measurement were performed on the same blood samples to compare results. The two measures of adducts (ELISA and ^{32}P-postlabelling) and SCE all demonstrated significant association with exposure as well as seasonal variation. Adduct levels in industrialized residents were found to be 30.4 adducts/10^8 Jan-March and 4.2/10^8 in Sept-Oct whereas rural residents had levels at 11.01/10^8 and 3.0/10^8 respectively. This experiment was a follow up to a previous study which showed that residents of industrialized towns in Poland had adduct levels and patterns similar to those of coke-oven workers, but two to three times higher than those of residents in rural areas (Hemminki, 1990). The large interindividual variation revealed by the biomarkers indicates individual differences in exposure, metabolic activation of PAHs and/or repair of DNA. It is still unclear as to how the variations in DNA repair rates influence in determining tissue site and risk of cancer in the general population (Gorelick, 1989).

Figure 2.1 Metabolism of BaP



Chapter 3. Synthesis of Benzo(a)pyrene-deoxyguanosine

3.1 Introduction

As discussed in Chapter 2, the metabolism of BaP has been extensively studied. Both *in vitro* and *in vivo* studies have demonstrated that the major detectable BaP bound DNA adduct is formed as a result of the BPDEI metabolite binding though the exocyclic amino group of deoxyguanosine (Garner *et al.,* 1985). In order to measure BaP-DNA adducts in human samples, it is most important to have a method that has been validated. Since the goal is to measure BaP-DNA adducts it is necessary to have a BaP-dGuo adduct standard to validate the method. A standard had to be synthesized to determine the recovery of BaP adducts and the efficiency of the ADAM method.

There are numerous ways to generate a benzo(a)pyrene-deoxyguanosine adduct. The early process incubated DNA (calf-thymus or salmon sperm) with benzo(a)pyrene and 3-methylcholanthrene-induced rat liver microsomes. When radioactive BaP was used to generate ^{3}H-BaP-dGuo, usually < 1% BaP was bound to the DNA. When commercially available BPDE was reacted with calf-thymus DNA, polyG or oligonucleotides it resulted in 1-2% of the bases being modified (Pulkrabek, 1977) or a modification level of 1.3% ^{3}H-BaPDE-DNA (Tierney, 1977; Manchester, 1990).

This chapter describes the synthesis of the BaP-dGuo adduct. It was important that the

standard be in the nucleoside form so that the ADAM method could be used. In order to mimic *in vivo* conditions the method using rat liver microsomes and ^{3}H-BaP was chosen first. This was important for producing the HPLC chromatograms. The modified DNA was digested to nucleosides with a published hydrolysis procedure (Park *et al*, 1989) and an experimental one used with the ADAM procedure. Once the recovery throughout the modification, digestion, purification, and acylation process was determined, the goal was to scale up the production of the standard. A large quantity was necessary to obtain structural information.

Since the efficiency of the microsomal reaction was low, the next step was to use the procedure involving the commercially available anti-benzo(a)pyrene-diol-epoxide (anti-BPDE). BPDE was reacted with 2'-dGuo and synthesized oligonucleotides. Several micrograms of BaP-dGuo were produced to yield an electrospray-MS demonstrating the correct molecular weight. The scale-up of this method produced the larger quantities (1 mg) of material needed but was not pure enough for the NMR analysis of the acylation reaction products. Finally, it was possible to obtain purified milligram quantities of the BaP-dGuo standard through an organic synthesis procedure. The strategies and results of synthesizing the BaP-dGuo standard are reported here in Chapter 3.

3.2 Methodology

3.2.1 Chemicals and Enzymes

Benzo(a)pyrene was obtained from Aldrich Chemical Company (Milwaukee, WI) and ^{3}H-BaP from Amersham. Anti-benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±) is a product of Midwest Research Institute (Kansas City, MO) and Chemsyn Science Laboratories (Lenexa, KS) of NCI Chemical Carcinogen Reference Standard Repository (Frederick, MD). Rat liver cytosol was prepared from postmitochondrial supernatant fraction (SL-9) supplied by Molecular Toxicology (Annapolis, MD). All chemicals and solvents used were reagent grade. Calf thymus DNA, glucose-6-phosphate, glucose 6-phosphate dehydrogenase and Nuclease P_1 (from *Penicillium citrinum*) were purchased from Sigma Chemical Co. (St. Louis, MO). Snake venom phosphodiesterase from *Crotalus adamanteus* was a product of ICN (Aurora, OH) and alkaline phosphatase from bovine intestinal mucosa was purchased from Boehringer Mannheim (Indianapolis, IN). Sep-pak C18 environmental cartridge was purchased from Waters (Milford, MA);. Methanol was purchased from Baxter (McGaw, IL). Chloroform, benzene, dichloromethane, ether, ethanol, were purchased from Mallinckrodt (Phillipsburg, NJ).

3.2.2 Preparation of modified DNA

Figure 3.1 summarizes the different ways to synthesize the BaP-dGuo adduct. Each method is described below. Briefly, BaP modified DNA was made by incubating calf-thymus DNA with BaP and rat liver microsomes or with BPDE. The modified DNA was isolated via phenol:chloroform extraction and ethanol precipitation, followed by enzymatic hydrolysis to nucleosides. The use of 2'-deoxyguanosine (2'-dGuo) was also used so that the DNA isolation and enzymatic hydrolysis steps could be omitted.

Microsomal Activation of Benzo(a)pyrene

The method of synthesis of benzo(a)pyrene-deoxyguanosine was similar to the procedure previously described (Gorelick & Wogan, 1989). To prepare *in vitro* modified DNA, calf thymus DNA (2mg/ml) was shaken in HEPES pH 7.1 with 2.5-3.5 mg/ml microsomal protein, 0.8 mM $NADP^+$, 5 mM glucose-6-phosphate, 0.5 mM $MgCl_2$, 0.4 units/ml glucose-6-phosphate dehydrogenase, and 100 mM ^{3}H-BaP (0.5-3 Ci/mmol) or 83 mM BaP in DMSO (1% total volume). After 90 minutes, the reaction was terminated by the addition of SDS (to 1%) and NaCl (to 250 mM). DNA was isolated by two extractions with an equal volume of phenol/chloroform (1:1), followed by an extraction with chloroform alone until a clear interface was observed. DNA was precipitated in cold ethanol and rinsed sequentially with benzene, chloroform, dichloromethane, ethanol and ether before drying under nitrogen. The isolated DNA was dissolved in water. Recoveries and binding levels were determined from absorbance at 254 nm (20 OD units/mg DNA/ml) and the liquid scintillation counting of known amounts of DNA. The BaP-DNA adduct was calculated by using the molar extinction coefficient of ε=29,000 at 347nm (Weinstein, *et al.*, 1976).

Enzymatic Hydrolysis

Initially a published method (Park, *et al.*,1989) known to produce nucleosides from PAH-modified DNA was used. This method of enzymatic hydrolysis used SVPD, AP, DNAse, and spleen phosphodiesterase I (37°C, 2 hours). A single digestion procedure that could be used to analyze more than one type of adduct would greatly enhance the flexibility of the ADAM method. The method that was used in the Wogan laboratory for the optimization of the ADAM

method with 2-d-Guo and for the analysis of 4-ABP adducts is as follows: microsomally modified calf thymus DNA (<1.7 mg/ml) was digested to nucleosides with nuclease P_1 (76.5 units/mg), 0.450 ml 2 mM $ZnCl_2$, 0.060 ml 1M NaAc. The volume was brought up to 3 ml with distilled H_2O and incubated for 1 hour at 37°C with agitation, covered with foil. An equal volume of 1M Tris buffer or 1M carbonate buffer was added. Note: The carbonate buffer was later substituted for the Tris buffer because the Tris was found to have contributed to the ^{35}S background in the radio-chromatogram. Snake venom phosphodiesterase (0.879 units/mg) and alkaline phosphatase (16.9 units/mg) were added and the reaction was incubated another hour at 37°C, with agitation. The reaction mixture was stored at -20°C until ready for HPLC analysis. The RP-HPLC gradient used was Gradient A. In order to establish that the digestion yielded nucleosides, a different HPLC gradient (Gradient C as described in Table 3.1) was used and used acetonitrile and H_20, pH 4.4.

Reaction of isomeric benzo(a)pyrene-diol-epoxide with DNA or 2'-Deoxyguanosine

Modification of several methods (Weinstein *et al.*, 1976, Vahakangas *et al.*, 1985, Jeffrey *et al.*, 1976) were used for the reaction of BPDE with calf thymus DNA, as follows: 2'-deoxyguanosine (20 mg) dissolved in 20 ml H_2O was reacted with 5 mg benzo(a)pyrene-diol-epoxide in 10 ml THF. The reaction mixture was incubated for 3-6 hours at 37°C in a shaking H_20 bath, followed by extractions with 6 x 15ml H_20-saturated ethyl acetate and 2 x 15ml H_20-saturated diethyl ether. The aqueous phase was recovered. The amount of DNA or deoxyguanosine was determined with absorbance at 254 nm. BaP was calculated by using the molar extinction coefficient of 29,000 at 347 nm. The modified samples were scanned from 200-700 nm. Simultaneously, ^3H-2dGuo (10 nmol, 31 Ci/mmol) was reacted with BPDE (0.81

mg) and taken through the same procedure. The rest of the ^{3}H-dGuo-BaP was extracted and evaporated. The pear shaped flask was washed 3 times with methanol and 3 times with H_2O.

The amount of 2-dGuo was determined from absorbance at 254 nm (20 OD units/mg DNA/ml) or by scintillation counting for the ^{3}H-dGuo (31 Ci/mmole). BaP was calculated by using the molar extinction coefficient of ε=29,000 at 347 nm. The modified samples were scanned from 200-700 nm. The cold BPDE-dGuo was concentrated by evaporation unit and the pear shaped flask was washed 3 times with methanol and 3 times with H_20. The volume was brought to 1.5 ml and stored until ready for HPLC analysis. At this time a microcolumn (1 μm) was attached to the electrospray-MS. Gradient F was used at a flow rate of 0.1 ml/min.

Reaction of BPDE with oligonucleotides

Several researchers (Cosman, *et al.*, 1990 ;Mao *et al.*, 1995) found that the reaction of BPDE with an oligonucleotide gave a 30% yield of BaP-dGuo. Oligonucleotides (1M and 10M solutions) were purchased from MIT Biopolymers laboratory. A small scale reaction was performed to determine whether the reaction would produce results similar to those in published literature. The first oligonucleotide used was a 9mer d(ATATGTATA). A quantity of 1.5 μmol, dissolved in 20 mM sodium phosphate, was incubated with 3.0 μmol BPDE (ratio 2 BPDE:1 oligonucleotide) dissolved in THF. All procedures were done under yellow lights. The mixture was incubated overnight at room temperature, covered with foil and in the dark. The reaction was analyzed by RP-HPLC with a Zorbax ODS column 4.6 x 250 mm, at room temperature with a flow rate of 1 ml/min using Gradient D: A = 20 mM NaPhos pH 7.0, B = methanol, linear gradient 0-90%B in 60 minutes. The peak at 20-25 minutes was collected and digested to

nucleosides as described previously, and analyzed by HPLC. A large scale reaction used 3 reaction vials (15 ml polypropylene tubes) each containing 10 μmol of oligonucleotide in 5 ml NaPhos, pH 7.0 and 20 μmol of BPDE (6 mg) dissolved in THF and 1.5% TEA. Mao *et al.* (1995) found that the addition of TEA increased the modification level. The HPLC anaylsis of the reaction products used a C_{18} semi-preparative column at room temperature with a flow rate of 3 ml/min. Another oligonucleotide was found to yield a high percentage of BaP-dGuo (Liu, 1996). This 11mer d(CTAGGGTATC) 1.3 μmole was reacted with BPDE (2.6 μmole).

Organic Synthesis of BaP-dGuo

A protocol was established (Seyedi, unpublished) for synthesis of the BaP-dGuo adduct. The chemical strategy is shown in Figure 3.2. The BaP-dGuo was first protected with benzyl groups, then incubated with BPDE overnight at 37°C, in the dark. The mixture was deprotected and purified. The molecular weight was verified with electrospray-MS.

3.2.3 Chromatography

The samples were analyzed by reverse phase HPLC (model 126, Beckman Instruments, Inc, Columbia, MD) equipped with diode array detector (model 168, Beckman Instruments, Inc, Columbia, MD). A Zorbax-ODS 5 μm column (4.6 mm x 250 mm, MacMod Chadds Ford, PA) or an Microsorb 5 μm C_{18} column (4.6 mm x 250 mm, Rainin, Woburn, MA) was routinely used with the HPLC gradients are summarized in Table 3.1. The radioactivity was determined on-line with a Radio-Chromatography Detector (Radiomatic Series A-280). Several

co-injections of the BaP-dGuo adduct generated with either microsomal activation or with BPDE were analyzed via HPLC with the diode array detector fixed at 347 nm and 254 nm. The oligonucleosides would have an absorbance at 254 nm and BPDE would have an absorbance at 347 nm. A radioactive chromatogram was also generated when possible. The ^{3}H label on the benzo(a)pyrene, when reacted with calf thymus DNA or on the deoxyguanosine, when reacted with BPDE, would give a peak when present.

3.3 Results and Discussion

The reaction involving the modification of calf thymus DNA with ^{3}H-BaP and microsomes was repeated several times. Typically it resulted in a modification level of 1 BaP in 10^{5} bases. Table 3.2 summarizes several such reactions. The goal was to produce enough adduct to generate HPLC chromatograms while validating the ADAM method. The specific activity had to be high enough to be able to detect a signal. For example, a sample had to contain approximately 100,000 cpm to produce an acceptable chromatogram. The specific activity of the ^{3}H-BaP ranged from 125 mCi/mmole to 12,500 mCi/mmole. As the table shows, the higher the specific activity of the ^{3}H-BaP, the lower the modification level of the DNA. For example, the reaction using a specific activity of 12,500 mCi/mmole resulted in a modification level of 1 BaP per 700,000 bases, while a modification of 1 BaP in 11,000 was obtained with ^{3}H-BaP at 500 mC/mmole. Therefore, the specific activity of 3000 mCi/mmole was used for the rest of the microsomal modification experiments. It consistently yielded a modification level of 1 BaP in 12,000-14,000 bases. The

recovery of the ^{3}H-BaP-dGuo was monitored during the enzymatic hydrolysis and Sep-pak C18 cartridge purification step. Fifty percent of the original adduct in the DNA was determined to be recovered up to the step when it was ready for acylation.

Once the recovery of the processing was determined, the next step was to scale-up the synthesis of the adduct. The goal was to make milligram quantities for the validation of the ADAM method. This would allow structural characterization of the acylation products. A comparison of modification procedures is shown on Table 3.3. The modification level was increased by using the commercially available BPDE. BPDE reacted with 2'-deoxyguanosine resulted in the highest modification level (1:793). The modified samples were scanned from 200 nm to 700 nm revealing a doublet peak from 200 nm to 300 nm (DNA or deoxyguanosine) and between 320 nm to 350 nm (BaP). A typical scan is shown in Figure 3.3. The molecular weight was confirmed by electrospray-MS. The molecular weight of the adduct is 569 yielding a peak at m/z 570 in a protonated spectrum.

When the reaction of BPDE incubated with 2'-deoxyguanosine was scaled up to 100 mg of DNA and 25 mg of BPDE, the amount of adduct recovered (0.1 mg) in the aqueous portion after solvent extraction was considerably lower than expected (2 mg). The solvent extracts were found to contain the adduct as revealed by HPLC analysis. The adduct was HPLC purified and the molecular weight was determined by electrospray-MS. Even after HPLC purification, the adduct was not pure. At this time a microcolumn (1 μm) was attached to the electrospray-MS. Gradient F was used at a flow rate of 0.1 ml/min. There were 4 peaks detected with the same molecular weight (m/z 570) and these peaks correspond to the 4 seen in the HPLC profile (Figure 3.12). The timing on the gradient of the electrospray was different because of periodic machine failure;

however, the profile is the same. The two major peaks most likely correspond to the *trans* isomers and the two minor peaks correspond to the two *cis* isomers. All four stereoisomers have the same molecular weight.

3.3.1 HPLC analysis

In order to determine if the hydrolysis of the DNA yielded nucleosides, the hydrolysate containing 50 μg of DNA was injected on the HPLC using gradient C. The peaks early in the chromatogram correspond to dC, dG, dT and dA (Figure 3.4). The HPLC profiles/radio-chromatograms generated by the two different enzymatic hydolysis procedures are illustrated in Figure 3.5. The ^{3}H label on the benzo(a)pyrene give the signal designated as cpm on the Y-axis. A co-injection of the cold BPDE-dGuo and the ^{3}H-BaP-dGuo, generated with microsomes, gave the same UV profile at 347 nm and 254 nm and was similar to the radioactive chromatogram (Figure 3.6) ^{3}H-BaP-dGuo. There is a 2 minute delay between the UV-HPLC chromatogram and the start of the radio-chromatogram. The 1st peak at around 3.5 minutes was identified as deoxyguanosine or undigested material. The doublet peak at 24 minutes (26 minutes on the radio-chromatogram) is considered to be the BaP-tetrol. The doublet peak at 30 minutes (28 minutes on the radio-chromatogram) is the BaP-dGuo-adduct. These chromatograms are reproducible. Figure 3.7 is an HPLC radio-chromatogram of ^{3}H-dGuo-BaP after extraction and purification via Sep-pak C_{18} column. The peak at 35 minutes is considered the ^{3}H-BPDE-dGuo adduct. The change in retention time from 26-28 minutes to 35 minutes is due to the change in the column temperature. The column temperature was room temperature instead of 35°C which

resulted in the peak eluting later in the chromatogram.

Figure 3.13 shows the HPLC profile of the crude mixture of the oligonucleotide (9mer) modified with BPDE. The unmodified oligonucleotide eluted at 24 minutes. The peaks that eluted at 42 and 44 minutes had absorbance at 347 nm and were identified as products of the BaP-tetrol. The peak at 24 minutes corresponded to the modified nucleotide. This peak was collected, enzymatically hydrolyzed and RP-HPLC purified. Mao *et al.*, (1995) successfully modified the same oligonucleotide. When using the same HPLC gradient, analyzing with a Hypersil-ODS 5 μm column at 3 ml/min, the modified nucleotide eluted at 23-25 minutes. The peak was collected, enzymatically hydrolyzed and HPLC purified with the gradient 0-100% NaPhos/methanol in 60 minutes (1ml/min) the modified nucleosides eluted at 49 minutes. The unmodified nucleosides dC, dG, dT, dA eluted at 15 minutes, 20 minutes, 23 minutes and 25 minutes respectively. Following the guidelines of this experiment, the peak that eluted at 49 minutes was collected and the molecular weight was determined with electrospray-MS. However, upon examination with electrospray-MS, these peaks did not contain BaP-dGuo.

3.3.2 Electrospray-Mass Spectral analysis of BaP-dGuo

BPDE and DNA or 2-dGuo

The BaP-dGuo adduct was RP-HPLC purified using Gradient B. The UV chromatogram is shown in Figure 3.8. Two fractions were collected (I: 1-10 minutes, II: 20-31 minutes) into glass pear shaped flasks. The volume was reduced and brought to 50% methanol. Samples I and II were analyzed by Electrospray MS (Hewlett Packard). Fraction I (Figure 3.9) shows peaks at

m/z 152.1, *m/z* 268.2, *m/z* 326.2, *m/z* 535.2, *m/z* 802.2. The molecular weght of deoxyguanosine is 267 giving a protonated *m/z* 268. The peaks *m/z* 535.2, and *m/z* 802.2 were dimers and trimers of deoxyguanosine. Guanine was represented by *m/z* 152.1. Figure 3.10 shows the electrospray-MS of Fraction II. The molecular weight of deoxyguanosine-benzo(a)pyrene (BaP-dGuo) is 569 giving a *m/z* 570 in a protonated spectrum. This ion was present in the spectrum. When this ion was fragmented (Figure 3.11), it broke up into several peaks *m/z* 241.4, *m/z* 257.2, *m/z* 285.2, *m/z* 303.1, *m/z* 436.3, *m/z* 454.4, *m/z* 537.9, *m/z* 570.3. The peak *m/z* 454.4 corresponded to the loss of the sugar. The *m/z* 303.1 peak was identified as the benzo(a)pyrene-diol-epoxide (mol wt. 302).

The electrospray-MS was connected to an HPLC (Hewlett Packard 1190). As the RP-HPLC analysis progressed, the MS was analyzed for the *m/z* 570. Figure 3.12 shows the electrospray-MS generated with gradient F. The appearance of four peaks demonstrated that there were four compounds with the same molecular weight.

3.4 Conclusions

The reaction with microsomally activated ^{3}H-BaP and calf thymus DNA successfully yielded ^{3}H-BaP-dGuo. The higher the specific activity of the ^{3}H, the lower the modification level of the DNA. It was found that the use of BPDE to modify DNA resulted in a higher yield of BaP-dGuo adduct. The ^{3}H-BaP-dGuo was used to determine recovery. Seventy percent of the adduct was recovered after enzymatic hydrolysis and purification. Another 20% was lost during

the speedvacuum step. Therefore about 50% of the adduct was lost as it is prepared for the acylation reaction. This was higher than the 10-15% recovery reported for fluoranthene-DNA adducts (Gorelick, Ph.D. Thesis, M.I.T), another PAH.

There are 3 items of data that demonstrated that the BaP-dGuo adduct has been synthesized. Benzo(a)pyrene diol-epoxide was successfully reacted with calf thymus DNA to yield the same profile as when calf thymus DNA was modified microsomally. The HPLC profile was the same whether the adduct was generated with BPDE or with microsomes. A co-injection of ^{3}H-BaP adduct made by reacting ^{3}H-BaP with calf thymus DNA and cold BPDE reacted with cold 2-deoxyguanosine yielded the same HPLC profile. The co-injection provided 2 independent means to monitor the adduct. UV (347nm) and the radiolabeled carcinogen ^{3}H-BaP, demonstrated that the profile was reproducible. As determined by electrospray-MS, the *m/z* 570 peak showed that the compound had the right molecular weight. The BaP-dGuo adduct generated with microsomally activated BaP and calf-thymus DNA was used for most of the validation experiments. In addition a protocol had been established to successfully react benzo(a)pyrene-diol-epoxide with protected 2'-deoxyguanosine. The procedure to make the adduct by reacting BPDE with protected 2'-deoxyguanosine was used to generate a milligram of BaP-dGuo adduct.

