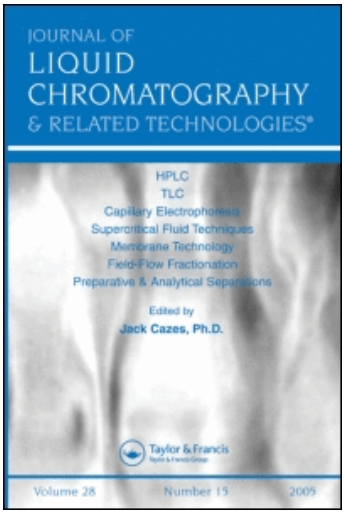

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Quantification of Benzo[a]pyrene-Guanine Adducts in *in vitro* Samples by LC Tandem Mass Spectrometry with Stable Isotope Internal Standardization

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Quantification of Benzo[a]pyrene-Guanine Adducts in *in vitro* Samples by LC Tandem Mass Spectrometry with Stable Isotope Internal Standardization

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Abstract: Benzo[a]pyrene, after metabolic activation, is known as one of the most potent polycyclic aromatic hydrocarbons that cause a carcinogenic effect. When benzo[a]pyrene is activated by one-electron oxidation to its radical cation, it binds to either the C-8 or N-7 of guanine on DNA and forms depurinated adducts. A sensitive liquid chromatography/tandem mass spectrometry method coupled with a stable isotope internal standard was developed for detection and quantitative analysis of benzo[a]pyrene with DNA adducts. *In vitro* samples were analyzed by our method. The method provides structural confirmation of the adduct, as well as quantitative analysis with accuracy, confidence, isomeric specificity, and precision to measure biologically relevant levels in small sample sizes.

Keywords: Benzo[a]pyrene, DNA adducts, *In vitro*, LC/MS/MS, Quantification

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INTRODUCTION

It is widely accepted that for chemical carcinogens to be effective, they must interact with cellular macromolecular constituents, including proteins, lipids, and more importantly, with nucleic acids.[1–5] One basic mechanism in chemical carcinogenesis is the formation of covalent bonds between chemical carcinogens and DNA, RNA, and protein to form adducts. These adducts are thought to interfere with DNA replication leading to point mutations or to deletion of a DNA sequence. Mutation is considered a critical step in the process of a tumor formation.[4] During the past 60 years or so, following the identification of the first pure chemical carcinogen,[6] no common factors or pathways for the mechanism of action of chemical carcinogens from different chemical classes have become evident. For this very reason, each class of carcinogen is often discussed separately. The potential for DNA adduct formation is recognized as a common property of most potent carcinogens and the formation of such adducts is the basis of several current strategies in molecular epidemiology and bio-monitoring.[7]

Despite this common aspect of the mechanisms for many chemical carcinogens, the complexities of metabolic activation and of the chemistry and stereochemistry of adduct formation have tended to keep some degree of compartmentalization in research and in literature reviews of DNA adduct formation by different classes of chemical compounds.[8] Typically, a single toxic chemical class, such as the polycyclic aromatic hydrocarbons (PAHs), induces many types of DNA damage. Particular adduct isomers may be the dominant product, however, many minor adducts are also formed. For example, even simple alkylating agents react with guanine and other bases at several sites.[9–11] In many cases, the pattern of DNA damage induced by an agent can serve as a distinctive "fingerprint" of exposure. In general, if such a pattern is distinctive, then it may be possible to work back from an observed spectrum of DNA adducts to deduce the nature of the chemical agent responsible and to prevent or repair the damage to DNA.[11] Most chemical carcinogens require metabolic activation to produce electrophilic intermediates in order for the tumor formation process to take place.[11] For example, the hypothesized mechanism for metabolic activation of polycyclic aromatic hydrocarbons (PAH), is that activation occurs by two main routes: one-electron oxidation to yield reactive intermediate radical cations[3,5,12–15] and monooxygenation to produce bay-region diol epoxides.[17–19] These activated intermediates then react with nucleotides to form covalently bonded compounds and cause a "molecular lesion". In many cases, stable DNA adducts can be found as evidence of these reactions.[20–38] The adducts formed by the above mechanisms can be either stable, remaining in DNA unless repaired by natural repair enzymes,

or depurinated and released from DNA by cleavage of the glycosidic bond between the purine base and deoxyribose.[11,39] Loss of adducts by depurination results in apurinic sites in the DNA, which, if not repaired, can also be mutagenic. Therefore, the importance of determination of structure of the DNA adducts formed in target tissues is a critical variable requiring extensive research. Researchers believe that study of DNA adducts will help to yield an understanding of the mechanisms of metabolic activation and, eventually, the mechanisms of tumor initiation.[40] The use of DNA adducts as bio-markers for risk assessment and environmental monitoring have caught researcher's interest.[41] The DNA adduct represents direct evidence of primary damage to DNA by a chemical. Therefore, measuring DNA adducts may prove to be more precise and reliable than measuring the external exposure.[42,43]

DNA adducts are typically big molecules, and therefore structure determination represents a very challenging problem in organic analysis. De-convolution of the chemical structures of DNA adducts is usually based on spectroscopic identification by nuclear magnetic resonance (NMR). However, NMR analysis is possible only with purification of many micrograms of pure adducts and, in many cases, such quantities are difficult to obtain. This is typically the case in living organisms. The most productive approach has been to prepare authentic DNA adduct standards by the reaction of synthetic reactive intermediates with target nucleotides or bases *in vitro*. Structural analysis is performed on the synthetic standards, and DNA adducts formed in cell culture or *in vivo* are identified by Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC) co-chromatography.[11] The detection and quantitation of DNA adducts by the direct methods described above is possible only when adducts are present in sufficiently large amounts (μmole/mole DNA). For samples with lower levels of DNA adducts, the radiolabeled xenobiotic method (^{14}C or ^{3}H) and ^{32}P postlabeling method (two-dimensional thin-layer chromatography) are often used.[11] However, each method has its own limitation. The GC and HPLC methods often lack confirmation information. The radiolabeling method is expensive and is limited by the relatively low specific activities of ^{14}C and ^{3}H. The ^{32}P postlabeling method also lacks confirmation information and accurate quantitative work is difficult by TLC.

