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Improved high-performance liquid chromatography analysis of ^{32}P-postlabeled 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine–DNA adducts using in-line precolumn purification

Robert J. Mauthe, Glenn A. Marsch, Kenneth W. Turteltaub*

Biology and Biotechnology Program, Lawrence Livermore National Laboratory, Livermore, CA 94550, USA

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Abstract

An improved HPLC-based ^{32}P-postlabeling assay has been developed for the analysis of DNA modified with the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Postlabeled samples are loaded onto a C_{18} precolumn and adducted bases are retained while excess radioactivity and unmodified DNA bases are eluted directly to waste through a switching valve. The use of this HPLC in-line precolumn purification (HIPP) technique allows entire postlabeled samples to be analyzed without prior removal of inorganic phosphate and unmodified DNA bases. The method has a sample to sample precision of 15% and accuracy of 20%, at adduct levels of 2 adducts/10^7 bases and shows a linear relationship between signal and adduction levels from 1 adduct per 10^4 to $\approx 2 \pm 1$ adducts per 10^9 bases. Individual postlabeled DNA samples can be analyzed by HPLC in less than 1 h, allowing high throughput. The use of calf-thymus DNA (CT-DNA), highly modified with PhIP, or DNA isolated from mice chronically fed a PhIP-modified diet shows two major PhIP–DNA adduct peaks and three additional minor adduct peaks when labeled under ATP-limiting conditions. Isolation of the HPLC purified peaks and analysis by thin layer chromatography (TLC) matches the five HPLC peaks to the spots typically seen by TLC, including N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP). Variations in digestion techniques indicate a potential resistance of the PhIP–DNA adducts to the standard enzymatic digestion methods. Attempts at adduct intensification by solid phase extraction, nuclease P1 enrichment or 1-butanol extraction decreased PhIP–DNA adduct peaks and introduced a large early eluting peak. Removal of the 3′-phosphate with nuclease P1 following the kinase labeling reaction simplifies the HPLC profile to one major peak (dG-C8-PhIP monophosphate) with several minor peaks. In addition to the high resolution provided by HPLC separation of the PhIP–DNA adducts, this method can be adjusted for analysis of other DNA adducts and is readily automated for high throughput.

Keywords: ^{32}P-Postlabelling; DNA adducts; 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine–DNA adducts

1. Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most prevalent of the amino-imidazoazaarene mutagens/carcinogens found in meats cooked at high temperatures [1–3]. PhIP, a moderate mutagen in the Ames assay [3], is a colon carcinogen in male rats, a mammary carcinogen in female rats [4] and causes lymphomas in male and female mice [5]. PhIP is activated to 2-hydroxy-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N^2-OH-PhIP) by microsomal monooxygenases, includ-

*Corresponding author.

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ing cytochrome P450 isozyme 1A2 [6]. The N^2-OH-PhIP is likely to be further activated to a more electrophilic derivative capable of forming stable adducts with cellular DNA [7]. In vitro studies using PhIP and N^2-OH-PhIP have identified the major PhIP–DNA adduct as N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine(dG-C8-PhIP) [8], but other adducts are likely to exist [9].

The ^{32}P-postlabeling protocol, developed by Randerath et al. [10], has undergone numerous modifications to achieve higher sensitivities and to maximize labeling efficiencies with a wide variety of chemicals. Several versions of the assay have been applied to the assessment of DNA adducts formed with PhIP [7,8,11].

In an effort to increase the chromatographic resolution, quantitation, throughput and safety of the ^{32}P-postlabeling method, several investigators have adapted the assay to HPLC [12–15]. After postlabeling with [^{32}P]ATP, a portion of the sample is either directly injected onto a HPLC column or is prepurified by removing excess ATP, unmodified bases and inorganic phosphate by one-dimensional TLC or column chromatography, prior to HPLC analysis. After elution of the adducted material, the sample is injected onto the reversed-phase HPLC column and the adducts eluted using standard reversed-phase solvents [12,13], a strong salt–low pH buffer [14,15], or an ion-pairing buffer [12,14].

In this study, we have improved the HPLC-based ^{32}P-postlabeling procedure by incorporation of an in-line precolumn technique that was used to remove inorganic phosphate and DNA bases not adducted to PhIP. Digestion and ^{32}P-labeling of PhIP-modified DNA were optimized and the peaks seen by HPLC compared to postlabeled samples analyzed by TLC. Additionally, several adduct enrichment procedures and additional enzymatic treatments have been investigated.

2. Experimental

2.1. Chemicals and enzymes

PhIP (>98% pure) was purchased from Toronto Research Chemicals (Downsview, Toronto, Canada). N^2-OH-PhIP (>98% pure) was obtained from SRI International. Spleen phosphodiesterase (SPDE; EC 3.1.16.11) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). T4 polynucleotide kinase (EC 2.7.1.78) was purchased from BRL Life Technologies (Gaithersburg, MD, USA). [^{32}P]ATP (6000 Ci/mmol, gamma label) was purchased from Amersham (Arlington Heights, IL, USA). Micrococcal nuclease (MN; EC 3.1.31.1), apyrase (EC 3.6.1.5), RNase A (EC 3.1.27.5), RNase T1 (EC 3.1.27.3), nuclease P1 (EC 3.1.30.1), proteinase K, ethylenediametetraacetic acid (EDTA), urea, dithiothreitol (DTT), 3-[N-morpholino]propanesulfonic acid (MOPS), Triton X-100, Tris, spermidine and phenol were purchased from Sigma (St. Louis, MO, USA). Other solvents (chloroform, isoamyl alcohol, 1-butanol) and reagents (sodium acetate, zinc chloride, ammonium formate, potassium phosphate, sodium chloride, sodium succinate, magnesium chloride, calcium chloride, tetrabutylammonium chloride and lithium chloride) were purchased from Aldrich (Milwaukee, WI, USA).