Figure 3.1 Scheme to make BaP-modified deoxyguanosine

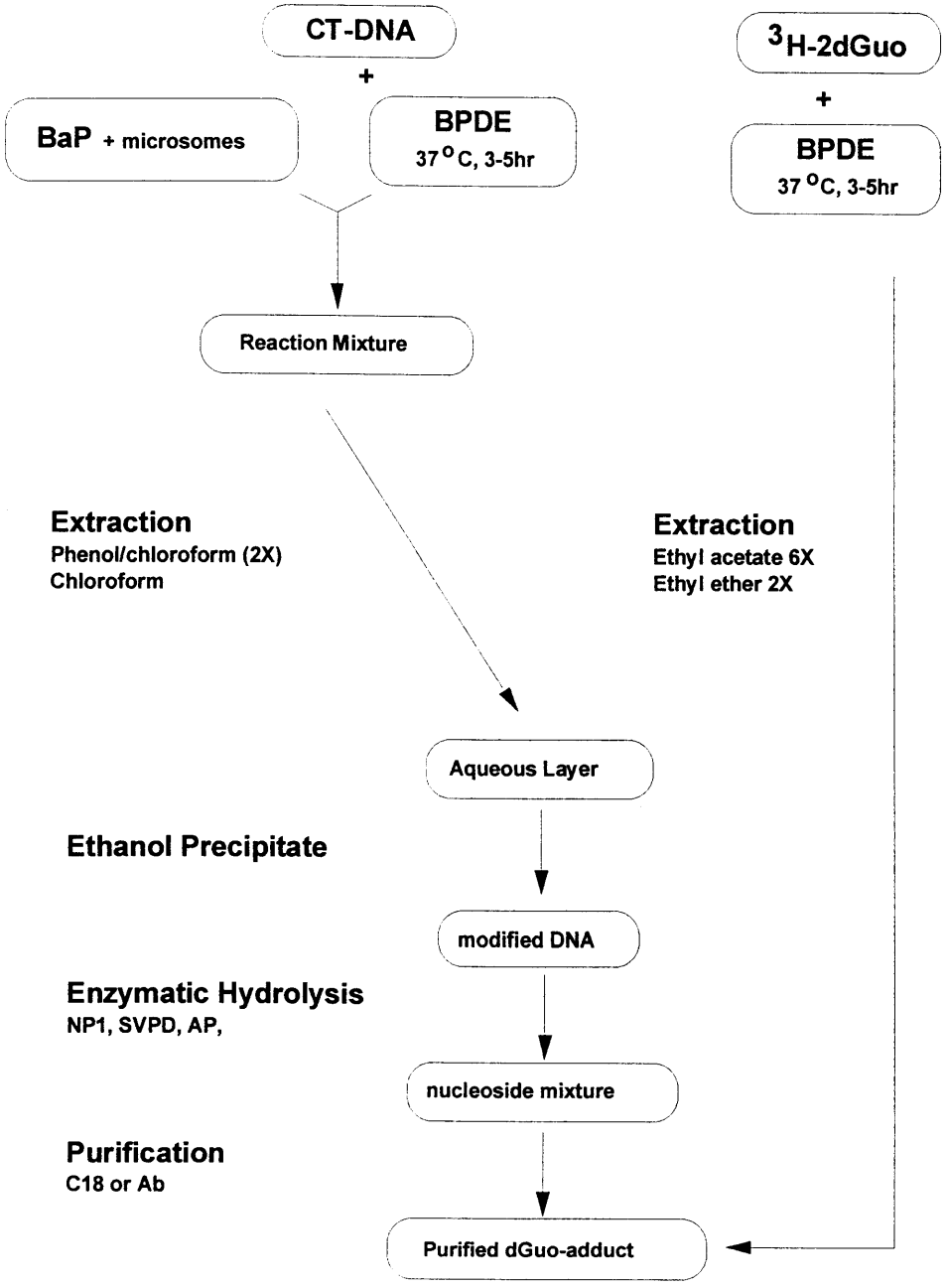


Figure 3.2 Organic synthesis of protected BaP-dGuo

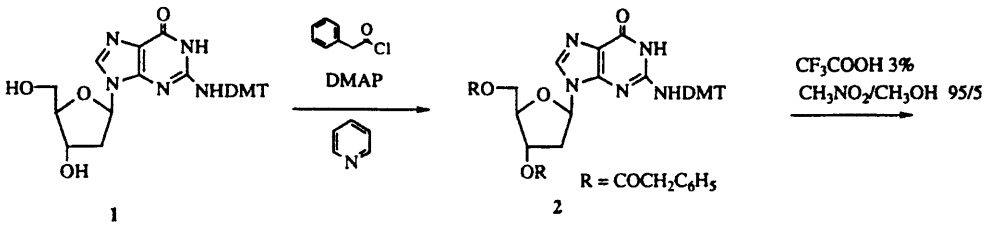


Figure 3.3 Spectral scan of BaP-modified calf-thymus DNA

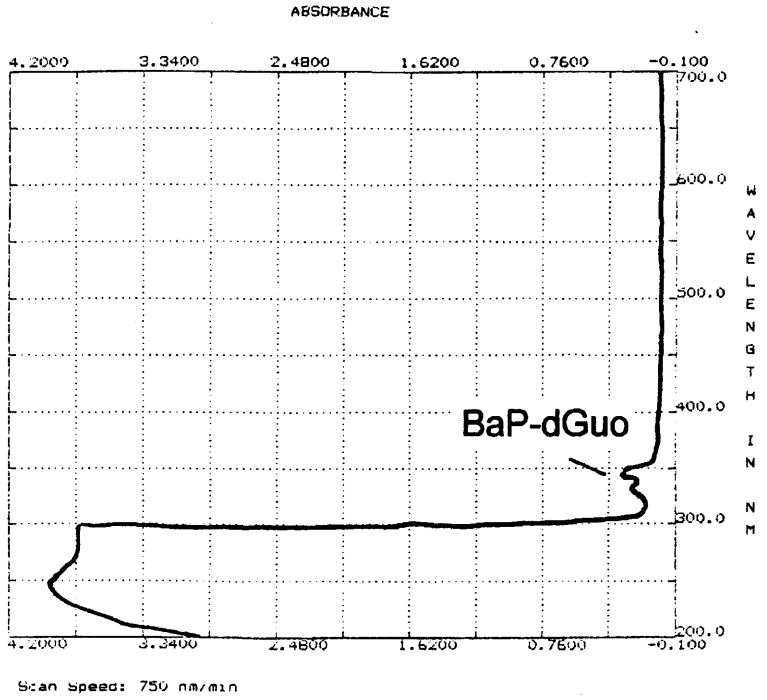


Figure 3.4 HPLC chromatogram of BaP-modified DNA digested to nucleosides

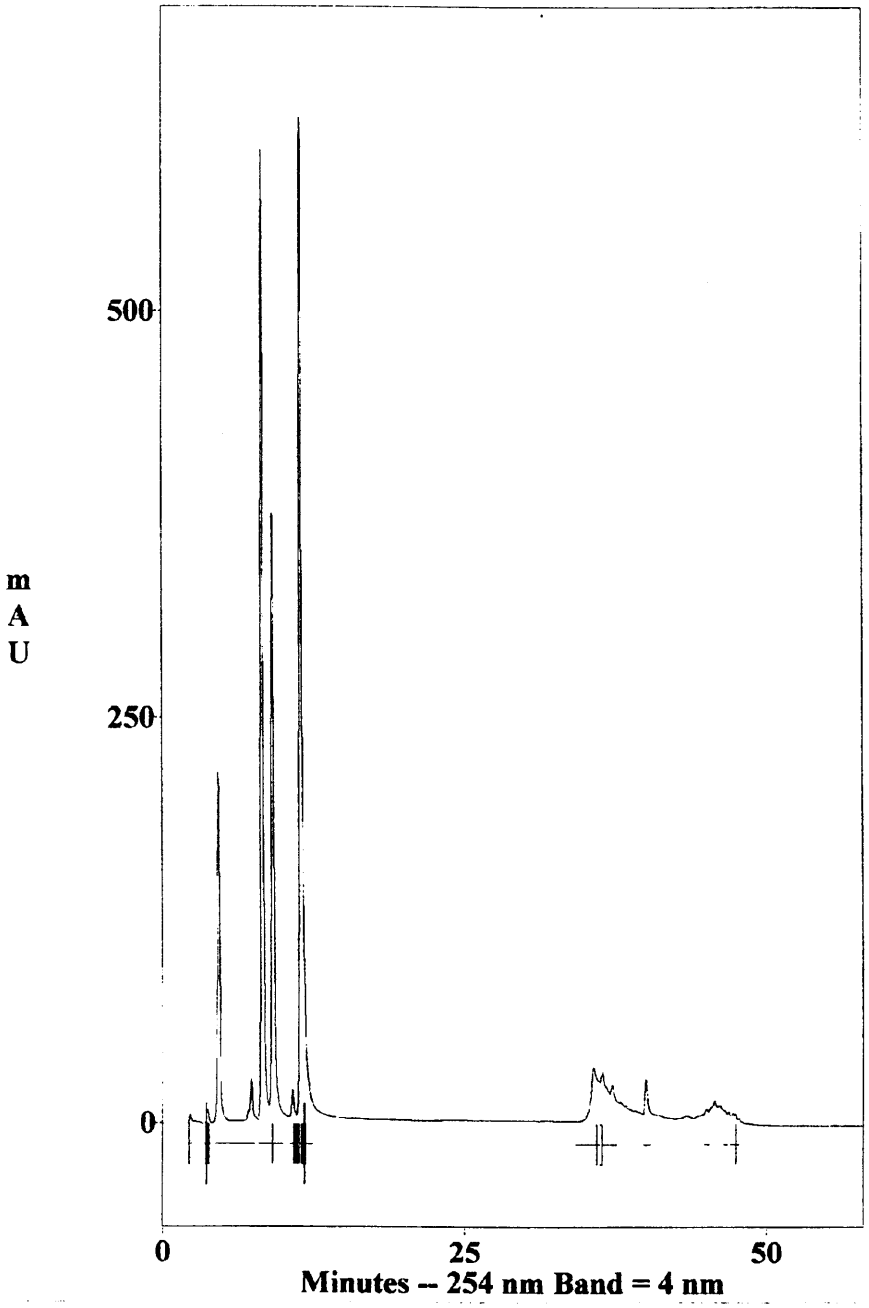
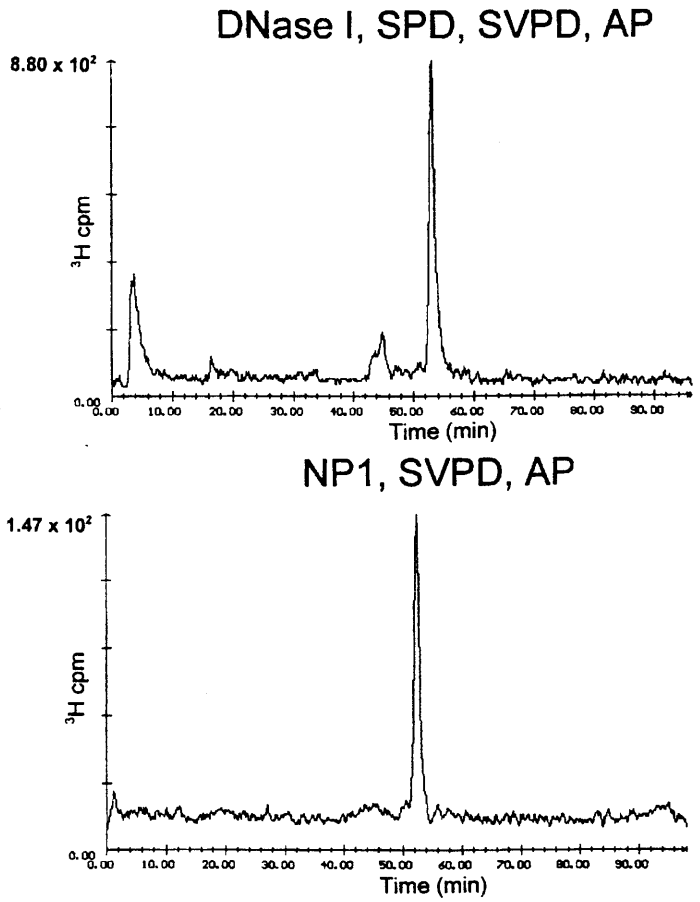


Figure 3.5 HPLC chromatograms of ^{3}H-BaP-dGuo after enzymatic hydrolysis



Major peak at 53 minutes
Top panel: BaP modified calf-thymus DNA digested with DNAase I, SPD,SVPD, & AP
Bottom panel: BaP modified calf-thymus DNA digested with NP1, SVPD & AP

Figure 3.6 HPLC chromatograms of ^{3}H-BaP-dGuo co-injected with cold BaP-dGuo

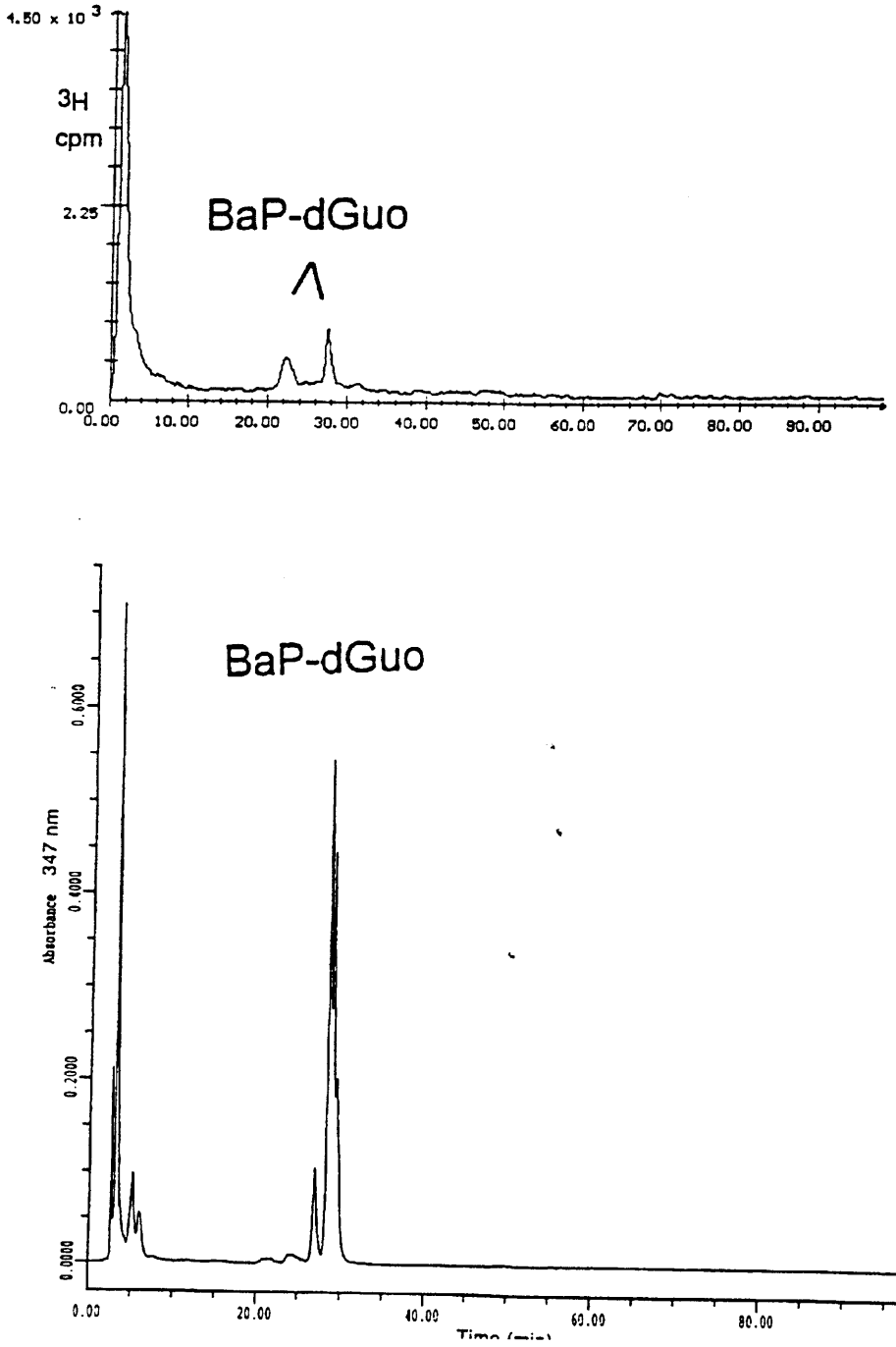
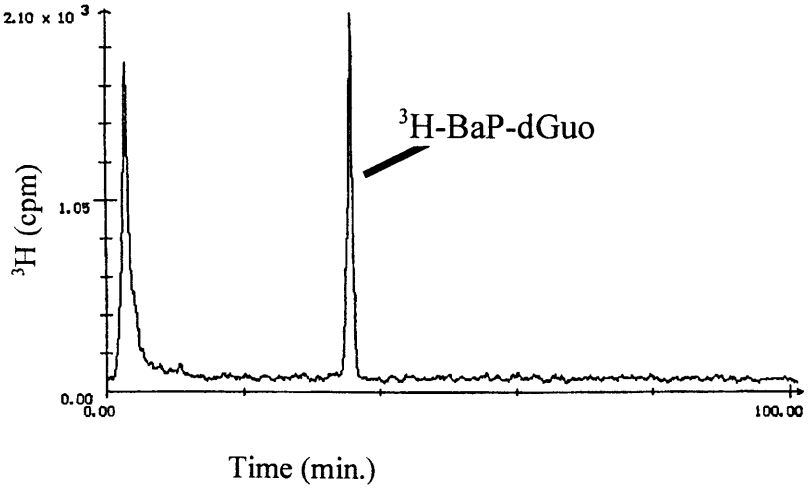
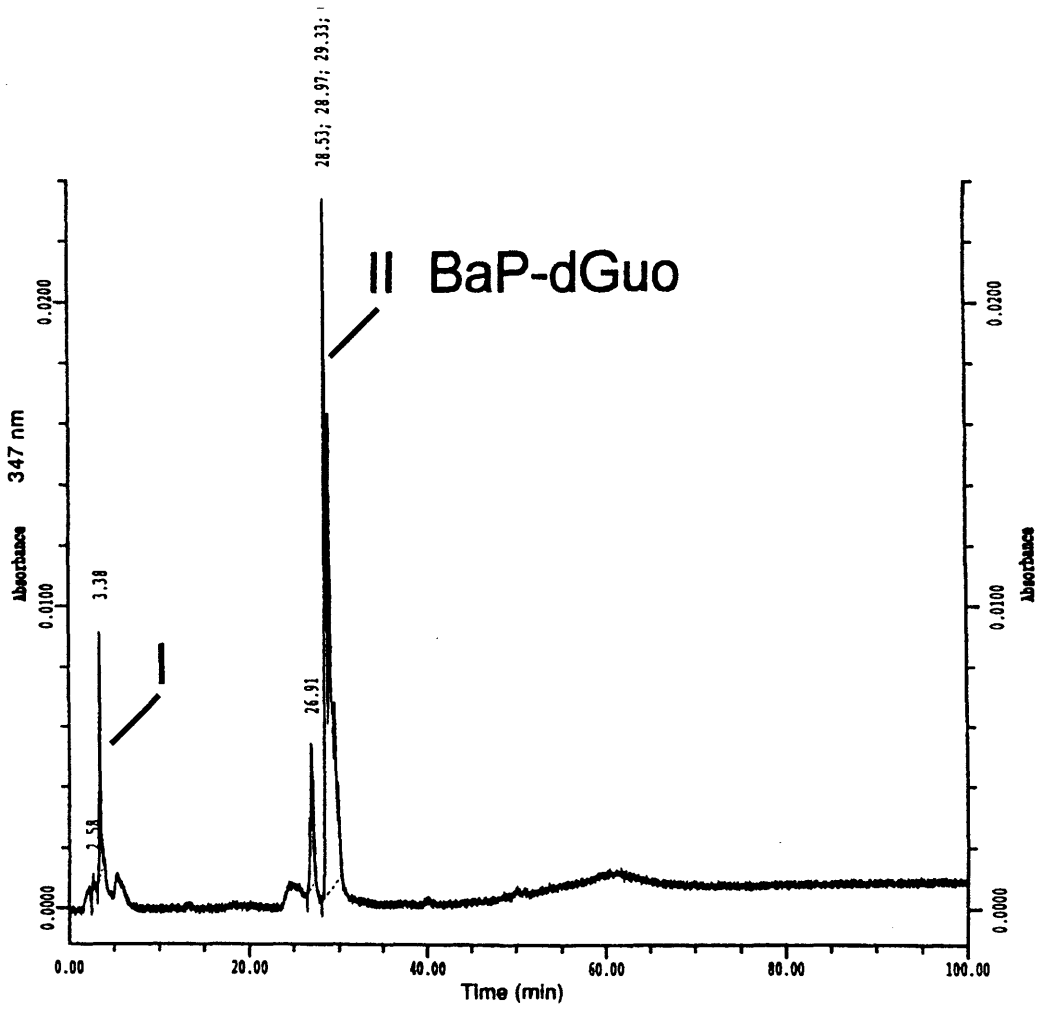


Figure 3.7 HPLC chromatograms of ^{3}H-BaP-dGuo after extraction and purification via sep-pak C18 column



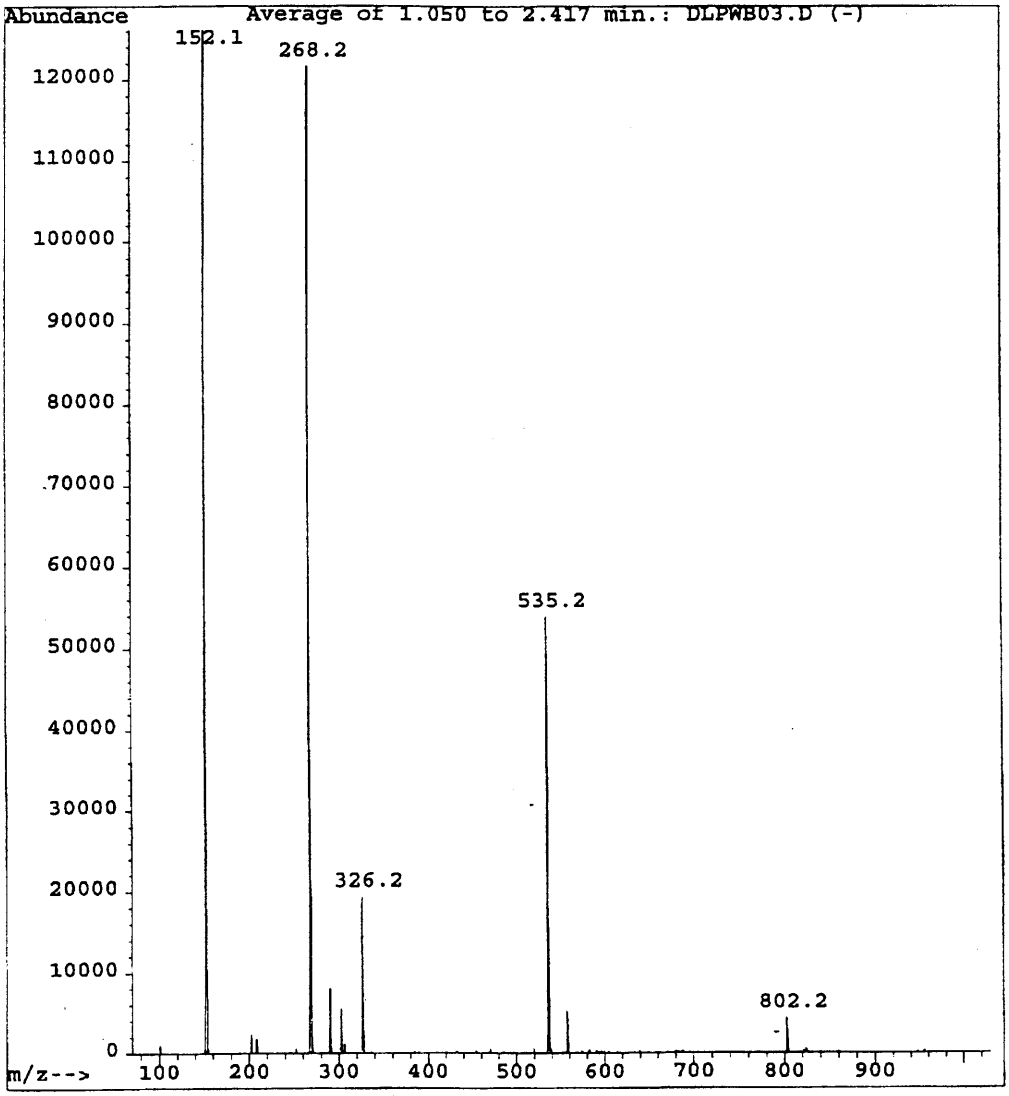
The peak at 35 minutes is identified as the ^{3}H-BaP-dGuo adduct
The adduct was made by reacting BPDE with ^{3}H-dGuo