Our research is focused upon the analysis of the benzo[a]pyrene-DNA/guanine adducts at trace levels by modern instrumentation. The ultimate goal of this study is to provide a better method of detecting the "bio-markers" for cancer formation. In this work we describe a method detecting DNA adducts formed via the free-radical activation pathway with great confidence. This will enable future researchers to gain a better understanding of cancer formation, which will in turn lead to better treatments of the disease.

EXPERIMENTAL

Materials and Methods

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

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

Synthesis and Purification of Analytical Standards and Internal Standards

BaP-C8Gua and BaP-N7Gua

The Rogan and Cavialier electrochemical synthesis method[3] was used for preparing BaP-C8Gua and BaP-N7Gua. The electrochemical synthesis device was assembled in our laboratory. Briefly, DMF (20 mL,

dried by sodium sulfate and degassed) containing 0.5 M $KClO_4$ as electrolyte was pre-electrolyzed at +1.45V verses the standard calomel reference electrode for 30 minutes with argon purging. The pre-dried (80°C under vacuum for 24 hours) deoxyguanosine monohydrate (1 mmol) and benzo[a]pyrene (0.1 mmol) were added to the solution and the resulting solution was stirred until all materials dissolved. The bubbling of argon was stopped and the electrode potential was gradually raised from zero to 1.1 V verses the standard calomel reference electrode (current is usually around 20–30 mA). The reaction was stopped when the current dropped to around 1 mA (usually after 2 hours). The dark brown, crude solution was then transferred to a separatory funnel along with 100 mL 0.05% formic acid in Milli-Q water. The solution was extracted three times with 150 mL of $CHCl_3$. The combined extract was dried on a rotary evaporator and re-dissolved in 5 mL DMSO. A C18 Sep-Pak cartridge was conditioned by first eluting with 6 mL of acetonitrile and then followed by 6 mL of water. Approximately 0.25 mL of the DMSO solution was then loaded on a pre-conditioned C-18 Sep-Pak cartridge and washed with 10 mL of water followed by 5 mL of 10% methanol in water to remove unreacted deoxyguanosine. Adducts were eluted with 15 mL of 50/50 (% v/v) acetone: methanol (both containing 0.1% formic acid) and collected. The procedure was repeated for the remaining DMSO solution. All adduct fractions were combined and dried on a rotary evaporator and then re-dissolved in 5 mL of DMSO for further HPLC purification. HPLC purification of BaP-C8Gua and BaP-N7Gua was conducted using a Waters HPLC system equipped with a Waters 600-MS multisolvent delivery system and manual injector, and a Hewlett Packard 1040 A DAD detector set to monitor the absorbance at 300 nm. Solvent A was 0.1% formic acid in acetonitrile and solvent B was 0.1% formic acid in Milli-Q water. Two HPLC steps were used for purifying the DNA adducts.

The first purification step was performed on a semi-preparative reversed phase C_{18} column (250 × 9.4 mm, 5 μm) (Phenomenex, Terrance, CA). In general, 250 μL of the DMSO (post Sep-packs) solution was injected onto the column and eluted with the following linear gradient program at a flow rate of 2.0 mL/min: 0 minute, 20% A; hold for 5 minutes; 75 minute, 100% A; hold for 10 minute; 85.1 minute, 20% A. The BaP-C8Gua eluted at 37 minutes and BaP-N7Gua eluted at 42 minutes. BaP-C8Gua and BaP-N7Gua were collected individually and dried under vacuum. The procedure was repeated for the remaining samples. Finally, all BaP-C8Gua fractions and all BaP-N7Gua fractions were combined, respectively, and dried under vacuum. Approximately 0.5 mL of DMSO was added to each sample to dissolve adducts for the second HPLC purification.

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

BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}

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

MS/MS/MRM (Multiple Reaction Monitoring) Optimization

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

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

For the quantitative study of adducts, a LC/MS/MS/MRM (mutiple reaction monitoring) method was developed and used. For the mass

spectrometer, the first quadrupole of the instrument was set only to pass the MH^+at m/z 402 for both adducts and m/z 413 for both deuterated internal standards. The collision induced fragmentation occurred at the second quadrupole. The product ions (daughters) were separated in the third quadrupole. Argon was used as the collision gas, with a gas cell pressure of $\sim 2.5 \times 10^{-3}$ mbar. The collision energy was set at 40 V. The quantitative analysis of adducts was done by MS/MS/MRM mode by monitoring their daughter ions. For the authentic adducts, (BaP-C8Gua and BaP-N7Gua) m/z $402 \rightarrow 277$ and m/z $402 \rightarrow 360$ transitions were monitored. For the internal standards (BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}) m/z $413 \rightarrow 287$ and m/z $413 \rightarrow 371$ transitions were monitored.

For the HPLC, a Zorbax SB-CN column (250×4.6 mm, 5 μm) fitted with a Zorbax SB-CN guard column was used for all the sample analysis. The flow rate was 0.7 mL/min. Formic acid, 0.2% in water, was used as solvent A and 0.2% formic acid in acetonitrile was used as solvent B. A linear gradient was run as follows: 0 minutes, 48% B; 5 minutes, 48% B; 20 minutes, 100% B; 25 minutes, 100% B; 25.1 minutes, 48% B; 30 minutes, 48% B. The BaP-C8Gua and BaP-C8Gua-d_{11} were co-eluted at approximately 7 minutes, and the BaP-N7Gua and BaP-N7Gua-d_{11} were co-eluted at approximately 10 minutes under the above conditions. For a typical analysis, 10 μL of sample was injected. A standard curve was constructed by replicate analysis of solutions containing BaP-C8Gua and BaP-N7Gua standards from 1 to 32 ng per injection, and the internal standards BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} at 2 ng per injection in all of the solutions.