2.2. Reaction of calf-thymus DNA with PhIP

Calf-thymus DNA (CT-DNA) modified by N-acetoxy-PhIP was used as a standard for methods development. DNA modification by N-acetoxy-PhIP was performed as previously described [9]. Briefly, N^2-OH-PhIP was N-O-acetylated to N-acetoxy-PhIP with acetic anhydride, which was then added dropwise to calf-thymus DNA in 20 m*M* MOPS buffer (pH 6.5) supplemented with 100 m*M* NaCl and 1 m*M* EDTA. This reaction mixture was then incubated for an additional 30 min at 37°C in the dark. The solution was stirred continuously and the reaction vessel was purged with nitrogen during the reaction to prevent oxidative decomposition. The DNA was then purified by repetitive extractions with 1-butanol (water-saturated) followed by ethanol precipitation in order to remove unadducted carcinogen. Adduct levels, as determined by ^{32}P-postlabeling, UV absorbance and fluorescent spectroscopic methods [9], were ≈1 adduct per 600 DNA bases. This DNA was used either directly (for HPLC peak collection and analysis by TLC) or was diluted with 5–10 μg of calf-thymus DNA (final modification

level of 0.02–200 adducts/10^7 bases) for use in this study.

2.3. Isolation of mouse tissue DNA

DNA was isolated from the tissue of mice chronically fed a diet supplemented with 400 ppm PhIP as follows: Tissues were lysed (0.5 ml of lysis buffer/g of tissue) in 10 m*M* Tris–HCl (pH 8), 4 *M* urea, 1% Triton X-100, 10 m*M* EDTA, 100 m*M* NaCl, 10 m*M* DTT, and 800 μg of Proteinase K/ml of lysis buffer at 37°C for 18 h in a shaking water bath. The lysate was then treated for 1 h at 37°C with RNase (500 μg of RNase A and 0.5 μg of RNase T1/ml of lysis buffer) followed by extraction with equal volumes of phenol, phenol–chloroform (1:1, v/v) and phenol–chloroform–isoamyl alcohol (25:24:1, v/v). The combined organics were then back-extracted with 1/10 volume of water. Aqueous samples were combined and the DNA precipitated by mixing with 1/20 volume of 5 *M* NaCl and two volumes of 100% ethanol. The DNA was spooled on a glass disposable pipette, washed twice with 70% ethanol in water and once with 100% ethanol. The DNA was redissolved in water and quantitated by measurement of UV absorbance at 260 nm. All DNA samples used in the study had an A_{260}/A_{280} ratio of between 1.7 and 1.9, indicating highly pure DNA and the efficient removal of proteins and organic extraction solvents.

2.4. Digestion of DNA

PhIP-modified DNA purified in vitro or in vivo was dissolved in 20 m*M* sodium succinate, pH 6.0, supplemented with 10 m*M* $CaCl_2$. Digestion of DNA with MN–SPDE was optimized for postlabeling conditions. Enzyme conditions were varied from 2.5 to 10 U of MN and 3 to 12 μg of SPDE per 5 μg DNA sample. The length of incubation in a shaking Eppendorf heater was extended from 30 min to 18 h. The overnight incubations were also tested at 4, 25 and 37°C. The optimal conditions for a 5-μg DNA sample were found to be 5 U of MN and 6 μg of SPDE at 25°C for 18 h. For experiments where larger DNA samples were digested, proportionately larger amounts of enzyme were also used.

2.5. ^{32}P-Postlabeling reaction

The digested DNA samples were then concentrated to approximately 10 μl under vacuum. The pH was adjusted to 8.5 with 2 μl of 0.5 *M* Tris–HCl (pH 8.5) and labeled under ATP-limiting conditions (15 nmol DNA bases, 35.7 pmol [^{32}P]ATP) with 1.5 μl of γ[^{32}P]ATP (6000 Ci/mmol, 0.143 mCi/μl), 2 μl of kinase buffer (0.5 *M* Tris–HCl, pH 8.5, 0.1 *M* $MgCl_2$, 30 m*M* DTT and 8 m*M* spermidine) and 0.4 μl of T4 polynucleotide kinase (4 U) for 120 min at 37°C. Any remaining ATP was hydrolyzed by addition of apyrase (0.1 U) and incubation at 37°C for 45 min. In addition to the ATP-limited labeling, standard ^{32}P-postlabeling experiments, using equimolar amounts ATP and DNA base, were also performed by addition of unlabeled ATP (15 nmol) or labeling of less DNA (35.7 pmol). The samples were frozen at −35°C until analyzed by HPLC. The efficiency of the postlabeling procedure was calculated by comparison of ^{32}P counts in postlabeled PhIP–DNA adducts and known adduct levels determined by spectroscopic methods [9].