Figure 3.8 HPLC chromatogram of BaP-dGuo



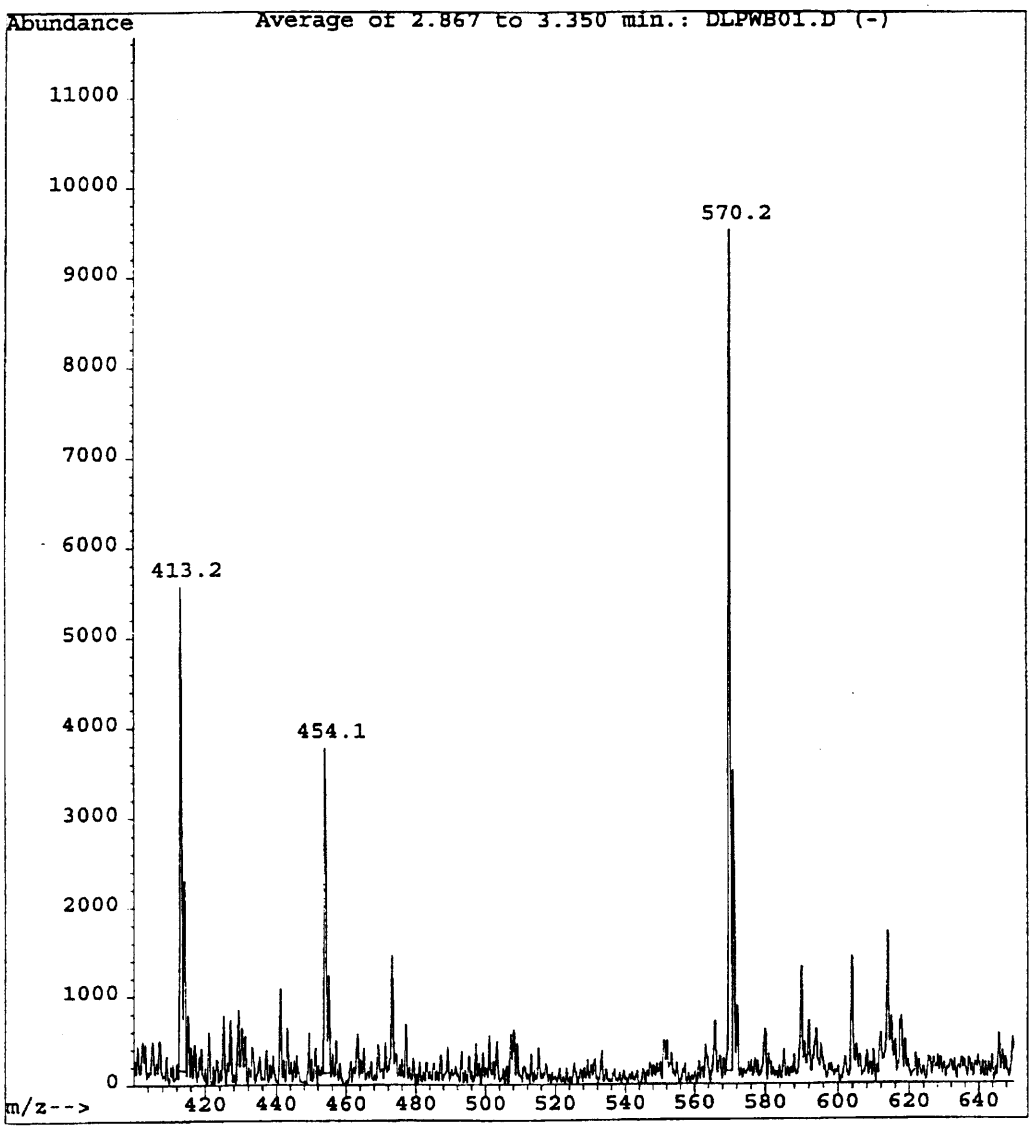
Fraction I: 2'-deoxyguanosine
Fraction II: BPDE modified 2'-deoxyguanosine

Figure 3.9 Electrospray-MS of Fraction I: deoxyguanosine



Deoxyguanosine m/z 151

Figure 3.10 Electrospray-MS of Fraction II: Benzo(a)pyrene-deoxyguanosine



BaP m/z 570

Figure 3.11 Electrospray-MS Fragmentation of m/z=570 peak from Fraction II

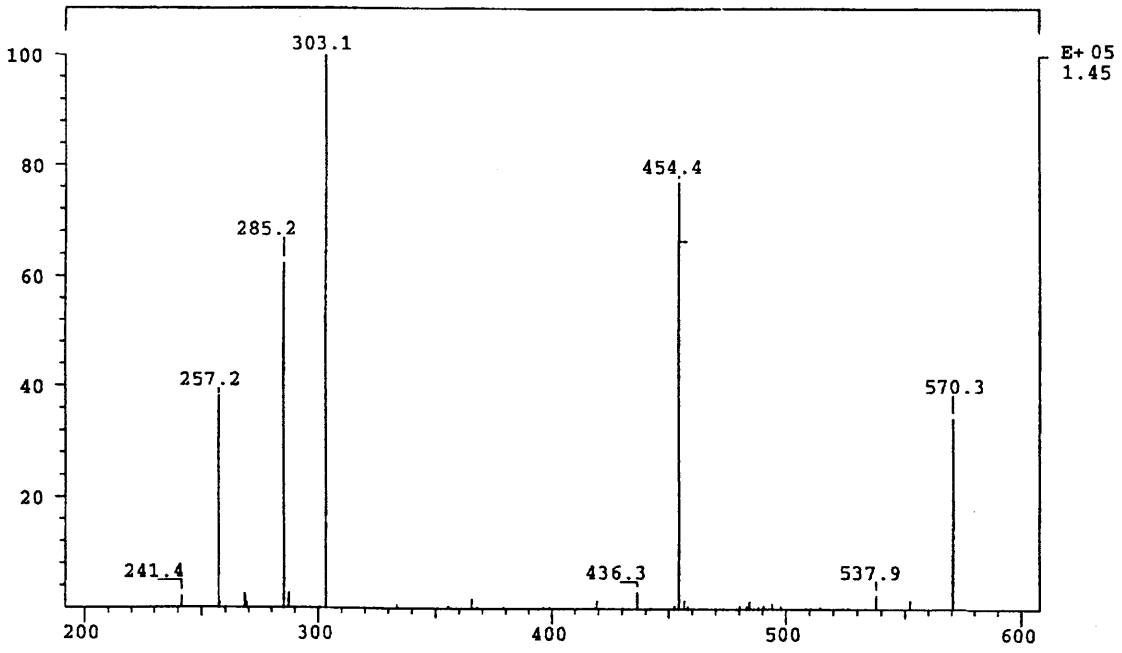
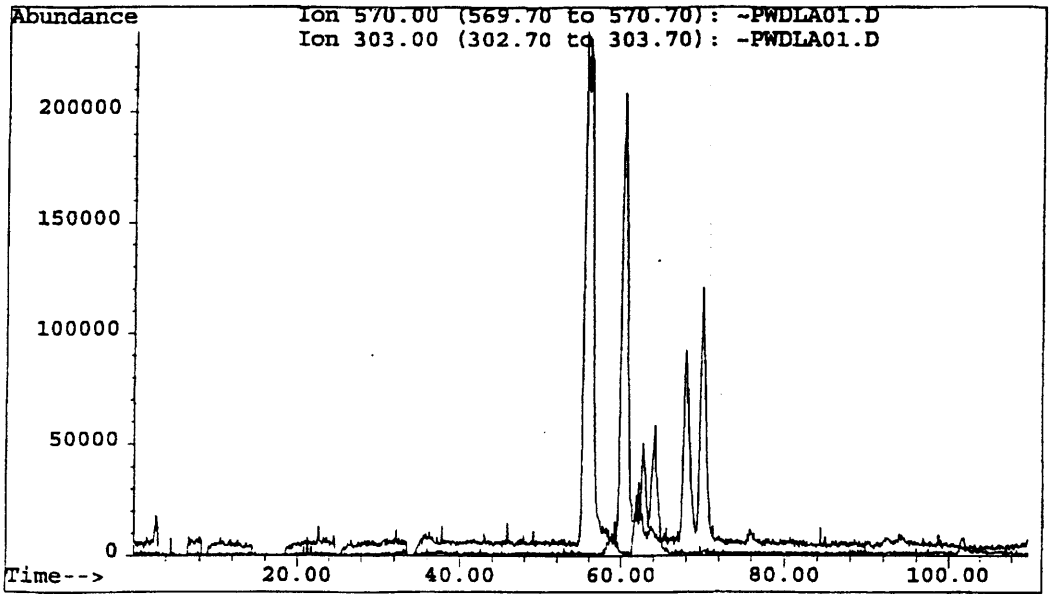
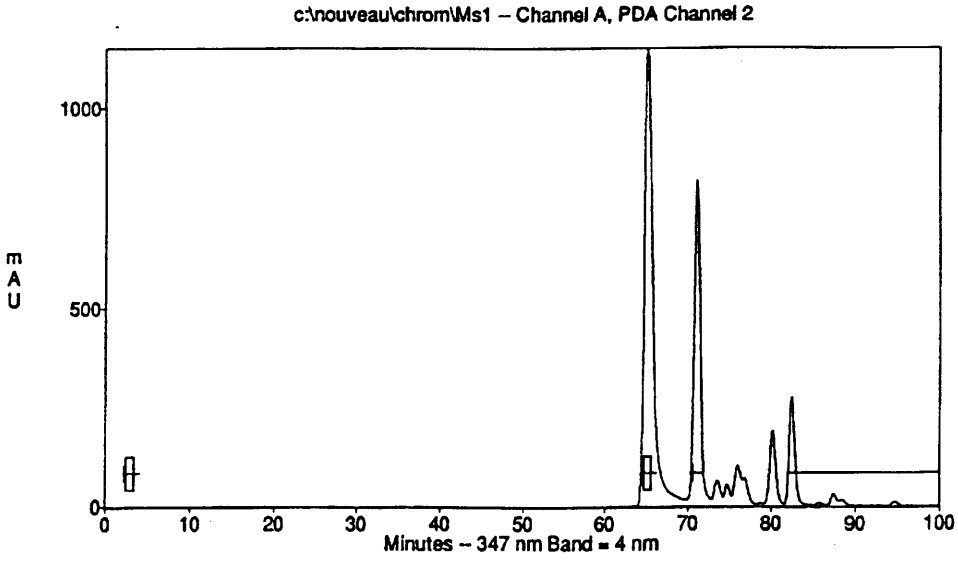


Figure 3.12 Electrospray-MS / HPLC analysis of BaP-dGuo





Top panel: Electrospray-MS
Bottom panel: HPLC chromatogram

Figure 3.13 HPLC chromatogram of oligonucleotide modified with BPDE

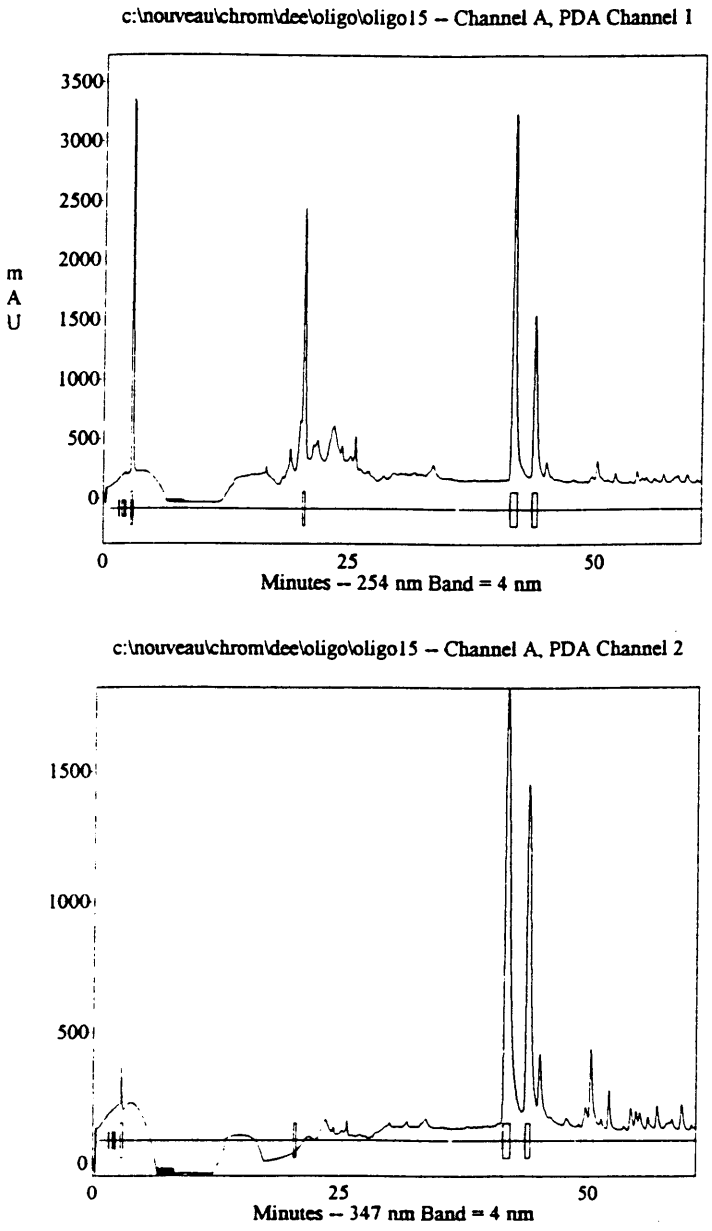


Table 3.1 Summary of HPLC solvent gradients

Name	Column	Solvents A:B	Gradient	Flow Rate ml/min.	Column Temp
A	Mac Mod Zorbax ODS 5μm 4.5x250mm	water:methanol	linear 0-25% B in 10' linear 25 -80%Bin 80' linear 80 -100% B in 10' isocratic 100% B for 10'	1	35°C
B	Rainin Microsorb C18 5μm 4.5x250mm	water:methanol	isocratic 40% for 13' linear 40-60% B in 10' isocratic 60% B for 12' linear 60-100% B for 20' isocratic 100% B for 25'	1	35°C
C	Mac Mod Zorbax ODS 5μm 4.5x250mm	water, pH 4.4 Buffered with acetic acid :acetonitrile	linear 2-20%B in 25' isocratic 20% B for 15' linear 20-60% B in 2' isocratic 60% B for 8' linear 60-100%B in 2' isocratic 100% B for 16'	1	RT
D	Phenomenex Ultramex 5 C18 5μm 250 x 10mm	20mM NaPhos pH7.1:methanol	linear 0-100%B in 60'	3	RT
E	Rainin Microsorb C18 5μm	20mM NaPhos pH7.1:methanol	linear 0-90%B in 60'	1	RT
F	Phenomenex Selectosil 5 C18 110A 5μm 150 x 1 mm	water:methanol Both with 0.5% acetic acid	linear 20-80%B in 80' linear 80-100% B in 10'	0.1	RT
G	Rainin Microsorb C18 5μm	water:methanol	isocratic 60% for 10' linear 60-80% B in 10' linear 80-100% B in 20' isocratic 100% B for 20'	1	35°C
H	Mac Mod Zorbax ODS 5μm 4.5x250mm	water:methanol	linear 25 -80%Bin 80' linear 80 -100% B in 10' isocratic 100% B for 30'	1	35°C
I	Mac Mod Zorbax ODS 5μm 4.5x250mm	water:methanol	linear 0-25% B in 10' linear 25 -80%Bin 80' linear 80 -100% B in 10' isocratic 100% B for 10'	1	35°C
J	Rainin Microsorb C18 5μm	water:methanol	isocratic 7% for 20' linear7-20% B in 15' linear 20-100% B in 20'	1	35°C

Note RT= room temperature

Table 3.2 Summary of microsomal activation of BaP in the presence of calf thymus DNA

Enzymatic Modification in DNA with ^{3}H-BaP					
Exp #	DNA (mg)	^{3}H-BaP	Recovery of ^{3}H-BaP(%)	Sp. Act. mCi/mmol	Modification Level
1				400	1:40,000
2	240	50 μCi	7 pmol (0.5)	12,500	1:700,000
3	4.5	50 μCi	88 pmol (6.3)	2,000	1:52,000
	4.5	50 μCi	115 pmol (8.2)	1,000	1:38,000
	4.5	50 μCi	350 pmol (20)	500	1:11,000
	4.5	50 μCi	276 pmol (20)	250	1:16,000
	4.5	50 μCi	160 pmol (11)	125	1:30,000
4	40	5 μCi	8 nmol	3000	1:12,000
5		2.5 mCi		833	1:28,000

Table 3.3 Modification with BaP

Substrate	Form of BaP	Recovery of BaP	Modification Level
CT-DNA	microsomes & ^{3}H-BaP	1.0%	1:50,000
CT-DNA	microsomes & BaP	2.0%	1:2,915
CT-DNA	BPDE	2.4%	1:592
2'-dGuo	BPDE	3.9%	1:563

Chapter 4 BaP-DNA Adduct Detection by Acylation With Methionine (ADAM)

4.1 Introduction

The ADAM method, introduced by our laboratory (Sheabar et. al., 1994), described the acylation of dGuo with ^{35}S-NHS-TBM. It was followed by a paper describing the application of this method to measure 4-ABP-dGuo adducts. The structural analysis of the reaction products showed that the 4-ABP-dGuo adducts can be measured by this method.

This chapter describes the further development of this method for detection of BaP-DNA adducts. The *in vitro* modified calf-thymus DNA described in Chapter 3 was used to measure the recovery and efficiency of the acylation reactions. First radioactive BaP-dGuo adduct was incubated with non-labelled NHS-TBM. The appearance of more non-polar reaction products made the application to measure BaP-dGuo seem feasible. The optimal conditions such as the equivalent molar ratio, temperature, solvent and time were established experimentally.

This chapter also describes difficulties encountered when with the use of ^{35}S-NHS-TBM. There were many sources of contamination that interfered with quantifying the reaction products such as poor purity of the labelling reagent, the use of Tris buffer, etc. As a result, an improvement of the method was introduced. This involved *in situ* synthesis of ^{35}S-TBM from ^{35}S-methionine which greatly improved the efficiency of the reaction.

The ADAM procedure (Sheabar, 1994) which subjects nucleoside adducts to acylation

with ^{35}S- methionine was successfully used to postlabel the BaP-dGuo adduct. Figure 4.1 includes a description of the ADAM method. The top panel shows the reaction using commercially available ^{35}S-TBM-NHS. Since BaP-dGuo is an N^2-adduct, the 3' and 5' hydroxyl groups are both available for acylation. An improvement of the ADAM method has been the result of using TBM for the acylation reaction instead of NHS-TBM. In addition to simplifying the reaction, the synthesis and purification of ^{35}S-TBM in the laboratory made it possible to control the purity and specific activity of the ^{35}S labeling reagent. The bottom panel of Figure 4.1 illustrates the acylation reaction involving the commercially available NHS-TBM or the *in situ* synthesized TBM and the nucleoside adduct. Both reactions produced more nonpolar reaction products that were analyzed via HPLC, because the reaction products were less polar, they eluted later than the labeling reagent and the starting adduct material. The results of applying the ADAM method to measure BaP-dGuo adducts are described here.

4.2 Materials

4.2.1 Chemicals

2'-deoxyguanosine and Sephadex LH-20 were purchased from Sigma Chemical Co (St. Louis, MO). 4-aminobiphenyl (4-ABP), triethylamine (TEA) and N',N'-diisopropylcarbodiimide (DIC), tetrahydrafuran (THF), pyridine, di-*tert*-butyldicarbonate, triethylamine (TEA), dimethylfomamide (DMF) were purchased from Aldrich Chemical Co. 2'deoxy-1',2'-

[^{3}H]guanosine 5'-triphosphate ([^{3}H]dGTP0 (31-37 Ci/mmol) were products of Amersham Co.; ^{35}S -Methionine (5mCi, 1000mCi/mmol) was purchased from NEN/Dupont Boston, MA). t-butoxycarbonyl-L-methionine,N-hydroxysuccinimidyl ester,TBM-NHS was purchased from Accurate Chemicals (Westbury, NY). t-butoxycarbonyl-L[^{35}S]-methionine,N-hydroxysuccinimidyl ester was purchased from Boehringer-Mannheim (Indianapolis, IN)

4.2.2 Chromatography

HPLC analyses were performed on a Hewlett Packard model 1090 M liquid chromatograph equipped with a Radiomatic flow-through radioactivity detector model A250. (Beckman HPLC model 126 equipped with a Radiomatic flow-through radioactivity detector model A280). Chromatographic conditions were as follows (Table 3.1): gradient B: isocratic 40% methanol/water, 13 min: linear gradient of 40-60%, 10 min; isocratic 60%, 12 min; linear gradient of 60 to 100% methanol, 20 min; and isocratic 100% methanol, 25 min; gradient G: isocratic 60% methanol/water, 10 min; linear gradient of 60-80%, 10 min; 80-100%, 20 min; and isocratic 100% methanol, 20 min. Flow rate was 1 ml per min for all gradients and the column temperature was 35°C.

4.2.3 ADAM method

Figure 4.2 describes the necessary preparation of the nucleoside adduct for the ADAM method. The DNA must be isolated, digested to nucleosides, purified and acylated with

^{35}S-labeling reagent. In order to isolate specific adducts of interest in a complex mixture, immunoaffinity chromatography can be introduced at several steps, such as purification of the nucleosides, before the acylation reaction and/or after the acylation reaction. However, immunoaffinity chromatography was not used in these experiments for the preparation of the BaP-adduct used for the acylation reactions. It was purified with a Sep-pak C18 column.

4.3 Acylation of benzo(a)pyrene-deoxyguanosine with TBM-NHS

4.3.1 Acylation with unlabelled TBM-NHS

Many parameters involved in the acylation reaction can be adjusted. The main limitation is that the adduct and solvents must be free of water. If there is water present its OH groups will compete for the labelling reagent and the reaction will not be efficient. The components of the reaction consist the dried adduct, nonaqueous solvent for the reaction, a base to establishe the appropriate pH, and the labelling reagent. DIC was later added to the reaction to regenerate the NHS-TBM. Other variables include that incubation time and temperature. The published method dissolved the adduct in THF, used TEA as a base and determined 50,000 molar equivalents of NHS-TBM. The reaction was incubated at 37°C for 2 hours.

Chomatographically pure ^{3}H-BaP-dGuo adduct (270 pmole) and nonradioactive dGuo-BaP were acylated as follows. Aliquots of dGuo-BaP and dGuo-^{3}HBaP(pmol) were dissolved in pyridine:THF (1:1) and acylated in the presence of TBM-NHS (50,000 molar equivalents), TEA

(1μl) and DIC (1μl) under anhydrous conditions. After incubation for 2 hours at 37°C, samples were cooled, dried *in vacuo* and stored at -20°C. Samples were redissolved in 50% methanol/water and analyzed by RP-HPLC. Three solvents, namely THF, pyridine and DMSO, and three temperatures (room temperture, 37°C and 75°C) were tried to acylate BaP-dGuo.

