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Development of a ^{32}P-Postlabeling/HPLC Method for Detection of Dehydroretronecine-Derived DNA Adducts in Vivo and in Vitro

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Pyrrolizidine alkaloids are naturally occurring genotoxic chemicals produced by a large number of plants. Metabolism of pyrrolizidine alkaloids in vivo and in vitro generates dehydroretronecine (DHR) as a common reactive metabolite. In this study, we report the development of a ^{32}P-postlabeling/HPLC method for detection of (i) two DHR−3′-dGMP and four DHR−3′-dAMP adducts and (ii) a set of eight DHR-derived DNA adducts in vitro and in vivo. The approach involves (1) synthesis of DHR−3′-dGMP, DHR−3′-dAMP, and DHR−3′,5′-dG-bisphosphate standards and characterization of their structures by mass and ^{1}H NMR spectral analyses, (2) development of optimal conditions for enzymatic DNA digestion, adduct enrichment, and ^{32}P-postlabeling, and (3) development of optimal HPLC conditions. Using this methodology, we have detected eight DHR-derived DNA adducts, including the two epimeric DHR−3′,5′-dG-bisphosphate adducts both in vitro and in vivo.

Introduction

Pyrrolizidine alkaloids are a class of heterocyclic compounds that are common constituents of hundreds of plant species around the world (*1*−*3*). Many pyrrolizidine alkaloids are highly toxic, causing tremendous livestock loss due to liver and pulmonary lesions (*4*−*9*). A number of pyrrolizidine alkaloids, including monocrotaline, retrorsine, isatidine, lasiocarpine, clivorine, and riddelliine, have been found to induce liver tumors in rats (*2*, *10*−*17*). Nevertheless, the mechanisms leading to carcinogenesis have not been established. Human foodstuffs, such as herbs, milk, and honey, may also be contaminated by pyrrolizidine alkaloids, which can cause human health problems (*2*, *5*). The herbal tea "gordolobo yerba", popular in the American southwest, and "bush tea", used to treat children for colds in Jamaica, may contain pyrrolizidine alkaloids (*11*, *18*, *19*). In addition, pyrrolizidine alkaloids have been found in dietary supplements, such as comfrey and coltsfoot, available from commercial sources (*20*−*22*). Comfrey, a popular herbal tea in the world and used for healing broken bones, ulcers, bruises, and the digestive tract (*21*), was found by Betz et al. to contain pyrrolizidine alkaloids in ranges from 0.1 to 400 ppm (*21*).

Pyrrolizidine alkaloids require metabolic activation to exert their toxicities (*5*, *6*). The pyrrole metabolites (dehydropyrrolizidines), resulting from hydroxylation of pyrrolizidine alkaloids followed by dehydration, have been found to be capable of binding to DNA, and these compounds are responsible for most of the genotoxic activities of the parent pyrrolizidine alkaloids (*1*, *2*, *6*, *11*−*14*, *23*−*27*). Many of the more tumorigenic pyrrolizidine alkaloids are macrocyclic diesters of the necine base, in particular of the retronecine base. Dehydropyrrolizidines are highly electrophilic and also highly unstable. Thus, like most dehydropyrrolizidines, retronecine-derived dehydropyrrolizidines bind to DNA to form DNA adducts which undergo hydrolysis to release the corresponding necic acids and produce the dehydroretroneciene (DHR)[1]-derived DNA adducts. Alternatively, retronecine-derived dehydropyrrolizidines are first hydrolyzed to DHR which subsequently binds to DNA, forming the DHR-derived DNA adducts. Consequently, the resulting DHR-modified DNA adducts could be responsible for tumor initiation of a number of tumorigenic pyrrolizidine alkaloids. DHR has been found as a common metabolite of a large number of pyrrolizidine alkaloids in vitro and in vivo, including retrorsine (*13*, *28*), monocrotaline (*13*, *28*−*31*), senecionine (*13*, *32*, *33*), and indicine (*13*). DHR itself is a tumorigen that induces rhabdomyosarcomas in rats (*29*, *34*) and skin tumors in mice (*30*). DHR has also been demonstrated to be capable of covalently binding to DNA, nucleosides, and nucle-

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[1] Abbreviations: MN, micrococcal nuclease; SPD, spleen phosphodiesterase; DHR, dehydroretronecine [7-hydroxy-1-(hydroxymethyl)-6,7-dihydro-5*H*-pyrrolizine]; 3′-dGMP, 2′-deoxyguanosine 3′-monophosphate; 5′-dGMP, 2′-deoxyguanosine 5′-monophosphate; DHR−3′-dGMP adducts (I and II), 3′-monophosphate of 7-(deoxyguanosin-N^2-yl)-dehydrosupinidine; DHR−3′,5′-dG-bisphosphate adducts (I and II), 3′,5′-bisphosphate of 7-(deoxyguanosin-N^2-yl)dehydrosupinidine; 3′-dAMP, 2′-deoxyadenosine 3′-monophosphate; DHR−3′-dAMP adduct, 3′-monophosphate of 7-(deoxyadenosin-N^6-yl)dehydrosupinidine; DHR−3′,5′-dA-bisphosphate adduct, 3′,5′-bisphosphate of 7-(deoxyadenosin-N^6-yl)dehydrosupinidine; PNK, cloned T4 polynucleotide kinase; DTT, dithiothreitol; PB microsomes, liver microsomes of female F344 rats pretreated with phenobarbital; NTP, National Toxicology Program; NCTR, National Center for Toxicological Research.

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otides (*25*−*27*, *35*−*39*). In this paper, we report the development of a ^{32}P-postlabeling/HPLC methodology for detecting and quantifying DHR-derived DNA adducts, including DHR−3′-dGMP and DHR−3′-dAMP adducts, and use of this method in quantifying DHR-derived DNA adducts obtained in vitro and in vivo.

Materials and Methods

Materials. Riddelliine was obtained from the National Toxicology Program (NTP). Phenobarbital (sodium salt), calf thymus DNA (sodium salt, type I), 2′-deoxyguanosine 3′-monophosphate (sodium salt) (3′-dGMP), 2′-deoxyguanosine 5′-monophosphate (free acid) (5′-dGMP), adenosine 5′-triphosphate (disodium salt) (ATP), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate ($NADP^+$), nuclease P1, micrococcal nuclease (MN), spleen phosphodiesterase (SPD), bicine, spermidine, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Monocrotaline, *o*-bromanil, and barium hydroxide octahydrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Cloned T4 polynucleotide kinase (PNK) was obtained from U.S. Biochemical Corp. (Cleveland, OH). Adenosine [γ-^{32}P]-5′-triphosphate ([γ-^{32}P]ATP) (specific activity of >7000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). All other reagents were obtained through commercial sources and were the highest quality available. All solvents were HPLC grade.