Adducts Stability Evaluation and Method Suitability

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

The adduct stability and the Sep-Pack procedure efficiency were demonstrated by treating adducts at extreme conditions, spiking the internal standards into the post Sep-Pack fraction, and assaying for recovery. Duplicate samples were prepared for each level of the study. The samples were prepared by spiking 15, 75, 150, 300, and 450 ng of authentic adducts into separate test tubes with 2 mL of 0.02 N HCl. The spiked concentrations were equal to 1, 5, 10, 20, and 30 ng on the column, based on a final volume of 150 μL and 10 μL injection. The solutions were hydrolyzed at 75°C for 1.5 hours. After cooling, each

sample was neutralized to pH 7.0 by addition of NaOH. The C_{18} Sep-Pak cartridges were activated by first rinsing with 6 mL of acetonitrile followed by 6 mL of purified water. Each sample was then loaded onto a separate cartridge and washed with 5 mL of purified water. Adducts were eluted with 3 mL of methanol. The methanol fraction for each sample was collected separately and spiked with 30 ng of the deuterated internal standards (both BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}). The final methanol fraction was dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μL of DMSO for LC/MS/MS/MRM analysis.

The method validation was demonstrated by measuring adduct levels in rat liver DNA samples spiked with a known amount of authentic standards. Duplicate samples were prepared for each spiked concentration level. Rat liver DNA was obtained as described in the protocol provided by Life Technologies Co. For each spiked sample, 1.0 gram of liver was minced and homogenized in 30 mL of DNAzol reagent using a hand held glass homogenizer for approximately 30 seconds. The solution was then centrifuged at 10,000 × g for 10 minutes to remove insoluble tissue fragments, RNA, and excess polysaccharides. The top layer was transferred to a new tube and the DNA was precipitated by the addition of a half volume of absolute ethanol. DNA was recovered by centrifugation at 1700 × g for 2 minutes and washed three times with 70% ethanol. The DNA pellet was air dried briefly, dissolved in 8 mM sodium hydroxide solution and the pH was then adjusted to 7.0 with HEPES and centrifuged at 10,000 × g for 2 minutes to remove any impurities. The DNA was then reprecipitated by addition of ethanol and recovered by centrifugation at 1700 × g for 3 minutes. The DNA pellet was then briefly air dried, and then redissolved in 8 mM sodium hydroxide solution and the pH was adjusted to 7.0 with HEPES. The quantity of DNA was estimated by reading the absorbance (1 AU = 50 μg DNA/mL) at 260 nm. The purity of the DNA was estimated by scanning the UV spectrum from 200 to 400 nm and calculating the 260/280 ratios. The typical yield of each preparation was approximately 3.7 to 4.1 mg of DNA per 1000 mg of liver tissue (3.7 ~ 4.1 μg/mg).

For each spike, 15, 75, 150, 300, and 450 ng of adducts were added separately into individual DNA solutions (approximately 4 mg equal to prepare from 1 g of liver tissue). The spiked levels were equal to 1, 5, 10, 20, and 30 ng on the column, based on a final sample volume of 150 μL and 10 μL injection per sample. The pH of each spiked solution was adjusted to approximately 2.5 with HCl and hydrolyzed at 75°C for 45 minutes.[11,43–45] After cooling, each sample was neutralized to a pH of 7.0 by the addition of sodium hydroxide solution, and then spiked with 30 ng of the deuterated internal standards (both BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}). The C_{18} Sep-Pak cartridges were activated by first rinsing with 6 mL of acetonitrile and followed by 6 mL of purified water.

Approximately 1/3 of the sample was then loaded on a cartridge and washed with 5 mL of purified water. Adducts were eluted with 3 mL of methanol. The procedure was repeated for each the sample and methanol fractions were then combined and dried under ultrapure nitrogen. Finally, the residue was redissolved in 150 μL of DMSO for LC/MS/MS/MRM analysis. Blank rat liver DNA samples were prepared by following the procedure described above. Neither authentic nor internal standards were added for the blank preparations in order to assess the possible bias.

In Vitro Sample Preparation and Analysis

The *in vitro* experiment was performed by following the procedure of Rogan and Cavalieri[3] and slightly revised. Rat liver DNA (isolated and purified in our lab) and calf thymus DNA (Sigma-Aldrich) were used for our in vitro experiments. Briefly, 50 mg of DNA was dissolved in 60 mL of 60 mM sodium hydroxide solution and then the pH was adjusted to 7.0 with H_3PO_4. Horseradish peroxidase (TypeII, Sigma-Aldrich) was added at 0.1 mg/mL with the presence of 0.5 mM of H_2O_2. Benzo[a]pyrene was added at 10 mM by spiking with a 2.5 mg/mL mL stock DMSO solution. The above solution was purged with argon, sealed, and then incubated at 37°C for a period of 30 minutes. The solution was cooled to room temperature and the procedure was repeated once with the same amount of HRP, H_2O_2, and benzo[a]pyrene and incubated at 37°C for another 30 minutes. At the end of the reaction, DNA was precipitated and collected by addition of 2 volumes of ethanol and then followed by centrifugation at 1700 × g for 2 minutes. For the depurinating adducts, the supernatant was used and the DNA pellet was extracted with 3 × 5 mL of $CHCL_3$. The supernatant and $CHCL_3$ extracts were pooled and evaporated under vacuum and the soluble residue was redissolved in 1 mL of 50/50 DMSO: water; 30 ng of deuterated internal standards were added. C_{18} Sep-Pack cartridges were activated by first being rinsed with 6 mL of acetonitrile and then followed by 6 mL of purified water. About half of the above sample (0.5 mL) was then load onto a cartridge and washed with 5 mL of purified water. Adducts were eluted out by 2.5 mL of methanol. The procedure was repeated for the rest of the sample and MeOH fractions were combined, dried under ulterapure nitrogen. Finally, the residue was redissolved in 150 uL of DMSO for LC/MS/MS/MRM analysis.

The stable DNA adducts were released by acid hydrolysis.[11,45] The DNA was dissolved in 25 mM NaOH and the pH of the solution was adjusted to 1.0 with HCL and hydrolyzed at 75°C for 45 minutes. After cooling, the sample was neutralized to 7.0 by addition of NaOH and

spiked with 30 ng of the deuterated internal standards. C_{18} Sep-Pack cartridges were activated by first being rinsed with 6 mL of acetonitrile and then followed by 6 mL of purified water. Approximately half of the above sample was load on a cartridge and washed with 5 mL of purified water. Adducts were eluted out by 2.5 mL of methanol. The procedure was repeated for the rest of the sample and MeOH fractions were combined and dried under ulterapure nitrogen. Finally, the residue was redissolved in 150 uL of DMSO for LC/MS/MS/MRM analysis. In order to assess the possible interference and bias, blank samples were prepared. Both calf thymus DNA and rat liver blank were prepared by following the procedure described above, but without the addition of benzo[a]pyrene for the reaction. Internal standards were not added in the final sample preparations in order to obtain the full background.