4.3.2 Acylation of BaP-dGuo adduct with ^{35}S-TBM-NHS

The ^{35}S-TBM-NHS was supplied at a specific activity of >800 Ci/mmol. The same conditions found successful for the acylation with unlabelled TBM-NHS were used for acylation of 5 pmole of the same adduct with ^{35}S-TBM-NHS ester. A molar equivalent of 400,000 was maintained for the reactions and the adduct was dissolved in pyridine. The mixture was incubated at 37°C for 2 hours.

After the acylation reaction, a solvent extraction clean-up step (Figure 4.3) was necessary to remove contaminants that could hide the signal given by the reaction products. Samples acylated with ^{35}S-labeling reagent were reconstituted in 50 μl methanol (2 times), 20 μl methanol, 250 μl H_20 (2 times) and transferred into 15 ml polypropylene tubes. The samples were then extracted 7 times with 3 ml of toluene. The toluene layer was discarded and the remaining aqueous portion was transferred to a 600 μl tube. The volume was reduced via rotary evaporation for 15-30 minutes and analyzed by HPLC. Hexane was later used, instead of toluene, as a solvent to clean up the reaction product contaminants. The recovery of the adducts through this process was determined for [^{3}H]dGuo, 8-oxo-[^{3}H]dGuo, [^{3}H]dGuo-4ABP and [^{3}H]BaP-dGuo. Since the purity of the ^{35}S-labelling reagent could not be guaranteed to be more than 40%

pure, often contaminants that were present obscured the signal that would be used to detect the carcinogen. A Sep-pak step was added after the solvent extraction step.

In order to determine the signal to noise ratio, 7.5 pmol of ^{3}H-BaP-dGuo was acylated with ^{35}S-TBM-NHS (3.1 mCi/mmol). A molar ratio of labelling reagent was 400,000 times the amount of adduct. Each reaction mixture was incubated for 37°C for 2 hours, extracted with either toluene or hexane and analyzed by RP-HPLC, using gradient H.

4.3.3 Results and discussion

Several variables of reaction conditions were modified, including solvent, amount of base, amount of labelling reagent, temperature, and length of incubation time. The incubation at 75°C resulted in the degradation of the BaP-adduct, while incubation at 37°C worked very well. The BaP-dGuo adduct did not dissolve completely in THF, but went into solution quickly with pyridine. However, the TBM-NHS required the addition of THF in the reaction to be completely dissolved. Figure 4.4 illustrates the successful acylation of BaP-dGuo with TBM-NHS. The top panel shows the reaction with the adduct dissolved in pyridine and incubated with TEA and DIC, but no TBM-NHS. The bottom panel shows the same reaction, but incubated in the presence of TBM-NHS (molar ratio 1:400,000).

The ^{35}S-TBM-NHS reaction products co-elute with the ^{3}H-BaP-dGuo adduct reaction products at 96 and 98 minutes (Figure 4.5). This is strong evidence to support the interpretation that the later appearing peaks were the expected reaction products: Figure 4.6 illustrates ^{35}S-TBM-NHS incubated in the presence of 5 pmol of ^{3}H-BaP-dGuo adduct (panel A) and incuabated alone (panel B). The peaks present in the 95 minute to 100 minute region were

acylated products. When the amount of ^{3}H-BaP-dGuo adduct was increased (0.2 pmole-5pmole), the amount of ^{35}S-acylated products increased linearly (Figure 4.6, panel C). Figure 4.7 and 4.8 show the HPLC chromatograms of the ^{35}S-TBM-NHS reagent control (no adduct in the mixture) and the reaction mixture in the presence of the adduct. In both cases, the addition of the adduct to the mixture resulted in the appearance of later appearing peaks. The extraction with the toluene is easier than the extraction with hexane followed by Sep-pak procedure. But the amount of acylation products lost during the clean up step was also examined. Table 4.1 shows the recovery of the different adducts during the solvent extraction process after being acylated with TBM-NHS. The toluene extraction resulted in more of the acylation products being lost (16%) versus only 3% lost in the hexane extraction clean-up step.

The signal to noise ratio is summarized in Table 4.2. The acylated reaction products appear in the region of 95-100 minutes. The background ^{35}S radioactivity was determined for the region of 85-90 minutes. In the case where no adduct was added to the reaction, the background ^{35}S radioactivity ranged from 29,264 to 41,560 cpm. The radioactivity present in the region, where acylated products would appear, ranged from 28,776 to 49,504. The average net ^{35}S radioactivity was 2828cpm. In the case where the acylation products of the adduct were toluene extracted, the net ^{35}S radioactivity was 25,406 cpm and 48,820 cpm ($\bar{x}$=37,113). The net cpm of the adduct acylation products after hexane extraction were in the same range (40,920 cpm and 53,616 cpm; $\bar{x}$=47,268). The signal is at least one order of magnitude above the background ^{35}S noise.

Optimal reaction conditions were as follows: The temperature of 37°C and incubation for 2 hours was used for the rest of the reactions. The reaction mixture did not require TEA, so it

was eliminated as the reaction condition requirement. DIC was needed to enhance the reaction. The labelling reagent was dissolved in THF and the reaction mixture maintained a 1:1 THF:pyridine ratio. The amount of molar equivalents needed for the reaction was also examined. The acylation reaction plateaued at 50,000 molar equivalents; therefore, the use of 400,000 was not necessary and when analyzing human samples would greatly decrease the specific activity and as a result, the sensitivity would be greatly comprimised. In addition, it was extremely difficult to get the entire sample into solution for HPLC analysis. As a result 50,000 molar equivalents were used in all further reactions.

4.4 Acylation of benzo(a)pyrene with TBM

An improvement of the ADAM method has been the result of using TBM for the acylation reaction instead of NHS-TBM. In addition to simplifying the reaction, the synthesis and purification of ^{35}S-TBM in the laboratory made it possible to control the purity and specific activity of the ^{35}S labeling reagent. Figure 4.1 illustrates the acylation reaction involving the commercially available NHS-TBM or the laboratory synthesized TBM and the nucleoside adduct. Both reactions produce in more nonpolar reaction products which can be analyzed via HPLC.

4.4.1 Synthesis of ^{35}S-TBM

The protection reaction of ^{35}S-methionine was performed as follows (Figure 4.9):

^{35}S-methionine (5mCi, 1000Ci/mmol) was thawed and transferred to a 5 ml glass tube. The solution was evaporated dry (2-3 hours) and kept on dry ice. Di-tert-butoxy-dicarbonate (5 µl), DMF (50µl), TEA (1µl) were added the mixture and incubated for 15 minutes at room temperature. Purity and radioactivity determination were determined from an aliquot (1 ml) was removed from the mixture and added to 5 ml H_20. The reaction mixture was evaporated (70 minutes), redissolved in 2 ml methanol and 200 ml H_20 and further purified via HPLC. Fraction 2 was collected and concentrated. The H_20:methanol HPLC gradient (gradient J) used for purity analysis and purification is was 7% MeOH isocratic for 20 minutes, isocratic 20% meOH for 15 minutes, linear gradient to 100% over 20 minutes.

4.4.2 Acylation of BaP-dGuo with ^{35}S-TBM

Cold BaP-dGuo adducts (2 nmol), dissolved in a mixture of pyridine:THF, was reacted with ^{35}S-TBM (1000 Ci/mmol) for 2 hours at 37°C. The reaction mixture was cooled, vacuum dried and the samples were incubated for an additional 30 minutes, 37°C before being stored at -20°C. The reaction products were hexane extracted and analyzed via RP-HPLC.

After the acylation reaction, a solvent extraction clean-up step is necessary to remove contaminants that would hide the signal given by the reaction products. Samples acylated with ^{35}S-labeling reagent were reconstituted in 50 ml methanol (2 times), 20 ml methanol, plus 250 ml H_20 (2 times) into 15ml polypropylene tubes. The samples were then extracted 7 times with 3ml of hexane. The hexane layer was discarded and the remaining aqueous portion was transferred to a 600 ml tube. The volume was reduced via rotary evaporation for 15-30 minutes and analyzed

by HPLC.

A radio-chromatogram, via the on-line radioactivity detector and the UV chromatogram, via the diode array detector were generated simultaneously (Figure 4.10). The acylation products as determined by absorbance at 347nm and ^{35}S both co-elute at 58-60 minutes. This provided additional evidence that the reaction was producing acylated BaP-dGuo adduct.

To further investigate the acylation reaction of BaP-dGuo with TBM, various amounts (fmole-pmole) ^{3}H-BaP-dGuo were dried and reacted with *in situ* synthesized and purified ^{35}S-TBM. The specific activities ranged from 0.4mCi-1000Ci/mmol). The ratio for the sample with the most adduct was calculated. The ^{35}S-TBM was prepared and 10µl of the mixture was added to each sample already dissolved in pyridine:THF. The samples were incubated at 37°C for 2 hours. The reaction mixture was cooled, vacuum dried and the samples were incubated for an additional 30 at 37°C before being stored at -20°C.

4.5 Results and discussion

A reproducible linear response was observed between the amount of substrate (^{3}H-BaP-dGuo) and the area of ^{35}S peaks (95 minutes-100 minutes). The ratio of 1:400,000 was used for the 2 different specific activities. Various amounts of dried ^{3}H-BaP-dGuo (0 fmol, 5 fmol, 10 fmol, 20 fmol, 50 fmol, 100 fmol and 1000 fmol) were reacted with ^{35}S-TBM at a specific activity of 1000 Ci/mmol. This produced a linear response (R=0.9279). This linear response was reproduced (R=0.860) using different amounts of ^{3}H-BaP-dGuo i.e., 50 fmol, 100

fmol, 200 fmol, 500 fmol and 1000 fmol, and a specific activity of 10 Ci/mmol. (Figure 4.11). These results demonstrated a linear and highly significant correlation between the amount of adduct reacted and total ^{35}S radioactivity detected in the acylated products.

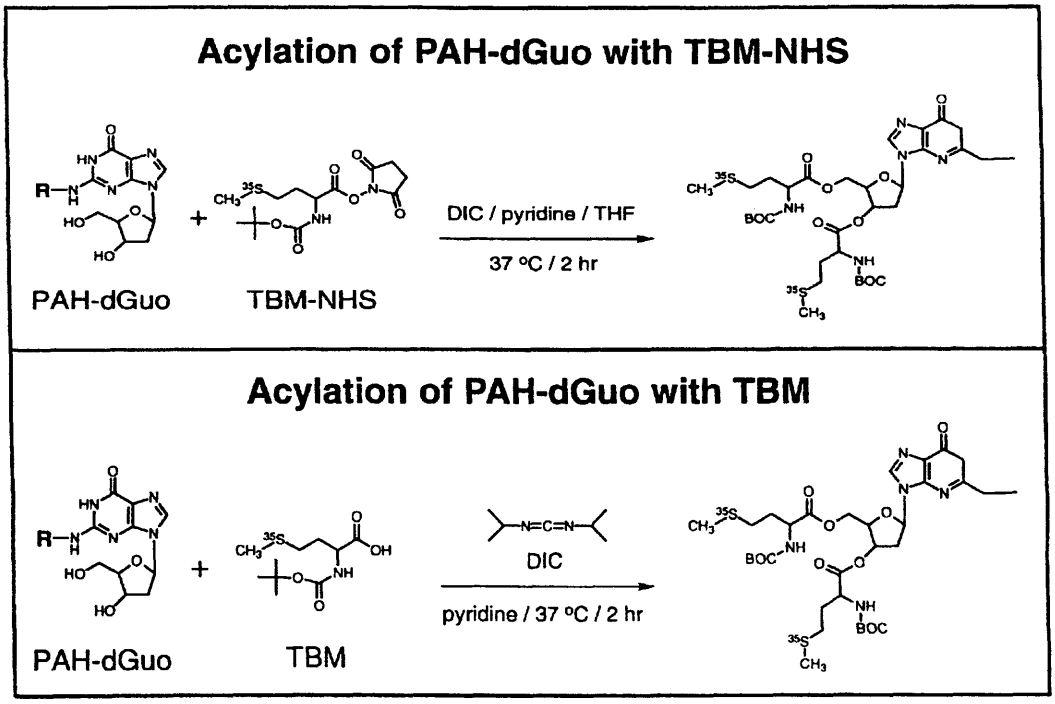
Recently, the cleanup procedure, performed after the acylation reaction, was abandoned. HPLC purification of the ^{35}S-TBM, prior to acylation, seemed to be adequate for detection of carcinogen-adducts. The acylation reaction of the BaP-dGuo standard made with protected 2'-dGuo is shown in Figure 4.11. The acylation products eluted at the same retention time as the acylation products of the standard made with calf-thymus DNA.

4.6 Conclusions

It was demonstrated that incubation of chomatographically pure ^{3}H-BaP-dGuo adduct (270 pmole), dissolved in pyridine:THF (50:50), in the presence of unlabeled TBM-NHS (1:50,000) for 2 hours at 37°C and analyzed by RP-HPLC produced reaction products at a 70% efficiency. The peaks between 55 minutes-60 minutes co-eluted in both the liquid chromatogram and the radio-chromatogram. The reaction products co-eluted with those obtained by acylating 5 pmol of ^{3}H-BaP-dGuo with ^{35}S-TBM-NHS. In addition there was a linear correlation between the amount of ^{3}H-BaP-dGuo used for the reaction and the area of the ^{35}S-acylated products. Specific activities of ^{35}S-TBM-NHS from 0.4 mCi/mmol to 10 Ci/mmol to 1000 Ci/mmol were found to be successful. The use of hexane or toluene, after acylation with ^{35}S-TBM-NHS proved useful to get rid of contaminants that would interfere with the detection of the acylation products.

^{35}S-TBM can be synthesized and purified in our laboratory. A linear relationship was observed between the amount of adduct versus the amount of ^{35}S acylated products when ^{3}H-BaP was reacted with ^{35}S-TBM. The acylation of BaP-dGuo was successful. Now that the purity of the reagent can be controlled, the solvent extraction performed after acylation is no longer necessary.

Figure 4.1 Description of Adduct Detection by Acylation with Methionine (ADAM)



Top panel: Reaction with commercially available ^{35}S-TBM-NHS
Bottom Panel: Reaction with ^{35}S-TBM synthesized from methionine