Retronecine was prepared via barium hydroxide-catalyzed hydrolysis of monocrotaline using the procedure of Hoskins and Crout (*40*). A solution of monocrotaline (1 g, 3.1 mmol) and barium hydroxide octahydrate (2 g, 6.3 mmol) in water (10 mL) was heated at reflux for 2 h. The solution was cooled, treated with carbon dioxide (dry ice), filtered, acidified with 1 N hydrochloric acid to adjust the pH to 3−4, and then extracted repeatedly with ethyl ether. The aqueous phase was collected and concentrated under reduced pressure. The residue was then passed through a column of AG 1X8 ion-exchange resin (20 g, OH^- form, 200−400 mesh, Bio-Rad Laboratories, Hercules, CA) and eluted with H_2O until the eluate was neutral. The combined eluates were evaporated and extracted three times with hot acetone. After filtration to remove the precipitate, the filtrate was collected and the solvent removed under reduced pressure. The resulting residue was crystallized from acetone to give 392 mg of retronecine in an 81% yield.

According to Mattocks et al. (*41*, *42*), DHR can be prepared by either dehydrogenation of retronecine or dehydration of retronecine *N*-oxide. Dehydrogenation of retronecine was chosen for the DHR preparation. Briefly, to a solution of retronecine (100 mg, 650 μmol) in chloroform (30 mL) in an ice bath was added *o*-bromanil (300 mg, 708 μmol) in $CHCl_3$ (6 mL) dropwise with stirring, over the course of 2 min. The hydroquinone byproduct was removed by extraction with anion exchange AG 1X8 resin (200−400 mesh, OH^- form). The organic phase was separated and concentrated under reduced pressure, providing practically pure DHR, which was recrystallized from ether and light petroleum giving pure DHR as white prisms in a 40% yield.

Animals. Female F344 rats were obtained from the NCTR breeding colony as weanlings. Liver microsomes of female F344 rats treated with phenobarbital (PB microsomes) were prepared according to published procedures (*43*). The rats were injected intraperitoneally with phenobarbital (75 mg/kg of body weight/day, in 0.5 mL of H_2O) for three consecutive days. Twenty-four hours following the final injection, rats were sacrificed by CO_2 inhalation. The livers were perfused with cold 1.15% KCl via the portal vein and immediately stored at −78 °C. The liver microsomes were prepared from thawed tissue by differential centrifugation methods (*44*) and stored at −78 °C prior to use. Protein concentrations were determined using a protein assay based on the Bradford method using a Bio-Rad protein detection kit (Bio-Rad Laboratories).

Synthesis of the 3′-Monophosphate of 7-(Deoxyguanosin-*N*^{2}-yl)dehydrosupinidine Adducts (DHR−3′-dGMP). A solution of 3′-dGMP (20 mg, 60 μmol) in 4 mL of 20 mM K_2CO_3 (pH 8.0) was purged with argon for 5 min. DHR (18 mg, 120 μmol) was added, and the resulting solution was stirred anaerobically at 60 °C for 6 h. The reaction mixture was then filtered through a 0.22 μm Millipore filter. The filtrate was concentrated to half of the volume under reduced pressure, and the adducts were purified by HPLC on a Whatman Partisil ODS-3 (4.6 mm × 250 mm) column eluted with 20 mM K_2CO_3 (pH 8.0) isocratically at a flow rate of 1 mL/min, and monitored at 254 and 220 nm. The DHR−3′-dGMP adducts were further purified on an analytical Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) eluted isocratically with 10% methanol in 20 mM NH_4OAc (pH 7.0) at a flow rate of 1 mL/min. The synthesis was repeated on a larger scale so that a greater quantity of DHR−3′-dGMP adducts was obtained for structural determination and for use as external standards for ^{32}P-postlabeling and HPLC.

Synthesis of DHR−2′-Deoxyguanosine 3′,5′-Bisphosphate Adducts (DHR−dG 3′,5′-Bisphosphate). A solution of 10 nmol of DHR−3′-dGMP in 50 μL of water and a 50 μL reaction mixture containing 0.4 μmol of ATP, 150 units of PNK, 40 mM bicine-NaOH (pH 9.5), 20 mM $MgCl_2$, 2 mM spermidine, and 20 mM dithiothreitol (DTT) was incubated at 37 °C for 40 min. The resulting products were separated by HPLC using a Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) and eluted isocratically with 10% methanol in 20 mM NH_4OAc at a flow rate of 1 mL/min, and monitored at 254 nm with a Waters 996 photodiode array detector.

Synthesis of DHR−2′-Deoxyguanosine 5′-Monophosphate Adducts (DHR−5′-dGMP). Following the procedure of Wickramanayake et al. (*37*), the DHR−5′-dGMP adduct was synthesized by reaction of DHR (18 mg, 120 μmol) with 5′-dGMP (64 mg, 180 μmol) in aqueous K_2CO_3 at pH 7.4 and 60 °C for 6 h. The precipitate was removed by filtration, and the products in the filtrate were separated by semipreparative HPLC. The crude DHR−5′-dGMP adducts were isolated using a Prodigy 5 μm ODS column (Phenomenex, 10 mm × 250 mm) equilibrated with 20 mM NH_4OAc (pH 7.0). After the sample had been applied, the column was eluted with a linear gradient from 20 mM NH_4OAc to 50% methanol in 20 mM NH_4OAc for 40 min with a flow rate of 2 mL/min. The adducts were further purified with an analytical Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) eluted isocratically with 20% methanol in 8 mM NH_4OAc (pH 7.0) at a flow rate of 1 mL/min. The collected fractions were lyophilized and stored at −70 °C until they were used.

Synthesis of the 3′-Monophosphate of 7-(Deoxyadenosin-*N*^{6}-yl)dehydrosupinidine Adducts (DHR−3′-dAMP). Like the DHR−3′-dGMP adduct, the DHR−3′-dAMP adduct was prepared by reaction of 3′-dAMP (43 mg, 130 μmol) in 8 mL of 20 mM K_2CO_3 (pH 8.0) with DHR (40 mg, 267 μmol) at 60 °C for 40 h. The resulting products were separated by HPLC using a Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) eluted at a flow rate of 1.0 mL/min with a linear gradient from 20 mM NH_4OAc to 50% methanol in 20 mM NH_4OAc in 40 min. The collected adducts were further purified by reversed-phase HPLC using the same conditions for the purification of the DHR−3′-dGMP adduct.