RESULTS AND DISCUSSION

Synthesis of BaP-C8Gua and BaP-N7Gua

Electrochemical synthesis of BaP-C8Gua and BaP-N7Gua was very successful. The structures of the synthesized adducts were elucidated by a combination of analytical instrumentation, which includes NMR, MS (ESI and APCI), and MS/MS. They perfectly matched the literature values.[3] Figure 1 shows the structure and numbering of BaP-C8Gua. The mass spectrum of BaP-C8Gua by electrospray showed a rational match with literature values.[3] The mass spectrum of BaP-C8Gua by electrospray showed an $(M+H)^+$ ion of m/z 402. The mass spectrum

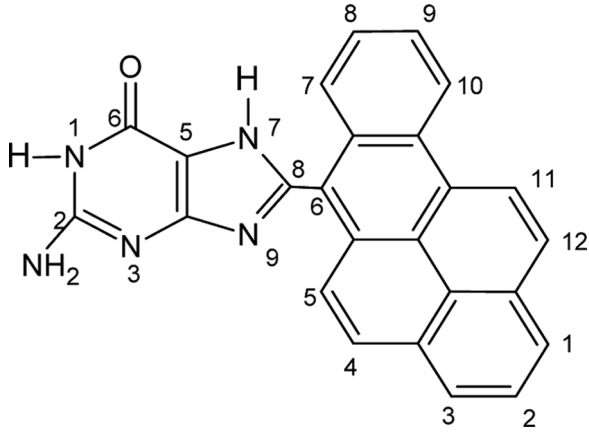

Figure 1. Structure and numbering of BaP-C8Gua.

of BaP-C8Gua by ESI is shown in Figure 2. The molecular weight of BaP-C8Gua ($(M+H)^+$ ion of m/z 402) was confirmed by APCI. The vaporization temperature was set at 450°C and 5 μA discharge current was used for APCI operation. Electrospray (ESI) was used for all sample analysis instead of APCI. The high vaporization temperatures used for APCI cause a bio-sample's matrix to decompose, therefore, a very frequent ion source and compartment clean up would have been needed if APCI was used. The MS/MS (ESI) spectrum of BaP-C8Gua was obtained by selectively passing the m/z 402 ion through the first quadrupole, bombarding the ion with argon gas in the second quadrupole, and then analyzing the daughter ions with the third quadrupole. Upon collision (collision energy 35 V), the 402 ion decomposes to produce the most abundant ion at m/z 277, which is likely to be BaP-CN^+. The greater abundance of the m/z 277 ion in the spectrum of BaP-C8Gua as compared with BaP-N7Gua is attributed to the greater stability of BaP-CN^+ versus BaP-NC^+.[3] The MS/MS spectrum of BaP-C8Gua by ESI is shown in Figure 3.

The greater abundance of the m/z 277 ion in the spectrum of BaP-C8Gua compared with BaP-N7Gua is very unique. Thus, it provides a rapid distinguishing characteristic between BaP-C8Gua and BaP-N7Gua. In addition to the above, the fragment ion of m/z 385 is assigned as $(M+H\text{-}NH_3)^+$ and the m/z 360 ion is assigned as $(M+H\text{-}NH_2CN)^+$. The BaP-N7Gua NMR spectrum correlates very well with literature values. Figure 4 shows the structure and numbering of BaP-N7Gua. The mass spectrum of BaP-N7Gua by electrospray shows perfect correlation with literature values as well.[3] The mass spectrum of

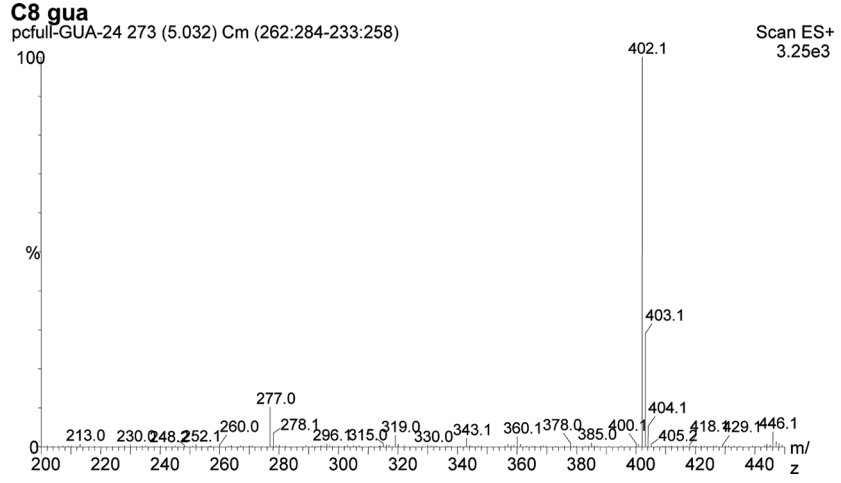

Figure 2. Mass spectrum of BaP-C8Gua by ESI.

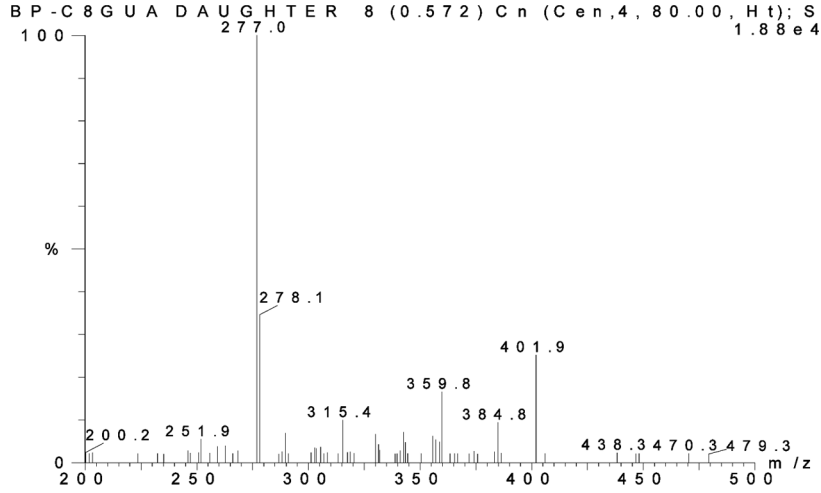

Figure 3. Daughter Mass spectrum (MS/MS) of BaP-C8Gua by ESI. Collision energy set at 35 V.

