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PRINCIPAL INVESTIGATOR: Peter G. Shields, M.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

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Peter G. Shields, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: pgs2@georgetown.edu

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13. ABSTRACT *(Maximum 200 Words)*

Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. The study of carcinogen-DNA adducts can provide corroborative evidence for the importance of genetic susceptibilities in breast cancer risk. We are establishing new assays for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies, and determine adduct levels in relation to metabolizing gene polymorphisms. The assays are novel because one uses a new chemical postlabeling method and quantitates adducts by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). The second uses capillary HPLC and laser-induced flourescence. Once validated, we will learn the variability for DNA adduct levels in the population as it relates to age, gender, race, and smoking in breast tissues from 235 donors (200 women, 35 men). We also will develop breast strains from normal donors, determine in vitro adduct formation levels and correlate these levels with p53 induction. Thus, this study will provide new information about genotype-phenotype relationships.

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(4) Introduction

Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Currently, many ongoing breast cancer studies are exploring risks related to genetic polymorphisms in these genes. Yet these studies by themselves do not provide absolute proof of etiology or causality. Thus, corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. We are focusing on carcinogen-DNA adducts because they are promutagenic and lead to alterations in cancer susceptibility genes. They also serve as a marker of the biologically effective dose of a carcinogen, indicating a person's phenotype for metabolism, DNA repair and apoptosis. Several available carcinogen DNA-adduct assays might be useful here, but they are not sufficiently specific and/or sensitive for testing mechanistic hypotheses in humans. We are attempting to establish a new assay for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies. This assay is novel because it uses a new chemical postlabeling method and quantitates adducts by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). We will develop and rigorously validate the assays using benzo(a)pyrene- (BPDE) and 4-aminobiphenyl (4-ABP)-related adducts as prototypes. Other methods are being explored in the event that finalization of the proposed method is not possible or if other, less labor intensive methods, can be done. With the development of an assay, we will learn the variability for DNA adduct levels in the population as it relates to age, gender, race, and smoking in breast tissues, and explore relative levels in liver tissue from autopsy donors. From a subset, 30 matched blood samples will be used to determine the relationship of breast levels (i.e., the target organ) to blood (i.e., the surrogate tissue). Finally, in these subjects, we will perform assays for genetic polymorphisms, to assess the association of "at risk" genetic variants with higher breast adduct levels. We also will test genotype-phenotype relationships by culturing primary breast cells from the same women, correlate adduct levels from in vitro carcinogen exposure and determine p53 response. This will allow us to establish the variability in the population for p53 induction from carcinogens, and might imply an independent risk for women with a low response.

During the interim between awarding the grant and receiving the funding, Dr. Shields decided to move his research laboratory from the intramural program of the National Cancer Institute to the Lombardi Cancer Center (LCC) of Georgetown University. Thus, it was decided to delay the implementation of the project until the budget and project design could be reorganized. First, it was inefficient to begin the project at NCI, which would last only three months and then require an interruption of the work and change in personnel. Upon arriving to the LCC, there was additional time needed to hire a postdoctoral fellow and establish the laboratory, although some work began almost immediately by Drs. Shields and Goldman. Thus, this project was not fulling implemented until about 6 months after the initial project date. All needed equipment, reagents and tissue samples were either transferred from the NCI, or purchased new at the LCC.

It should also be noted with the transfer of the grant from the NCI to LCC, there was a loss of available money because the NCI does not require overhead costs, while the LCC does. The DOD was requested to increase the cost of the total award to cover the additional overhead. This was denied and so there was a required rebudgeting to allow for the overhead, which reduced the available direct monies by almost 30%. Thus, some tasks may not be accomplished by the end of the three year grant period.

(5) Body

Task 1: **To develop a ^{14}C-postlabeling method with acetic anhydride using micropreparative techniques for chemical specificity and AMS for sensitivity (CAP). BPDE and 4APB adducts will be used as prototypes. (Months 1 -18)**

As will be described, we have been simultaneously developing the CAP method for BPDE and 4ABP. And we have been pursuing a capillary HPLC and laser induced fluorescence method (LIF) for the detection of BPDE adducts, because we expect that this will be simpler and less labor intensive. Start-up funding from LCC enabled the purchase of this state-of-the-art equipment.

CAP for BPDE adducts

During the initial start-up period, a publication resulted from the preliminary data presented in our grant proposal. A percentage of the effort by Dr. Shields and Goldman was devoted to confirming experiments and revising the manuscript. This manuscript is included in the appendix. There, we described the method of postlabeling carcinogen-DNA adducts by acetylation with ^{14}C-acetic anhydride combined with quantitation of ^{14}C by accelerator mass spectrometry (AMS). For this purpose, adducts of benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (BPDE) with DNA and deoxyguanosine (dG) were synthesized. The most promutagenic adduct of BPDE, 7R,8S,9R-trihydroxy-10S-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG), was HPLC purified and structurally characterized. Postlabeling of the BPdG adduct with acetic anhydride yielded a major product with a greater than 60% yield. The postlabeled adduct was identified by liquid chromatography-mass spectrometry as *pentakis*(acetyl) BPdG (AcBPdG). Postlabeling of the BPdG adduct with ^{14}C-acetic anhydride yielded a major product coeluting with an AcBPdG standard.

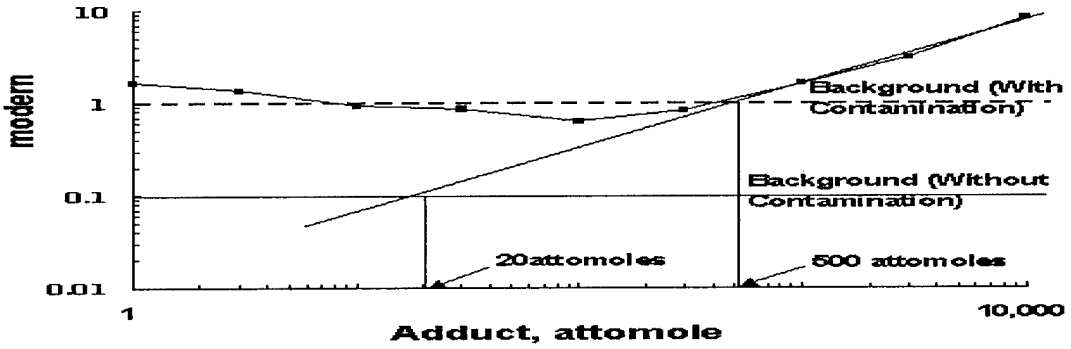
Following that paper, an important effort ensued that would allow to improve the sensitivity of the assay by reducing the background. As in Figure 1 (Figure 3 of the paper), we were able to bring the limit of detection to about 500 attamoles, but the 14C background was too high to do better. Therefore our next efforts were directed toward reducing background. This was first attempted by optimizing the clean-up procedures after postlabeling. As a standard, we synthesized and purified acetylated ^{3}H-BPdG standard (^{3}H-AcBPdG). 1400 dpm of the standard, 1000mCi/mmol, was used for each experiment. The cleanup was optimized using ^{14}C-acetylated nucleotide mix (^{14}C-AcdN) or ^{14}C-acetylated BPdG (^{14}C-AcBPdG). 10^6 dpm of the ^{14}C-labeling reaction was applied to Seppak cartridges. The recovery was determined by liquid scintillation counting. All Seppak cartridges were from Waters.