2.6. HPLC analysis of PhIP–DNA adducts

Adducted DNA was analyzed using a modification of the technique described by Pfau et al. [14]. A binary Rainin HPLC system was attached to a Rainin UV detector (Rainin Instruments Company, Woburn MA, USA) and a Radiomatic Flow-one radioisotope detector (Packard, Meriden, CT, USA) with a 0.5-ml sample cell in series (Fig. 1). Rainin Data Acquisition was used for gradient control and data acquisition. An entire ^{32}P-postlabeled sample (214 μCi ^{32}P) was loaded onto a Rainin Dynamax precolumn (3 μm C_{18} coated packing material, 25×4.6 mm I.D.) using a Rheodyne injector equipped with a 50-μl injection loop to pre-enrich for adducted DNA [16,17]. HPLC conditions were optimized using 0.25 *M* KH_2PO_4, pH 2.1 (buffer A) and methanol (buffer B). Inorganic ^{32}P and unmodified DNA bases were eluted directly to waste with 15% B at a flow-rate of 3 ml/min for 5 min. The flow was then redirected to a MVC_{18} analytical column (3 μm packing material, 100×4.6 mm I.D.) using a Rheodyne 7000 switching valve and the flow-rate was changed to 1 ml/min. The adducted DNA bases were eluted with a gradient

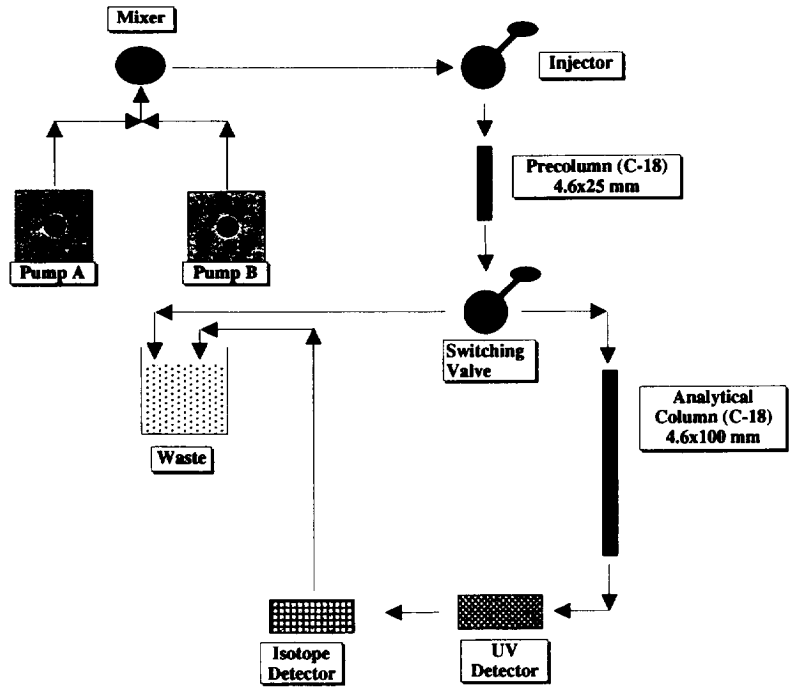

Fig. 1. Schematic diagram of the HPLC system. The microsorb 25×4.6 mm, 3 μm C_{18} precolumn retains adducted nucleotides, while unmodified DNA bases along with excess unbound radioactivity elute directly to waste. The Rheodyne 7000 switching valve allows change of flow to the Microsorb 100×4.6 mm, 3 μm C_{18} analytical column which is used to separate PhIP-modified mononucleotides. ^{32}P content is measured in a Radiomatic Flow-one beta counter by Cerenkov counting using a 500-μl cell.

as follows: 15% B from 0 to 5 min; 15–35% B over 5 min and 35–45% B over 25 min. When analyzing the monophosphate DNA adduct samples, the method following the 5 min wash with 15% methanol was: 15% B for 0 to 5 min; 15–35% B over 5 min; 35–45% B over 25 min; 45% for 5 min and 45–60% B over 5 min. For HPLC runs of previously purified peaks, the same conditions were used except that the splitter was not used and the flow-rate for the first 5 min was 1 ml/min.

2.7. TLC analysis of PhIP–DNA adducts

The ^{32}P-labeled DNA digest was spotted on polyethyleneimine–cellulose (PEI–cellulose) sheets and eluted in four dimensions using similar techniques to those previously published [6,10]. PEI–cellulose sheets were prepared as previously described [6]. Development in D1 (bottom to top) was overnight in 2.3 *M* sodium phosphate (pH 5.8). Development in D3 (bottom to top) was with 2.7 *M* lithium formate, pH 3.35, and 7 *M* urea. D4 (rotated 90°) was with 0.5 *M* Tris–HCl (pH 8.0), 4.2 *M* lithium formate and 7 *M* urea. D5 (same direction as D4) was with 1.7 *M* sodium phosphate, pH 6.0. Screen-enhanced autoradiography (−80°C) was carried out for 1–72 h depending on the level of DNA modification and the amount of DNA used for postlabeling analysis. After autoradiography, spots were cut out and ^{32}P levels determined by Cerenkov counting.

2.8. Enrichment of PhIP–DNA adducts

In an effort to enrich the PhIP–DNA adducts prior to postlabeling, three enrichment procedures were used; removal of the normal DNA bases by disposable C_{18} Sep-Pak chromatography, extraction of the adducted DNA bases into 1-butanol or removal of the 3′-phosphate of normal DNA bases with nuclease

P1 treatment. For solid phase extraction of the DNA digest, a disposable C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) was prepared with 10 ml of methanol, 10 ml of water and 10 ml of 0.25 *M* KH_2PO_4 (pH 2.1). The DNA digest sample, diluted in 1 ml of 0.25 *M* KH_2PO_4 (pH 2.1), was loaded onto the column over 1 min and reloaded. The C_{18} cartridge was rinsed with 5 ml of 0.25 *M* KH_2PO_4 (pH 2.1), 5 ml of water and 1 ml of 5% methanol in water. The adducted DNA was then eluted in 2 ml of 100% methanol. The eluted sample was dried under vacuum and resuspended in 20 μl of water prior to ^{32}P-postlabeling.