Figure 4.2 Flow diagram for carcinogen-nucleoside labelling with ADAM

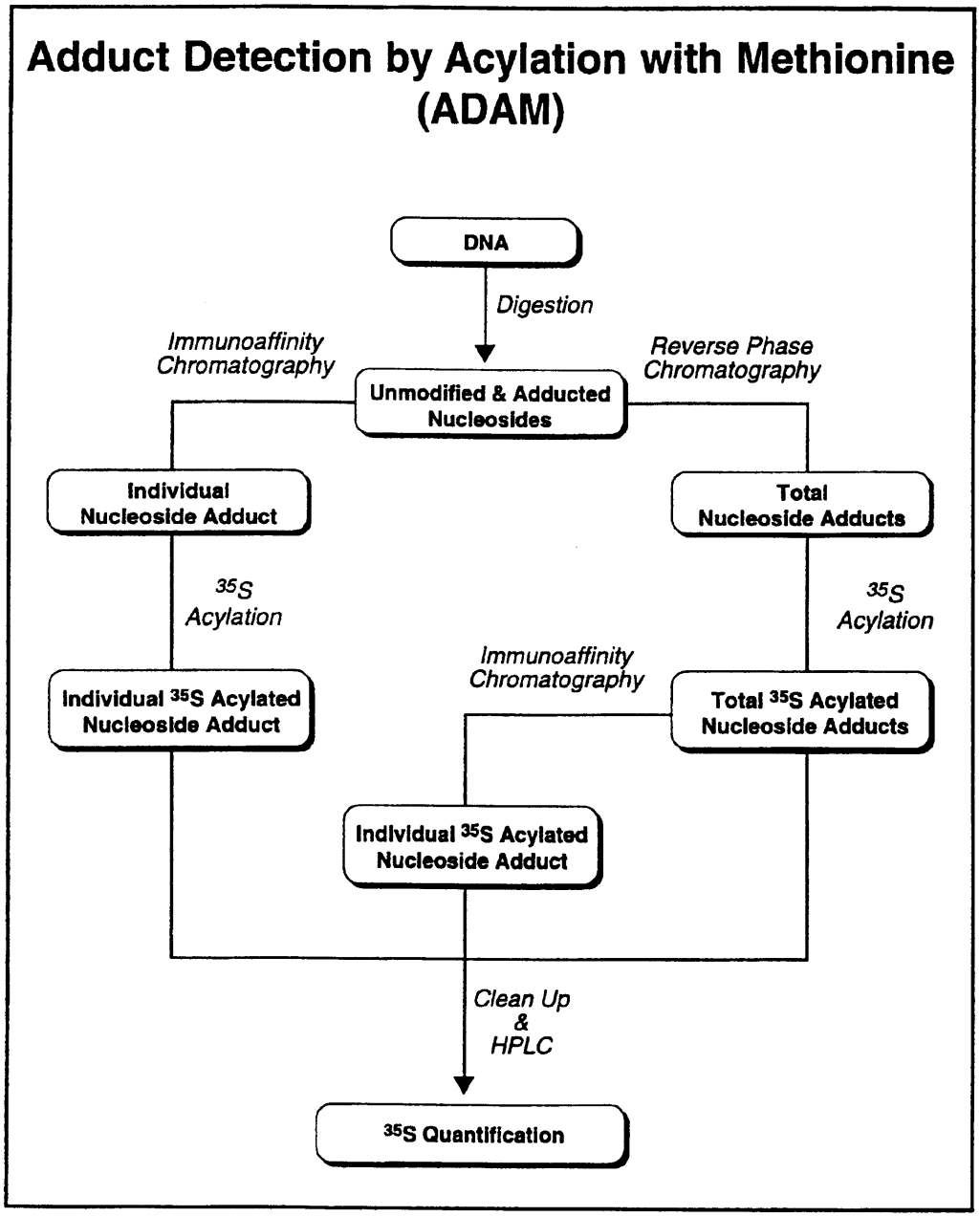


Figure 4.3 Flow diagram for solvent extraction of acylated products

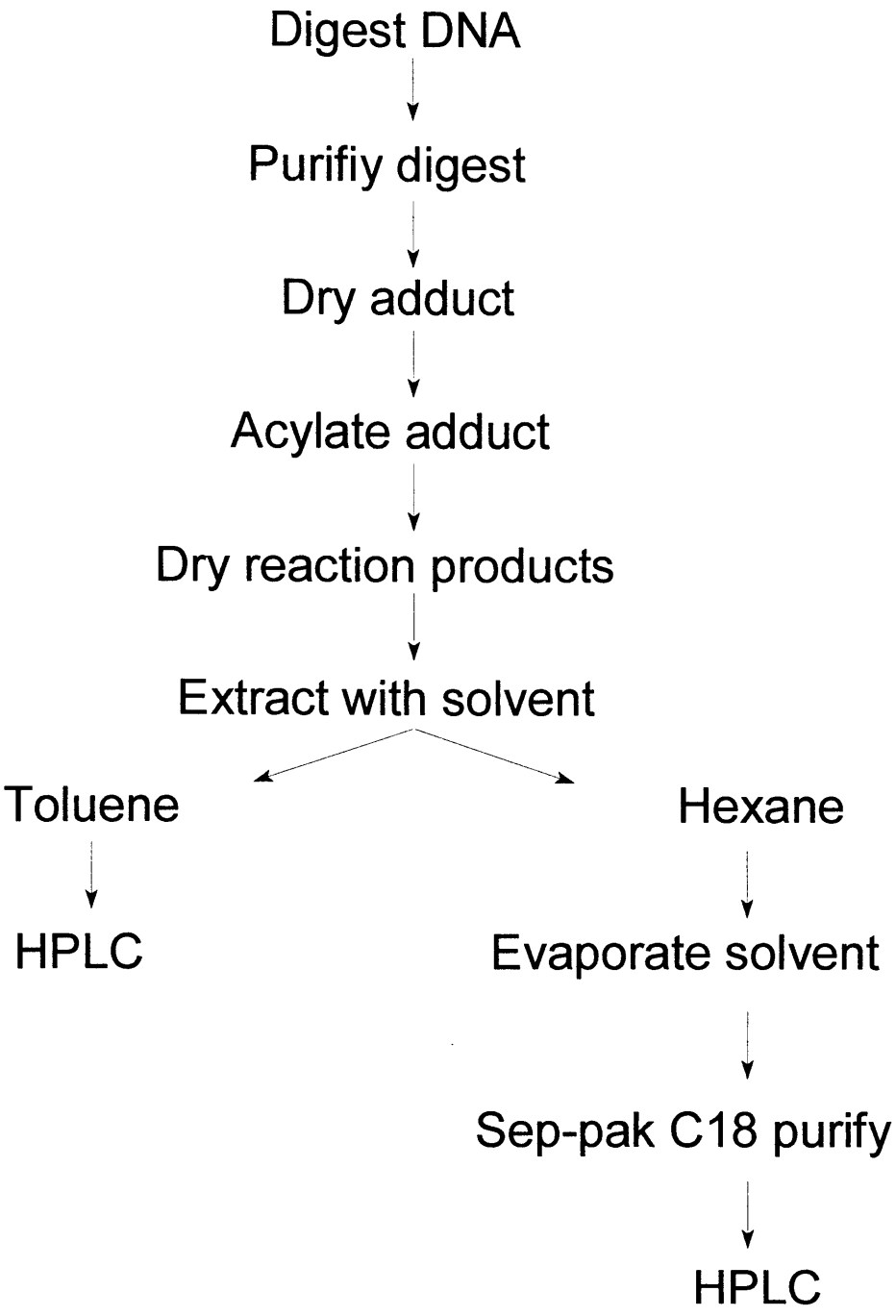


Figure 4.4 Acylation of BaP with unlabelled TBM-NHS

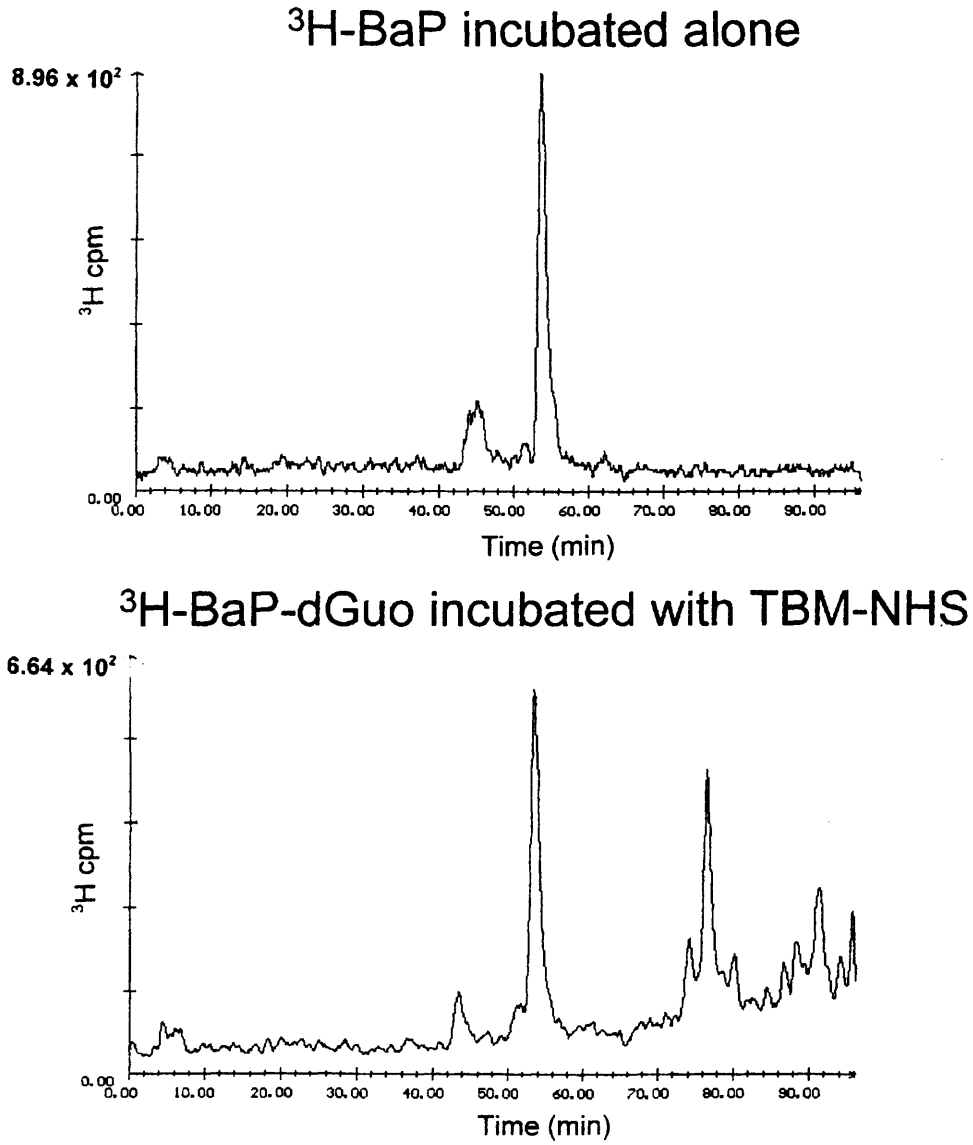
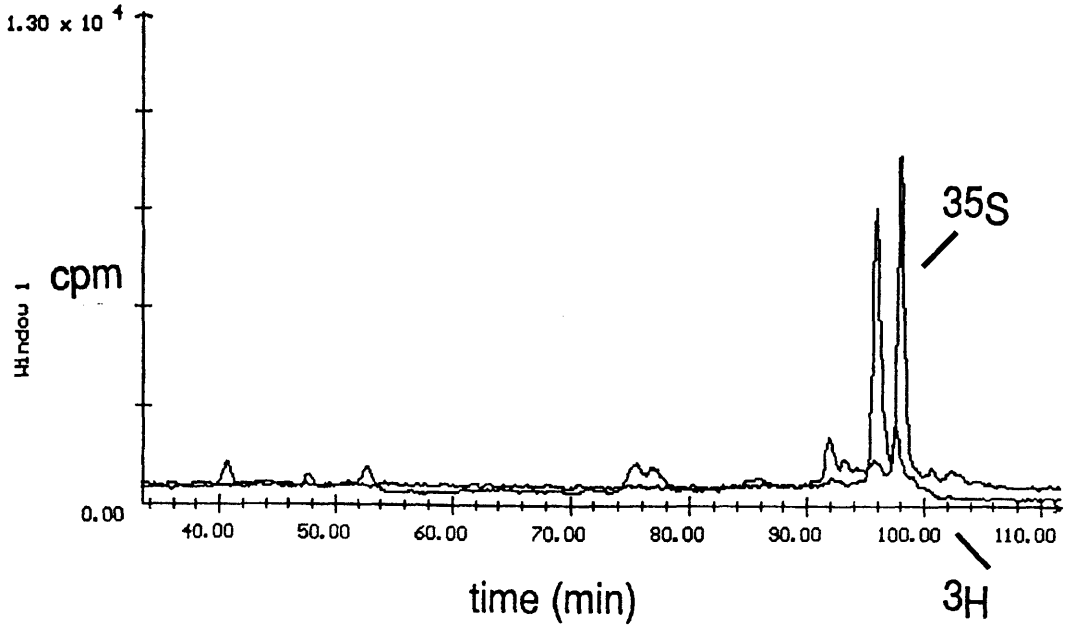
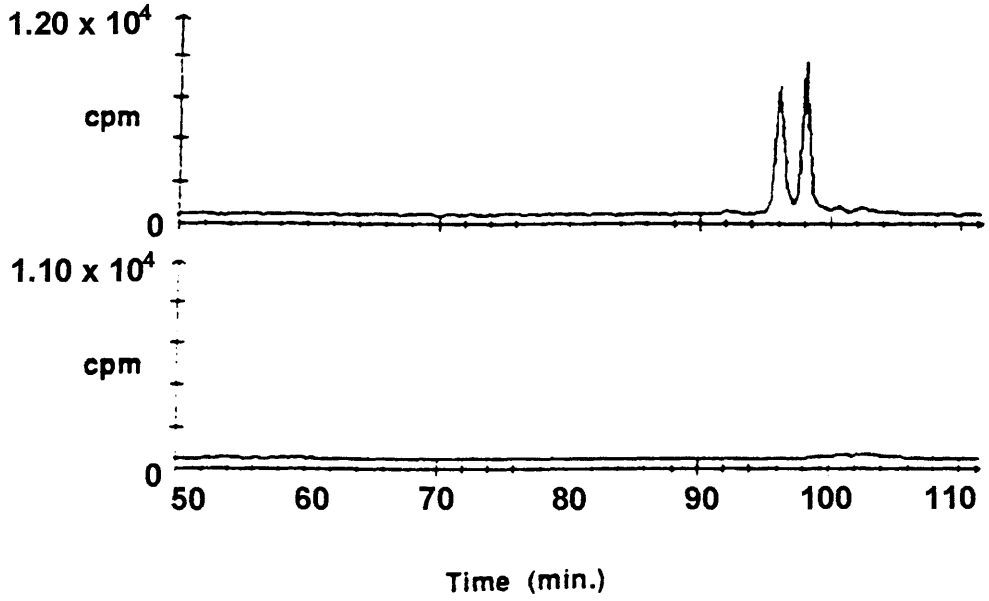


Figure 4.5 Co-elution of reaction products after acylation of BaP-dGuo with TBM-NHS



Top line: BaP-dGuo acylated with ^{35}S-TBM-NHS
Bottom line: ^{3}H-BaP-dGuo acylated with unlabelled TBM-NHS

Figure 4.6 Acylation of BaP-dGuo with ^{35}S-TBM-NHS



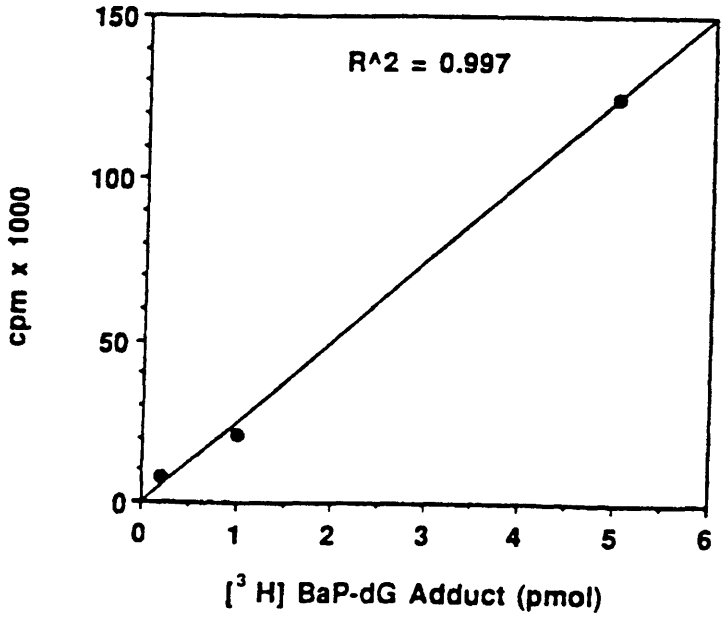


Figure 4.7 HPLC chromatogram of BaP-dGuo acylated with ^{35}S-TBM-NHS after toluene extraction

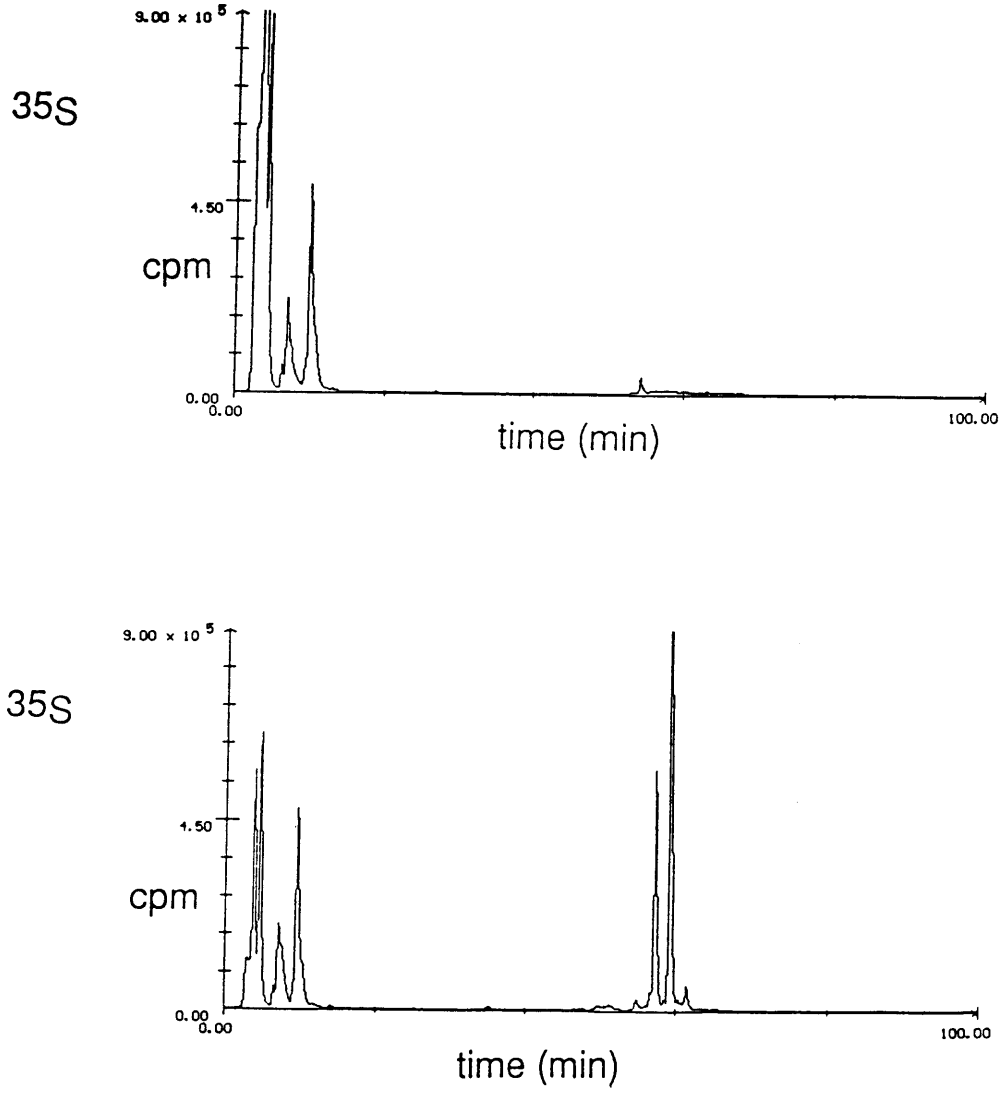
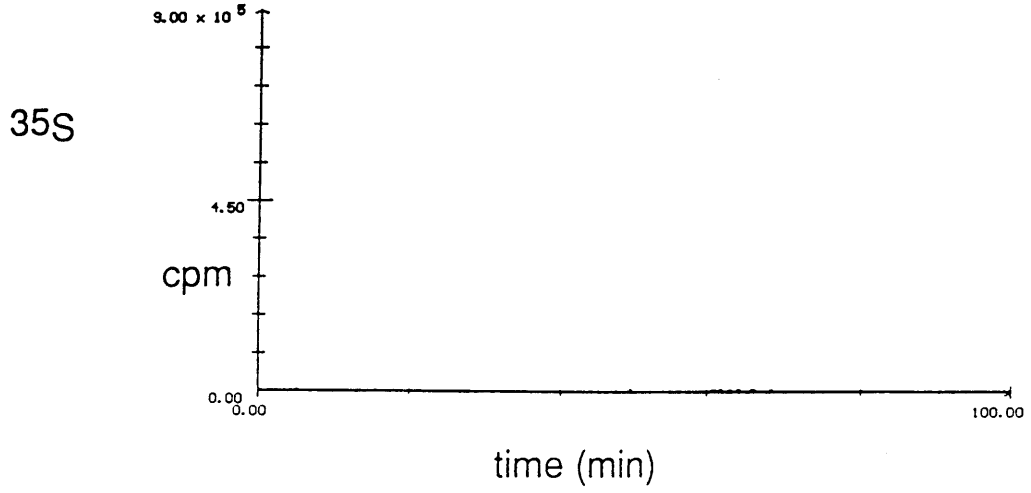


Figure 4.8 HPLC chromatogram of BaP-dGuo acylated with ^{35}S-TBM-NHS after hexane extraction and Sep-pak purification



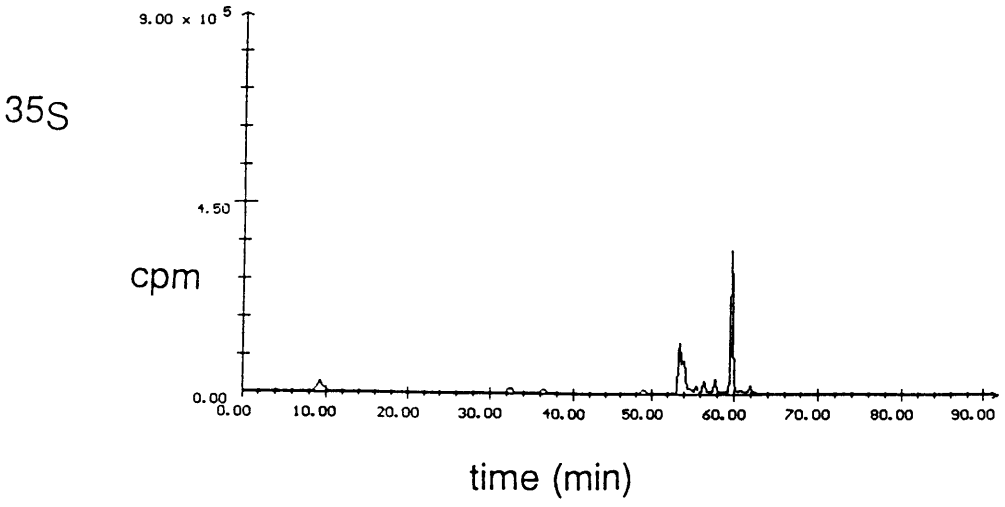


Figure 4.9 *In situ* synthesis of ^{35}S-TBM

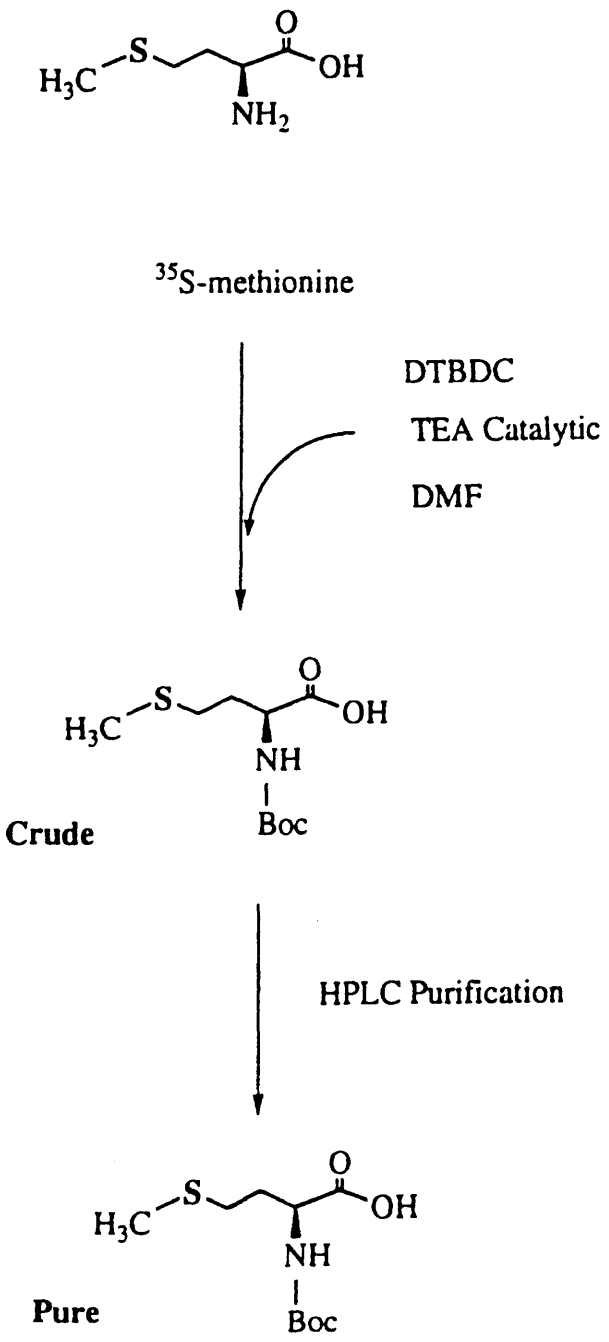
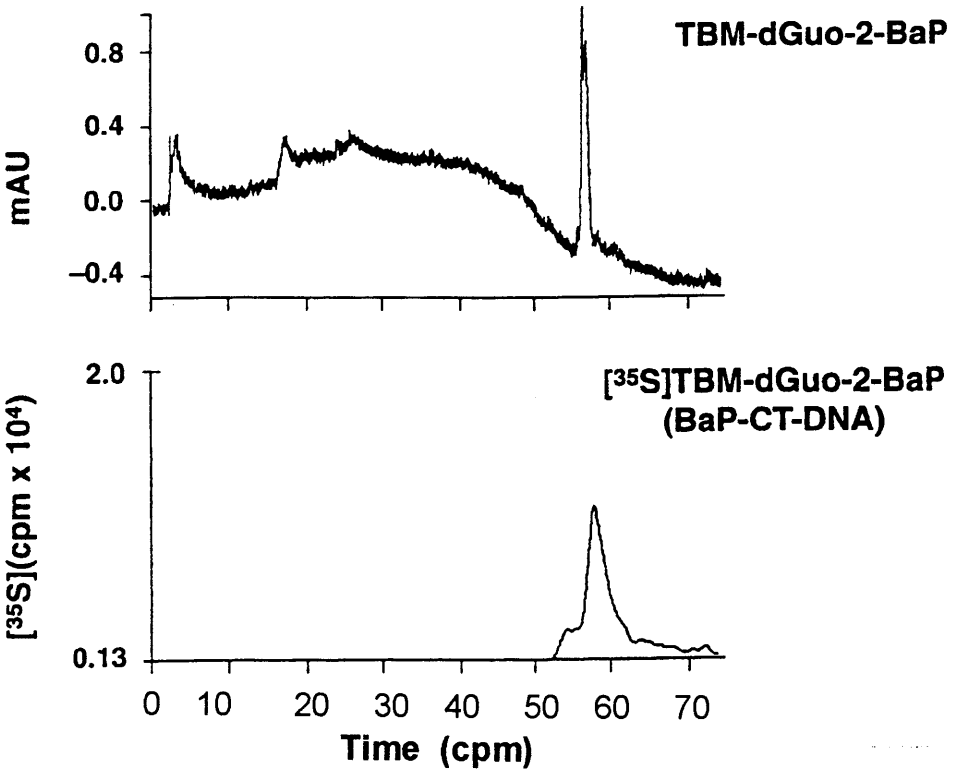


Figure 4.10 HPLC chromatogram of BaP-dGuo acylated with ^{35}S-TBM



Top panel: UV HPLC chromatogram 254 nm
Bottom panel: ^{35}S (cpm)

Figure 4.11 Linear correlation between ^{3}H-BaP-dGuo and ^{35}S acylated products

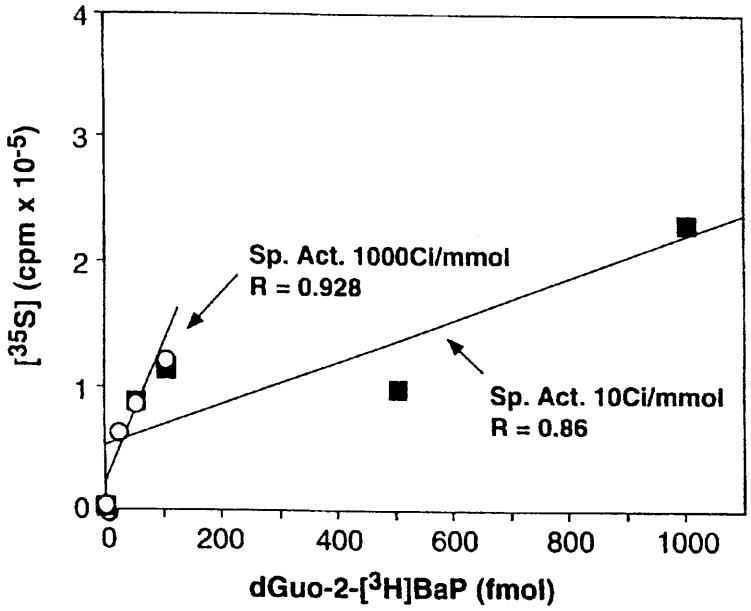


Table 4.1 Recovery of adducts during solvent extraction after acylation with ^{35}S-TBM-NHS

Acylation Products Lost during Extraction (%)

	[^{3}H]dGuo	8-Oxo-[^{3}H]dGuo	[^{3}H]dGuo-8-ABP	dGuo-2-[^{3}H]BaP
Toluene	36.1	15.8	58.5	18.9
	27.2	21	84.2	13.9
	27.8		26.9	
	13.2		45.8	
	12.3		90.3	
	41.3		51.9	
			76.2	
	26.3±11.8	18.4±3.7	62.0±22.7	16.4±3.5
Hexane	1.6	N.D.	10.4	3.2
	1		11.6	3.2
	1.4		6.3	
	0.9		3	
	1.8		3.8	
			2.3	
			6.8	
			7.2	
	1.3±4.4		6.4±3.4	3.2±0

Table 4.2 Signal to noise ratio

^{35}S acylated products: Toluene vs. Hexane Extraction				
Adduct	Solvent	Signal 95'-100'	Noise 85'-90'	Net Cpm
none	hexane	41,184	41,560	-376
none	hexane	49,504	41,408	8,096
none	toluene	28,776	29,264	-488
none	toluene	34,112	30,032	4,080
7.5 pmol	toluene	96,900	48,080	4,8820
7.5 pmol	toluene	64,936	39,520	25,406
7.5 pmol	hexane	109,360	55,744	53,616
7.5 pmol	hexane	100,208	59,288	40,920

Chapter 5. DNA Adducts of Benzo(a)pyrene: Animal Studies

5.1 Introduction

Since BaP is an animal carcinogen, mice were treated with BaP, to test the applicability of the ADAM method to BaP-DNA adducts formed in living tissues. The optimal acylation conditions for the analysis of BaP-dGuo were determined with *in vitro*-modified calf thymus DNA. The molar ratio needed to obtain the best reaction efficiency was 1:50,000, incubated at 37°C for 2 hours. These reaction conditions were to be used for *in vivo* samples.