We tested the following:

QMA/OASIS/C18: Combination of three columns in series, each treated as described below. The 70% CH_3OH eluent from QMA, 1ml, was loaded directly on the OASIS column. The 1ml CH3OH eluent from the OASIS Seppak was concentrated on speedvac prior to transfer to the C18 (5mg) cartridge.
QMA/OASIS: Combination of the QMA anion exchange seppak with the OASIS reverse phase polymer in series (see below).
QMA: The QMA anion exchange seppak, 130mg, was equilibrated with 5ml 70% CH_3OH. Analytes were eluted with 70% CH_3OH and the first 1ml fraction was collected.
OASIS: The OASIS reverse phase polymeric seppak, 100mg, was conditioned with 5ml CH_3OH/5ml dH_2O and nonspecific binding was blocked with acetylated nucleotide-mix (AcdN), 1mM, 50ml. Analytes were added in 50% CH_3OH, washed with 25ml of each a. Na-acetate, 50mM/50% CH_3OH pH 5.3 b. Na_2CO_3 (50mM)/50% CH_3OH pH9.3 c. 0.01%

tween20 and d. dH_2O. Analytes ware eluted with 1ml CH_3OH.
C18 (5mg): Columns were prepared by wetting 100mg of C18 reverse phase resin in 1ml

Figure 1



methanol and by packing of 50ml of the suspension onto a 1.5ml polypropylene column. AcdN, 1mM, 50ml was applied to block nonspecific binding. Analytes were loaded, washed on a vacuum suction apparatus with 20ml 25% DMSO, 20ml 0.0025% tween20, and 10% aq. CH_3OH, eluted with 1ml 100% CH_3OH, and dried on a speedvac.
C18 (50mg): The C18 reverse phase seppak, 50mg, was treated as above for the C18 (5mg).
NP: The silica normal phase seppak, 160mg, was conditioned with 1ml hexane, 1ml CH_2Cl_2 and again 1ml hexane, and equilibrated with CH_2CL_2(80)/ ethyl acetate(15)/ methanol(2). ^3H- or ^{14}C-labeled analytes were loaded on the seppak in CH_2CL_2(80)/ethyl acetate(15)/ methanol(2) and the first 1ml of eluent was used for further experiments.
Immunoaffinity chromatography (IAC) w/o Resin: Antigen was added directly to a polypropylene column with 2 frits (without resin). Column was washed with 25ml ammonium acetate, 30mM, pH 7.0 followed by 25ml dH_2O, and eluted with 1ml CH_3OH.
IAC w/tween: 8E11 antibodies were immobilized on an Immunopure® Protein G IgG Orientation kit (Pierce). A 60ml aliquot of the resin was packed on a 1.5ml polypropylene column. The antigen was loaded directly on the column, washed with 25ml ammonium acetate, 30mM, pH 7.0, followed by 0.0025% tween20 in ammonium acetate, 30mM, pH 7.0, and 25ml dH_2O. The antigen was eluted with 1ml CH_3OH.
IAC: As above without the wash with 0.0025% tween20/ammonium acetate.

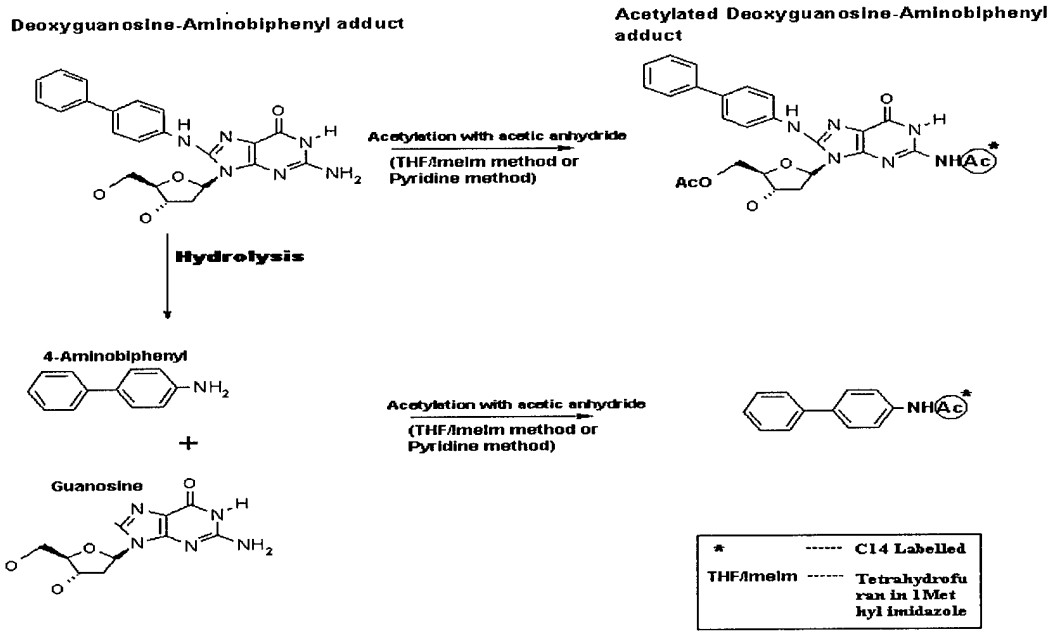
Unfortunately, while we were able to retain good adduct recovery for almost all the methods, we were not able to improve the decrease of the background. This remains the major problem. Our plans to alter the chemical conditions for postlabeling, in order to use less starting material, chemically bind or precipitate the remaining labeled acetic acid, or better optimize that IAC. However, due to the decrease in funding from the loss of indirect monies, we have temporally put the BPDE CAP on hold to pursue 4ABP CAP, because the chemical conditions here might be sufficiently different that would allow for better recovery and cleanup. Separately, we are optimizing a Capillary HPLC and LIF method that would allow for the detection of BPDE adducts.

CAP for 4ABP adducts: This method also is based on postlabeling the carcinogen-DNA adducts with ^{14}C-acetic anhydride (**Ac_2O**) coupled with accelerator mass spectroscopy (AMS). Briefly the

method the method involves digesting the sample DNA to yield a mixture adducted and non-adducted nucleotides. Purifying the adducted nucleotides by immunopurification procedure follows this. Subsequently the carcinogen-base adducts are subjected to an acylation procedure using ^{14}C-acetic anhydride. The products are isolated using HPLC followed by detection using AMS procedure. For this purpose, adducts of 4-aminobiphenyl with guanosine have already been synthesized by us. The authenticity of these materials have been confirmed during this grant period by mass spectroscopy.

We have adopted a two-fold approach to towards standardizing the detection of ABP-DNA adducts in patient samples. One is detecting ABP-guanosine adducts as such by obtaining digested products of DNA samples followed by immunopurification. Secondly, examine the possibility of hydrolyzing the 4-aminobiphenyl from the bases itself, thus trying to do away with process of immunopurification procedure (Figure 2). Also, this would make the methodology more specific. The present report is based on the second approach that has given promising leads.

Figure 2



Standardizing the methodology involved the following important steps.

1. Normalize the acetylation procedure of the starting material (4-ABP/ABP-Gu adduct) to obtain maximal yields. Standardize the reaction conditions with respect to temperature, duration of reaction, and amount of acetic anhydride required for acetylation (reactions would be initially carried out using cold acetic anhydride). Use HPLC as means for detection of products. Once the profiles the compounds have been set, scale down the amount of starting material (4-ABP) and the amount acetic anhydride. Subsequently go in for standardizing using ^{14}C labeled acetic anhydride.
2. Validate the ability of 4-ABP to bind to antibodies generated against ABP-Gu adducts. (Antibodies have been obtained from both Gerald Wogan and Regina Santella for two attempts to have suitable antibodies for IAC.) Bind the antibodies to columns (obtained commercially). Calibrate the binding capacity of both 4-ABP and ABP-Gu using CapLC with appropriate controls. Should the binding not be possible or efficient enough, go in for making antibodies against 4-ABP itself. Prepare standards of ABP-Gu adduct by established protocols (from existing literature) and use this in validating binding efficiencies of antibodies.

3. Standardize the hydrolysis step of ABP-Gu.
4. Perform AMS.

The yields of acetylation were initially standardized (with non-radioactive acetic anhydride). The yields are compared with 4-aminobiphenyl-guanosine as a standard using HPLC. The acetylation has been carried out using two methods

1) Acetic anhydride dissolved Tetrahydrofuran containing 16% methyl imidazole
2) Acetic anhydride dissolved in pyridine.

Reaction protocol:

1) 1 : 1 of the starting material (4ABP-Gu) was dried in vacuum for ~15-20 min (drying time was standardized and its effect on elution profile was also verified to be OK).
2) 500 : 1 if THF/MeIm was taken in a dry vial(20 : 1 of anhydrous pyridine in the alternative method). 3 : 1 of anhydrous acetic anhydride was mixed with this and instantly vortexed. 30 : 1 (from the THF/MeIm mix) or the entire pyridine/Ac.An mix was then added to 4ABP-Gu (4-ABP alone).
3) The reaction was allowed to proceed for 20 mins (THF/1MeIm) [2hrs in case of pyridine method].
4) The reaction was terminated by adding 70 : 1 of water. Subsequently, the samples were dried in vacuum for ½ hr. The samples were redissolved in 100% methanol, dried. The samples were dissolved 5-20% acetonitrile before HPLC analysis.

For the 4-ABP acetylation, we have thus far mostly focused on the pyridine based method, while for the dG-8-ABP adduct acetylation both the methods (Pyridine and THF/1MeIm) have been used. Elution profile of 4-aminobiphenyl as characterized by the gradient profile is given below

C18 column; acetonitrile (5%) and water (95%).