Chemical Reaction of DHR with Calf Thymus DNA. Purified calf thymus DNA (2.5 mg, 7.5 μmol) in 2.5 mL of 20 mM K_2CO_3 (pH 7.5) was reacted with 64 nmol of DHR at 37 °C for 40 min. After incubation, the reaction mixture was extracted twice with 2.5 mL of a chloroform/isoamyl alcohol mixture (24/1, v/v). The DNA in the aqueous phase was precipitated by adding 250 μL of 3 M sodium acetate followed by an equal volume of cold 2-propanol and washed with 70% ethanol. After the DNA had been redissolved in 20 mM K_2CO_3 (pH 7.5), the DNA concentration and purity were analyzed spectrophotometrically. The DNA was stored at −78 °C prior to ^{32}P-postlabeling/HPLC analysis.

Development of ^{32}P-Postlabeling/HPLC Methodology for Analysis of DHR−3′-dGMP and DHR−3′-dAMP Adducts. (1) Enzymatic Digestion of DHR-Derived DNA. Initially, conventional ^{32}P-postlabeling enzymatic digestion procedures were employed (*45*−*59*). Briefly, 10 μg of DNA (in 10 μL of distilled water) from the reaction of DHR and calf thymus DNA was enzymatically hydrolyzed to the corresponding 2′-deoxyribonucleoside 3′-monophosphates at 37 °C for 4 h by 1.25 units of MN and 62 milliunits of SPD contained in a 20 μL solution of 20 mM sodium succinate and 10 mM calcium chloride (pH 6). Two enrichment methods, nuclease P1 treatment and *n*-butanol extraction, were employed. For the nuclease P1 method, the MN/SPD-digested DNA solutions were incubated at 37 °C for 40 min with nuclease P1 (8 μg, in 4 μL of buffer containing 0.24 M sodium acetate and 2 mM $ZnCl_2$ at pH 5) to remove the normal 3′-monophosphate of 2′-deoxyribonucleosides. The resulting incubation mixture was then evaporated to dryness under reduced pressure and redissolved in 10 μL of distilled water for ^{32}P-postlabeling.

For the enrichment by *n*-butanol extraction, the incubation mixture (30 μL) was extracted with water-saturated *n*-butanol (2 × 100 μL) in the presence of phase-transfer agent tetrabutylammonium chloride. A buffer solution (4 μL) containing 50 mM bicine (pH 9.0) and 0.1 M DTT (2/1, v/v) was added, and the *n*-butanol was removed under reduced pressure. The samples were redissolved in 25 μL of water for ^{32}P-postlabeling.

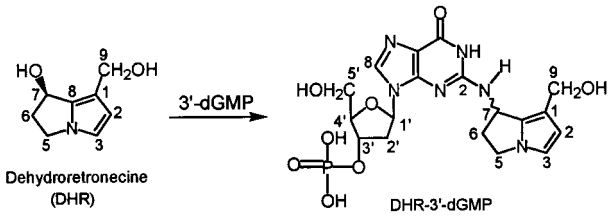
(2) Effect of MN and SPD on DHR-Derived DNA Adduct Detection. To determine the optimal conditions for DNA digestion and adduct enrichment, enzymatic digestion of DNA from reaction of DHR and calf thymus DNA was also conducted with different quantities of digestion enzymes, including using $^1/_4$, $^1/_8$, $^1/_{16}$, and $^1/_{32}$ of the MN and SPD quantity employed above. To determine whether MN and SPD enzymes would affect the yield of DHR-derived DNA adducts, incubation of the synthetically prepared DHR−3′-dGMP and DHR−3′-dAMP adducts (30−60 fmol) with MN and SPD was compared.

(3) Analysis of [^{32}P]DHR−3′,5′-dG-Bisphosphate and [^{32}P]DHR−3′,5′-dA-Bisphosphate Adducts by HPLC. Each of the MN/SPD-digested DNA samples was dissolved in 10 μL of distilled water and ^{32}P-phosphorylated by incubating with 10 μL of PNK mix containing 100 μCi of [γ-^{32}P]ATP (specific activity of >7000 Ci/mmol), 12 units of PNK, and 2 μL of 10× PNK buffer [200 mM bicine-NaOH (pH 9.6), 100 mM DTT, 100 mM $MgCl_2$, and 10 mM spermidine] at 37 °C for 40 min. The labeled mixture was injected onto a Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) and eluted isocratically with 20 mM NaH_2PO_4 (pH 4.5) for 10 min, followed by a linear gradient of 20 mM NaH_2PO_4 (pH 4.5) to 15% methanol in 20 mM NaH_2PO_4 for 60 min. The HPLC flow rate was 1.0 mL/min, and the scintillation fluid flow rate was 3.0 mL/min. To avoid interference by the high level of radioactivity of the free ^{32}P and the unreacted [γ-^{32}P]ATP, the on-line FLO-ONE radioactivity detector (Radiomatic Instruments, Tampa, FL) was equipped with a diverter, and the eluent from the first 40 min was diverted away from the radioactivity detector.

(4) ^{32}P-Postlabeling/HPLC Analysis of the DHR−3′-dGMP Adduct Using Smaller Amounts of DNA. Besides using 10 μg of DNA, ^{32}P-postlabeling/HPLC analysis of the DHR−3′-dGMP adduct was also conducted with 5, 3, 1, and 0.5 μg of DNA under conditions similar to those described above.

(5) ^{32}P-Postlabeling/TLC Analysis of the DHR−3′-dGMP Adduct. An aliquot containing 10 μg of DNA from the reaction of DHR and calf thymus DNA was digested by MN and SPD, enriched by nuclease P1, and ^{32}P-postlabeled as described above. As a control experiment, 8.3 fmol of the synthetic DHR−3′-dGMP adduct was also ^{32}P-postlabeled in parallel under the same conditions. To analyze the [^{32}P]DHR−3′,5′-dG-bisphosphate adducts, the labeled mixture was applied onto 10 cm × 10 cm PEI-cellulose plates (Macherey-Nagel) and a two-dimensional development was carried out as previously described (*45*, *46*). Autoradiography was performed on Dupont Cronex films, and the radioactivity on the TLC spots was quantitated by Cerenkov counting.