This chapter describes two animal experiments. In the first experiment, mice were given a large dose of highly radioactive benzo(a)pyrene and the samples were taken 24 hours later. The use of radioactivity (^{3}H) provided an independent means to monitor the recovery of the BaP-dGuo throughout the process of DNA isolation up to the acylation products. In addition, a radio-chromatogram was generated from the ^{3}H-BaP given to the animals. The second purpose of this experiment was to determine if DNA could be isolated from paraffin embedded tissues. Most studies which used postlabelling procedures analyzed fresh or frozen tissue. Many pathology laboratories store tissue biopsies embedded in paraffin. Goelz (1995) showed that DNA can be isolated from wax-embedded tissues and used to detect point mutations in specific genes. It would be valuable to use the vast resource of archival material

to detect the presence of carcinogen-DNA adduct to obtain evidence of past exposure. ^{32}P-postlabelling analysis of fixed tissues was carried out in rats treated with BaP and AAF (Hewer and Phillips,1993). The adduct levels were shown to be stable even in tissues fixed for 92 days and then embedded. In pathology laboratories, the time of fixation can be indefinite. In the experiment reported here, the livers from the animals were divided into 4 sections. One section was frozen the other 3 sections were fixed in 10% formalin for 3 different time periods. The chromatography and acylation results are reported here. The amount of ^{35}S incorporated in authentic ^{3}H-BaP-DNA adduct samples were used for the calibration and quantification of the BaP-DNA adduct levels in both the frozen and embedded tissues.

The purpose of the second experiment was to analyze BaP-DNA adducts in mice that were treated before being weaned with a dose of BaP that will cause tumors. The newborn mouse lung adenoma assay (Busby, 1984) was used for 3 reasons: (1) The assay has proven to successfully produce tumors in mice treated with polycyclic aromatic hydrocarbons; (2) The results can be seen within a short period of time. Lung tumors appear within 6 months; (3) Adduct levels can be compared with tumor frequency. The target tissues in this assay are the lungs (primary) and liver (secondary). Previous results (Busby, unpublished) involving the tumorigenicity of BaP showed that a total dose of 210 μg produced 3.85 lung tumors/mouse and 28% liver tumors in males. The total dose of 150 μg produced ~2 lung tumors/mouse. In this study, lung tumors were produced in all of the male litters. The DNA was isolated from the tissues. The ADAM method was used to determine the level of modification in the lungs and livers and are recorded here.

Chemicals

Benzo(a)pyrene was obtained from Aldrich Chemical Company (Milwaukee, WI) . Rat liver cytosol was prepared from postmitochondrial supernatant fractions (SL-9) supplied by Molecular Toxicology, Inc. (Annapolis, MD). ^{3}H-BaP was purchased from Amersham Corporation (Arlington Heights, IL). anti-benzo(a)pyrene-r-7, t-8-dihydrodiol-t-9, 10-epoxide (±) was purchased from NCI Chemical Carcinogen Reference Standard Repository (Frederick, MD). Dimethylsulfoxide (DMSO) was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI) and stored under argon. ^{35}S -Methionine (5mCi, 1000mCi/mmol) was purchased from New England Nuclear Products/Dupont (Boston, MA). *t*-butoxycarbonyl-*L*-methionine, N-hydroxysuccinimidyl ester, TBM-NHS was purchased from Accurate Chemicals (Westbury, NY). Sep-pak C18 environmental cartridge were obtained from Waters (Milton, MA). Nuclease P_1 (NP_1 from *Penicillium citrinum*) was purchased from Sigma Chemical Company (St. Louis, MO). Snake venom phosphodiesterase from ICN Pharmaceuticals (Costa Mesa, CA). Proteinase K (from Tritirachium album), Rnase A and Alkaline Phosphatase from calf intestine were purchased from Boerhinger Mannheim Biochemicals (Indianapolis, IN). Flo-Scint III scintillation fluid was purchased from Packard Instrument Company (Downers Grove, IL). N', N',-diisopropylcarbodiimide was obtained from Aldrich. An LKB Liquid Scintillation Counter was used (Turku, Finland). Solvents used were HPLC grade.

5.2 Short term dosing/ Acute Exposure

5.2.1 Animal Treatment

Forty-eight female CD-1 mice (6-8 weeks old) from the Charles River Laboratory, Wilmington, MA were housed in metabolic cages, 6 per cage. Food and water were given *ad libitum* and animals were housed in 12-h dark cycle for 24 h. Mice were each injected i.p. with ^{3}H-BaP (24 mg/kg b.w., 117.04 mCi/mmole) dissolved DMSO (100μl) or solvent only. Animals were sacrificed 24 hours later. The livers were collected and washed with RSB buffer and divided into 4 parts. 1 part was frozen in liquid nitrogen and stored at -100°C. The remaining 3 parts were fixed and embedded in paraffin.

5.2.2 Fixation and embedding of liver samples

The remaining 3 parts of liver from each animal were put in fixative cassettes. One of each was fixed in 10% formalin for 24, 48 and 168 hours before embedding in paraffin. Prior to embedding the tissues in paraffin, the tissues were processed for 15 hours in preservatives using an automatic tissue processor (Tipshaw, Trimatic Model 2500, Detroit, MI). They were then positioned into blocks of paraffin and stored. The samples of a given fixation time were process simultaneously.

5.2.3 Deparaffinization of liver tissues

The excess paraffin was removed with a razorblade. The samples were cut into 5 pieces and transferred into new cassettes, tightly closed and placed into an erlenmeyer flask. Xylene (200 ml) was added and the erlenmeyer was placed under vacuum and immersed in a water bath at 47°C for 45 minutes. The xylene was discarded and the process was repeated for an additional 45 minutes. Cassettes were then successively soaked twice in 100%, 95%, and 75% ethyl alcohol and water for 5 minutes. Samples were stored at -20°C until ready for DNA isolation.

5.2.4 DNA isolation

DNA was isolated from the livers according to the method of Davis et al (1986). Briefly, the homogenates were incubated with proteinase K at 37°C, extracted with phenol:chloroform:isoamyl alcohol. The DNA was ethanol precipitated and dissolved in SSC buffer (5.4 mM trisodiumacetate, 30mM NaCl). The DNA was treated with DNase free-RNase for 30 minutes at 37°C. The DNA was extracted with phenol:choroform, ethanol precipitated, washed with 70% ethanol, dried and dissolved in 1 ml 10mM Tris buffer (pH 7.4) and stored at 4°C overnight. DNA concentration was determined by UV absorbance. The ratios of A_{260nm}/A_{280nm} ranged from 1.83-1.90. The DNA was then enzymatically hydrolyzed as described previously.

5.2.5 Enzymatic hydrolysis of DNA

DNA samples were hydrolyzed to mononucleosides using NP_1, snake venom phosphodiesterase and alkaline phosphatase. 200μg DNA in 0.1 M sodium acetate buffer were hydrolyzed with NP_1 (84.5 units NP_1/mg DNA) for one hour at 37°C. Snake venom phosphodiesterase (0.9 SVPD units/mg DNA) and alkaline phosphatase (16.7 units AP/mg DNA) were then added and the incubation was continued at 37°C for one hour.

5.2.6 Purification of enzymatic digests

The enzymatic hydrolysates were loaded onto a C18 Sep-Pak cartridge, preconditioned with methanol (20ml) and water (20 ml), and recycled through 4 more times. The column was washed with water (20 ml) and the modified nucleosides were eluted with methanol (20 ml). The solvent was removed in vacuo. The nucleosides were redissolved in 40% methanol H_2O (vol/vol), passed through Millipore cellulose filters (nominal molecular weight limit 30,000) and stored desiccated at -20°C. Samples were then acylated with ^{35}S-TBM-NHS and/or analyzed by RP-HPLC.

5.2.7 HPLC analysis

HPLC analyses of BaP adducts from calf thymus DNA modified under *in vitro* conditions and from mouse liver DNA were performed on a Hewlett Packard (model 1090 M)

liquid chromatograph equipped with a Radiomatic Flo-One (Meriden) flow-through radioactivity detector (model A250). A Microsorb 5μ C_{18} column was used with the following gradients with a 1 ml/min flow rate at 35°C: Gradient I: linear gradient of 0-25% methanol/water, 10 minutes; linear gradient of 25%-80%, 80 minutes; linear gradient of 80-100% methanol, 10 minutes; and isocratic 100% methanol, 10 minutes. ^{35}S-acylation products were analyzed by RP-HPLC with the same conditions as above, using the following gradient. Gradient B: isocratic 40% methanol/water, 13 minutes: linear gradient of 40-60% 10 minutes; isocratic 60%, 12 minutes; linear gradient or 60-100% methanol, 20 minutes; and isocratic 100% methanol 25 minutes.

5.2.8 Acylation with ^{35}S-TBM-NHS

Liver samples were acylated according to the method described by Sheabar (1994). ^{3}H-BaP-dGuo adduct samples (5 pmol) were prepared as mentioned above from *in vitro* modified calf thymus DNA and liver tissues were acylated with ^{35}S-TBM-NHS. Purified DNA of control animals were also spiked with adduct from DNA modified under *in vitro* conditions and acylated. Acylation was carried out for 2 hours at 37°C in the presence of ^{35}S-TBM-NHS ester (50,000 mol-equivalents) in THF (50 μl), pyridine (50 μl), DIC (1 μl). Liver DNA samples isolated from animals treated with DMSO only and containing amounts of adduct equivalent to those used in the acylation were similarly treated and acylated. Following the acylation, samples were dried under reduced pressure for 30 minutes, redissolved in methanol (100 μl) and water (500 μl), purified by extraction from hexane (5 x 5

ml) and analyzed by HPLC, using gradient B.

5.3 Results And Discussion

5.3.1 Chromatography

The isolated DNA from 6 mice were pooled to generate a HPLC profile of the ^{3}H-BaP-DNA (Figure 5.2) using Gradient I. Sixty-seven percent of ^{3}H-BaP-DNA adducts were recovered from the enzymatic hydrolysate of the DNA after chromatography by C18 sep-pak cartridges. The major HPLC peak eluted at 52 minutes. The HPLC profiles of digests of BaP-DNA samples isolated from tissues fixed for 0-168 hours differ from that of frozen tissue (Figure 5.2). A new peak appeared at 8 minutes after fixation for more than 24 hours. The acid hydrolysis of this peak (0.1N HCl, 6 hours, 80°C) reveals the appearance of new peaks (using Gradient A) at 24, 38 and 64 minutes. The peak detected from the enzymatic digestion of calf thymus DNA modified under in vitro conditions were different from these retention times. This suggests that the peak that appears at 8 minutes represents undigested oligonucleotide. It appears that the the efficiency of enymatic digestion was affected by length of fixation. On the contrary, Hewer and Phillips (1993) used gel electrophoresis to examine the completion of enzymatic digestion in tissues fixed in formalin for 0-92 days and found that the enzymatic hydrolysis was not affected by the fixation in formalin.

5.3.2 Adduct Levels

Samples acylated by ^{35}S-TBM-NHS were analyzed by RP-HPLC. It revealed a peak at 52 minutes. Figure 5.3 shows the radio-chromatograms of samples acylated by ^{35}S-TBM-NHS. The peaks at 50 minutes and 55 minutes displayed in the radio-chromatogram of the treated mouse samples do not appear in the reagent control (Figure 5.3a) or the DMSO control mice (Figure 5.3b). When the DMSO control sample is spiked with the internal ^{3}H-BaP-dGuo internal standard (Figure 5.3c) before acylation, the peaks have the same retention time as the BaP treated mouse sample (Figure 5.3d).

The modification levels were found to increase significantly with increase in the length of fixation and reached a plateau after ≥48 hours of fixation. (data not shown). The results of the modification levels (Table 5.1) were calculated on the basis of the radio-labeled ^{3}H-BaP and with the amount of ^{35}S incorporated into the acylation products. There was a good correlation between the modification levels regardless of whether calculations were based on ^{3}H or ^{35}S. Although the modification level looked like it increased by 20-fold after fixation for 168 hours, the amount of picomole of BaP based on the internal standard was found to be < 5 pmole. Therefore, the calculation of total nucleotides is incorrect. There appears to be a selection of BaP-modified DNA that occurs during the isolation, hydrolysis, purification and acylation of DNA from formalin-fixed paraffin embedded tissues. If the numbers were taken at face value, it would give a inflated impression of the modification level. The inflated numbers are due to the loss of, or inability to recover unmodified DNA. One explanation could be that the crosslinking of the proteins in the DNA (ex. histones) is virtually irreversible

making it extremely difficult to isolate and digest unmodified DNA. Therefore, when computing modification levels in fixed-embedded tissues, from other animal samples or human samples, the numbers of BaP adducts/ 10^8 nt should be divided by 20.

5.4 Newborn Mouse Lung Adenoma Assay

5.4.1 Animal Treatment

Pregnant CD-1 mice obtained from Charles River Breeding Laboratory (Kingston, NY) approximately 7 days before the end of gestation were housed individually in polycarbonate cages equipped with dust filters and contained hardwood chip bedding (Sani-Chips; P.J. Murphy Co., Moonachie, NJ) in laminar airflow isolation cubicles under controlled temperature (22±1°C), light (12h light-dark cycle) and humidity (50±10%). NIH open formula diet (NIH-07 Rat and Mouse Feed; Zeigler Bros, Inc., Garners, PA) and distilled water were supplied *ad libitum*.

Animals were dosed with benzo(a)pyrene as previously described with a dose of 210 μg benzo(a)pyrene /35 μl DMSO three times over a two week period (Figure 5.4) Freshly prepared preparations of BaP dissolved in DMSO were protected from light until administration to animals. Newborn mice were injected i.p. on day 1 with 5 ml DMSO containing 1/7 of the total dose, followed by a second treatment on day 8 containing 2/7 of the dose dissolved in 10 μl DMSO, and the third treatment on day 15 with the remaining 4/7 of the dose dissolved in 20 μl DMSO. ^{3}H-benzo(a)pyrene (240 mCi/mole) was used for two reasons: (1) provide a means of determining modification level and following recovery and

(2) provide an independent means of verification for carcinogen detection by postlabeling methods. Control mice were given DMSO in the same volumes. At least 50 animals were included in each treatment group. Tissues for DNA adduct analysis were collected from animals sacrificed by decapitation at 24 hours, 72 hours, 1 week, and 5.5 months after the last injection. Lungs, livers, and kidneys were quickly excised and rinsed in 1X RSB (10mM Tris-HCl, pH 7.4, 10 mM NaCl and 25 mM Na_2EDTA, pH 7.4). After blotting dry, organs were individually frozen in liquid nitrogen and pooled in polyethylene scintillation vials in dry ice according to tissue, sex and litter. The tissues were stored in polyethylene vials at -100°C until DNA could be isolated.

5.4.2 Isolation of DNA

DNA was isolated as follows. Pooled tissues (<0.400g) were homogenized with a motor driven Polytron in 19ml of lysis buffer (800 mM GuHCl, 20 mM EDTA, 30mM Tris/HCl, 5% Tween-20, 0.5% Triton X-100, pH 8.0) containing RNase A (200 μg/ml). The homogenate was digested with Proteinase K (1mg/ml) at 50°C for 2 hours or until clear. Unclear lysates were centrifuged 1,500 *g* for 10 minutes. The clear lysate was loaded on to a Qiagen genomic-tip-500/G anion exchange column (Qiagen Inc. Chatsworth, CA). After washing twice with 15 ml of wash buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0), the DNA was eluted with 15ml of 1.25 M NaCl, 50mM Tris/HCl, 15% ethanol, pH 8.5). The DNA was precipitated with 0.8 volume of isopropanol and centrifuged (3,000 *g* for 1 hour). The pellet was washed twice with 1 ml of ice-cold 70% ethanol, air dried and dissolved in

water. DNA purity was determined by the A_{260nm}/A_{280nm} ratio and the concentration was calculated by absorbance assuming an O.D. of 1.0 equal to 50 μg/ml DNA. DNA that did not have a A_{260nm}/A_{280nm} ratio of at least 1.7, was and retreated with proteinase K at 50°C overnight, then dissolved in 750mM NaCl, 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 15% ethanol) and purified with as previously using a Qiagen genomic-tip-500/G.

5.4.3 Enzymatic hydrolysis of DNA

DNA samples were hydrolyzed to mononucleosides using NP_1, snake venom phosphodiesterase and alkaline phosphatase. 200μg DNA in 0.1 M sodium acetate buffer were hydrolyzed with NP_1 (84.5 units NP_1/mg DNA) for 3 hours at 37°C. Snake venom phosphodiesterase (0.9 SVPD units/mg DNA) and alkaline phosphatase (16.7 units AP/mg DNA) were then added and the incubation was continued at 37°C for 3 hours.

5.4.4 Immunoaffinity chromatography

Immunoaffinity chromatography of BaP DNA adducts from preweanling mice: Hydrolyzed DNA was separated into modified and unmodified nucleosides via immunoaffinity chromatography. 200μl columns were poured using immobilized 8E-11 antibodies. Hydrolysate was recycled through the column 3 times, washed with 5ml of water (25 column volumes) and modified nucleosides were eluted with 2ml of methanol (10 column volumes). The volume was divided into triplicate samples in amber 500 ml polypropylene tubes (VWR)

and dried *in vacuo*.

5.4.5 Manufacturing of ^{35}S-TBM

The protection reaction of ^{35}S-methionine was performed as follows: ^{35}S-methionine (5mCi, 1000Ci/mmol) was thawed and transferred to a 5ml glass tube. The solution was evaporated dry (2-3 hours) and kept on dry ice. Di-tert-butoxy-dicarbonate (5 µl), DMF (50 µl), TEA (1µl) were added the mixture and incubated for 15 minutes at room temperature. Purity and radioactivity were determined from an aliquot (1 µl) which was removed from the mixture and added to 5 ml H_20. The reaction mixture was evaporated (70 minutes), redissolved in 200 µl methanol and 200 µl H_20 and further purified via HPLC. The water:methanol HPLC gradient (gradient J) used for purity analysis and purification was: 7% meOH isocratic for 20 minutes, isocratic 20% meOH for 15 minutes, linear gradient to 100% over 20 minutes.

5.4.6 Acylation of DNA from mouse samples with ^{35}S-TBM

The dried samples were resuspended in pyridine containing TBM ($5x10^4$ molar equivalents) and DIC (ratio of 1:16, TBM:DIC) and the mixture was incubated for 2 hours at 37°C. Similar conditions were used to acylate dGuo-^{3}H-BaP isolated from calf thymus DNA adducted *in vitro* and synthesized dGuo-BaP standard. The samples were dried under reduced pressure and stored at -20°C until analysis. The samples were resuspended and

analyzed via RP-HPLC. The water:methanol gradient (Gradient B) used was isocratic 40% methanol for 13 minutes, linear gradient of 40-60% methanol for 10 minutes, isocratic at 60% for 12 minutes, linear gradient of 40-60% for 20 minutes, and isocratic 100% for 25 minutes.

5.5 Results and Discussion

5.5.1 Tumor Incidence

The purpose of this experiment was to test the applicability of the ADAM method to BaP-DNA adducts formed in living tissues. The newborn mouse lung adenoma assay successfully produced tumors in mice treated with the total dose of 150 μg of BaP. The total number of animals that developed at least one lung tumor (top panel) or liver tumor (bottom panel) 5.5 months after dose of 210 μg BAP are displayed in Table 5.2. No liver tumors were detected in the female mice. Tables 5.3 & 5.4 display the tumor frequency resulting from the newborn mouse assay. The tumor range for the male mice (Table 5.3) was 3-29 tumors/animal and 0-5 liver tumors/animal. The female mice (Table 5.4) displayed a tumor range of 0-5 lung tumors/animal and there were no liver tumors detected. This difference in the tumor frequency, although the dose was the same may be attributed to litter variance in the formation of tumors.

5.5.2 Adduct Levels

Since the tumor incidence in the male mice, the male samples were acylated. The DNA (200μg) was isolated from the lung and liver tissues. After the DNA was extracted from the tissues enzymatically hydrolyzed and immuno-affinity purified, the samples were acylated. A positive control, 100fmole of BaP modified calf thymus DNA was run along side of the samples. Figure 5.5 displays a representative chromatogram of an acylated mouse sample. The top panel is the DMSO control sample. The bottom panel displays the acylation of a lung sample from a 72 hour treated mouse. The region from 55-60' was determined after the subtraction of the paired DMSO control. The amount of BaP present was calculated based on the radioactivity of the positive control. Conversion of molar amounts of adducts to DNA modification level assumed an average molecular weight of 324 per nucleotide. The levels and persistence of the BaP-DNA adducts in the male mice is shown in Figure 5.6. BaP-dGuo adducts persisted at detectable levels in the DNA from the tissues. Maximum adducts in the lungs were found 165 days post-treatment. The maximum levels in the livers occurred three days post-treatment. By 165 days following treatment, BaP-dGuo adducts decreased in the liver 9.3%. The phenomenon of PAH adduct levels reaching maximum levels at 3 days was also described by Wang *et al.*, (1995). The same dosing regimen was used for fluoranthene. The *anti*-FADE adducts reached maximum levels after 3 day in lungs, livers, spleen/thymus and kidney. The lung had 0.5 fmole/μg DNA. The liver and kidneys had 0.13 fmole/μg DNA. In a similar study, (Melikian, *et al.*, 1989), where the metabolism of ^{3}H-BaP in newborn mice was studied, the levels of organic soluble radioactive metabolites reached maximum levels at day 2 for the lung and day 4 for the liver. The maximum levels reached after 3 days possible reflect the saturation of the capacity of newborn mice to absorb,

transport, metabolize and eliminate the large dose of PAH administered and also to remove the DNA adducts formed through metabolic activation of the PAH (Wang *et al.*, 1995).

5.6. Conclusions

The levels of BaP-DNA adducts were investigated in treated mice. The 24 hour exposure to ^{3}H-BaP revealed the applicability of the ADAM method to *in vivo* samples and the correlation of the adduct levels based on ^{3}H and ^{35}S validated the method. It was demonstrated that DNA can be isolated from paraffin embedded tissues and analyzed for BaP-dGuo adducts. Adduct levels of BaP isolated from fixed-embedded tissues must be divided by 20 to correct for the nucleotide levels.

The use of 210μg BaP in the newborn mouse lung adenoma assay successfully produced tumors. Samples were collected for analysis of BaP-DNA adducts in lungs and livers. The persistence of adduct levels in mice dosed in the newborn mouse assay was determined by the ADAM method.