Gradient profile in HPLC:	0-2 min	-	45% acetonitrile
	2-5 min	-	grad 45-75% acetonitrile
	5-9 min	-	75%
	9-11 min	-	grad 75-95%
	11-13 min	-	95%
	13-15 min	-	grad 95-45%
Run parameters: Flow rate		-	1ml/min
Resting backpressure		-	82-85 bars
Injection volume		-	10 : 1

With the above parameters, the compound 4-aminobiphenyl eluted out at 7.76 min. The absorption maxima was ~280 nm. A small related peak could be seen at 7.06 min. The ABP-Gu adduct eluted at ~3.5 min. The absorption maxima was ~300 nm.

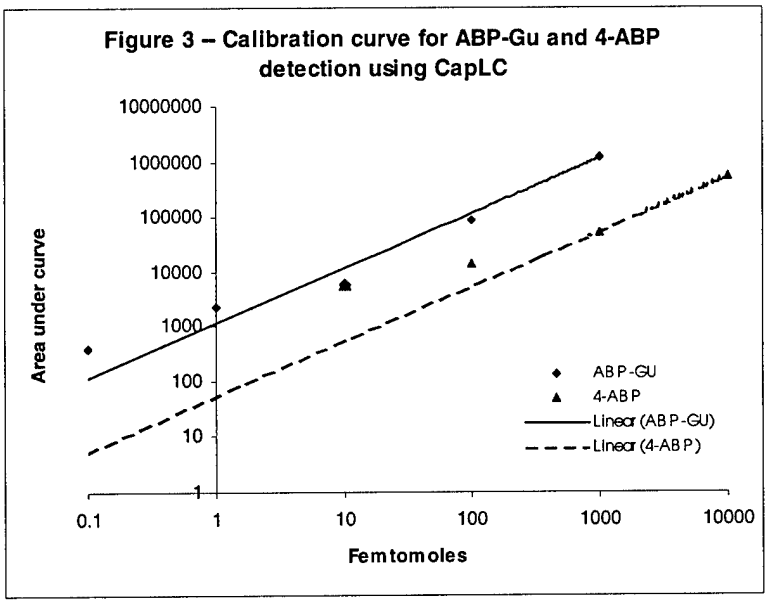
The yields of acetylation were standardized for 4-ABP. In case of ABP-Gu adducts the yields were not efficient (<50% yield of the acetylated product – as measured by UV detection in HPLC). Since the yields were better in case of 4-ABP (~ same as starting material), the focus was shifted to 4-ABP acetylation.

The optimal conditions to achieve acetylation of 4-ABP was observed to 37°C with incubation time of ~2hrs. Mass spectroscopy was used for the acetylated 4-aminobiphenyl and

the control-unacetylated samples at the facility in Pharmacology department using an HP1100 GC MS instrument. Samples were injected directly into the MS for analysis based on run parameters standardized earlier for 4-ABP. The presence of the acetylated compound was inferred using the anticipated molecular weight. A near quantitative yield of the product from the starting material was observed and there were no other side products. The MS of 4-aminobiphenyl alone also showed the presence of a 212 molecular weight peak that corresponded to the acetylated product. Interestingly, the HPLC profile also showed a peak at the RT where the product elutes out.

The existing reaction protocol gave about 2000-fold excess of Ac_2O over starting material (1 nanomole). This meant one vial of labeled acetic anhydride could successfully acetylate about 10 DNA samples. The amount of Ac_2O was scaled down serially to observe the minimum requirement for acetylation. The yields still stood the same when the amount of Ac_2O was diluted up to ten fold below the existing amount. This meant greater amount of samples could be acetylated for the same of labeled Ac_2O. Since the amounts of 4-ABP are expected to much lower than the presently used concentration, more samples can be expected to processed if the amount Ac_2O could be further scaled down. However, when the amount of starting material and the Ac_2O amount were further scaled down, there were problems with yields. This problem is presently being addressed.

A calibration curve was established (Figure 3)



Antibodies to ABP-Gu adducts were obtained from Dr. Regina Santella at Columbia University in the form of ascites and were stored at -70°C. The antibodies were bound onto a column containing immobilized protein-G (commercially obtained kits) using the following procedure:

1) Load 10ml of ascites solution (~10mg of Ab) onto the column.
   a. Equilibrate the column with 5 ml of Antibody Binding/washing buffer.
   b. 10 ml of ascites solution + 10 ml of PBS buffer.
   c. Load 2-4ml onto a column – gentle inversion – tap end of bottom.
   d. Mix by gentle inversion for 30 minutes @ room temperature.. Remove top and bottom caps sequentially and allow passing out the remaining solution.
   e. Repeat procedure 'c' and 'd' until all the ascites has been loaded onto the column
   f. Wash extensively with Bind/wash buffer (4-5 times).

2) Cross Link the bound antibody with DSS
   a. One vial content of DSS/1 ml of DMSO. Mix with 1.5 ml cross linking buffer (from kit).
   b. Load onto column, mix and allow settling.
   c. Wash with 1 x 5ml of cross-linking buffer.
3) Block the remaining active sites
   a. Load 2ml of blocking buffer onto column, mix by inversion, allow settling the column and allow the solution to pass out.
   b. Wash with elution buffer and allow flowing through (5 ml first time, 2ml next time).

Store the column @ 4°C (with 0.02% NaN_3)

Aliquots from this column were used load small columns that were used to check for binding of capacities. The following protocol was used to run the samples and process them for detection by CapLC electrophoresis (using UV detection).

1) Elute out the old solution containing NaN_3. wash the column with 2-5ml of bind/wash buffer.
2) Take 20:1 of gel from the already prepared Immunoaffinity column into fresh smaller columns.
3) Wash with bind/wash buffer twice .
4) Load a known amount of ABP-Gu std (100 femtomole) from a diluted stock onto the column and collect the fractions.
5) Gently place the white frit on top of the column.
6) Mix by gentle inversion for 1 hr and allow to settle.
7) Dry down the sample and redissolve in water. Same as above but sample not passed through column.- to rule out non specific binding.
8) Do the same as in '6)' for 4-aminobiphenyl.
9) ABP-Gu and 4-ABP – Measure concentration before loading. After loading and collecting, determine efficacy of generated antibodies.
10) Use appropriate starting material and determine background and recovery of CAP labeled adducts.

A calibration curve using tritiated label adducts was generated for detection of 4-ABP and ABP-Gu. 4-ABP with a suggested limit about 10 femtomoles/l (Figure 3). Using this, around 1 picomole was selected to monitor the binding capacities for IAC optimization. There have been problems presently carrying out this protocol. Binding of either ABP-Gu adducts or 4-aminobiphenyl could not be observed thus doubting the presence/binding efficiencies of the generated antibodies. The problem is being tackled by checking if antibodies have bound to the columns (assay the amount of Antibodies in ascites as well as columns using ready-made kits). Also, there has been a problem of drying out of the samples after they have been eluted out in methanol. Presence of small amounts fat like material is suspected. This is being tackled by using Tween-20 (<5%) and 20% methanol.

We are currently estimating the antibodies in the ascites solution and preparing new columns and checking for binding efficiencies. If antibodies bind only to adduct, then we will concentrate on standardizing acetylation efficiencies of the adduct alone. We will continue with standardization of acetylation efficiency for 4-ABP and ABP-Gu and start with 14C labeling for acetylation purposes. In the next year we expect to complete standardizing acetylation procedures for ABP-Gu adducts.