Scheme 1. Synthetic Preparation of DHR−3′-dGMP Adducts



(6) Analysis of [^{32}P]DHR−5′-dGMP Adducts by HPLC. The labeled mixture containing [^{32}P]DHR−3′,5′-dG-bisphosphate adducts obtained above was adjusted to pH 5.0 with 4 μL of 0.4 M acetic acid and then 3′-dephosphorylated with 17.5 μg of nuclease P1 (5 μg/μL in 0.42 M sodium acetate and 2 mM $ZnCl_2$) at 37 °C for 5 h. The dephosphorylated mixture was then subjected to HPLC analysis under the conditions described in part 3.

Instrumentation. A Waters HPLC system consisting of a model 600 controller, a model 996 photodiode array detector, and a pump was used for the separation and purification of DHR-derived DNA adducts. Electrospray (ES) mass spectrometry was performed using a Platfoum II single-quadrupole instrument (Micromass, Inc., Altrincham, U.K.). ES tandem mass spectrometry was performed using a Quattro liquid chromatograph (Micromass). Separate MS functions were used to acquire full scan data at a low and high cone voltages in a single chromatographic run (e.g., 20 and 40 V, respectively, for *m*/*z* 100−600). ES tandem mass spectrometry was performed using the negative and/or positive ion mode with a source temperature of 80 °C for infusion with a syringe pump. Product ion scans were obtained from CID of selected ions using a cone voltage between 37 and 40 V and collision energies between 24 and 31 eV. The collision gas was Ar at pressures between 2 and 4 × 10^{-3} mbar. LC/MS samples (5 μL injection volume) were introduced into the ES probe following separation with a Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) and eluting with the conditions previously described, split to approximately 0.2 mL/min entering the probe. The ^{1}H nuclear magnetic resonance (NMR) experiments were carried out on a Bruker AM 500 MHz spectrometer (Bruker Instruments, Billerica, MA) at 301 K. Samples were dissolved in 0.6 mL of deuterated water (D_2O). The D_2O peak was assigned a resonance of 4.7 ppm. Typical NMR spectral acquisition parameters were as follows: data size, 32K; flip angle, 90°; sweep width, 6000 Hz; and relaxation delay, 1 s. NOE difference and homonuclear coupling NMR experiments were conducted for assisting in proton resonance assignment. Circular dichroism (CD) spectra of DHR−3′-dGMP adducts were determined with a quartz cell with a path length of 1 cm at ambient temperature on a Jasco 500A spectropolarimeter. CD spectra are expressed by ellipticity (in millidegrees) for 20 mM NH_4OAc solutions that read 1.0 absorbance unit in a UV/visible spectrophotometer at the wavelength of maximum absorption in a quartz cell with a path length of 1 cm.

Results

Synthesis of Retronecine and Dehydroretronecine (DHR). Retronecine was prepared from barium hydroxide-catalyzed hydrolysis of monocrotaline by the procedure of Hoskins and Crout (*40*). DHR (Scheme 1) was synthesized by dehydrogenation of retronecine with *o*-bromanil in $CHCl_3$ (*41*). Its structure was confirmed by ^{1}H NMR spectral analysis: ^{1}H NMR (DMSO-d_6): δ 6.53 (d, $J_{2,3}$ = 2.5 Hz, 1, H_3), 6.01 (d, $J_{2,3}$ = 2.5 Hz, 1, H_2), 4.99 (m, 1, H_7), 4.90 (d, 1, OH_7), 4.36 (m, 1, H_9), 4.32 (m, 1, H_9), 3.97 (m, 1, H_5), 3.77 (m, 1, H_5), 2.61 (m, 1,

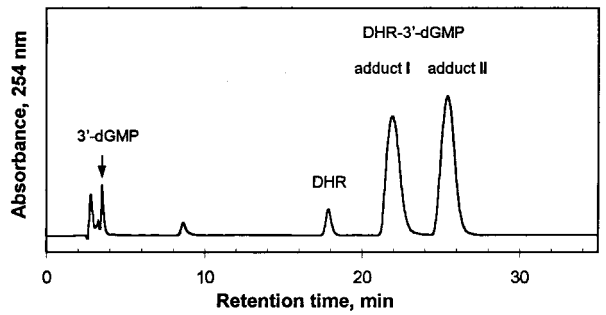


Figure 1. Reversed-phase HPLC purification of the synthetically prepared DHR−3′-dGMP adducts I and II, employing a Prodigy 5 μm ODS column (4.6 mm × 250 mm) eluted isocratically with 10% methanol in 20 mM NH_4OAc (pH 7.0) at a flow rate of 1 mL/min.

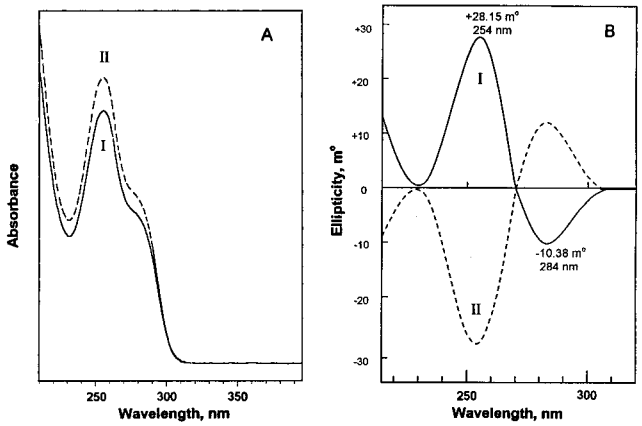


Figure 2. (A) Ultraviolet/visible absorption and (B) CD spectra of the synthetically prepared epimeric DHR−3′-dGMP adduct I and DHR−3′-dGMP adduct II obtained from the HPLC purification described in the legend of Figure 1. These adducts eluted at 21.9 and 25.5 min, respectively.

H_6), 2.17 (m, 1, H_6). The NMR chemical shift assignment of DHR was determined by nuclear Overhauser enhancement (NOE) difference experiments, homonuclear coupling experiments, peak splitting, and peak integration.