The following procedure was used for the extraction of PhIP-modified DNA bases into 1-butanol: to each 20 μl digestion sample was added 25 μl of 100 m*M* ammonium formate (pH 3), 15 μl of 50 m*M* tetrabutylammonium chloride and 10 μl of water [18]. This solution was extracted twice with 70 μl of 1-butanol (water-saturated). The combined butanol solutions were back-extracted with 125 μl of water (1-butanol-saturated). After addition of 3 μl of 200 m*M* Tris (pH 8.5), the sample was dried and resuspended in 20 μl of water.

For digestion with nuclease P1 to remove the 3′-phosphate from unprotected DNA bases, the MN–SPDE digested samples were adjusted to pH 5 with 0.1 *M* HCl and 2 μl of 10×buffer (250 m*M* sodium acetate, pH 5; 300 m*M* $ZnCl_2$) and 12.5 μg of nuclease P1 were added [19]. The samples were incubated at 37°C for 15 min and concentrated to ≈20 μl under vacuum.

2.9. Nuclease P1 treatment following postlabeling

Postlabeled 3′,5′-bisphosphate samples were digested with nuclease P1 to remove the 3′-phosphate [14]. After adjustment of the sample to pH 5 with 0.25 *M* HCl, 2 μl of nuclease P1 buffer and 8 μg of nuclease P1 were added. The samples were incubated for 45 min at 37°C and then were frozen at −35°C until analyzed by HIPP.

2.10. Data analysis

All data and statistical analyses were carried out using Microsoft Excel 5.0 for the Macintosh.

3. Results and discussion

3.1. HPLC technique

The use of a switching valve to direct the flow of HPLC eluents from the precolumn to either waste or to the analytical column (Fig. 1) allowed the injection of entire ^{32}P-postlabeled DNA samples without contamination or increased background of the analytical column over more than 200 HPLC runs. Over 99% of the 214 μCi [^{32}P]ATP per run was removed from the sample directly to waste. Furthermore, a single precolumn, used to retain the adducted DNA bases, could be used for more than 100 runs without a significant increase in ^{32}P background levels or a decrease in retention of the adducted DNA bases. The efficiency of the in-line precolumn chromatography was compared to other methods used to remove excess radioactivity, including disposable C_{18} cartridges and one-dimensional ion-exchange TLC. Identical samples (10 μg of mouse spleen DNA with a modification level of ≈52 PhIP/10^7 bases) were postlabeled and contaminating radioactivity either removed by C_{18} solid phase extraction [12], or eluted off the origin after one-dimensional TLC with 4 *M* pyridinium formate [14], by 2-propanol–25% ammonium hydroxide (1:1) [13], or analyzed by the HIPP method described here. The HIPP method yielded 4.4±1 fmol PhIP adduct while solid phase extraction, elution of the TLC origin with 2-propanol–ammonium hydroxide and elution of the TLC origin with 4 *M* pyridinium formate all showed significantly lower yields (1.2±0.4, 0.8±0.2 and 1.8±0.6 fmol, respectively). The disposable C_{18} cartridge enrichment did not effectively remove sufficient ^{32}P to be analyzed by non-splitting HPLC without overloading the beta-detector. Additionally, the one-dimensional TLC samples contained large highly hydrophilic breakthrough peaks while the in-line precolumn method had low to moderate breakthrough peaks (chromatograms not shown). In a separate experiment, dG-C8-PhIP 3′,5′-bisphosphate adduct that had previously been purified by HPLC, was used to determine the percentage of compound retained on the C_{18} precolumn. UV absorbance with and without the in-line precolumn enrichment procedure described here

showed that less than 1% of the compound was lost in the splitting process (data not shown).

3.2. Sensitivity and reproducibility of assay

The sensitivity and reproducibility of this method is illustrated in Fig. 2. Intersample variations are less than 15% for samples with adduct levels >1 adduct/10^7 bases labeled in parallel, on the same day, with the same digestion and labeling conditions and materials. Samples postlabeled on different days fell within 20% variation. Repeated labeling of 1 ng of modified calf-thymus DNA diluted in 5 μg of unmodified DNA (2 adducts/10^7 bases) yielded 0.39±0.07 fmol of [^{32}P]DNA adduct when independently labeled on different days (n=10). The response of the technique described here is linear from 1 adduct/10^4 bases to approximately 2 adducts/10^9 bases, with the limit of sensitivity being 1500 dpm detectable above background (500 dpm). The measured error in samples increased to 50% (n=8) in the lowest detectable samples (Fig. 2B; 0.02 adduct/10^7 bases), because the signal from labeled adducts was barely above the ^{32}P baseline. At 0.2 adduct/10^7 bases (Fig. 2C) inter-sample variability was 20% (n=6). HPLC run times of under 50 min yield a sample throughput of greater than eight samples per day without automation. Use of an autosampler and pneumatic switching valve should double the throughput.