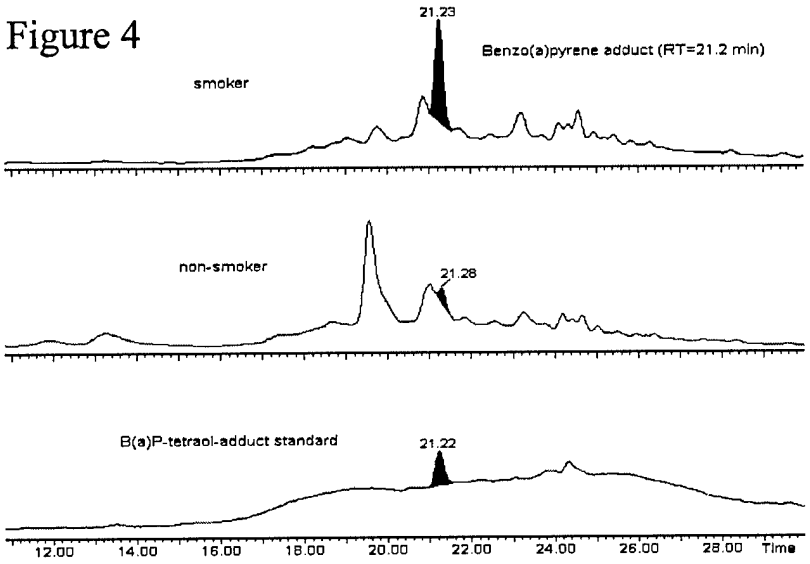
Capillary liquid chromatography with laser induced fluorescence (capLC-LIF)

One of the best methods for B(a)P quantification is the fluorescent detection of B(a)P-tetraol following HPLC separation of the acid hydrolyzed B(a)P adduct. The B(a)P tetraol has strong fluorescence (λex=345 nm; λem=390 nm) which allows direct detection of the compound

without labeling. We are taking advantage of this native fluorescence and further modifying the method to improve the detection sensitivity. To this purpose, we have recently installed a capillary LC system (Waters Inc., Milford, MA) together with a laser-induced fluorescence detector (Picometrics, Ramonville St Agne, France). The capLC decreases typical HPLC flow rates 1000 fold (from 1ml/min to 1μl/min). This promises an approximately 100 fold improvement in sensitivity over the HPLC/fluorescence when combined with the laser induced fluorescence detection (which can be focused on the capillary). Both the capLC and the fluorescence detector are new instruments that were not available a year ago. The capLC has a unique syringe pump design that assures reproducible flows at this incredibly low flow rate. The detector is interfaced with a 325 nm argon laser. This powerful combination has great sensitivity. We have been able to detect 100 attomol (100x10-18 mole) of the B(a)P tetraol with signal to noise 7:1. The dose response was linear over 3 order of magnitude. This should allow detection of less than 1 adduct in 109 nucleotides with 100 micrograms of DNA extracted from the tissue!!!

Frozen autopsy tissues (several grams of each specimen) are available for this study together with paraffin-embedded tissue blocks and microscopic slides prepared from them. We have extracted DNA from two of each lung, liver, and breast samples. The six samples yielded at least 100mg of DNA per gram of tissue (several 100 mg can be obtained from each available specimen). This quantity is sufficient to carry out the B(a)P adduct measurements.

Figure 4



To further verify the applicability of the method to human samples, we have analyzed two human samples with B(a)P adducts previously measured by 32P-postlabeling. One sample was lung tissue of a smoker with high B(a)P adduct level by 32P-postlabeling, the second lung sample was a nonsmoker with B(a)P adduct below the detection limit of the 32P-postlabeling method. 0.05 mg of each DNA was hydrolyzed by 0.1N HCl for 4 hours at 90^{0}C. The DNA lysate was applied to a C18 seppak cartridge (Alltech, State College, PA) and washed with 5ml of distilled water. The retained hydrophobic compounds including the B(a)P tetraol were eluted in 1ml of 100% acetonitrile and dried. The sample was reconstituted in 20μl of 25% aqueous methanol and injected into the capLC using an autosampler. The analytes were separated on a β-basic 100 x 0.3 mm column (Keystone Scientific, Bellefonte, PA) using a linear gradient of water and acetonitrile from 5% to 50% acetonitrile over 45 minutes. The B(a)P tetraol was identified by retenion time corresponding to the retention time of an authentic B(a)P standard (Chemsyn, Lenexa, KS). The result is shown in Figure 4. High adduct level by 32P-postlabeling was clearly higher by fluorescence (Figure 4). The sample undetectable by 32P-postlabeling was still measurable. Further experiments are needed to determine optimal extraction and separation conditions as well as validate the quantification with known satandards of B(a)P-derivatized DNA.

We have shown that the proposed method for B(a)P-adduct detection is extremely sensitive (<100amol B(a)P-tetraol) and applicable to human samples. The sensitivity of the detection system is only one important parameter. It is equally important to retrieve the adduct in high yield and without interfering impurities so that the decrease in the signal to noise ratio is minimal. The method needs to be also robust and reproducible to withstand the application to a

human sample-set. In the next year we will optimize the method and determine the reproducibility. Because it is expected that adducts of other polycyclic aromatic hydrocarbons (PAHs) will be present in the human DNA exposed to complex PAH mixtures, IAC will be incorporated as a micropurification method before CapHPLC.

Task 2: To demonstrate the use of the new adduct detection method in human breast tissues, livers, and blood from autopsy and surgical reduction mammoplasty donors. (Months 18 - 36)

Autopsy samples as described in the grant proposal, collected at the NCI, are now at LCC. We have samples from matched breast, liver and lung samples. We also have samples from subjects who underwent reduction mammoplasty, and have developed primary strains from these same women. At the LCC, we have established the same study, where we recruit women who are undergoing reduction mammoplasty (about 1-2 per week), have them provide a 2 hour long personal interview, and provide us with breast tissue and blood. We then culture the tissue as well as bank fresh frozen tissue. Thus far, 8 subjects have been recruited.

Task 3: To use a novel breast primary epithelial culture system will be used to demonstrate the variability of p53 in the population.

To determine p53 response in 40 human primary breast epithelial cells following exposure to benzo(a)pyrene (Months 30-36)
We have transferred 66 HMEC strains from the NCI. There is now an ongoing collection of tissues, with routine culturing at LCC (about 1 per week). p53 data in response to irradiation has been tested in 11 of those NCI strains and one of the LCC strains. The NCI strains were used initially to develop the protocol for p53 protein measurement, and once that was established, data was collected on strains that were subsequently unfrozen. Many of the experimental problems have been worked out, i.e. the best way to culture the frozen strains for p53 experiments, the amount of tissue I need from LCC patients to establish a strain. The main problem that still exists with the NCI frozen strains is that many did not survive the freezing process, however, since we have multiple tubes for each strain, we are hoping that at least one tube is viable, and we will be able to get data for each strain. If this fails, there is sufficient accrual at LCC, with fresh tissue, that the total number of subjects is not problem.

Tissue is collected from disease-free women undergoing reduction mammoplasty within 4 hrs. following surgery. The tissue is digested with a collagenase/hyaluronidase mixture into organoids. The organoids are plated in flasks following a 24 hour digestion period. The flasks are maintained in serum-free mammary epithelial cell growth medium and fed three times a week. Epithelial cells are passaged at confluency, which they typically reach 3-4 weeks following digestion. Frozen strains are thawed and maintained in the same manner. Experiments to measure p53 are done at the second or third passage. The cells are plated in equal amounts in 35 mm dishes at about 70% confluency (usually about 1 X 10^5 cells/per dish). Cells are then exposed to gamma-irradiation in a JL Sheperd Mark I irradiator at doses of 5, 10 or 20 Gy. Controls are sham-irradiated cells.

Four hours following irradiation, proteins are extracted from the cells and used in western analysis. p53 is measured using the DO(1) antibody according to standard protocols. Beta-actin protein levels are also measured. The results are quantitated using densitometry and the p53 protein levels are normalized to the beta-actin protein levels to obtain the level of p53 induction. In some experiments, 4-aminobiphenyl (4-ABP) was applied to cells cultures. A cytotoxicity assay was used to determine the appropriate concentration of 4-ABP and the cells were incubated in 0.3 uM, 30 uM (the ED_{10}), or 3mM 4-ABP. Non-treated cells were used for controls. P53 and beta-actin proteins were measured as above.

DNA damage resulting from chemical carcinogens and ionizing radiation has been shown to induce p53 in several experimental models. However, to our knowledge this has not yet been demonstrated in normal human mammary epithelial cells (HMECs). We have performed primary

cultures of HMECs from six different cancer-free women undergoing reduction mammoplasties. The cells were passaged at confluency and experiments were performed at the third passage. The environmental carcinogen 4-aminobiphenyl (4-ABP) was prepared as a 10-fold dilution series with concentrations ranging from 30 mM to 30 nM and applied to the HMECs. Following a 24-hour incubation period, cell death was measured using a fluorescence assay. There was an increase of cell death at all concentrations of 4-ABP as compared to non-treated controls with a maximum two-fold increase in death with 30 mM 4-ABP. Using Western analysis, we have also shown an approximate two-fold increase in p53 protein in the HMECs of the one strain tested thus far with 0.3 uM 4-ABP. Additionally, we investigated the effect of γ-irradiation on the six HMEC cell strains. The HMECs were each exposed to γ-irradiation at doses of 5, 10, and 20 Gy. p53 protein was detectable by Western analysis at all levels of irradiation, and at baseline in non-irradiated controls. An approximate 2-fold increase was demonstrated at the highest level of irradiation (20 Gy) from control levels (0 Gy) in all six strains. However, there was a differential amount of p53 induction among the strains at lower doses of γ-irradiation (5 and 10 Gy). The six strains can be separated into three distinct groups for response (none, low, and high) with three strains showing no significant increase in p53 at 5 and 10 Gy; two strains demonstrating a dose-dependent increase in p53 at 5 and 10 Gy; and one strain showing an initial 2-fold increase in p53 at 5 Gy and then leveling off at 10 and 20 Gy. These results indicate that the p53 response to DNA damaging agents differs in the breast and we hypothesize that such differences might influence breast cancer risk.