Synthesis of DHR−3′-dGMP Adducts. DHR−3′-dGMP adducts were synthesized by the reaction of DHR with 3′-dGMP (Scheme 1). The resulting reaction products were purified by HPLC, first onto a Whatman ODS-3 column to remove most of the excess 3′-dGMP (data not shown) and further with a Prodigy 5 μm ODS column (Phenomenex) (Figure 1). The materials contained in the chromatographic peaks that eluted at 21.9 and 25.5 min exhibited a baseline separation and exhibited identical UV/visible absorption spectra (Figure 2A) which were identical to that of the DHR−5′-dGMP adduct reported by Wickramanayake et al. (*37*). These products were also characterized from the ES-MS negative product ion spectra, which were identical (Figure 3). The deprotonated molecule, $(M - H)^-$, was at *m*/*z* 481 and product ions corresponding to the deprotonated base (*m*/*z* 285), deoxyribose − monophosphate (*m*/*z* 195), deoxyribose − monophosphate − H_2O (*m*/*z* 177), and ions derived from phosphate ion (*m*/*z* 97 and 79). The positive ion product spectrum (not shown) contained the protonated molecule, $(M + H)^+$, at *m*/*z* 483, loss of H_2O (*m*/*z* 465), the protonated DHR − guanine (*m*/*z* 269), dGMP (*m*/*z* 348), guanine (*m*/*z* 152), and DHR − H_2O (*m*/*z* 136). These data suggested that the materials contained in the chromato-

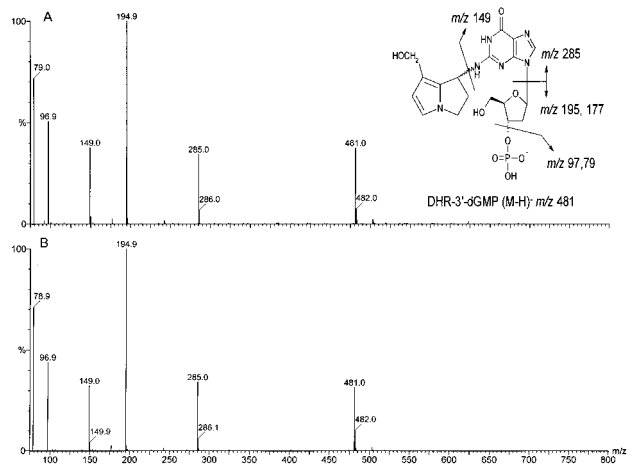


Figure 3. Negative ion electrospray mass spectra of the synthetically prepared (A) DHR−3′-dGMP adduct I and (B) DHR−3′-dGMP adduct II.

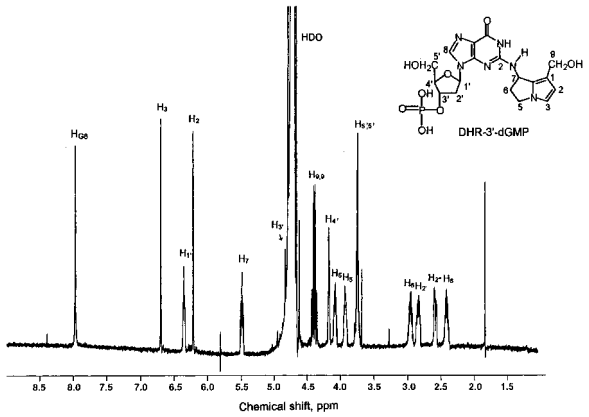


Figure 4. Proton NMR spectrum (500 MHz) of the synthetically prepared DNA adduct standards identified as the mixture of DHR−3′-dGMP adducts I and adduct II.

graphic peaks at 21.9 and 25.5 min shown in Figure 1 were racemic DHR−3′-dGMP adducts.

The CD spectra of both adducts were determined (Figure 2B). The CD Cotton effects of the first adduct were a mirror image of those of the second adduct, indicating that these two adducts were a pair of epimers. These CD spectra also had Cotton effects similar to those of 7-(deoxyguanosin-N^2-yl)dehydrosupinidine previously reported by Wickramanayake et al. (*37*). The ^1H NMR spectrum of the adduct mixture was determined by NMR decoupling and NOE techniques as well as by comparison of the NMR data of the DHR and 3′-dGMP measured under identical conditions (Figure 4 and Table 1).

Consequently, on the basis of mass, CD, and NMR spectral analysis, the structures of these two products were characterized as a pair of epimeric DHR−3′-dGMP adducts.

On the basis of the analysis by proton NMR using an internal standard (1,4-dioxane), the quantity of these adducts obtained from the organic reaction followed by repeated purification was 0.91 mg. Using the UV/visible absorbance measurement in water, the molar extinction coefficient of DHR−3′-dGMP adducts at 254 nm was determined to be 4.7×10^4 M^{-1} cm^{-1}.

Reactions of DHR and 3′-dGMP were carried out three times, with the molar ratio of DHR to 3′-dGMP being 1/1, 2/1, and 3/1. The reaction with a ratio of 2/1 provided the highest yield (2.5%).

Table 1. ^{1}H NMR Spectral Data (500 MHz) of DHR, 3′-dGMP, thr DHR−3′-dGMP Adduct, 5′-dGMP, and the DHR−5′-dGMP Adduct

	chemical shift (ppm)				
	DHR	3′-dGMP	DHR−3′-dGMP	5′-dGMP	DHR−5′-dGMP
H2	6.01		6.29		6.31
H3	6.53		6.76		6.73
H5	3.97		4.13		4.17
H5	3.77		3.99		4.15
H6	2.61		2.96		3.02
H6	2.17		2.51		2.59
H7	4.99		5.56		5.57
H9	4.36		4.41		4.71
H9	4.32		4.36		4.24
OH7	4.90				
H8		7.97	8.01	8.77	8.14
H1′		6.27	6.41	6.35	6.46
H2′		2.62	2.88	2.72	2.87
H2′		2.74	2.59	2.60	2.59
H3′		4.82	4.75	4.63	4.77
H4′		4.21	4.25	4.25	4.24
H5′		3.76	3.83	4.04	4.49
H5′		3.77	3.83	4.09	4.49
	coupling constant (Hz)				
	DHR	3′-dGMP	DHR−3′-dGMP	5′-dGMP	DHR−5′-dGMP
$J_{1',2'}$		7.0		7.0	
$J_{2,3}$	2.5		2.5		2.5
$J_{9,9}$			12.1		

Synthesis of DHR−5′-dGMP Adducts. Like the synthesis and HPLC purification of the DHR−3′-dGMP adduct, the DHR−5′-dGMP adduct was prepared by reaction of DHR with 5′-dGMP followed by two HPLC separations. The HPLC profile of the second HPLC purification provided a baseline separation of the DHR−5′-dGMP adducts (Figure 5). Negative ion ES mass spectral analysis showed that both these adducts had product ion spectra that were identical to those of the DHR−3′-dGMP adducts (Figure 6). The positive ion product spectrum (not shown) contained the protonated molecule, $(M + H)^+$, at *m/z* 483, loss of H_2O (*m/z* 465), the protonated DHR − guanine (*m/z* 269), dGMP (*m/z* 348), guanine (*m/z* 152), and DHR − H_2O (*m/z* 136).

