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An improved liquid chromatography–tandem mass spectrometry method for the quantification of 4-aminobiphenyl DNA adducts in urinary bladder cells and tissues

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ABSTRACT

Exposure to 4-aminobiphenyl (4-ABP), an environmental and tobacco smoke carcinogen that targets the bladder urothelium, leads to DNA adduct formation and cancer development [1]. Two major analytical challenges in DNA adduct analysis of human samples have been limited sample availability and the need to reach detection limits approaching the part-per-billion threshold. By operating at nano-flow rates and incorporating a capillary analytical column in addition to an online sample enrichment step, we have developed a sensitive and quantitative HPLC–MS/MS method appropriate for the analysis of such samples. This assay for the deoxyguanosine adduct of 4-ABP (dG-C8-4-ABP) gave mass detection limits of 20 amol in 1.25 μg of DNA (5 adducts in 10^9 nucleosides) with a linear range of 70 amol to 70 fmol. 4-ABP-exposed human bladder cells and rat bladder tissue were analyzed in triplicate, and higher dose concentrations led to increased numbers of detected adducts. It was subsequently established that sample requirements could be further reduced to 1 μg digestions and the equivalent of 250 ng DNA per injection for the detection of low levels of dG-C8-4-ABP in a matrix of exfoliated human urothelial cell DNA. This method is appropriate for the characterization and quantification of DNA adducts in human samples and can lead to a greater understanding of their role in carcinogenesis and also facilitate evaluation of chemopreventive agents.

1. Introduction

All living organisms are constantly exposed to toxic agents, endogenous and exogenous. In humans, such exposures may originate from a variety of sources including automobile and industrial exhausts, dietary sources and cigarette smoke [2,3]. These agents or their electrophilic metabolites can bind covalently to deoxyribonucleic acid (DNA) to form addition products commonly referred to as DNA adducts whose formation has long been associated with carcinogenesis [4–7]. It has been postulated that when DNA adducts are not efficiently repaired, alterations in the DNA sequence may occur during replication that can lead to mutation and ultimately cancer [8]. Many arylamines and nitrosamines are potent mutagens and have been implicated in chemical carcinogenesis. Prominent among them is 4-aminobiphenyl (4-ABP), an environmental carcinogen found in cigarette smoke, paints, food colors, hair dyes and fumes from heated oils and fuels [9–13]. 4-ABP targets the bladder urothelium and has been linked to human bladder cancer, a major world health problem [1,14–16]. While an indirect measurement of 4-ABP exposure only serves as an estimate of potential DNA damage, a direct measurement of 4-ABP DNA adducts accounts for inconsistencies in adsorption, metabolism, detoxification, and DNA repair and is consequently more relevant to assessing the effect of exposure on bladder cancer [17]. Several studies have also shown that after cases were adjusted for exposures, higher levels of DNA adducts were observed in cancer patients than non-cancer patients [18–22]. Accordingly, DNA adducts serve as both biomarkers of exposure and susceptibility to cancer and for that reason their measurement has significant implications for disease risk assessment [23].

A major challenge in the analysis of DNA adducts has been the low levels at which they typically occur *in vivo*. They are found in a complex matrix of protein, ribonucleic acid (RNA), and salt as well as excess unmodified bases, which in the case of 4-ABP is a million to a billion-fold [17,24]. These adduct quantities

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have been observed in a number of studies employing a wide array of distinct analytical methodologies that include ^{32}P-postlabeling [25–28], tissue immunohistochemical staining [29–33], gas chromatography–mass spectrometry (GC–MS) [15,34], and high-performance liquid chromatography–mass spectrometry (HPLC–MS) [35,36]. In addition to the complex matrix, often only a small quantity of DNA may be obtainable for analysis from *in vivo* samples, necessitating minimal analyte loss during sample handling. A method suitable for the analysis of 4-ABP DNA adducts at levels compatible with human exposure must simultaneously tolerate the constraints of limited sample availability and detection limits approaching the part-per-billion threshold.

Mass spectrometry-based approaches, most notably HPLC–MS, which combine the features of high sensitivity with structural information have assumed a leading role in this area. A detailed and comprehensive review of the recent literature on the status of HPLC–MS for the analysis of DNA adducts can be found in an article by Singh and Farmer [37]. Additional and somewhat more focused reviews on this subject are also available [38–45].