(6) Key Research Accomplishments:

1. The method for CAP detection of BPDE adducts is feasible, but there remains substantial problems in reducing background. One manuscript has been published.
2. The method for CAP detection of 4ABP adducts is being established. Labeling of the adducts is possible, with reproducibility and authenticity. Micropurification methods are now being developed.
3. Capillary HPLC and LIF is being developed for BPDE adducts. Preliminary data from human tissues indicates that this method is feasible.
4. An epidemiological infrastructure has been established at LCC to continue accrual of women undergoing reduction mammoplasty.
5. Breast strains have been cultured, exposed to gamma radiation and 4-aminobiphenyl and differential p53 responses have been found. We hypothesize that women with better responses might be at reduced breast cancer risk.

(7) Reportable Outcomes

1. One manuscript has been published, which appears in the appendix.
2. One abstract has been submitted to the AACR. It describes the preliminary results for cultured breast strains and p53 response. Eight cell strains have been established and frozen.
3. Tissue repository of breast tissues from women undergoing reduction mammoplasty has been established.
4. An R21 NIH grant proposal was submitted to further develop the capillary HPLC/LIF method by Dr. Goldman.

(8) Conclusions

This grant supports the development of BPDE and 4ABP adduct detection methods, which will be applied to breast tissues in order to examine genotype-phenotype relationships. It also supports the establishment and use of breast epithelial strains for examining the genotype-phenotype relationships and the p53 response based on adduct levels. Thus far, we have determined the basic CAP methods for the BPDE and 4ABP, but there are problems for the former with limitations in clean-up of contaminants. We are currently determining if we will have the same problems with the latter. As an alternate strategy, we are developing a capillary HPLC and LIF method for BPDE adducts. The preliminary data in human samples is very promising, but the quantitative accuracy still needs to be determined. We have established an epidemiological study and tissue repository of women undergoing reduction mammoplasty. In addition to the samples collected while at the NCI, this will ensure sufficient tissues for this study. The initiation of this project was delayed because Dr. Shields moved his laboratory, but the project is now fully implemented. Importantly, due to the change in institutions, less money is available for research because of loss to indirect costs. Thus, it is not clear is all tasks will be completed, but thus far there is not yet reason to believe that this will be the case.

(9) References

Goldman, R., Day, BW, Carver, T.A., Mauthe, R.J., Turltetaub, K.W., Shields, P.G.: Quantitation of benzo[*a*]pyrene-DNA adducts by postlabeling with ^{14}C-acetic anhydride and accelerator mass spectrometry, Chemico-Biological Interactions. 126:171-183. (2000)

APPENDICES

Related Journal Articles

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Chemico-Biological Interactions 126 (2000) 171–183

Chemico-Biological Interactions

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Quantitation of benzo[*a*]pyrene-DNA adducts by postlabeling with ^{14}C-acetic anhydride and accelerator mass spectrometry

Radoslav Goldman [a,1], Billy W. Day [b], Tonya A. Carver [c], Robert J. Mauthe [d], Kenneth W. Turteltaub [c], Peter G. Shields [a,*]

[a] *Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

[b] *Departments of Environmental and Occupational Health and of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15238, USA*

[c] *Lawrence Livermore National Laboratory, Livermore, CA 94551, USA*

[d] *Pfizer Inc., Groton, CT 06340, USA*

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Abstract

Quantitation of carcinogen-DNA adducts provides an estimate of the biologically effective dose of a chemical carcinogen reaching the target tissue. In order to improve exposure-

Abbreviations: AMS, accelerator mass spectrometry; AP, alkaline phosphatase; BPDE, benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide; BPdG (RSRS), 7*R*,8*S*,9*R*-trihydroxy-10*S*-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene; CD, circular dichroism; dG, deoxyguanosine; DMSO, dimethylsulfoxide; LC–MS, liquid chromatography–mass spectrometry; LSC, liquid scintillation counting; MeIm, 1-methylimidazole; NP1, nuclease P1; PB, phosphate buffer; RP-HPLC, reverse phase-high performance liquid chromatography; SVPD, snake venom phosphodiesterase; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran; TLC, thin layer chromatography.

* Corresponding author. Present address: Cancer Genetics and Epidemiology Program, Lombardi Cancer Center, Georgetown University Medical Center, Research Building W315, 3970 Reservoir Road, NW, Washington, DC 20007, USA. Tel.: +1-202-6870003; fax: +1-202-6870004.

E-mail addresses: rg26@gunet.georgetown.edu (R. Goldman), pgs2@gunet.georgetown.edu (P.G. Shields)

[1] Present address: Cancer Genetics and Epidemiology program, Lombardi Cancer Center, Georgetown University Medical Center, Research Building W301, 3970 Reservoir Road, NW, Washington DC 20007, USA.

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assessment and cancer risk estimates, we are developing an ultrasensitive procedure for the detection of carcinogen-DNA adducts. The method is based upon postlabeling of carcinogen-DNA adducts by acetylation with ^{14}C-acetic anhydride combined with quantitation of ^{14}C by accelerator mass spectrometry (AMS). For this purpose, adducts of benzo[*a*]pyrene-r-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BPDE) with DNA and deoxyguanosine (dG) were synthesized. The most promutagenic adduct of BPDE, 7*R*,8*S*,9*R*-trihydroxy-10S-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG), was HPLC purified and structurally characterized. Postlabeling of the BPdG adduct with acetic anhydride yielded a major product with a greater than 60% yield. The postlabeled adduct was identified by liquid chromatography–mass spectrometry as pentakis(acetyl) BPdG (AcBPdG). Postlabeling of the BPdG adduct with ^{14}C-acetic anhydride yielded a major product coeluting with an AcBPdG standard. Quantitation of the ^{14}C-postlabeled adduct by AMS promises to allow detection of attomolar amounts of adducts. The method is now being optimized and validated for use in human samples.

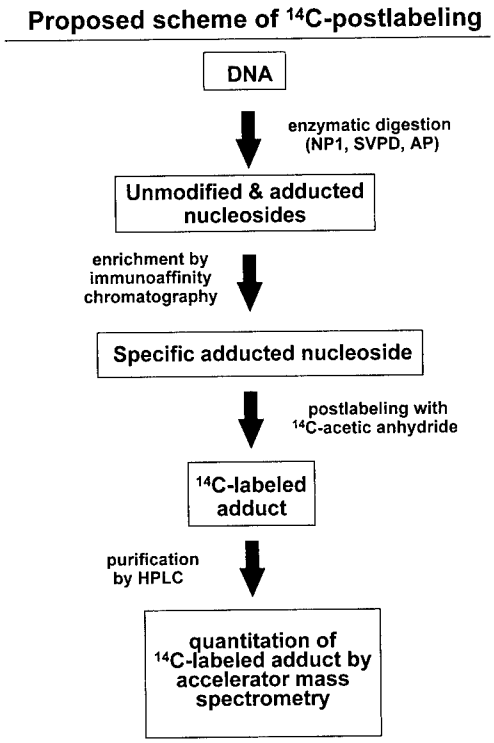
Keywords: Molecular epidemiology; Carcinogenesis ; ^{14}C-postlabeling; DNA adduct; Benzo[*a*]pyrene

1. Introduction

Epidemiological studies indicate that a substantial portion of human cancer derives from exposures to chemical carcinogens [1]. Most carcinogens form DNA adducts as a common pathway to mutagenesis [2–4]. The detection of carcinogen adducts in human tissues is a central strategy in molecular epidemiology [5] allowing: (1) estimates of the effective dose of a carcinogen in the target tissue; and (2) correlation of exposure with the incidence of disease.