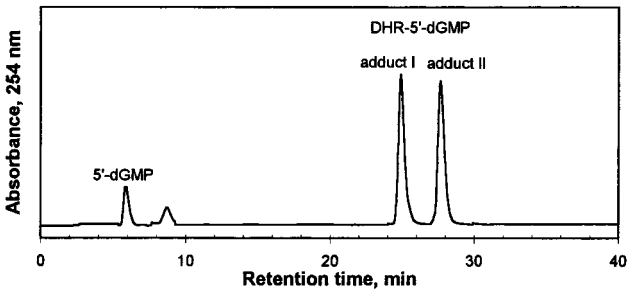


Figure 5. HPLC purification of the DHR−5′-dGMP adduct with a Prodigy 5 μm ODS column (4.6 mm × 250 mm) eluted isocratically with 8 mM NH_4OAc (pH 7.0) at a flow rate of 1 mL/min.

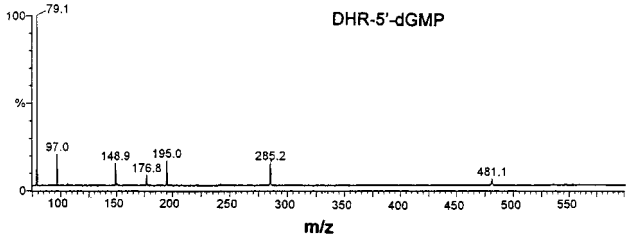


Figure 6. Negative ion electrospray mass spectrum of the DHR−5′-dGMP adduct.

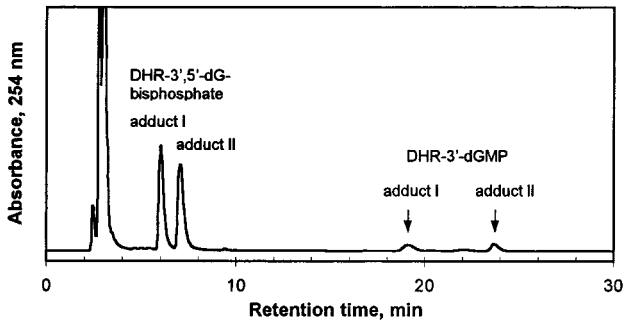


Figure 7. HPLC separation of DHR−3′,5′-dG-bisphosphate adducts formed from the reaction of the DHR−3′-dGMP adduct with cold ATP catalyzed by PNK following the HPLC conditions described in the legend of Figure 1.

The ^{1}H NMR spectrum was assigned by NOE difference experiments, homonuclear coupling experiments, peak splitting, and peak integration. H1′ (6.35 ppm) to H5″ (4.04 ppm) and H8 (8.77 ppm) were assigned by NOE techniques. The chemical shift and coupling constant assignments are shown in Table 1.

There was a very strong similarity between the chemical shifts of 3′-dGMP and the DHR−3′-dGMP adduct, and between 5′-dGMP and the DHR−5′-dGMP adduct. The biggest difference between the 3′-dGMP and 5′-dGMP chemical shifts was seen along the backbone H3′, H5′, and H5″ NMR chemical shifts. There was a very strong similarity between the DHR NMR chemical shifts of the DHR−3′-dGMP adduct and the DHR−5′-dGMP adduct.

These adducts had UV/visible absorption spectra identical to those of the DHR−5′-dG adduct reported by Wickramanayake et al. (*37*). Thus, on the basis of UV/visible absorption and mass and NMR spectral analysis, the compounds contained in chromatographic peaks that eluted at 25.1 and 27.7 min in Figure 5 were identified as DHR−5′-dGMP adducts. The reaction yield was 0.9%.

Synthesis of the DHR−3′,5′-dG-Bisphosphate Adduct. The DHR−3′,5′-dG-bisphosphate adduct was synthesized by 5′-phosphorylation of the synthetically prepared epimeric DHR−3′-dGMP adducts with cold (nonradioactive) ATP catalyzed by PNK. The resulting reaction mixture was purified by reversed-phase HPLC (Figure 7). The chromatographic peaks that eluted at 19.2 and 23.8 min contained the two recovered substrates, epimeric DHR−3′-dGMP adduct I and adduct II, respectively. The materials contained in chromatographic peaks that eluted at 6.2 and 7.3 min, respectively, had UV/visible absorption spectra similar to that of the DHR−3′-dGMP adduct (Figure 2A). These materials were characterized by analysis of their identical negative product ion ES mass spectra (Figure 8). The deprotonated molecule, $(M - H)^-$, was at *m/z* 561 and product ions corresponding to loss of H_2O (*m/z* 543), loss of one H_3PO_4 (*m/z* 463), loss of the DHR moiety (*m/z* 426), loss of two H_3PO_4 (*m/z* 365), the ribose 3′,5′-bisphosphate ion (*m/z* 275), loss of H_2O from the ribose 3′,5′-bisphosphate ion (*m/z* 257), ribose − the monophosphate ion (*m/z* 195), $H_3P_2O_7^-$ (*m/z* 177), $HP_2O_6^-$ (*m/z* 159), $H_2PO_4^-$ (*m/z* 97), and PO_3^- (*m/z* 79). On the basis of UV/visible absorption and mass spectral analysis, the structures of these two reaction products were identified as DHR−3′,5′-dG-bisphosphate adduct I and DHR−3′,5′-dG-bisphosphate adduct II, respectively.

Synthesis of DHR−3′-dAMP Adducts. Like the DHR−3′-dGMP adducts, the DHR−3′-dAMP adducts

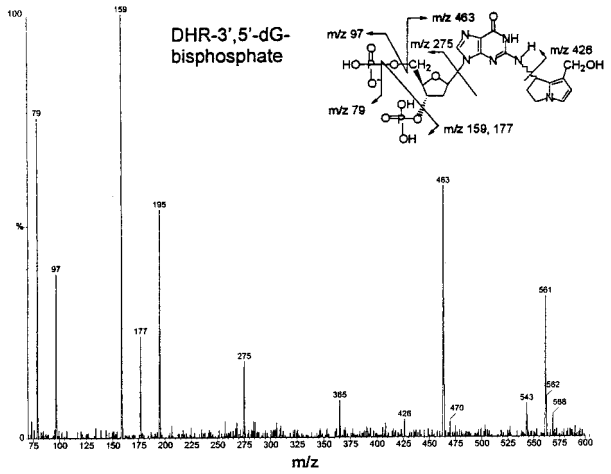


Figure 8. Negative ion electrospray proton ion mass spectrum of the DHR−3′,5′-dG-bisphosphate adduct.