Figure 5.1 Protocol for 24 hr exposure of mice to ^{3}H-BaP

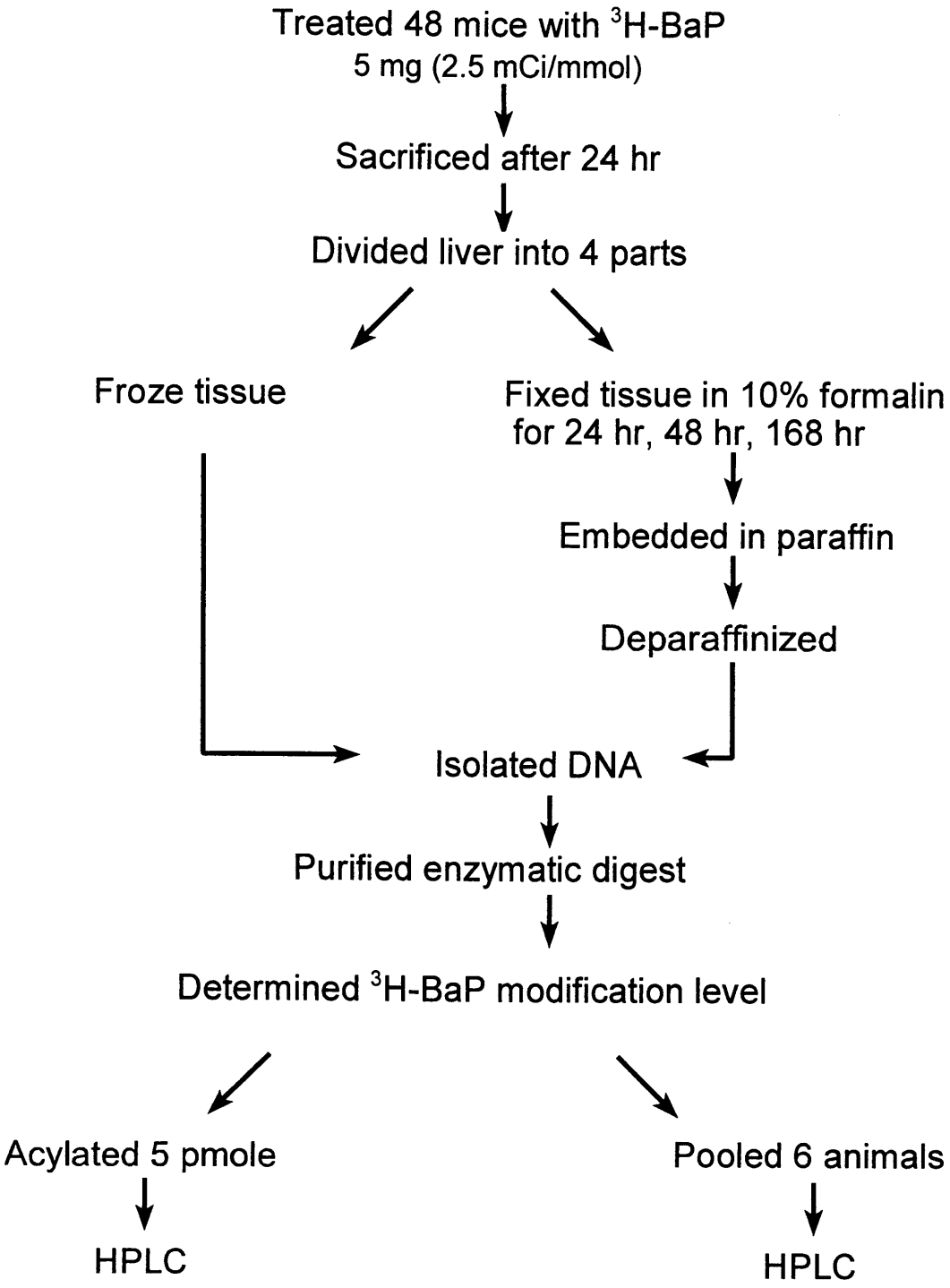


Figure 5.2 HPLC radio-chromatogram of ^{3}H-BaP-DNA adducts

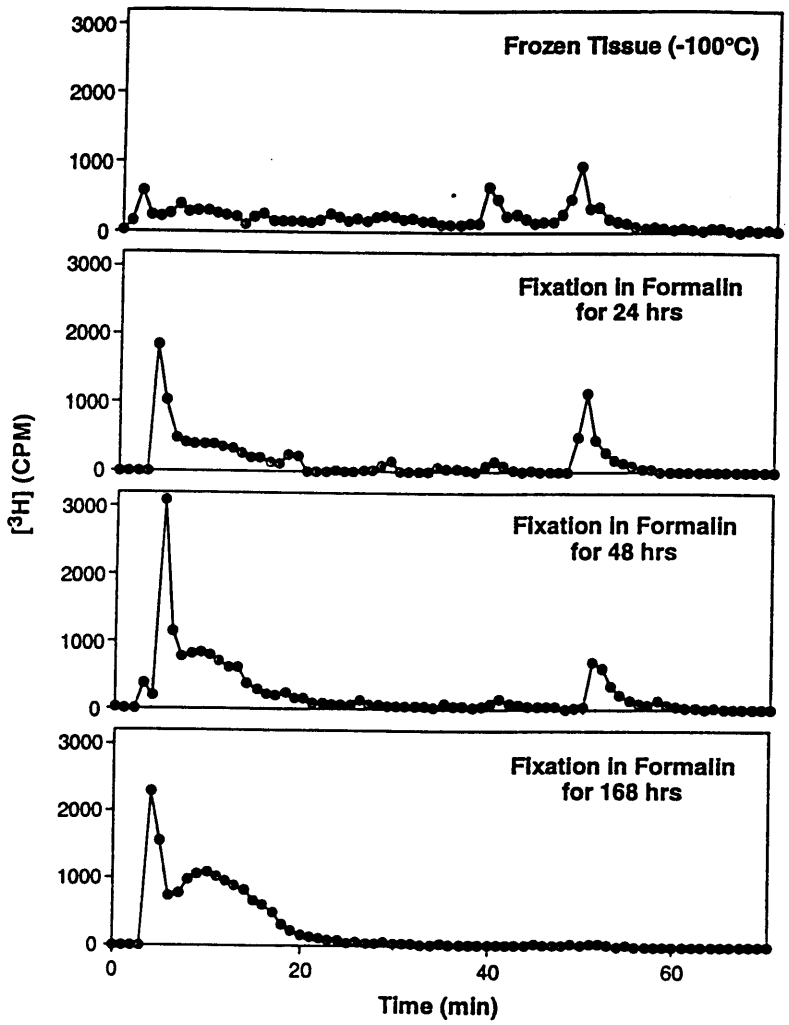
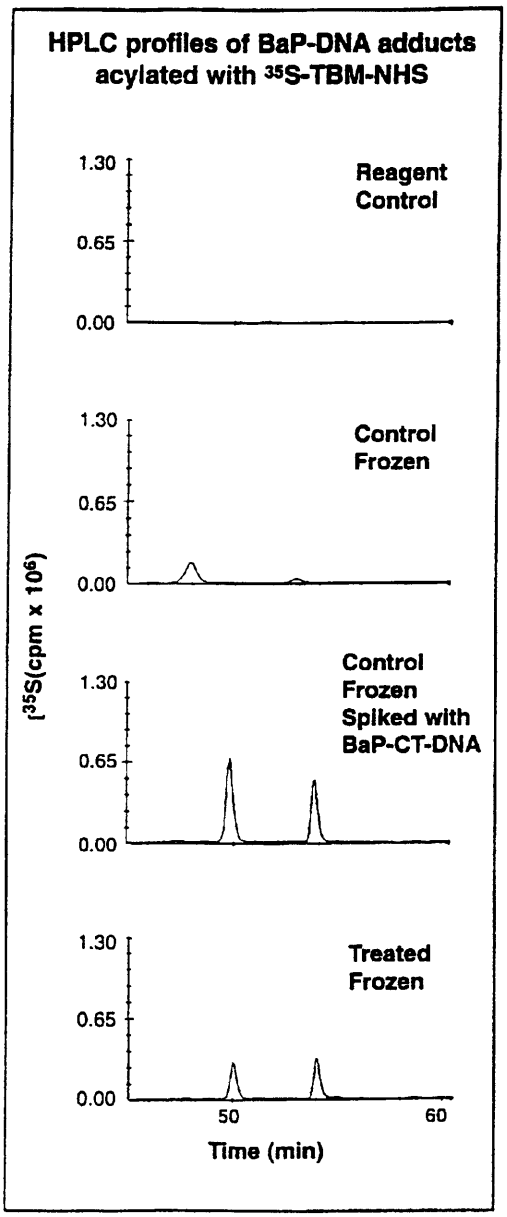


Figure 5.3 HPLC profiles of mouse DNA acylated with ^{35}S-TBM-NHS



Panel A: Reaction with all reagents for ADAM analysis but no nucleoside.
Panel B: Control animals treated with DMSO.
Panel C: Control sample spiked with internal standard ^{3}H-BaP-dGuo.
Panel D: Animal treated with ^{3}H-BaP.
Acylated products appear at 50 minutes and 55 minutes.

Figure 5.4 Dosing regimen for the newborn mouse lung adenoma assay

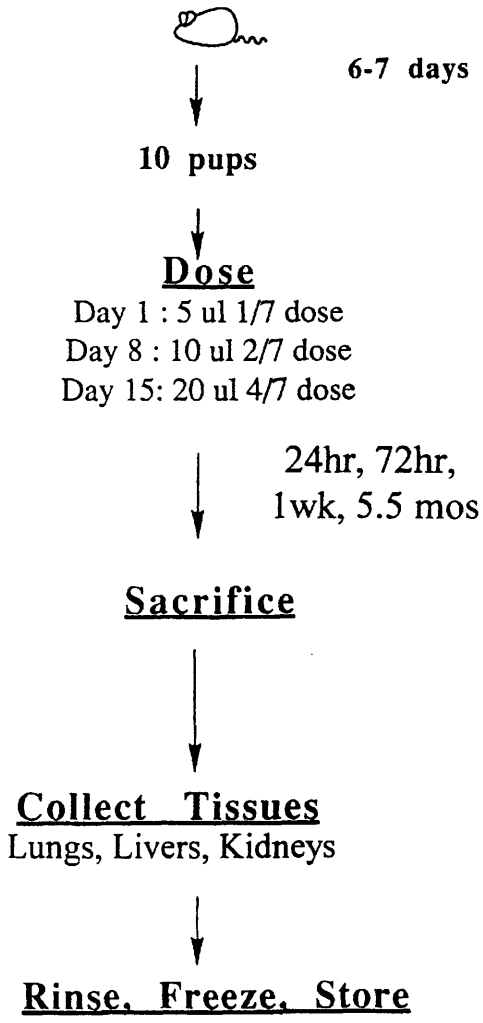


Figure 5.5 HPLC chromatogram of acylated mouse sample from newborn mouse lung adenoma assay

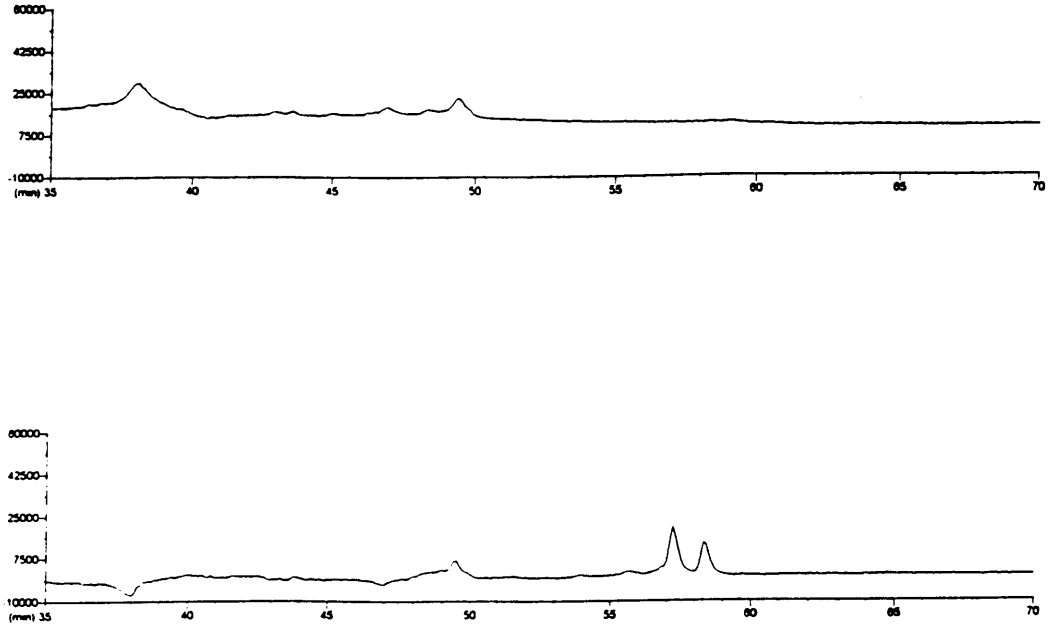


Figure 5.6 Persistence of BaP-DNA adduct in male mice

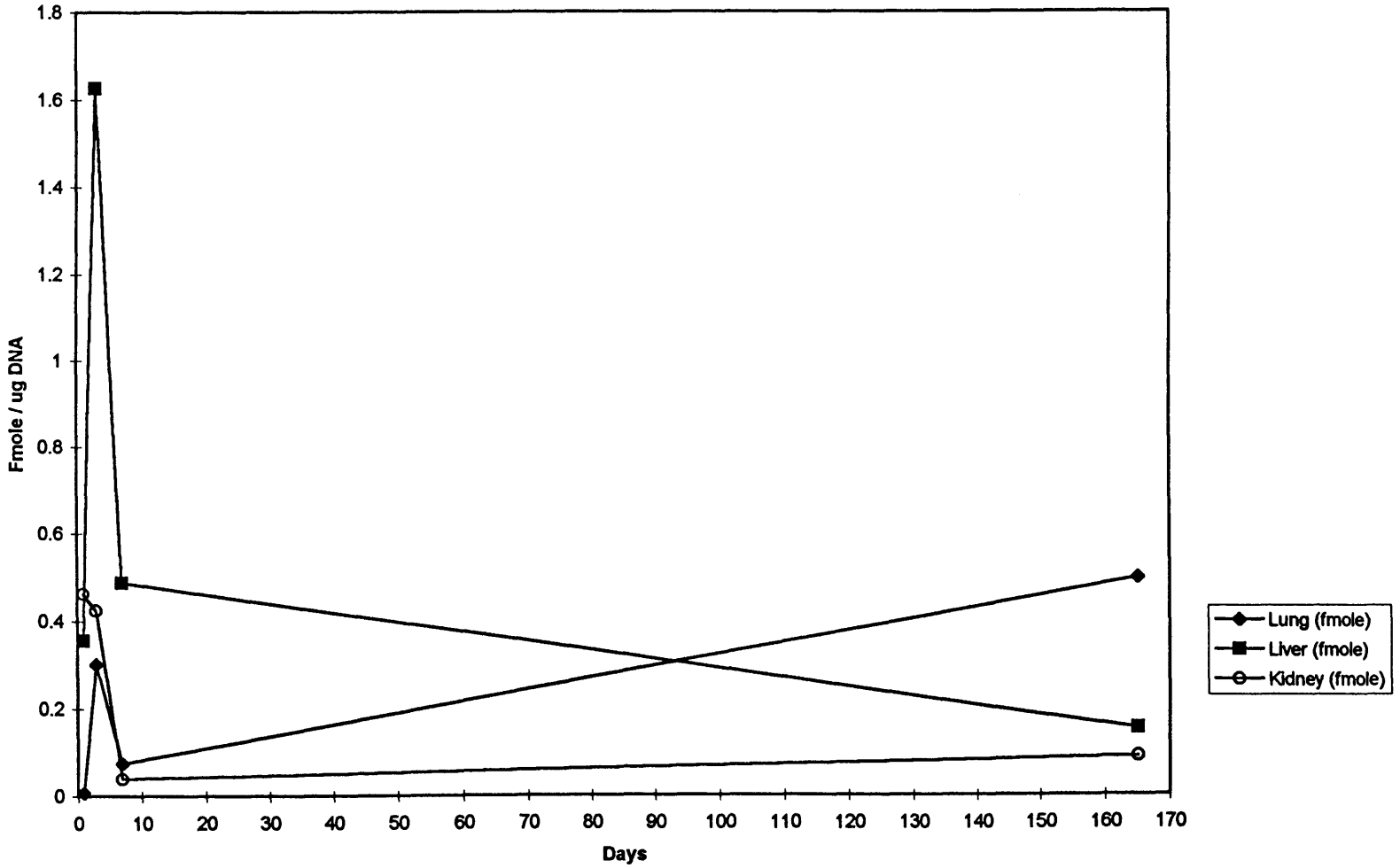


Table 5.1 Modification levels in BaP treated mouse liver determined on the basis of the 3H-BaP internal and the ^{35}S-acylation products.

Treatment	Adduct (pmol ^{3}H-B(a)P in the reaction)	Acylated Adduct (pmol) Based on IS	Modification Level (Adducts: 10^{8} nucleotides) ^{3}H	^{35}S
Treated Frozen	5	2.65	2,097	2,208
Treated Fixed (24hrs)	5	3.67	29,167	28,230
Treated Fixed (48 hrs)	5	4.08	43,333	50,936
Treated Fixed (168 hrs)	5	3.81	40,000	45,738

Table 5.2. Summary of the percentages of animals with at least one tumor.

Total # of Mice with Lung Tumors

	Litter 5	Litter 40
Male	4/4 (100%)	5/5 (100%)
Female	4/6 (67%)	6/6 (100%)

Total # of Mice with Liver Tumors

	Litter 5	Litter 40
Male	4/4 (100%)	2/5 (40%)
Female	0/6 (0%)	0/6 (0%)

Table 5.3. Lung and liver tumor frequency after 5.5 months in male mice dosed with 210 μg BaP in newborn mouse lung adenoma assay.

Tumorigenicity in Male CD-I Mice				
	Litter 5		Litter 40	
	# of Tumors per animal			
Animal	Lung	Liver	Lung	Liver
1	10	5	1	1
2	29	1	3	3
3	3	0	2	2
4	7	4	4	4
5	-	-	5	5

Table 5.4. Lung and liver tumor frequency after 5.5 months in female mice dosed with 210 μg BaP in newborn mouse lung adenoma assay.

Tumorigenicity in Female CD-I Mice				
	Litter 5		Litter 40	
	# of Tumors per animal			
Animal	Lung	Liver	Lung	Liver
1	5	0	1	0
2	3	0	3	0
3	5	0	1	0
4	0	0	2	0
5	1	0	2	0
6	0	0	1	0

Chapter 6. DNA Adducts of Benzo(a)pyrene: Human Studies

6.1 Introduction

There is extensive evidence from ^{32}P-postlabelling studies and immunological data demonstrating that BaP-DNA adducts are present in humans. Even in these cases, it was difficult to distinguish one related adduct from another. Additionally, care must be taken when interpreting fluorescence data because the formation of tetraols can disproportionately contribute to the overall fluorescence yield (Geacintov *et al.*, 1987). Dipple (1994) stated "It is perhaps unreasonable to expect that specific hydrocarbon-nucleoside adducts can be detected in man, but with improved methods this aim may be realized sooner than expected". This chapter describes another step to address this issue through the application of the ADAM method to detect BaP-DNA adducts in human tissue DNA samples.

The ADAM procedure has been shown to successfully analyze BaP-modified calf-thymus DNA. These optimal conditions were also used to analyze *in vivo* tissues from BaP-treated mice. Because of the promising results seen in the mouse samples, the next step was to investigate human tissues. This chapter describes use of the ADAM method to analyze 2 sets of human samples for BaP-dGuo adducts. One set of human samples involved tissue that was collected during surgery to remove lung tumors. The other set of samples was breast tissues removed from cancer patients, fixed then embedded in paraffin. The previous chapter described the success of to DNA isolation

from embedded tissues and the ability to detect BaP-dGuo adducts with the ADAM method. This knowledge could then be applied to embedded human samples. Since pathology laboratories treat tissue biopsies of cancerous and noncancerous tissues with fixative and embed them in paraffin, a large number of embedded human tissues are stored in pathological archives. These samples are valuable because they often have a medical history attached to each tissue.

This chapter describes the detection of BaP-dGuo in human tissues with the ADAM method. Figure 6.1 describes the strategy for the detection of BaP-DNA adducts in human samples. If the tissues are fixed-embedded, the paraffin was removed and treated the same as the frozen tissues. The tissues were homogenized, the DNA was isolated and enzymatically hyrolyzed. The modified nucleosides were purified with immunoaffinity chromatography, spiked with an internal standard and acylated with ^{35}S-TBM. The acylation products were quantified and the results are reported here.

6.2 Materials and Methods

SL-9 (MolTox); calf thymus DNA, glucose-6-phosphate, glucose 6-phosphate dehydrogenase, Nuclease P_1, alkaline phosphatase from bovine intestinal mucosa (50% glycerol) (Sigma Chemical Co.); Benzo(a)pyrene (Aldrich); ^{3}H-BaP (Amersham); anti-benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±) (NCI Chemical Carcinogen Reference Standard Repository); ^{35}S -Methionine (5mCi, 1000mCi/mmol) (NEN/Dupont); snake venom phosphodiesterase (ICN); Sep-pak C18 environmental cartridge (Waters); methanol (Baxter); xylene, chloroform, benzene,

dichloromethane, ether, ethanol, tetrahydrafuran, pyridine di-*tert*-butyldicarbonate, triethylamine, dimethylfomamide, (Mallinckrodt).

6.2.1. Acylation reagent preparations

All reagents were purified and rigorously dried prior to use in the acylation. DIC was distilled under reduced pressure, and TEA was distilled over sodium metal. Both reagents were chromatographed on Sephadex LH-20. THF and pyridine were distilled over sodium metal and benzophenone (as an indicator) and calcium hydride for 2 and 10 days, respectively. All purified reagents were aliquoted into dry ampules (prewashed with methanol, dried at 500°C for 4 hours and aspirated with argon), sealed under vacuo and stored at room temperature. Aliquots of approximately 0.1g TBM were weighed, dried in vacuo for 5 hours and stored in a dessicator at 4°C until used. Prior to use in the acylation reaction, the TBM was dissolved in pyridine at a concentration of 2M.

6.2.2 Removal of Paraffin (from archival tissues)

Paraffin embedded human breast tissues (provided by Dr. F. Perera) were stored at ambient temperatures until the paraffin was removed with xylene (1.2ml, 47°C for 135 minutes). The samples were centrifuged 14,000 rpm for 2 minutes. The solvent was then removed, 1.2ml of xylene was replaced and further incubated for 15 minutes. This process was repeated once more before successively soaking the samples twice for 5 minutes in 1ml of 100%, 95%, and 75%

ethanol and water. Samples were temporarily stored at 4°C for 18 hours. Each sample was washed with 1 ml H_20 and stored at -20°C until ready for DNA isolation.

6.2.3 DNA isolation

DNA was isolated from the frozen human lung samples and fixed human breast samples according to the method of Davis et al (1986). The breast tissues were provided by Dr. P. G. Shields. Fifty μg of the human lung samples were analyzed. When possible 100 μg of the human embedded breast tissues were analyzed. Samples that did not contain 100 μg, all of the DNA was analyzed. Briefly, the homogenates were incubated with proteinase K at 37°C, extracted with phenol:chloroform:isoamyl alcohol. The DNA was ethanol precipitated and dissolved in SSC buffer (5.4 mM trisodiumacetate, 30mM NaCl). The DNA was treated with DNase free-RNase for 30 minutes at 37°C. The DNA was extracted with phenol:choroform, ethanol precipitated, washed with 70% ethanol, dried and dissolved in 1 ml 10mM Tris buffer (pH 7.4) and stored at 4°C overnight. DNA concentration was determined by UV absorbance. The ratios of A_{260nm}/A_{280nm} ranged from 1.83-1.90.

6.2.4 Enzymatic hydrolysis of DNA

DNA samples were hydrolyzed to mononucleosides using NP_1, snake venom phosphodiesterase and alkaline phosphatase. 200 μg DNA in 0.1 M sodium acetate buffer were hydrolyzed with NP_1 (84.5 units NP_1/mg DNA) for one hour at 37°C. Snake venom

phosphodiesterase (0.9 SVPD units/mg DNA) and alkaline phosphatase (16.7 units AP/mg DNA) were then added and the incubation was continued at 37°C for one hour.

6.2.5 Immunoaffinity chromatography

Before acylation with ^{35}S-TBM, the eznymatic digests were purified via immunoaffinity chromatography. Immunoaffinity columns (400 µl) containing PAH specific monoclonal antibody were prepared with gel stored at 4°C for several days prior to the purification. Each column was equilibrated with H_20 before the DNA digests were diluted in 5ml H_2O and loaded 3 times. The columns were washed with 20 ml H_2O before being eluted with 100% (8 ml) methanol. The sample volume was reduced to ~100 µl and spiked with 25 fmol of ^{3}H-BaP-dGuo as an internal standard. The samples were dried under reduced pressure and stored in a dessicator at -20°C until acylation.