Tracing the links from carcinogen exposure, DNA binding, resultant mutations and cancer contributes to an understanding of cancer etiology and the design of prevention strategies [6,7]. This task, however, is inherently difficult as humans are typically exposed to only low level complex carcinogen mixtures [8,9]. Difficulties for carcinogen-DNA adduct detection arise, in part, due to insufficient sensitivity, specificity, and reproducibility of the available analytical methods [10,11]. Detection methods based on immunoassay or ^{32}P-postlabeling may be sensitive enough [12], but lack the desired specificity unless combined with micropreparative steps (e.g. immunoaffinity chromatography) [13–15]. These additional analytical steps tend to reduce sensitivity and increase labor and/or cost beyond acceptable limits [16,17].

In our previous studies, chemical-specific measurements of benzo[*a*]pyrene-DNA adducts in human tissue reached the detectable limit in approximately 25% of examined samples [16]. This prompted us to develop a more sensitive method with retained chemical specificity for high throughput epidemiological studies. The chosen approach combines postlabeling of carcinogen-DNA adducts with [^{14}C]acetic anhydride with quantitation of ^{14}C by AMS (Scheme 1). AMS is an ultrasensitive ^{14}C detection method with documented sensitivity of zeptomole (10^{-20} mole) quantities of ^{14}C [18]. This leads to a theoretical detection limit of 1 adduct in 10^{12} to 10^{14} nucleotides based on studies with ^{14}C-labeled carcinogens [19]. This limit translates into at least 1000-fold improved sensitivity over currently



Scheme 1.

available methods [11]. We are attempting to combine this unmatched sensitivity of AMS with the versatility of the postlabeling methods. The approach is particularly promising because the postlabeling by acetylation with [^{14}C]acetic anhydride has an established chemical precedent [20]. The method should be also easily adaptable to a wide range of compounds.

The method is being developed on the most carcinogenic stereoisomer of the adduct formed by benzo[*a*]pyrene diolepoxide with deoxyguanosine which is 7*R*,8*S*,9*R*-trihydroxy-10*S*-(N^2deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene, BPdG (RSSR). Postlabeling of this widely studied precarcinogenic adduct will allow comparison with established methods. It is expected that molecular epidemiological studies based on improved detection and quantitation of DNA-adducts (and other biomarkers) will provide new, invaluable information for early detection and prevention of cancer.

2. Materials

(±)-BPDE, (+)-BPDE, (−)-BPDE, and racemic ^{3}H-BPDE were purchased from Chemsyn (Lenexa, KS). AP, DMSO, SVPD, and dG were from Sigma (St.

Louis, MO). THF/MeIm was from Perkin-Elmer/ABI (Foster City, CA). [$^{14}C_4$]acetic anhydride was from DuPont-NEN (Billerica, MA); the sealed glass ampule was kept at −20°C until experimental use. Pyridine was distilled over sodium and stored at 24°C in sealed glass ampules. DMSO was distilled under reduced pressure. All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

3. Methods

3.1. Synthesis of BPdG (RSRS)

Two methods were used to obtain sufficient quantities of standards of BPdG. Method 1B, based on derivatization of dG with BPDE, has higher yield than method 1A which relies on adduction of BPDE to DNA followed by digestion to nucleosides.

3.1.1. Method 1A

The synthesis followed a previously published method with minor modifications [20]. Calf thymus DNA (3 mg) was reacted with (+)-BPDE (0.5 mg) or racemic (±)-BPDE (0.5 mg) in 3 ml of 0.1M phosphate buffer, pH 7.5, containing 0.1 ml DMSO and 0.3 ml ethanol at 37°C for 12 h. The reaction was extracted with diethyl ether, ethyl acetate, and n-butanol (3 × 10 ml each). The DNA was precipitated from the aqueous layer with 0.3 M sodium acetate, pH 5.2, containing 2.5 volumes of ethanol. The precipitate was washed with 7:3 ethanol–water, dissolved in 0.1 M sodium acetate, pH 5.3, with 0.2 mM $ZnCl_2$, and digested with nuclease P1 (77 U/mg) for 3 h at 37°C. The buffer was adjusted with 0.3 M Na_2CO_3 to pH 8.5, and made 2 mM in $MgCl_2$. Alkaline phosphatase, 17 U/mg, and SVPD, 0.9 U/mg, were added and the reaction was further incubated for 3 h at 37°C. The digest was cleaned on a C_{18} SepPak with water (30 ml) and eluted with 3 ml of methanol. The methanolic eluent was concentrated on a Speedvac and reaction products were separated by HPLC using method 4A (see below).

3.1.2. Method 1B

The synthesis was based on a previously published protocol [21]. (+)-BPDE or racemic (±)-BPDE (0.5 mg) was dissolved in 100 μl of DMSO and reacted with dG (70 mg) in 5 ml of TFE containing 63 μl of TEA and heated to 37°C for 5 h. The solvent was evaporated on a Speedvac. The resulting mixture was resuspended in distilled water and extracted with n-butanol (4 × 10 ml). The organic layers were pooled, washed with water (3 × 50 ml), and concentrated on a rotary evaporator. The residue was dissolved in methanol and separated by HPLC using method 4A (see below).

3.2. Acetylation of BPdG with acetic anhydride

The yield of acetylation was compared in two solvent systems. The reaction had a high yield in both pyridine and THF/MeIm, but the second method is faster and more efficient at low concentrations of starting material.

3.2.1. Method 2A

BPdG (RSRS) (10 nmol) was acetylated for 2 h at 35°C with acetic anhydride (3 μl) in 20 μl of anhydrous pyridine. The reaction was terminated by the addition of 20 μl of 50% aqueous methanol and evaporation of the solvents to dryness on a Speedvac. The product of the reaction was purified by HPLC method 4B (see below). The separated peaks were collected and analyzed by LC-MS.

3.2.2. Method 2B

Acetic anhydride (3 μl) was dissolved in 400 μl of THF/MeIm (84% THF, 16% MeIm). An aliquot of 20 μl was added to HPLC-purified BPdG (1-100 pmol). The reaction was carried out for 20 min at 22°C, stopped with 20 μl of 50% aqueous methanol, and evaporated to dryness on a Speedvac. The reaction products were analyzed by HPLC method 4B and the major product was further analyzed by LC-MS.

3.3. Acetylation of BPdG with [$^{14}C_4$]acetic anhydride

3.3.1. Method 3A

BPdG (RSRS) (2 nmol) was acetylated for 2 h at 35 °C with 8.4 mCi/mmol [$^{14}C_4$]acetic anhydride (3 μl) in 12 μl of anhydrous pyridine. The reaction was terminated by evaporation of the solvent on a Speedvac. The concentrated reaction mixture was dissolved in 200 μl of methanol and evaporated to dryness three times. The product of the reaction was purified by HPLC method 4C (see below). The peak corresponding to pentakis(acetyl) BPdG was collected and the column was washed with 200 ml of THF, 200 ml of water, and 200 ml of methanol. Aliquots of the purified [^{14}C]AcBPdG were reinjected on the washed HPLC column. The fractions co-eluting with AcBPdG standard were analyzed by AMS for ^{14}C-content (see below).

3.3.2. Method 3B

[$^{14}C_4$]acetic anhydride (3 μl) was dissolved in 400 μl of THF/MeIm (84% THF, 16% MeIm). A 20 μl aliquot was added to the HPLC-purified RSRS stereoisomer of BPdG (10 pmol) under a controlled anhydrous atmosphere in a polycarbonate reaction chamber (Coy Laboratories, Grass Lake, MI). The reaction was carried out for 20 min at 22°C, stopped by addition of 50% aqueous CH_3OH (20 μl), and evaporated to dryness on a Speedvac. The reaction products were loaded on a Waters C18 SepPak cartridge (100 mg) together with 5 pmol of AcBPdG standard. The SepPak was washed with 0.03% aqueous CH_3CO_2H (50 ml), 10% aqueous methanol (50 ml), 30% aqueous methanol (50 ml), and 20 mM ammonium acetate,

pH 7.4 (50 ml). [^{14}C]AcBPdG was eluted with methanol (2 ml). Aliquots of the methanolic eluent were analyzed by HPLC, method 4C, and the ^{14}C content of the HPLC fractions was measured by AMS (see below).