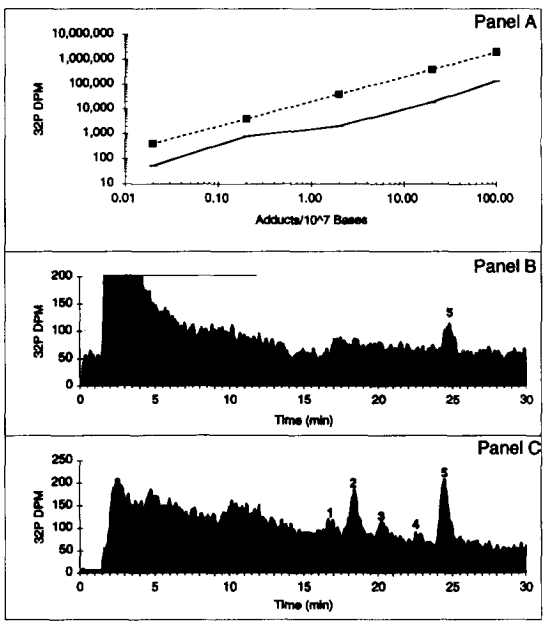

Fig. 2. Detection limits of assay. PhIP-modified calf-thymus DNA (CT-DNA) (≈1 adduct/600 bases) diluted with unmodified CT-DNA from 0.02 adducts/10^7 bases to 100 adducts/10^7 bases was labeled under ATP-limiting conditions, analyzed by HPLC and plotted as the adduct level versus total radioactivity in the PhIP–DNA specific peaks (Panel A - solid line; error bars show standard error, n=3). Representative chromatograms of the lowest detectable adduct level (Panel B - 0.02 PhIP/10^7 bases) and ten-fold higher adduct levels (Panel C - 0.2 PhIP/10^7 bases) are shown.

3.3. Correlation of HPLC peaks with TLC spots

To correlate the HPLC adduct peaks with TLC adduct spots, 1 μg of highly modified PhIP-DNA standard was digested, labeled and analyzed by HIPP. The HPLC peaks were collected and adjusted to pH 7 with 0.25 *M* NaOH. The ^{32}P activity eluting from the HPLC is shown in Fig. 3. Aliquots of the fractions corresponding to peaks 1 through 6 were

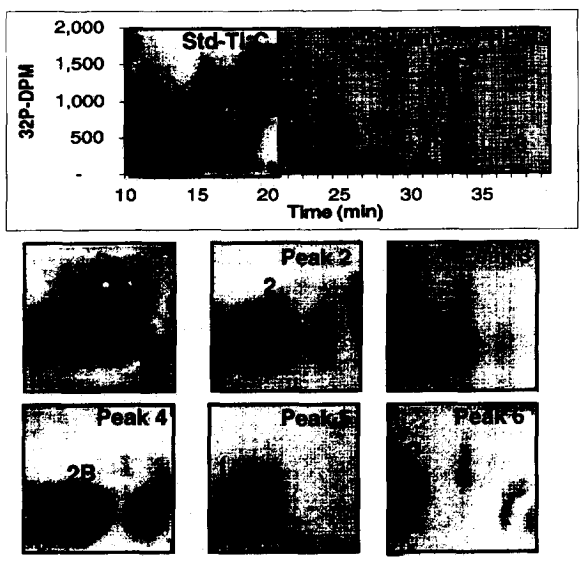

Fig. 3. Correlation of HPLC peaks and TLC spots. HPLC and TLC profile of 1 μg of CT-DNA (1 adduct/600 bases) ^{32}P-postlabeled under ATP-deficient conditions. An aliquot of the sample analyzed by HPLC along with aliquots of HPLC peaks 1–6 were spotted on TLC plates and eluted using ion-exchange TLC (eluted with D2, D4, D5). The dried plates were then exposed to X-ray film at −70° (peaks 2 and 5 for 3 h; all others for 28 h). TLC spot 1 corresponds to dG-C8-PhIP adduct.

analyzed by TLC and imaged by exposure to X-ray film (note exposure times). The TLC spots from the standard sample (2-μl aliquot of sample analyzed by HIPP) are labeled as spots 1, 2 and 3, with spot 1 identified as the dG-C8-PhIP adduct [8]. As seen in Fig. 3, several of the HPLC peaks migrate to the same areas of the TLC plate as the spots from the standard. HPLC peak 1 migrates to the same area as spot 3 from the standard. HPLC peaks 2 and 4 migrate to the same area as TLC spot 2 from the standard. HPLC peaks 3, 5 and 6 all migrate to the area of TLC spot 1. When large amounts of highly modified DNA (1 μg of DNA modified with 1 PhIP/600 bases) is digested and postlabeled by the HIPP method, the peaks corresponding to TLC spots 1 and 2 account for >90% of all adduct peaks, but as many as eight additional ^{32}P-labeled peaks are seen. The fast-eluting spots on the right of the TLC plates are not thought to be related to PhIP–DNA adduct as they are randomly seen and are not sample-dependent (Fig. 3).