BaP-N7Gua by electrospray showed an $(M+H)^+$ ion of m/z 402. The mass spectrum of BaP-N7Gua by ESI is shown in Figure 5. The molecular weight of BaP-N7Gua ($(M+H)^+$ ion of m/z 402) was confirmed by APCI. The same conditions as used for BaP-C8Gua were used. The

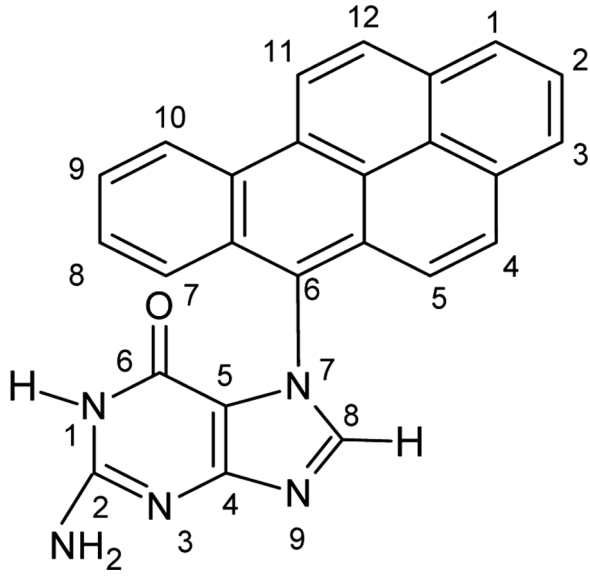

Figure 4. Structure and numbering of BaP-N7Gua.

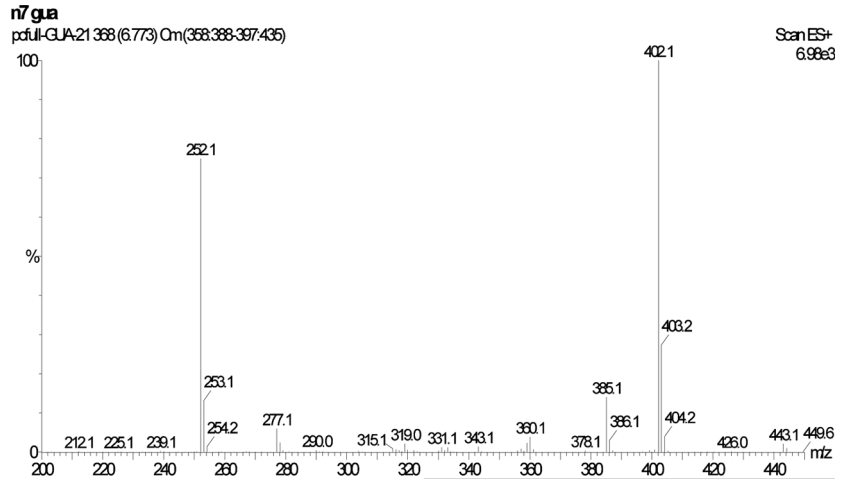

Figure 5. Mass spectrum of BaP-N7Gua by ESI.

MS/MS spectrum (ESI) of BaP-N7Gua was obtained by appling the same MS/MS parameters used for BaP-C8Gua. Upon collision, the m/z 402 ion decomposes to produce abundant ions of m/z 252 and 277. The m/z 252 ion is the benzo[a]pyrene moiety resulting from facile protonation at the N7 position of BaP-N7Gua, followed by a loss of the neutral guanine moiety. The m/z 277 ion is likely to be BaP-NC^+. The MS/MS spectrum of BaP-N7Gua is shown in Figure 6. The higher abundance of the m/z 252 daughter ions in the MS/MS spectrum of BaP-N7Gua compared with BaP-C8Gua is very unique. Thus, it provides a rapid distinguishing characteristic between BaP-C8Gua and BaP-N7Gua. In addition to the above, the fragment ion of m/z 385 is assigned as $(M + H\text{-}NH_3)^+$ and m/z 360 ion is assigned as $(M + H\text{-}NH_2CN)^+$.

Synthesis of BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}

Electrochemical syntheses of BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} were successful. The structures of the synthesized adducts were elucidated by MS and MS/MS. Both BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} mass spectrum obtained by electrospray showed excellent correlation with the spectrum of their non-deuterium forms. The $(M + H)^+$ ions of m/z 413 obtained for both BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} are eleven units greater than their non-deuterium forms, which indicates the eleven deuteriums on benzo[a]pyrene-d_{11} moiety. For their daughter (MS/MS) mass spectrum, the same procedure used for their non-deutrium form

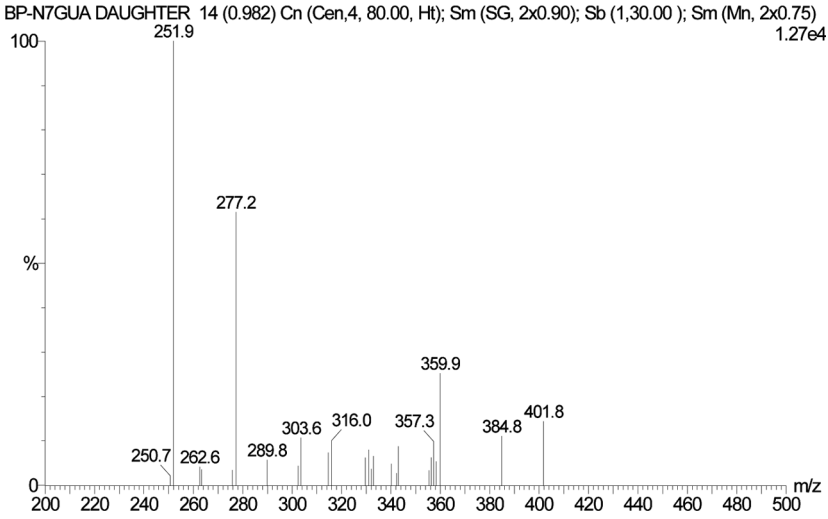

Figure 6. Daughter mass spectrum (MS/MS) of BaP-N7Gua by ESI. Collision energy set at 35 V.