3.4. Accelerator mass spectrometry

Measurement of the radiocarbon content of the samples was carried out using accelerator mass spectrometry (AMS) [18,22]. AMS measures the amount of radioisotope, e.g. ^{14}C relative to a stable isotope of the same element. In this study, ^{14}C was measured relative to ^{13}C and then normalized to the ratio of ^{14}C/^{12}C using the Australian National University sugar standard [23] as reference. For AMS analysis, HPLC samples were converted to graphite by a two step process involving combustion of the samples to CO_2 followed by reduction to filamentous graphite, as described previously [24]. Because the complete process to graphite works best with 1–2 mg of total carbon, and to insure a well-known and constant amount of carbon in each HPLC fraction, 2 mg of tributyrin (providing ca. 1 mg of carbon), well characterized with respect to radiocarbon content, was added to fractions as a carrier carbon prior to combustion. The samples were measured as graphite and converted to amol radiocarbon/mg sample by subtracting carbon-14 contribution from control material and carrier carbon [18,24].

3.5. Electrospray mass spectrometry

Mass spectrometric determinations were carried out on Perkin-Elmer/Sciex API 1 spectrometer equipped with an atmospheric pressure ionization source and an IonSpray interface which was maintained at 5 kV. The orifice was maintained at 70 V, high purity N_2 flowing at 0.6 L/min served as curtain gas, and high-purity air maintained at 40 psi was used for nebulization. Samples were introduced using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a diode array 1040A detector.

The analytes (BPdG or AcBPdG) were introduced into the mass spectrometer without column separation and without splitting of the effluent. As little as 2 pmol of analyte was injected into the ionization source in a 40 μl/min flowing stream of 50% aqueous acetonitrile containing 0.05% TFA. Mass spectra were acquired every 6-12 s over the range of m/z 300 to 1000 (m/z 0.1 resolution). Analytes were detected as their $[M + H]^+$, $[M + K]^+$, or $[M + Na]^+$ions.

3.6. HPLC separation of adducts

Analytes were injected with a Waters 717Plus autosampler into a Hewlett-Packard 1050 Series liquid chromatograph equipped with a diode array 1040A detector.

3.6.1. Method 4A

The separation of BPdG was achieved with a C_{18} column (Beckman Ultrasphere ODS, 5 μm particle size, 10 × 150 mm) using a gradient of methanol (solvent

A)–water (solvent B) (0–3 min, 40% A; 3–5 min, linear gradient to 50% A; 5–22 min, linear gradient to 55% A, 22–26 min, linear gradient to 99% A; 26–30 min isocratic at 99% A) as the mobile phase at a flow rate of 3 ml/min with UV monitoring at 279 and 344 nm. Under these conditions the retention time of BPdG (RSRS) was 18 min.

3.6.2. Method 4B

Acetylated BPdG was separated from other analytes on a C_{18} column (Beckman Ultrasphere ODS, 5 μm particle size, 4.6 × 250 mm) using a gradient of methanol (solvent A)-water (solvent B) (0–20 min, 70% A; 20–22 min, linear gradient to 90% A; 22–29 min, isocratic at 90% A; 29–30 min, linear gradient to 100% A; 30–35 min, isocratic at 100% A) as the mobile phase at a flow rate of 1 ml/min with UV monitoring at 279 and 344 nm. Under these conditions the retention time of pentakis(acetyl) BPdG (RSRS) was 22.9 min.

3.6.3. Method 4C

A longer gradient was used for cleanup of ^{14}C-acetylated BPdG using the Beckman Ultrasphere ODS column, 5 μm particle size, 4.6 × 250 mm. The gradient was as follows : methanol (solvent A)-water (solvent B) (0–10 min, 20% A; 10–13 min, linear gradient to 50% A; 13–25 min isocratic at 50% A; 25–28 min, linear gradient to 70% A; 28–38 min, isocratic at 70% A; 38–40 min, linear gradient to 90% A; 40–50 min, isocratic at 90% A; 50–53 min, linear gradient to 100% A; 53-60 min, isocratic at 100% A). The retention time of pentakis(acetyl) BPdG (RSRS) was 44.5 min under these conditions. Fractions of 1 ml were typically collected and further analyzed for ^{14}C-content by AMS as described above.

3.7. UV-Vis and CD spectra

All spectrophotometric measurements were carried out using a Beckman DU640 spectrophotometer. The concentration of BPdG was measured at 279 nm using $\varepsilon = 40\,984\ M^{-1}\ cm^{-1}$. CD spectra were recorded on a JASCO J-500A spectropolarimeter.

4. Results

Derivatization of DNA with (+)-BPDE or (±)-BPDE yielded one major product as previously described [20]. The product was purified from the enzymatic DNA digest by HPLC (Method 4A) and characterized. CD spectropolarimetry showed that the product was the RSRS stereoisomer of BPdG [20] (Fig. 1A). The mass spectrum of the purified adduct was consistent with the mass of a BPDE adduct with dG (BPdG) (Fig. 1B). Synthesis of BPdG from dG and (±)-BPDE was carried out in TFE/TEA [21]. The reaction afforded a product with the same spectral characteristics as the BPdG adduct isolated from BPDE-derivatized DNA (data not shown), but in higher yield. This method was used for the preparation of

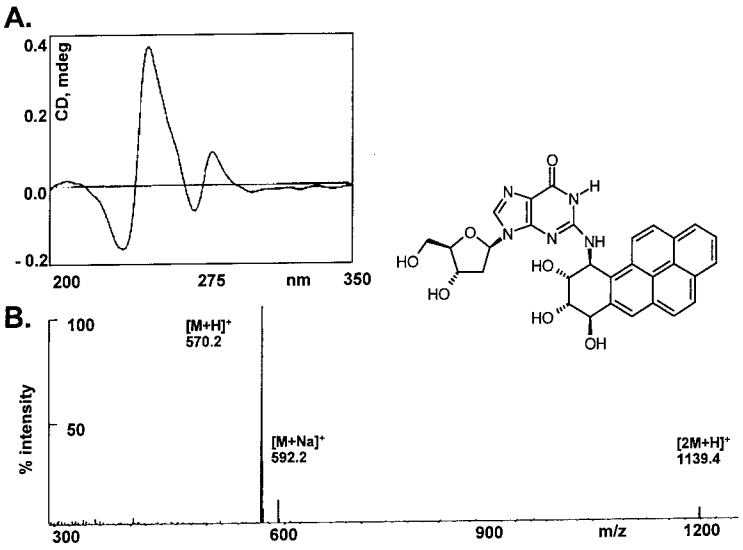


Fig. 1. Characterization of the major BPdG stereoisomer. (A) CD spectra of the HPLC-purified BPdG (RSRS) are identical with previously published CD spectra. (B) The quasimolecular ion determined by electrospray mass spectrometry is consistent with that of the BPdG adduct.

standards of the BPdG adducts. The adducts were used for the optimization of postlabeling reactions and as chromatographic standards.

Postlabeling of the BPdG (RSRS) adduct by acetylation with acetic anhydride was initially carried out as described previously [20]. Acetylation in pyridine yielded one major product in >60 % yield. The product was isolated by HPLC (Method 4B) (Fig. 2A). Its protonated quasimolecular ion (m/z 779; electrospray MS) of the major product was consistent with addition of five acetyl groups to BPdG (RSRS), i.e. pentakis(acetyl) BPdG (AcBPdG) (Fig. 2B). The AcBPdG was further used as a standard for HPLC separations of ^{14}C-AcBPdG.

Use of THF/MeIm as a solvent gave higher postlabeling yields when picomolar amounts of the BPdG adduct were acetylated [25]. Pentakis(Acetyl) BPdG was also the major product under these conditions as verified by LC-MS, but the reaction was faster and more efficient (data not shown).

Acetylation of BPdG (RSRS) at the nanomolar scale with excess [$^{14}C_4$]acetic anhydride yielded a major peak in the accelerator mass spectrometric (AMS) profile of the HPLC fractions (Fig. 3A). This radiolabeled product co-eluted with the AcBPdG standard. Aliquots of the isolated [^{14}C]AcBPdG product were further analyzed by AMS after extensive washing of the column to remove background ^{14}C (i.e. the unidentified ^{14}C-species retained on the HPLC column). The AMS analysis of samples obtained by serial dilution of the [^{14}C]AcBPdG using the washed column was carried out according to established procedures [18,24]. The AMS analysis fractions coeluting with the AcBPdG standard allowed construction of a theoretical dose-response curve for detection of the postlabeled adduct (Fig. 3B). The detection

limit under these conditions was 1 fmol of adduct, even though the fractions showed a 10-fold higher level of background ^{14}C than expected (Fig. 3B).