3.4. Optimization of digestion conditions

A number of factors, including digestion time and conditions, were found to alter peak intensities and relative peak ratios, indicating the need to carefully optimize digestion, enrichment and labeling conditions. DNA digested with MN–SPDE, with increasing time of incubation and at 4, 25 and 37°C, resulted in varying amounts of ^{32}P-postlabeled adducts being found by the HIPP method (Table 1). The relative peak areas did not change significantly. Using standard enzyme concentrations of 5 U of MN and 6 μg of SPDE per 5 μg of DNA sample at 37°C, maximum adduct levels were found after a 30 min digestion (≈12.6 fmol of DNA–PhIP adduct). Overnight digestion at 37°C caused a slight decrease in overall adduct levels (9.6 fmol), but overnight digestion at 25°C provided equivalent adduct profiles, and increased adduct yield (14.7 fmol). Optimal digestion conditions, based on reproducibility and overall peak area, were found to be an 18-h digestion at room temperature with enzyme concentrations of 5 U of MN and 6 μg of SPDE per 5 μg of sample (Table 1, Fig. 4A). Analysis of the digested DNA by HPLC with UV monitoring indicate complete hydrolysis to 3′-deoxyribonucleotides under these optimized conditions, but does not eliminate the possibility that small amounts of adducted oligomers remain. The use of alternative digestion procedures significantly alter the DNA–adduct peak profiles. Digestion of the DNA with doubled enzyme concentrations yielded 30% higher overall adduct levels, mainly by increasing the amount of peak 2 by 40%, resolving peaks 1 and 3 and maintaining the size of peaks 4 and 5 (Fig. 4B). Digestion of DNA samples with MN for 2 h at 37°C,

Table 1
PhIP–DNA adduct levels following digestion with MN–SPDE

Digestion time (h)	Incubation temperature (°C)	PhIP–DNA adducts (fmol±S.E.)
0	37	0.28±0.10
0.2	37	2.49±0.19
0.5	37	11.33±1.98
1	37	12.43±1.21
2	37	11.72±1.64
3.5	37	11.38±0.89
18	37	9.55±0.81
18	4	11.11±1.67
18	25	14.68±1.35[a]

Highly modified CT-DNA diluted into 5 μg of unmodified CT-DNA (see Section 2), to an adduct level of ≈1 adduct/10^7 bases. The samples were digested under the conditions stated in the table with 5 U of MN and 6 μg of SPDE and were labeled with [^{32}P]ATP under ATP-limiting conditions. The samples were then sequentially analyzed using the HPLC-based methods described in Section 2. PhIP–DNA adducts (fmol) were determined from the radioactivity measured in the PhIP–DNA adduct peaks. Values given are the average±standard error of three independently labeled samples.

[a]Significantly different than 1 h, 37°C digestion (Student's t-test, $p<0.05$).

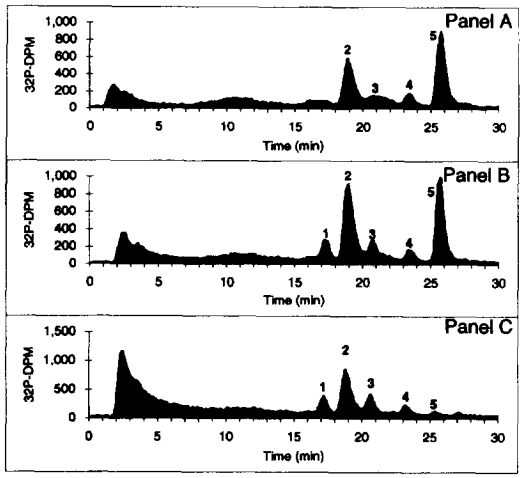

Fig. 4. Comparison of enzymatic digestion methods. Spleen DNA (5 μg) isolated from mice chronically fed 400 ppm PhIP-modified chow (≈50 PhIP/10^7 bases) was digested with 5 U of MN–6 μg of SPDE for 4 h at 37°C (Panel A); with 10 U of MN–12 μg of SPDE for 4 h at 37°C (Panel B) or with 5 U of MN for 2 h, then with 6 μg of SPDE for 2 h at 37°C (Panel C).

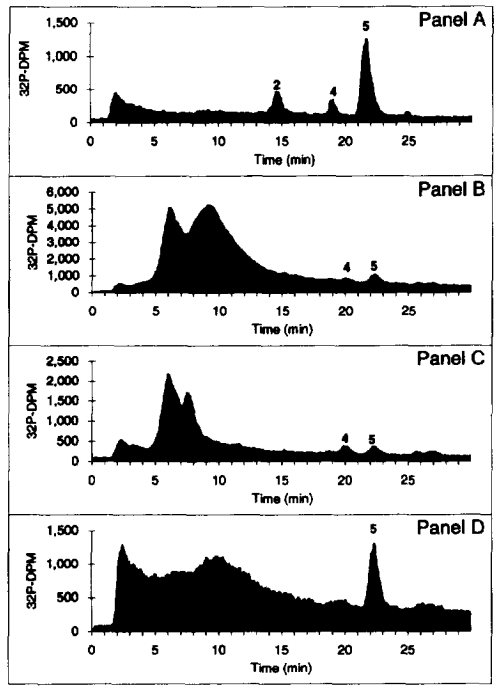

Fig. 5. Comparison of modified-DNA enrichment methods. HPLC profiles of 5 μg of mouse spleen DNA labeled under ATP-limiting conditions, with no enrichment procedure (Panel A), disposable C_{18} cartridge enrichment (Panel B), 1-butanol enrichment (Panel C) and nuclease P1 enrichment (Panel D).

followed by SPDE for 2 h at 37°C, reduced the overall adduct levels by 60% and nearly eliminated the dG-C8-PhIP adduct peak (peak 5), while increasing the level of peak 2 by 50% and resolving peaks 1 and 3 (Fig. 4C).