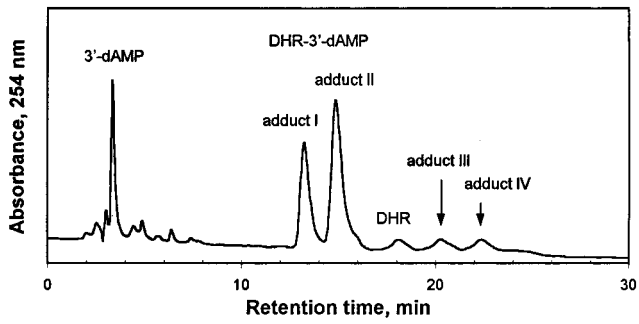


Figure 9. HPLC separation of DHR−3′-dAMP adducts with a Prodigy 5 μm ODS column (Phenomenex, 4.6 mm × 250 mm) eluted isocratically with 10% methanol in 20 mM NH_4OAc (pH 7.0) at a flow rate of 1 mL/min.

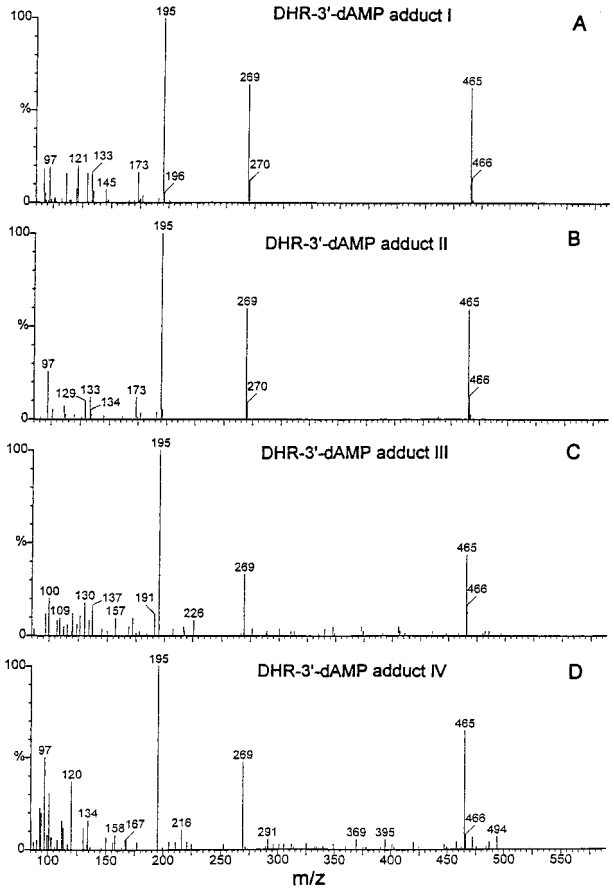


Figure 10. Negative ion electrospray mass spectra of four isomeric DHR−3′-dAMP adducts prepared from the reaction of DHR and 3′-dAMP followed by HPLC purification as described in the legend of Figure 1.

were prepared by reaction of DHR with 3′-dAMP followed HPLC separations. The HPLC profile of HPLC purification is shown in Figure 9. The chromatographic peak that eluted at 18.2 min contained the recovered substrate, DHR. The materials contained in the chromatographic peaks that eluted at 13.2, 15, 20.5, and 23.5 min were analyzed using LC/ES-MS. As shown in Figure 10A−D, these four products had identical mass spectra with the $(M - H)^-$ ion (*m*/*z* 465), M − deoxyribose − PO_4^- (*m*/*z* 269), deoxyribose − PO_4^- (*m*/*z* 195), and $H_2PO_4^-$ (*m*/*z* 97). Thus, on the basis of mass spectral analysis, they were all DHR−3′-dAMP adducts.

Development of a ^{32}P-Postlabeling/HPLC Method for Analysis of DHR−3′-dGMP Adducts. ^{32}P-postlabeling methodology was selected for detecting and quantifying DHR-modified DNA adducts in vitro and in vivo. The synthesized epimeric DHR−3′-dGMP adducts with a specific quantity ranging from 1 to 60 fmol were first employed to develop optimal conditions for the entire process, including DNA enzyme digestion, adduct enrichment, ^{32}P-postlabeling, and HPLC separation. To confirm the ^{32}P-postlabeling products, [^{32}P]DHR−3′,5′-dG-bisphosphate, the cold synthetically prepared DHR−3′,5′-dG-bisphosphate adducts I and II were cochromatographed by HPLC with the ^{32}P-postlabeling reaction product mixture.

(1) Effect of MN/SPD on ^{32}P-Postlabeling of DHR−3′-dGMP and DHR−3′-dAMP Adducts. Initially, 10 μg of DNA from the reaction of DHR and calf thymus DNA was digested with 1.25 units of MN and 62 milliunits of SPD, quantities commonly reported for enzyme digestion (*45-48*). No DHR−3′,5′-dG-bisphosphate adducts were detected by HPLC (Figure 11A). With a concern that the digestion enzymes may interact with DHR−3′-dGMP and/or [^{32}P]DHR−3′,5′-dG-bisphosphate adducts, optimal quantities of MN and SPD and an optimal digestion time were then pursued. Digestion of DNA was repeated by using (i) 312 milliunits of MN and 16 milliunits of SPD, (ii) 156 milliunits of MN and 8 milliunits of SDP, (iii) 78 milliunits of MN and 4 milliunits of SDP, and (iv) 39 milliunits of MN and 2 milliunits of SPD (panels B−E of Figure 11, respectively). As shown in panels C and D of Figure 11, there are eight chromatographic peaks that eluted at 47.6, 48.3, 51.4, 53.9, 55.3, 60.1, 61.0, and 62.6 min are designated as P1−P8, respectively. These chromatographic peaks were not detected from ^{32}P-postlabeling/HPLC analysis of the untreated calf thymus DNA (Figure 11F) or from incubation of riddelliine with calf thymus DNA in the absence of rat liver microsomes (data not shown). Thus, the eight chromatographic peaks, P1−P8, are all DHR-derived DNA adducts. As compared with the HPLC profile from ^{32}P-postlabeling/HPLC analysis of the synthetically prepared DHR−3′-dGMP adducts (Figure 12A), the DNA adducts designated as P4 and P6 are [^{32}P]DHR−3′,5′-dG-bisphosphate adducts derived from DHR−3′-dGMP adduct I and adduct II, respectively (each indicated with an arrow in Figure 11A−E). Thus, the results shown in Figure 11 clearly indicate that the use of 78 milliunits of MN and 4 milliunits of SPD provided the highest yield of DHR−3′,5′-dG-bisphosphate adducts (Figure 11D).