6.2.6 Acylation with of Human Tissues with ^{35}S-TBM

The dried samples were resuspended in pyridine containing TBM (5 x 10^4 molar equivalents) and DIC (ratio of 1:16, TBM:DIC). Self-manufactured ^{35}S-TBM was added and the mixture was incubated for 2 hours at 37°C. The samples were dried under reduced pressure and stored at -20°C until hexane extracted. The samples were resuspended and hexane extracted as previously described and analyzed via RP-HPLC. The water:methanol gradient used was isocratic

40% methanol for 13 minutes, linear gradient of 40-60% methanol for 10 minutes, isocratic at 60% for 12 minutes, linear gradient of 40-60% for 20 minutes, and isocratic 100% for 25 minutes.

6.2.7 Quantitation of BaP-DNA adducts

The amount of BaP-adduct present in the tissues was based on the radioactivity ^{35}S as determined by the following evidence: (1) the area under the peak between 58-60 minutes was considered to be the reaction product because the retention time is the same as that of the BaP-adduct reaction product; and (2) a linear correlation was found between the amount of ^{3}H-BaP-dGuo reacted and the amount of ^{35}S incorporated into acylation products.

The chromatograms generated by the reagent controls were subtracted from the radio-chromatograms generated by the samples. Since the samples were spiked with an internal standard, the area under the peak(s) of interest were calculated using the area of the acylated internal standard. The amount of of BaP-deoxyguanosine (fmol) was determined and the internal standard was subtracted from the total amount calculated. This value was translated to number of adducts per 10^8 nucleotides based on the amount of DNA used for the study. It was assumed that a human diploid genome contained $12x10^9$nt to calculate the number of adducts per cell. Simply, the number of adducts/10^8nt were multiplied by 120 to calculate the number of adducts per cell.

6.3 Results and Discussion

A total of 15 lung samples were analyzed. The results are summarized in Table 6.1. The range of the adduct levels found was 2-426 adducts/10^8 nt (0.05-147 fmole/μg DNA) with an average of 13,185 adducts per cell. Adducts were not detected in 1 sample. Figure 6.2 shows a representative chromatogram of an acylated adduct profile from lung DNA. (Samples that gave a negative number after subtracting the controls were denoted as ND.)

The HPLC profiles produced by the acylation of BaP-dGuo with ^{35}S-TBM have similar retention times as embedded human breast samples acylated with ^{35}S-TBM. Figure 6.3 shows a representative radio-chromatogram of a breast tissue DNA acylated with ^{35}S-TBM. A total of 14 embedded breast tissues were deparaffinized and acylated. The range of adduct levels shown in Table 6.2, was 35-1764 adducts/10^8 nt (1-54 fmole/μg DNA) with an average of ~44,000 adducts per cell. Adducts were not detected in 2 samples and denoted as ND. As described in chapter 5, when calculating the modification level, there was a 20-fold difference between frozen tissues and tissues fixed in formalin for 168 hours. Based on internal standards the calculated amount of BaP was accurate. As a result, the modification levels (Table 6.3) were divided by 20 to give a more accurate representation of what the adduct levels/cell might have been if the tissues had not been fixed and embedded.

The acylation reaction conditions found to be optimal for *in vitro* modified DNA and 2'-deoxyguanosine were found to produce comparable results with BaP-dGuo adducts in mouse and human tissues. In both cases of human sample analyses, the acylation products of human lung and embedded human breast samples have the same retention time as the peaks produced by the acylation of BaP-dGuo from modified calf thymus DNA and DNA from mice dosed with ^{3}H-BaP. It is reported here that DNA from embedded tissues can be isolated and analyzed for BaP-DNA

adducts by the ADAM procedure. The use of DNA from embedded tissues will allow the analysis of archival tissues.

In addition to being able to measure BaP-DNA adduct levels in human samples both frozen and embedded, the method has proven to be at least as sensitive as other DNA-adduct detection methods eg., ^{32}P-postlabelling, immunological methods and fluorometric methods. Bartsch *et al.,* (1992) examined carcinogen metabolism and human lung tissues and the effect of tobacco smoking. Since cigarette smoking is the strongest risk for lung cancer so far identified (IARC, 1986), ^{32}P-postlabelling was used to measure the adduct levels in smokers and ex-smokers. Smokers were found to have significantly higher levels of BaP-DNA adducts (mean of $5.38 \pm 3.19/10^8$ nt) than ex-smokers (mean of 1.09 adducts/10^8 nt $\pm$ 0.084). PAH-DNA levels were determined by ELISA in WBC taken from residents in Poland where the air pollution is high. The mean PAH-DNA level in exposed residents was 30.4 adducts/10^8nt (Perera *et al.*, 1992). In 1994, Rojas *et al.*, validated a new fluorometric that can measure 1 BPDE adduct per 10^8 unmodified nucelosides. The quantity of WBC DNA required varied between 5 and 500μg and the assay was applied to WBC DNA samples from lung cancer patients. High levels of BPDE adducts ranging from 62-533 adducts/10^8 nucleotides, were found in six out of the seven lung cancer patients. In WBC DNA samples from healthy individuals the BPDE-DNA adducts were much lower (4-10 adducts/10^8nt)

It has been shown that the ADAM method can be used to analyze BaP-DNA adducts in human samples and that the ADAM method is as sensitive as the above mentioned methods. It also has several advantages. (1) The method is easier than ^{32}P-postlabelling because the radioactive shielding is not required and the chemical acylation reaction is not impeded by the

limitations of enzymatic reactions; (2) The samples can be examined for more than one family of compounds. Since the eluate from the immunoaffinity purification was saved, other adducts could be isolated using a different antibody; (3) The method is not dependent on the availability of antibodies. The acylation reaction products must first be determined through the use of a standard for the compound of interest. The unmodified nucleosides can be removed from the enzymatic hydrolysate with a C18 column. The remaining modified nucleosides are acylated and the reaction products can be quantified; (4) Fluorescence techniques are sensitive, but require the compound of interest to have fluorescence characteristics. The ADAM method does not use fluorescence in the quantitation of the adducts and therefore, can be applied to compound that lack fluorescence. The use of the ADAM method to detect BaP-DNA adducts will be useful in analyzing random human lungs for BaP-dGuo adduct.

Figure 6.1 Scheme for the detection of BaP-DNA adducts from human tissues

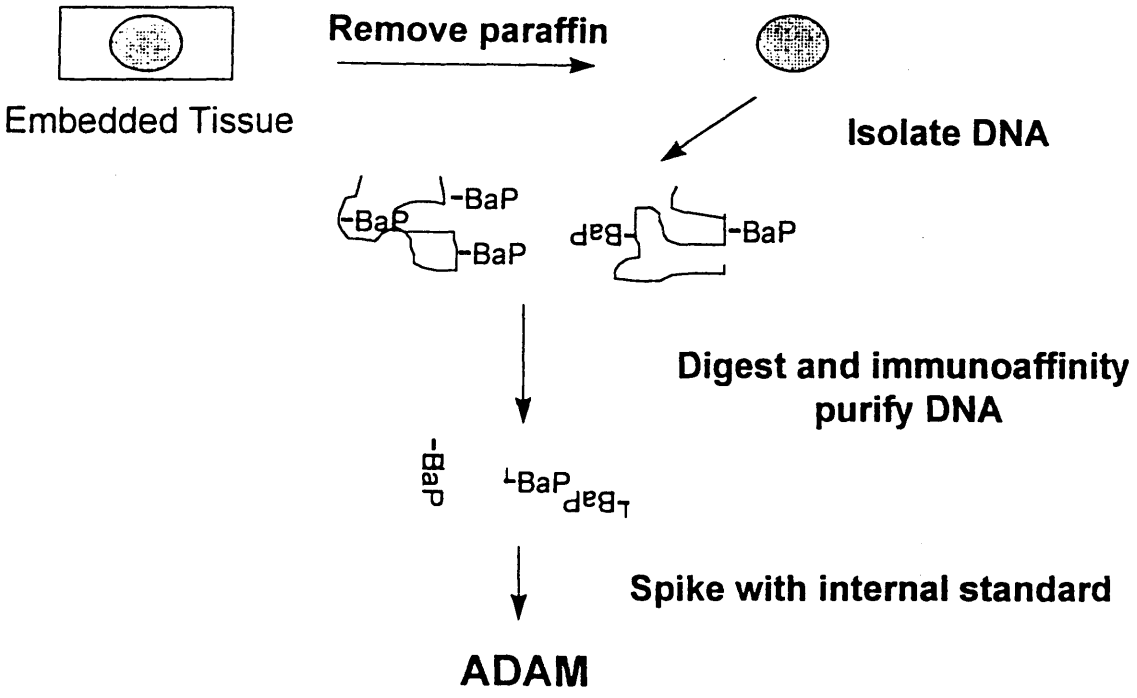


Figure 6.2 HPLC chromatograms of the acylation of ^{3}H-BaP-dGuo with ^{35}S-TBM

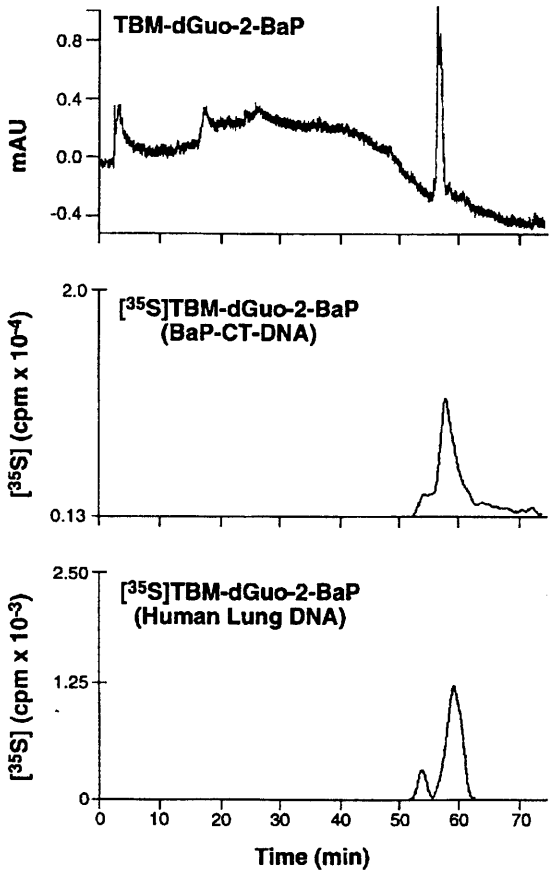


Figure 6.3 Radio-chromatograms of acylation products of DNA modified with BaP

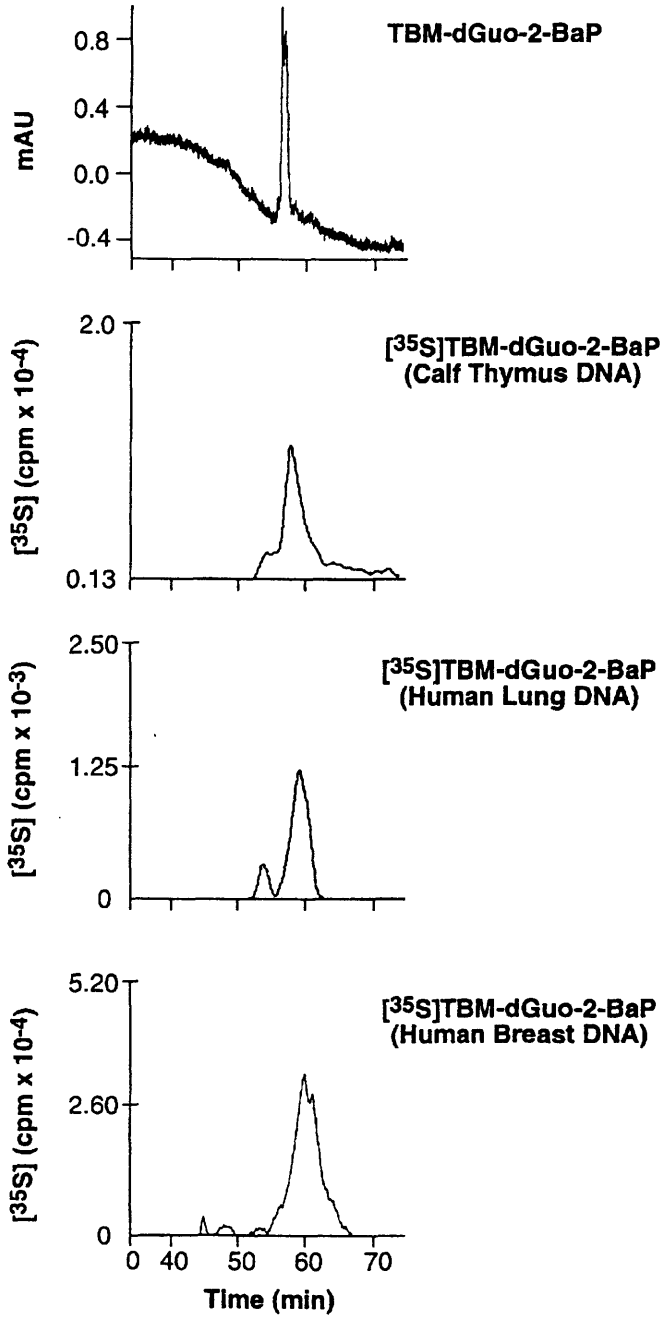


Table 6.1. BaP modification levels of human lung samples determined by the ADAM method. Fifty μg of DNA used for the analysis.

Human Lung			
Sample	**Adduct/10^8 nucleotides**	**Femtomole BaP/μg DNA**	**Adducts/Cell $\bar{x}$= 13,185**
F	426	14.7	51,120
K	58	2	6,960
P	2	0.05	240
R	210	7.56	25,200
N_1	12	0.36	1,440
N_2	55	1.64	6,600
C_1	ND	ND	ND
C_2	116	3.48	13,920

Table 6.2. BaP modification levels of human breast samples determined by the ADAM method.

Embedded Breast			
Sample	Adduct/10^8 nucleotides	Femtomole BaP/μg DNA	Adducts/Cell $\bar{x}$= 44,000
1	ND	ND	ND
2	484	15	58,080
3	35	1	4,200
4	ND	ND	ND
5	393	12	47,160
7	154	5	18,480
8	243	7	29,160
9	177	5	21,240
10	114	4	13,680
11	296	9	35,520
13	287	9	34,440
14	562	17	67,440
16	673	21	80,760
17	1764	54	211,680

Table 6.3. BaP adducts per cell in human breast samples determined by the ADAM method. The adducts are represented as the raw numbers and then divided by 20. The embedded tissue modification levels were divided by 20 to account for the 20-fold difference seen between those seen in frozen and those seen in fixed mouse tissues.

Embedded Breast

Sample	Adducts/Cell $\bar{x}$= 44,000	Adjusted Adducts/Cell $\bar{x}$= 2,221
1	ND	ND
2	58,000	2,904
3	4,200	210
4	ND	ND
5	47,160	2,358
7	18,480	924
8	29,160	1,458
9	21,240	1,062
10	13,680	684
11	35,520	1,776
13	34,440	1,722
14	67,440	3,372
16	80,760	4,038
17	211,680	10,584

Chapter 7 Conclusions and Recommendations

7.1 Summary of Thesis Results

The ability to measure individual exposure to carcinogens is valuable when trying to assess cancer risk. The use of carcinogen-DNA adducts, specifically BaP-DNA adducts, has been explored in this thesis to determine their usefulness in evaluating exposure to benzo(a)pyrene. An internal standard was generated to use as a control for the analytical experiments described. Several methods were explored to generate a BaP-dGuo standard. Benzo(a)pyrene diol-epoxide was successfully reacted with calf thymus DNA to yield the same profile as when calf thymus DNA is modified microsomally. This standard was used to further develop and adapt the ADAM method for the analysis of BaP-DNA adducts. A protocol has been established to successfully react benzo(a)pyrene-diol-epoxide with 2-deoxyguanosine.

Two major things were accomplished with the ADAM method. The first was further development and application of the ADAM method. The second was improvement of the ADAM with ^{35}S-TBM. The successful acylation of ^{3}H-BaP-dGuo with NHS-TBM is reported here as well. The improvement of the ADAM by the substitution of TBM in the place of TBM-NHS has had several advantages. The manufacturing, purity and specific activity can be controlled in the laboratory. In addition, the efficiency of the reaction is increased to 90%. As a result, the HPLC profile of the reaction products is much cleaner.

In vivo mouse samples were analyzed for BaP-DNA adducts with the ADAM method. The HPLC profiles produced by the acylation of BaP-dGuo with ^{35}S-TBM have similar retention times as embedded human breast samples acylated with ^{35}S-TBM. These have the same retention time as the peaks produced by the acylation of BaP-dGuo produced with calf thymus DNA, liver DNA from mice dosed with ^{3}H-BaP (data not shown) and human lung data. The acylation reaction conditions found to be optimal for *in vitro* modified DNA and 2'-deoxyguanosine have been found to produce comparable results with BaP-dGuo adducts in tissues. It is reported here that DNA from embedded tissues can be isolated and analyzed for BaP-DNA adducts by the ADAM procedure.

This work also included investigation of BaP-DNA adducts in mice and use of the ADAM method with human samples. The acylation products of BaP-dGuo, regardless of the source, elute at the same retention time. The use of 210 μg BaP in the newborn mouse lung adenoma assay successfully produced tumors. Samples were collected for analysis of BaP-DNA adducts in target and nontarget organs. It is possible and successfully shown here that DNA can be isolated from paraffin embedded tissues and analyzed for BaP-dGuo adducts.

7.2 Conclusions

Several conclusions arise from the work. First, ^{35}S content of acylated products was linearly correlated with the nucleoside adduct concentration over the range from 20 to 1000 femtomoles. Second, adduct levels determined on the basis of ^{35}S acylation were highly correlated with those determined from the ^{3}H-BaP internal standard in ^{3}H-BaP treated mice. Third, HPLC

profiles ^{35}S acylation products of adducts in DNA isolated from mouse tissues and human lung tissues were identical to authentic standards.

The major conclusions of this thesis research can be summarized as follows:

(1) A BaP-dGuo standard was generated for HPLC analysis.

- The HPLC profile was the same regardless of whether the adduct was generated with BPDE or with microsomes.
- A co-injection monitoring the UV absorbance (A_{347nm}) and the radio-labeled carcinogen ^{3}H-BaP provided two independent measures of the adduct and demonstrated a reproducible profile.
- The electrospray-MS analysis confirmed that the compound had the correct molecular weight *m/z* 570.

(2) The ADAM method was further developed and applied to measure BaP-dGuo adducts.

- The acylation of ^{3}H-BaP-dGuo with ^{35}S-TBM-NHS was successful over a range of specific activities from 10 Ci/mmol to 1000 Ci/mmol.
- Detection of BaP in a range of 20-1000 femtomoles of BaP-dGuo was accomplished.

(3) The ADAM method was improved by the substitution of ^{35}S-TBM which can be synthesized and purified in our laboratory.

- A linear relationship was observed between the amount of adduct versus the amount of ^{35}S acylated products when ^{3}H-BaP was reacted with ^{35}S-TBM.

- The improved method has several advantages. The manufacturing, purity and specific activity can be controlled and reaction efficiency increased to 90%.

(4) The levels of BaP-DNA adducts were investigated in treated mice.

- The 24 hour exposure to ^{3}H-BaP revealed the applicability of the ADAM method to *in vivo* samples and the correlation of the adduct levels based on ^{3}H and ^{35}S validated the method.
- Reaction products not present in the reagent control proved promising in the possibility of analyzing BaP-DNA adducts in tissues.
- It was demonstrated that DNA can be isolated from paraffin embedded tissues and analyzed for BaP-dGuo adducts.
- The use of 210 μg BaP in the newborn mouse lung adenoma assay successfully produced tumors. Samples were collected for analysis of BaP-DNA adducts in target and nontarget organs.
- The persistence of adduct levels in mice dosed in the newborn mouse assay was determined based on the ^{3}H-BaP treated mice.

(5) The ADAM method was used to quantify BaP-DNA adducts in human samples.

- The adduct levels examined are from DNA isolated from human lung and embedded breast tissues.

- The HPLC profiles of the acylation products had the same retention time as the peaks produced by the acylation of BaP-dGuo produced from calf thymus DNA and treated mice.

7.2 Recommendations for future work

Further optimization of the ADAM procedure for analysis of BaP-dGuo adducts

This research has produced an optimal method for measuring benzo(a)pyrene-dGuo adducts. However, there is always the potential for simplifying the method to increase productivity and ease of use. An improvement in the yield of the adduct during the hydrolysis and purification steps would be useful. Other areas to explore include adjusting the HPLC chromatography for the analysis of the acylation products by adjusting the HPLC profile by changing the column temperature or solvent gradient. For example, a shorter gradient would make it more efficient to analyze more samples.

Obtain additional informationufrom the tissues collected in the newborn mouse assay

In terms of the newborn mouse lung adenoma assay, there are a few areas that may be of interest. An examination of DNA isolated from female mouse samples from the newborn mouse assay would determine if there is a relationship between the DNA-adduct levels and tumor frequency in the liver. Since there were no liver tumors produced in the female mice, it would be interesting to determine if adduct levels in female were the same or different than the adduct levels in the male mice.

Analyze human samples for additional adducts

A study of the adduct levels in the heart would be a line of experimentation because it has been reported that other laboratories conducting similar experiments with PAHs found high adduct levels in the heart but no tumors in the heart. Is this because the turnover cell is much slower than a liver or lung cell or is better repair by the heart cell the key? These and other questions could be explored.

It would also be possible to examine histone-BaP adducts in newborn mouse lung adenoma assay. The histone adducts are the result of the carcinogen covalently binding to the proteins which the DNA is coiled around. This can be measured with a laser enhanced procedure specific for benzo(a)pyrene adducts (Ozbal, unpublished data). This would allow comparison of the two adducts and the carcinogenic correlation of each. The type of relationship which exists will provide valuable insight into the significance of the adduct levels and the formation of tumors.

The use of DNA from embedded tissues will allow the processing of stores of archival tissues. The advantages are that medical information can be obtained from the patient to get more information about the samples and that once the tissues are fixed and embedded, they can last indefinitely. The phenomenon of the amplified/selection of BaP-modified DNA from fixed-embedded tissues needs to be better characterized.

After anti-body purification, Ab column eluates were preserved. These washes can be repurified using antibodies for other compound. This would allow the examination of the same human lung and breast tissues for several different families of adducts. These might include heterocyclic ammines (PhIP, MeIQx), akylating agents, and possibly aflatoxins.

Broader goals include measuring more human samples in cancer and noncancer patients to see if there is any relationship on the levels of DNA adducts and tumor formation. It would be valuable to examine the same tissue to try to detect more than one type of adduct.

The need for more accurate measures of exposure is critical. Each individual has a unique set of levels of exposure to environmental carcinogens as well as a unique set of susceptibilities to these carcinogens. The variety may be due to age, sex, or even genetic background. Currently, one is not able to determine an individual's level of carcinogenic exposure and assess the relative risk of cancer for that individual with certainty. The generation of tissue specific DNA adducts, will provide a data base in correlating molecular biomarkers, specifically DNA adducts and cancer. Although it is clear the carcinogenesis is a multi-step process involving mutations, ineffective repair, etc. and eventually uncontrollable growth, an established relationship of biomarkers to exposure will pave the way to accurately answer questions of individual human exposure and may provide a basis for qualitative risk assessment.

This thesis focused on the use of the ADAM method to measure BaP-dGuo adducts. The ADAM method can be applied to other nucloside adducts as well. This method, with its improvements over previous approaches, will allow even more study of the role of PAHs in cancer formation in humans.

Chapter 8. References

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