3.5. Enrichment techniques

Attempts to enrich the adducted DNA bases prior to postlabeling with disposable C_{18} cartridges, 1-butanol extraction or nuclease P1 digestion yielded lower peak areas and altered peak profiles (Fig. 5). Spleen DNA, from mice chronically fed a diet supplemented with 400 ppm of PhIP, was digested using the optimized conditions and used in all three enrichment procedures. As seen in Fig. 5, all three enrichment techniques yield a large early-eluting set of hydrophilic peaks, with the disposable C_{18} cartridge forming the largest peak, followed by extraction with 1-butanol and the smallest early-eluting peaks resulting from the nuclease P1 digestion. Attempts to remove unmodified bases with a C_{18} cartridge resulted in lowering the overall adduct peak areas by approximately 65%, decreasing peak 5 by 60%, while peak 4 remained unchanged and quantitation of peak 2 was not possible due to the large tail of the early-eluting peak (Fig. 5B). Likewise, extraction with 1-butanol to remove unmodified bases resulted in 78% less radioactivity in adduct peaks, increasing peak 4 by 10%, but decreasing peak 5 by 86%, and peak 2 again could not be quantified (Fig. 5C). Attempts to use the nuclease P1 enhancement produced 25% less radioactivity in DNA adduct peaks with only peak 5 being identified (Fig. 5D). Additionally, when the kinasing reaction was performed using equimolar concentrations of ATP and PhIP–DNA adduct, similar peak profiles were seen, but at significantly lower levels overall.

In an effort to identify the early-eluting hydrophilic peak present in the 1-butanol-extracted spleen DNA sample (Fig. 5C), aliquots were collected, dried and either reanalyzed directly by HPLC (Fig. 6A) or digested with MN (5 U) and SPDE (6 μg) (Fig. 6B), snake venom phosphodiesterase (0.04 U)

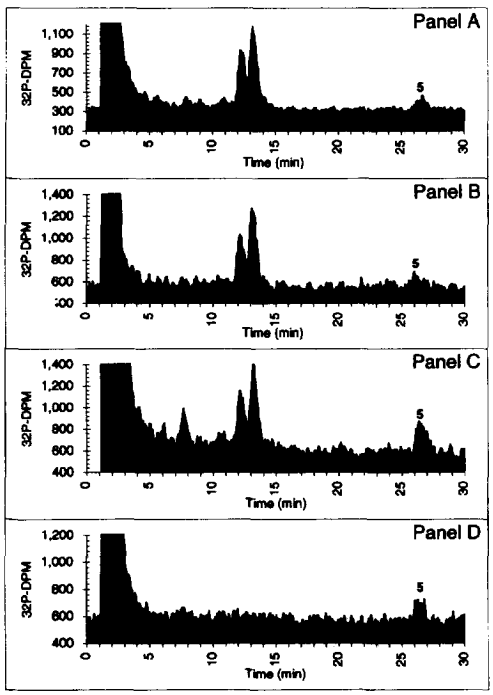

Fig. 6. Enymatic digest of an unknown early-eluting contaminating HPLC peak from Fig. 5(B–D). The early-eluting HPLC peak from a 5-μg mouse spleen DNA sample enriched using the 1-butanol procedure and labeled under ATP-limiting conditions was collected, neutralized and reanalyzed by HPLC (Panel A), or treated with 5 U of MN and 6 μg of SPDE (Panel B), 0.01 U of snake venom phosphodiesterase (Panel C) or with 5 μg of nuclease P1 (Panel D). The HPLC methods used were identical to those used in the collection of the peak except for the removal of the 5 min inital split to waste.

(Fig. 6C) or with nuclease P1 (25 μg) (Fig. 6D) and then analyzed by HPLC. The splitter used in previous HPLC experiments was not used for these runs. The HPLC profiles of the untreated, MN–SPDE, and snake venom phosphodiesterase samples were very similar, with the majority of the counts eluting in the breakthrough peak, a small amount eluting at the same retention time as the original isolated peak and a small amount of dG-C8-PhIP adduct. Digestion with nuclease P1 eliminated the early-eluting peak and decreased the dG-C8-PhIP adduct. Analysis of the early-eluting peak from the disposable C_{18} cartridge enriched spleen DNA sample yielded similar results (data not shown).

3.6. Analysis of 5′-monophosphate adducts

Treatment of postlabeled samples, adjusted to pH 5 with HCl and incubated with 15 μg of nuclease P1 for 45 min at 37°C followed by HPLC analysis, resulted in the appearance of a new later-eluting peak and the reduction of early-eluting peaks (Fig. 7). A peak eluting very close to the original C8-dG bisphosphate adduct could not be positively identified as residual C8-dG bisphosphate adduct, or as a new monophosphate adduct derived from an earlier eluting bisphosphate adduct. While others have reported the simplification of the PhIP–DNA adduct profile to one peak/spot with nuclease P1 treatment [14], or nuclease P1 and phosphodiesterase I [20], we find one major peak and several unidentified minor peaks.