An additional cleanup step with a C_{18} SepPak cartridge preceding the HPLC separation eliminated the background ^{14}C. Postlabeling of picomolar amounts of BPdG (method 3B) combined with SepPak cleanup and HPLC separation yielded AMS profiles with the major peak co-eluting with the AcBPdG standard (Fig. 4A). Analyses of aliquots of this reaction allowed construction of a new dose-response curve for detection of the postlabeled [^{14}C]AcBPdG (Fig. 4B). The detection reached the theoretical limit of 100 attomoles of adduct using these conditions.

5. Discussion

Cancer results from an as yet unidentified interplay of environmental/endogenous exposures and genetic susceptibilities [5,6]. Many ongoing studies in our laboratory [26] and elsewhere [27,28] examine the contribution of genetic polymorphisms to the variability of function in both 'caretaker' (metabolism, detoxification, DNA repair) and 'gatekeeper' (cell cycle control, apoptosis) cancer susceptibilty genes [29]. Carcinogen-DNA adducts serve as biomarkers that link genetic susceptibility with an exposure [10]. For example, in lung [30], bladder [31,32], and other tissues [33],

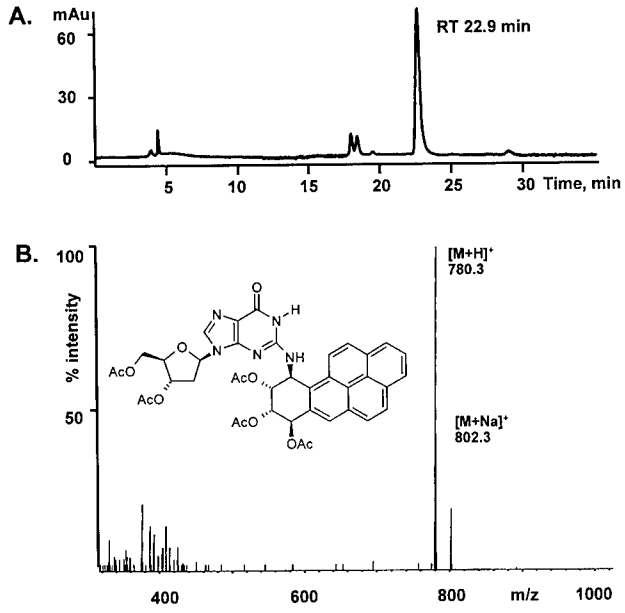


Fig. 2. Characterization of the BPdG postlabeled by acetylation (AcBPdG). (A) The HPLC profile (monitored at 344 nm) shows one major product with a retention time of 22.9 min (Method 4B) (B) The mass spectrum of the HPLC purified peak (22.9 min) is consistent with the pentakis(acetyl) BPdG adduct.

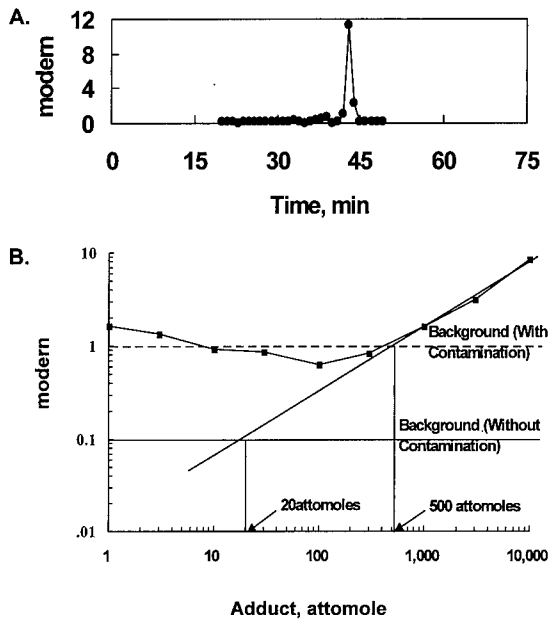


Fig. 3. Analysis of the ^{14}C-acetylated BPdG (nanomolar scale). (A) HPLC profile of the [^{14}C]AcBPdG with AMS detection (Method 4C). The major ^{14}C-peak, RT 44.2 min, coelutes with the synthesized AcBPdG standard. Modern is defined as 5.9×10^{10} $^{14}C/g$ of carbon (approximately the concentration of ^{14}C in the atmosphere in 1950 [23]). (B) Dose-response of [^{14}C]AcBPdG aliquots of the major peak. The graph (log/log scale) shows that the background in the samples is about ten-fold higher than the background ^{14}C in the tributyrin solvent. The squares represent individual measured values. The straight line is an extrapolation to background without contamination.

DNA adduct levels are higher in persons with hypothesized 'at risk' genetic variants. This shows that the carcinogen-DNA adducts estimate the total burden of a particular exposure in the target tissue and suggest a link to cancer risk [30,34]. The chemical specificity and sensitivity of the DNA adduct-detection are two key issues limiting the application of most currently available methods to large epidemiological datasets. The ^{14}C-postlabeling is an alternative method with the potential to be both specific and sensitive. The procedure, is being developed using the well known carcinogen benzo[*a*]pyrene which is related to important environmental and occupational exposures including smoking.

The acetylation reaction yielded one major product, pentakis(acetyl) BPdG (AcBPdG), in high yield. Postlabeling by acetylation with [^{14}C]acetic anhydride was analogous to the labeling with acetic anhydride as expected. The major peak of ^{14}C detected by AMS co-eluted with the AcBPdG standard in the HPLC profile (Fig. 3A and Fig. 4A). The area under the peak was proportional to the amount of postlabeled adduct injected on the column (Fig. 4B). It is important to separate the excess ^{14}C from the ^{14}C-postlabeled adduct prior to the HPLC analyses (Fig. 3B). Impractical amounts of solvent (> 500 ml) had to be used for column washing when > 100 dpm of ^{14}C was applied to the column. The cleanup was still incom-

plete after the wash with 600 ml of solvents when $> 10^6$ dpm of ^{14}C was loaded on the column (Fig. 3B). The excess ^{14}C-label was therefore washed out on a C_{18} SepPak cartridge prior to the HPLC analysis (Fig. 4). The procedure provided sufficient cleanup without affecting recovery. The recovery tested with synthesized ^{3}H-AcBPdG was always greater than 90% (data not shown). The current limit of detection in the attomolar range promises excellent sensitivity for the detection of adducts in human DNA, provided that the reactions/cleanup proceed without additional complications at a smaller scale. Optimization of the washing step and of the reaction at femtomolar and attomolar starting amounts of adduct are underway.

This report summarizes the development of a novel postlabeling method based on acetylation with ^{14}C combined with AMS quantitation. The presented preliminary results are encouraging. The HPLC separation of postlabeled adducts provides chemical specificity which will be further increased when combined with immunoaffinity chromatography for isolation of the adduct of interest from DNA. The assay also promises excellent sensitivity. The quantitation of the ^{14}C by AMS does not rely on radioactive but rather determines the ratio of ^{14}C to ^{12}C. This provides a significant increase in sensitivity compared to the short-lived ^{32}P isotope used in classical postlabeling. The projected sensitivity should allow detection of adducts in the range of 1 adduct in 10^{12} nucleotides. We should stress that this report is preliminary, that the optimization and validation steps will be crucial to prove the utility of this method, and that the ultimate test will be the analysis of human samples.

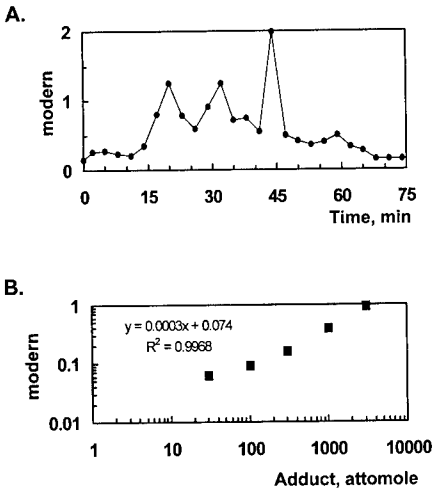


Fig. 4. Analysis of the ^{14}C-acetylated BPdG (picomolar scale). (A) HPLC profile of the [^{14}C]AcBPdG with AMS detection after preparatory cleanup on a C18 seppak cartridge (Method 4C). The major ^{14}C-peak (44.2 min) coelutes with the synthesized AcBPdG standard. Dose–response of ^{14}C-AcBPdG aliquots of the major peak. The background in the samples is similar to the background ^{14}C in the tributyrin solvent, i.e. about 0.1 Modern. The projected sensitivity is about 100 amol of adduct.

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