was used for both BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} by selectively passing m/z 413 ion through the first quadrupole, for the BaP-C8Gua-d_{11}, upon collision, the 413 ion form decomposes to produce the most abundant ion of m/z 287 and it is likely to be BaP-CN^+-d_{10}. The formation of BaP-CN^+-d_{10} (m/z 287) instead of BaP-CN^+-d_{11} (m/z 288) indicates a possible deuterium/hydrogen exchange during the fragmentation. Similar to the BaP-C8Gua spectrum, the greater abundance of the m/z 287 ion observed in the spectrum of BaP-C8Gua-d_{11}, as compared with BaP-N7Gua-d_{11}, is attributed to the greater stability of BaP-CN^+-d_{10} versus BP-NC^+-d_{10}. This unique difference provides a rapid distinguishing characteristic between BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}. In addition to the above, the fragment ion of m/z 396 is assigned as $(M + H\text{-}NH3)^+$ and m/z 371 ion is assigned as $(M + H\text{-}NH_2CN)^+$. The same condition was used for the BaP-N7Gua-d_{11}, the 413 ion decomposes to produce the most abundant ions of m/z 263 and 287. The daughter (MS/MS) mass spectrum of BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11} by ESI is shown in Figures 7 and 8.

MS/MS/MRM Optimization

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

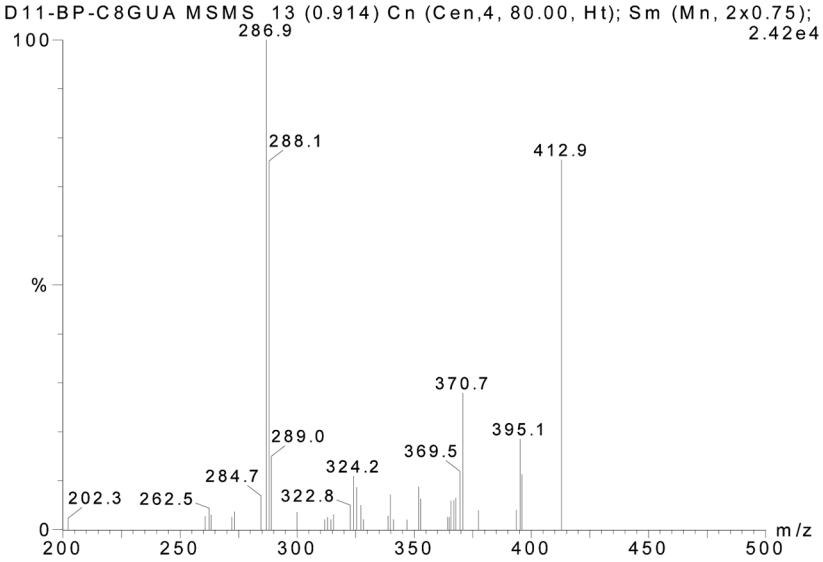

Figure 7. Daughter mass spectrum (MS/MS) of BaP-C8Gua-d_{11} by ESI. Collision energy set at 35 V.

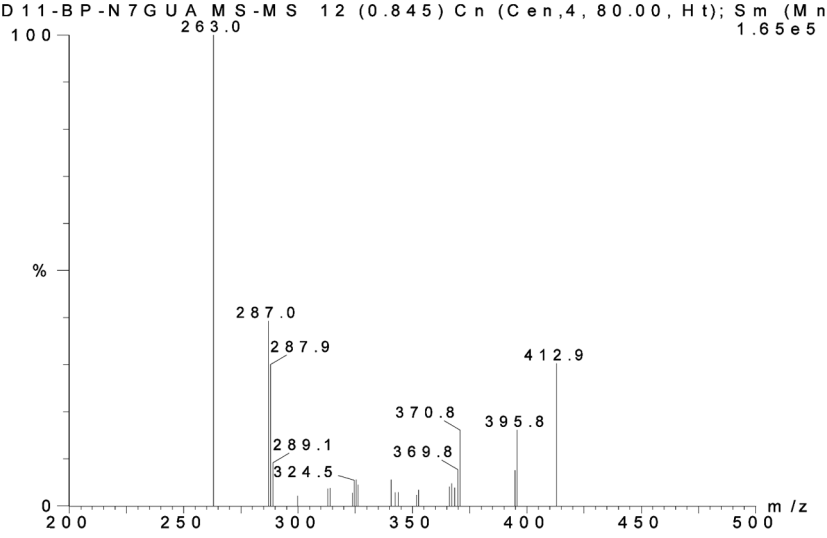

Figure 8. Daughter mass spectrum (MS/MS) of BaP-N7Gua-d_{11} by ESI. Collision energy set at 35 V.

The electrospray current for BaP-C8Gua and BaP-N7Gua, which gave the most intense signal at MH + (m/z 402) was 3 kV. The intensity of m/z 402 ion signal for both adducts decreased when lower ionization energy was applied. The decreased sensitivity may have been caused by incomplete sample ionization. The intensity of the m/z 402 ion signal for both adducts also decreased when a higher ionization energy was applied. The decreased sensitivity may have been caused by an arcing effect or sample break down. In addition to the above phenomenon, high background noise was observed when higher ionization energy was used. The collision energy for BaP-C8Gua and BaP-N7Gua, which produced the most intense signal for their respective daughter ions m/z 277 (most abundant fragment) was 40 V. The intensity of the m/z 277 ion for both adducts decreased when high collision energy was applied. The decreased sensitivity may have been caused by excessive fragmentation or further break down of the daughter ion. The optimized conditions were used for LC/MS/MS/MRM (m/z 402 → m/z 277) sample analysis in order to obtain the maximum sensitivity.