3.7. Effect of the DNA amount used in the postlabeling assay

The effect of digesting increasing amounts of DNA is shown in Fig. 8. Spleen DNA, from animals fed a 400 ppm PhIP-modified diet, was digested with linearly proportionate amounts of the MN–SPDE (see Section 2). The overall adduct levels increase by digestion of up to 25 μg, although not in a linear fashion. The use of increased DNA does not affect the relative ratio of adducts formed (data not shown). Experiments done using equimolar amounts of DNA

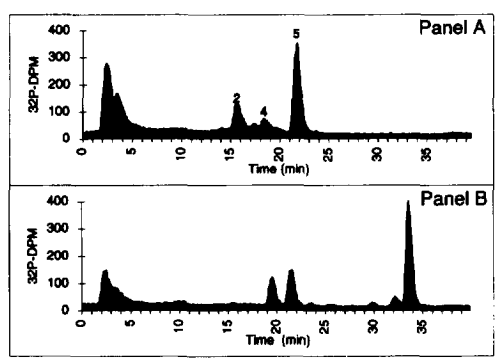

Fig. 7. Digestion of 3′,5′-bisphosphates to 5′-monophosphates. HPLC profiles of 5 μg of CT-DNA ($\approx$1 adduct/10^7 bases) without (Panel A) and with (Panel B) a 1-μg nuclease P1 treatment following ATP-limiting ^{32}P-postlabeling.

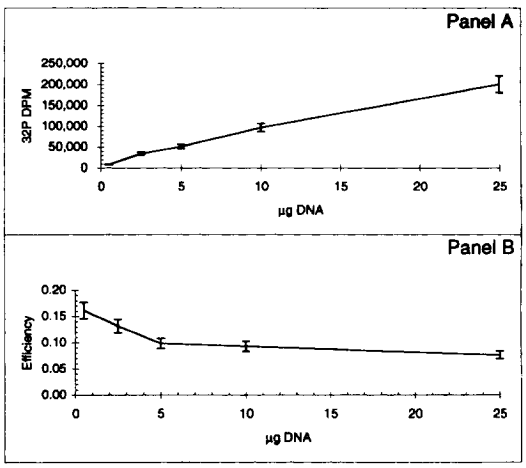

Fig. 8. Effect of increasing DNA amounts on the postlabeling assay. Increasing amounts of mouse spleen DNA were digested using MN–SPDE and analyzed under the same ATP-limiting conditions as described in Section 2. The results are plotted as the amount of DNA versus total ^{32}P DPM found in PhIP–DNA adduct peaks.

bases and ATP showed the same peak profiles and no increase in sensitivity.

4. Conclusions

The use of HPLC in-line precolumn purification (HIPP) ^{32}P-postlabeling allows the rapid high resolution detection of PhIP–DNA adducts with sensitivity of up to 2 ± 1 adducts/10^9 bases and less than 20% variation within samples labeled together at adduction levels greater than 1 adduct/10^8 bases. Analysis of the bisphosphate postlabeling mix shows the existence of two major DNA adducts with at least three additional minor adducts. Optimization of enzymatic digestion conditions was found to effect the overall labeling level and peak ratios, demonstrating the need to carefully optimize these aspects of the postlabeling procedure to ensure consistent results. Enrichment procedures or using equimolar concentrations of DNA bases and ATP were not able to increase the sensitivity of this assay, but increasing amounts of DNA in the assay did improve the overall amount of adduct measured at the cost of a lower labeling efficiency. The improved chromatographic separations of C_{18} HPLC over PEI–cellulose was established, as several of the HPLC peaks comigrate when spotted on the PEI–cellulose TLC plates.

While others have also found indications of incomplete enzymatic hydrolysis of PhIP-modified DNA, our experiments are not confirmatory. Digestion of the bisphosphate mixture with nuclease P1 does result in a simplified adduct pattern, but the area of the new monophosphate peak does not account for the combined area of the lost bisphosphate peaks. The symmetrical peak shapes also indicate pure adduct species, as a random modified oligomer would be expected to yield non-symmetrical peaks. Finally, fluorescence studies of the isolated HPLC peaks also indicate the presence of at least five different PhIP–DNA adduct species and is consistent with fluorescence studies on macromolecular DNA modified by PhIP (Marsch et al., manuscript in preparation). Thus, multiple lines of evidence support the existence of multiple PhIP–DNA adduct species.

The HIPP procedure provides a rapid analysis of DNA adducts with individual samples analyzed in less than 1 h. This readily automatable system allows rapid analysis of multiple samples with high resolution and low radiation exposures. The high amount of radioactivity (>200 μCi/run) and the use of low pH–high salt buffers does necessitate the daily cleaning of the HPLC system with a water–methanol mix, but column life does not appear to be diminished, as a single precolumn and column can last hundreds of runs without a loss of peak resolution or an increase in ^{32}P background.

The sensitivity of HPLC-based postlabeling is limited by the short counting interval (30 s in a 500-μl flow cell at a flow-rate of 1 ml/min) as the sample flows through the cell of the detector. For this reason, HPLC-based postlabeling is likely never to be as sensitive as analysis by TLC, where plates can be exposed to film for days. However, the HIPP method does provide a high throughput and is a flexible chromatographic technique for detecting and measuring DNA adducts to levels of 2 adducts/10^9 bases with high chromatographic resolution. Although most HPLC-based postlabeling methods have been non-quantitative or have only examined highly modified DNA, the sensitivity reported here with PhIP–DNA adducts is in agreement with reports of

other DNA adducts analyzed by HPLC [15]. This assay offers increased throughput, ease of peak collection, automation and chromatographic resolution over standard TLC-based postlabeling methods. This technique will be useful in future animal experiments, structural DNA adduct studies and in in vitro DNA reactions with PhIP and other DNA-binding compounds.

Acknowledgments

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