In analyses conducted on conventional bore 2 mm internal diameter (i.d.) HPLC–MS, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-4-ABP) was reported as the principal isomeric adduct formed in the reaction of calf-thymus DNA with *N*-hydroxy-4-ABP and was also isolated in hepatic DNA of mice treated with 4-ABP. The reported levels of dG-C8-4-ABP were between 1.8 and 430 adducts in 10^7 nucleotides in calf-thymus DNA modified *in vitro* through reaction with *N*-hydroxy-4-ABP and 4.9 and 30 in 10^7 nucleotides in hepatic DNA isolated from mice treated with 4-ABP [24]. Since these analyses were carried out with relatively large bore columns (≥2 mm i.d.) they required processing 0.1–1.0 mg or more DNA. This has been reduced as impressive improvements in overall sensitivity for the trace level detection of bulky DNA adducts have been achieved by coupling capillary separation methods to nanoelectrospray ionization (ESI) MS [46]. In their capillary LC–microESI-MS/MS technique developed for the analysis of dG-C8-4-ABP adducts in human pancreas DNA from a small set of smokers and non-smokers, Ricicki et al. achieved a limit of quantification (LOQ) approaching 1 adduct in 10^8 nucleotides using 100 μg of DNA per sample and only 13.3 μg of DNA per analysis [36]. This was further decreased to 2.50 μg of DNA per analysis in a subsequent study using a smaller i.d. capillary column (75 μm i.d. compared to 320 μm i.d. in the previous study) and a reduced flow rate of 200 nL/min compared to the previous 20 μL/min [47]. A major limitation in both of the latter two studies was the off-line solid-phase extraction (SPE) enrichment step requiring relatively large (100 μg) quantities of DNA in order to compensate for the inevitable analyte losses during sample processing. The method we present here integrates a 75 μm i.d. analytical column with online sample enrichment on a trap column prior to analysis, reducing both the quantity of DNA required and detection levels. This improved DNA adduct quantification methodology has a detection level of 5 adducts per 10^9 nucleosides using a total of 5 μg of DNA and the equivalent of only 1.25 μg of DNA per analysis.

2. Experimental

2.1. Chemicals and standards

Caution: 4-Aminobiphenyl and its derivatives are carcinogenic and should be handled carefully.

McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum from Life Technologies (Grand Island, NY), rat liver S9 from Moltox (Boone, NC), Nicotinamide adenine dinucleotide phosphate (NADP) from Amresco, Inc. (Solon, OH) and Harlan 7012 Nature Ingredient diet from Harlan Laboratories (Bartonsville, IL) were required during cell and animal dosing periods. The following chemicals were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO): D-glucose-6-phospate disodium hydrate, calf-thymus DNA, nuclease p1 from *penicillium citrinium*, deoxyribonuclease 1 (DNase I) type 2 from bovine pancreas, alkaline phosphatase from *Escherichia coli* (type IIIs), ethanol, magnesium chloride, dimethyl sulfoxide (DMSO), and 4-Aminobiphenyl (4-ABP). Hydrochloric acid was purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphodiesterase 1 (crotalus adamanteous venom) was purchased from USB Corporation (Cleveland, OH). For HPLC–MS/MS analysis, acetic acid (glacial, 99.99+%) was acquired from Aldrich Chemical Co. (Milwaukee, WI), and Burdick and Jackson solvents (methanol, acetonitrile, and water) were obtained from Thermo Fischer Scientific (Pittsburgh, MA) and were HPLC grade. *N*-(2-Deoxyguanosine-8-yl)-4-ABP (dG-C8-4-ABP) was acquired from Toronto Research Chemicals (North York, ON) and the deuterium labeled internal standard dG-C8-4-ABP-d_9 was previously synthesized and characterized in our laboratory [36].

2.2. Cell study

RT-4 human bladder carcinoma cells were grown in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum and maintained in a humidified incubator at 37 °C with 5% CO_2. Cells were plated at a concentration of 2.0 million cells per 10-cm dish with 10 mL growth medium overnight and then treated with 0, 0.5, 5.0, or 50 μM 4-ABP dissolved in DMSO for 3 h in the presence of 6% rat liver S9, 10 mM D-glucose-6-phospate disodium hydrate, and 5 mM NADP. Triplicate cultures were completed at each condition. Following treatment, cells were harvested by trypsinization, washed once with ice-cold PBS and stored at −80 °C until analysis. DNA was extracted using Qiagen Blood and Cell Culture DNA Midi Kits according to the manufacturer's instructions. Cell cultures of roughly 5 million cells yielded 20–40 μg of nuclear DNA.

2.3. Animal study

Twelve 2-month-old male F/344/NHsd rats (approximately 180 g of body weight) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The protocol was reviewed and approved by the Animal Care and Use Committee at Roswell Park Cancer Institute. Animals were acclimated for 1 week and were fed the Nature Ingredient diet (Harlan 7012) and water *ad libitum*. 4-ABP was prepared in DMSO and administered by IP injection at doses of 0, 25, 100, or 250 mg/kg of body weight in a final volume of 0.1 mL per rat. Animals were sacrificed 24 h after the dosing, and the urinary bladders were obtained immediately and stored at −80 °C. DNA was extracted with an Invitrogen Easy-DNA kit according to the manufacturer's instructions. Approximately 10–30 μg of DNA was isolated from rat bladder specimens of about 30 mg each.

2.4. Exfoliated human urothelial cell study

Four 150–250 mL urine samples from a lifetime non-smoker were collected and stored at −80 °C. Samples were thawed and urothelial cells were pelleted by centrifugation at 8000 × *g* for 10 min at 4 °C (Thermo Scientific, Sorvall RT-1). DNA was isolated using a Qiagen Blood and Cell Culture DNA Midi Kit with the following yields: 0, 1.3, 2.2 and 2.8 μg. One μg of DNA was removed from each sample, digested according to the procedure described below and reconstituted in 20 μL 10% methanol for three 5 μL injections per sample. DNA from two of the samples was pooled for a 1 μg digest to be spiked with IS and 2.24 fmol dG-C8-4-ABP before protein precipitation. The remainder of DNA from the third sample was reserved for testing digestion efficiency. Specifically, 1 μg