LC/MS/MS/MRM Method

Two fragment ions were selected for the LC/MS/MS/MRM analysis for both authentic and deuterated standards. For BaP-C8Gua and BaP-N7Gua, m/z 402 → 277(major) and m/z 402 → 360 (minor) transitions were monitored. For the internal standards (BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}) m/z 413 → 287 (major) and m/z 413 → 371(minor) transitions were monitored. The standard curves were constructed by three replicated analyses of solutions containing BaP-C8Gua and BaP-N7Gua, from 1 to 32 ng (1, 2, 4, 8, 16, and 32 ng) per injection and internal standards (BaP-C8Gua-d_{11} and BaP-N7Gua-d_{11}), containing 2 ng per injection in all of the solutions. Results from LC/MS/MS/MRM are very linear. The standard curve was constructed based on peak height instead of peak area to achieve a more accurate result. The linear equation of $y = 1.0967x$ and $R^2 = 0.9998$ were obtained with accuracy from 93% to 111% (Table 1). The standard deviation of three injections for all levels of standards was less than 5.6% and the highest percent relative standard deviation (%RSD) for all levels of standard injections was less than 5.5%. The limit of detection (LOD) for BP-C8Gua standards was 77 pg (192 *f*mole (S/N = 3) and limit of quantitation (LOQ) was 153 pg (381 mole) (S/N = 6). The response ratio of BaP-N7Gua to BaP-N7Gua-d_{11} was linear as well. The standard plot was constructed based on peak height instead of peak area to achieve a more accurate result. The linear equation of $y = 0.3945x$ and $R^2 = 0.9999$ were obtained with accuracy from 96% to 111% (Table 2). The highest standard deviation

Table 1. BaP-C8Gua LC/MS/MS/MRM linear plot accuracy calculation

BaP-C8Gua on column (ng)	BaP-C8Gua Calculated (ng)	Accuracy (%)	Average % accuracy ± standard deviation	RSD (%)
1.00	1.08	108		
1.00	1.05	105	105 ± 2.1	2.00
1.00	1.03	103		
2.00	2.09	104		
2.00	2.22	111	105 ± 4.2	4.00
2.00	2.02	101		
4.00	4.22	106		
4.00	3.73	93	101 ± 5.6	5.54
4.00	4.13	103		
8.00	8.51	106		
8.00	8.00	100	102 ± 3.1	3.04
8.00	7.90	99		
16.00	16.23	101		
16.00	16.50	105	102 ± 2.5	2.45
16.00	15.79	99		
32.00	30.78	96		
32.00	33.32	104	99 ± 3.4	3.43
32.00	31.37	98		

of three injections for all standard injections was less than 4.1% and the % RSD for all levels of standards was less than 3.9%. The limit of detection (LOD) for BaP-N7Gua standards was 96 pg (239 *f*mole) (S/N = 3) and limit of quantitation (LOQ) was 192 pg (477 *f*mole) (S/N = 6). These results indicate that the method is accurate and precise. Our LC/MS/MS/MRM method demonstrated the ultimate advantage over traditional UV-VIS or FLR detection methods by having low LOD and LOQ values. Most importantly, since stable isotopes were used as internal standards to avoid all possible errors, confidence of data was greatly enhanced compared with other methods. Such advantages are important for trace level analysis, especially, when analyzing bio-samples where interferences are often abundant.

Chromatographic separation between BaP-C8Gua and BaP-N7Gua peaks was very good. The resolution between the two peaks was 6.36. The tailing factor for BP-C8Gua was 1.07 and 1.24 for BaP-N7Gua at 5% peak height. Both the resolution and tailing factor were calculated based on the 8 ng of analytes on column standard injection. The above result showed improvement when compared with similar literature methods.[3,46–49] Representative chromatograms of standard injections are illustrated in Figure 9.

Table 2. BaP-N7Gua LC/MS/MS/MRM linear plot accuracy calculation

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

Adducts Stability Evaluation and Method Suitability

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

The recovery for BaP-C8Gua was from 95% to 105% and the average recovery was 98%. The recovery for BaP-N7Gua was from 97% to 107% and the average recovery was 101%. The above data indicates that both adducts are very stable at elevated temperature, and the acid hydrolysis procedure does not cause significant break down of the adducts. The approximate 100% recovery for both adducts indicates no adduct loss during the Sep-Pack clean up. The internal standards added into the post-Sep-Pak column served as an indicator (fixed numerator). Therefore, any adduct lost during the Sep-Pak clean up procedure would be detected by a decreasing ratio. The excellent recovery demonstrated a

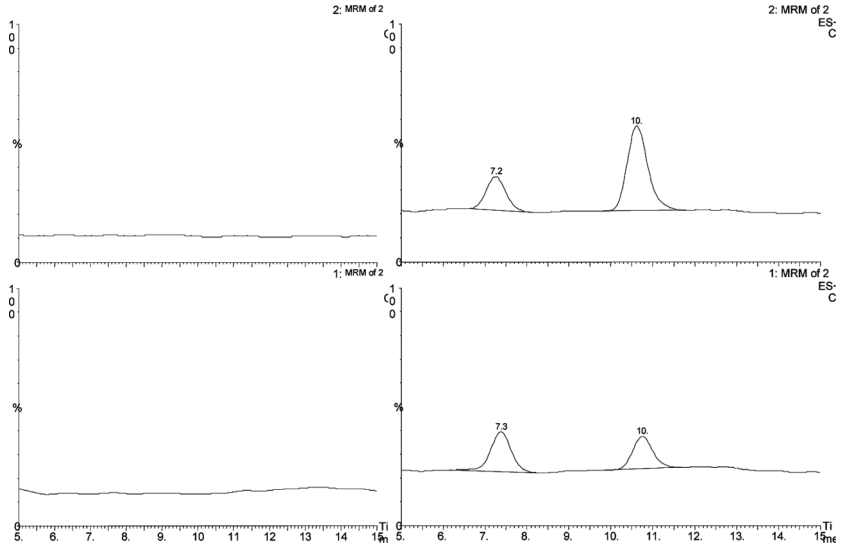

Figure 9. Representative LC/MS/MS/MRM chromatograms of the blank and standard injections. (From left to right, Blank injection, Std injection (1 ng on column per adduct). Top channel is the transition for internal standards (m/z 413 → 287 and 371) and the bottom channel is transition for adducts (m/z 402 → 277 and 360). The early eluter is the BaP-C8Gua and the late eluter is BaP-N7Gua).

high efficiency for the Sep-Pak clean up procedure. The recovery of authentic adducts spiked in rat liver DNA was good and the blank rat liver DNA preparation showed no interference peak when compared with the spike. For BaP-C8Gua, the recovery range was from 82% to 101% and the average recovery was 93%. For BaP-N7Gua, the recovery range

Table 3. *In vitro* sample results

Sample ID		BaP-C8Gua (ng)/% total adduct	BaP-N7Gua (ng)/% total adduct
Calf Thymus DNA Sample	Calf Thymus DNA (Stable Adducts)	11.9/26.1	6.8/14.9
	Calf Thymus DNA (Depurinating Adducts)	5.0/11.0	21.9/48.0
Rat Liver DNA Sample	Rat liver DNA (Stable Adducts)	10.9/35.7	3.9/12.8
	Rat liver DNA (Depurinating Adducts)	4.5/14.8	11.2/36.7

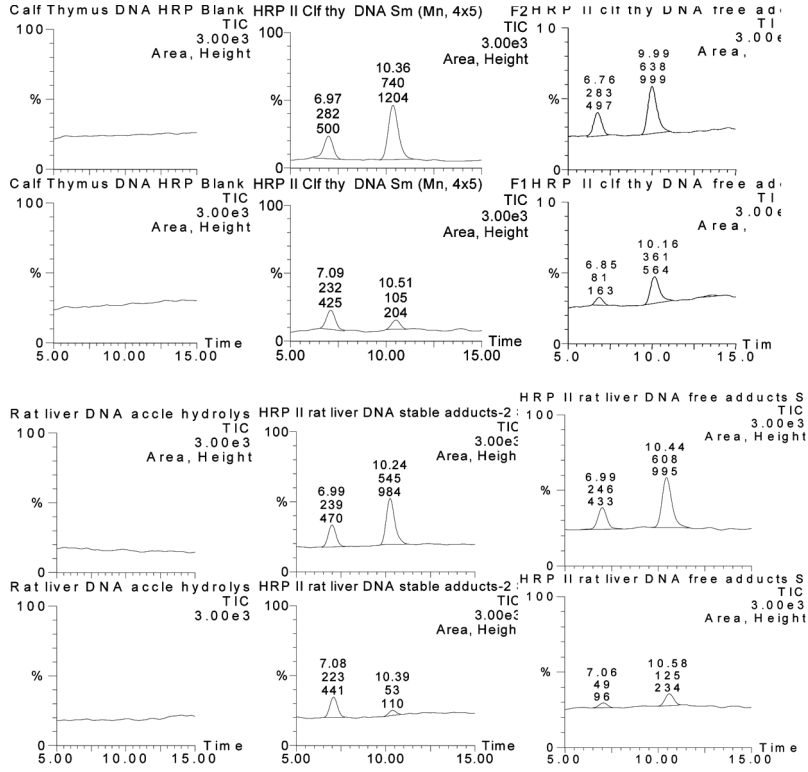

Figure 10. Representative LC/MS/MS/MRM chromatograms of the horseradish peroxidase experiments. (From left to right, row 1-calf thymus DNA: blank, stable adducts, depurinating adducts, row 2-rat liver DNA: blank, stable adducts, depurinating adducts. Top channel is the transition for internal standards (m/z 413 → 287 and 371) and the bottom channel is the transition for adducts (m/z 402 → 277 and 360). The early eluter is BaP-C8Gua and the late eluter is BaP-N7Gua).

was from 83% to 104% and the average recovery was 94%. For the BaP-C8Gua, the limit of detection (LOD) of rat liver DNA spike was 98 pg (61 mole/mg DNA) (S/N = 3) and limit of quantitation (LOQ) was 196 pg (122 *f*mole/mg DNA) (S/N = 6). For the BaP-N7Gua, the LOD was 99 pg (62 *f*mole/mg DNA) and LOQ was 198 pg (124 *f*mole/mg DNA). The LOD and LOQ for both adducts in rat liver DNA spikes were very close to the values that were calculated from their pure form injections. The above data indicates that the method is valid, rugged, free of bias, and well suited for DNA analysis. Furthermore, since stable isotopes were used in all steps as internal standards to avoid all possible errors, the highest data quality was ensured.

In Vitro Experiment Results

The *in vitro* experiment results indicate that BaP-N7Gua is the major depurinating adduct content for both calf thymus and rat liver DNA samples, while BaP-C8Gua dominated in the stable adduct analysis. Blank preparation showed no interference with our analysis and was bias free. The above result agrees with the finding of other researchers.[3,46] Once BaP-N7Gua is formed it will break away from DNA faster than the BaP-C8Gua. The *in vitro* results and additional details are presented in Table 3. LC/MS/MS/MRM chromatograms are presented in Figure 10.

CONCLUSION

High performance liquid chromatography coupled with tandem mass spectrometery has proven to be very powerful instrumentation in the analysis of DNA adducts in complex biological tissues. Our study was focused on the quantitative study of benzo[a]pyrene DNA nucleoside adducts in *in vitro* and *in vivo* samples by utilizing this technique. Most importantly, confidence of data was greatly enhanced since novel stable isotopes were used as internal standards in all processes to avoid any possible errors and interferences. Such advantages are important for trace level analysis, especially when analyzing bio-samples where interferences are often abundant.

We conclude that our LC/MS/MS with the stable isotopes internal standards method was suitable for bio-sample analysis and it delivers greater data confidence. Therefore, more research and application efforts should be made in liquid chromatography/tandem mass spectrometry with stable isotopes internal standards; the importance in utilizing such a technique in future research cannot be overemphasized. Furthermore, different PAHs should be included in the study to assess the risk of their carcinogenesis effects. By gathering the information of all these studies, more information about cancer formation could be provided and lead to better cures of the disease.

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