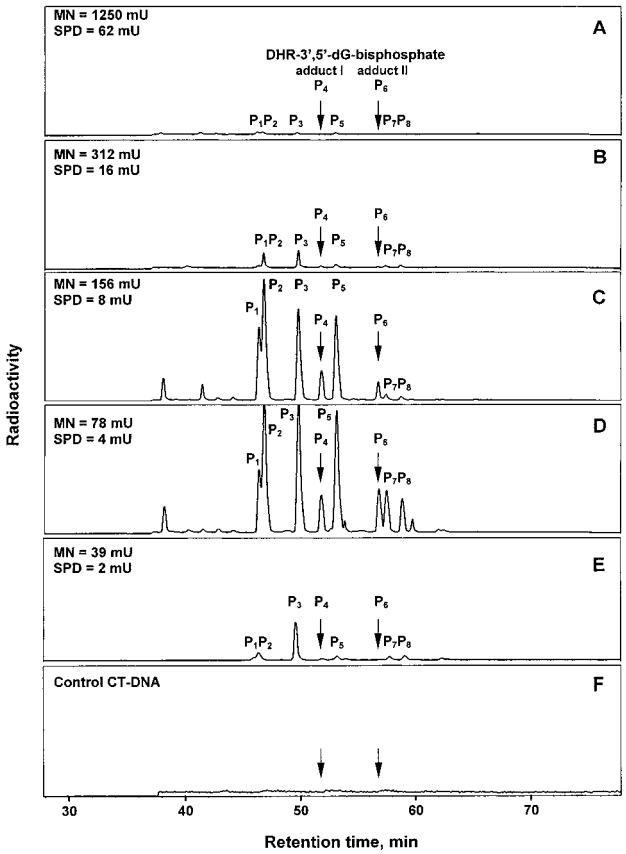


Figure 11. Effect of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD) concentration on ^{32}P-postlabeling/HPLC analysis of DHR-modified DNA adducts contained in DNA from the reaction of DHR and calf thymus DNA. Ten micrograms of DNA was digested by MN and SPD and postlabeled with ^{32}P by enzymatic phosphorylation in the presence of [γ-^{32}P]ATP and polynucleotide kinase. Various enzyme concentrations were used for DNA digestion. The ^{32}P-postlabeled adduct formation from the enzymatic digestion with (A) 1250 milliunits of MN and 62 milliunits of SPD, (B) 312 milliunits of MN and 16 milliunits of SPD, (C) 156 milliunits of MN and 8 milliunits of SPD, (D) 78 milliunits of MN and 4 milliunits of SPD, and (E) 39 milliunits of MN and 2 milliunits of SPD and (F) ^{32}P-postlabeling/HPLC analysis of untreated calf thymus DNA (10 μg) using 78 milliunits of MN and 4 milliunits of SPD.

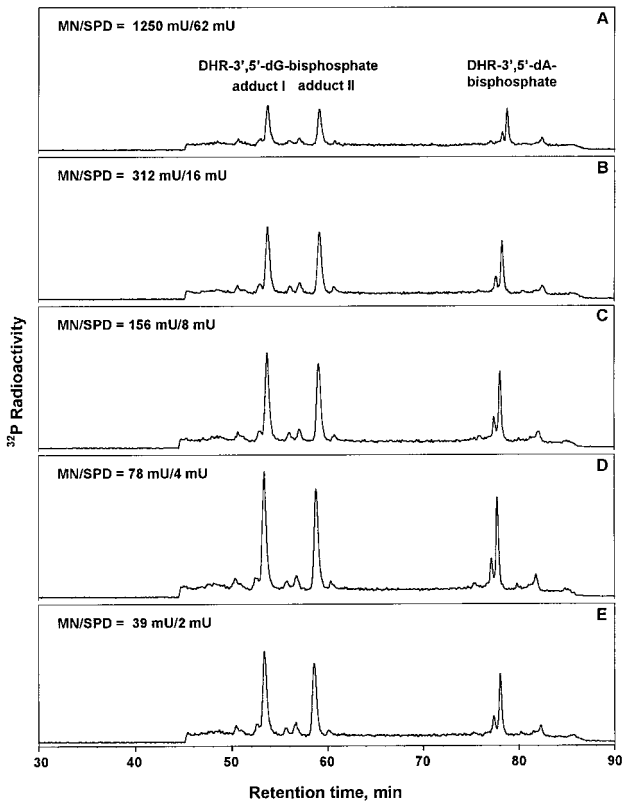


Figure 12. Effect of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD) concentration on ^{32}P-postlabeling/HPLC analysis of a mixture of 8.3 fmol of DHR−3′-dGMP and 20 fmol of DHR−3′-dAMP adducts. The DNA samples were digested with (A) 1250 milliunits of MN and 62 milliunits of SPD, (B) 312 milliunits of MN and 16 milliunits of SPD, (C) 156 milliunits of MN and 8 milliunits of SPD, (D) 78 milliunits of MN and 4 milliunits of SPD, and (E) 39 milliunits of MN and 2 milliunits of SPD.

The conditions for analysis of DHR−3′-dAMP adducts by ^{32}P-postlabeling were then pursued using different amounts of MN and SPD and different incubation times. As shown in panels A−E of Figure 12, like DHR−3′-dGMP adducts, the optimal conditions for analysis of DHR−3′-dAMP adducts were also the use of 78 milliunits of MN and 4 milliunits of SPD. These were also the optimal conditions for ^{32}P-postlabeling a mixture of DHR−3′-dGMP and DHR−3′-dAMP adducts (Figure 12A−E).

(2) Enrichment of DHR−3′-dGMP Adducts by Nuclease P1 and *n*-Butanol Extraction. DHR-derived DNA was obtained from the reaction of DHR with calf thymus DNA; 10 μg was enzymatically digested under optimal conditions (same conditions described in Figure 11D), and the resulting DHR−3′-monophosphate deoxyribonucleoside adducts were enriched with various amounts of nuclease P1 and with an incubation time ranging from 20 to 40 min. It was found that use of 8 μg of nuclease P1 and incubation for 20 min at 37 °C provided the best enrichment.

Enrichment by *n*-butanol was similarly studied. Following the conventional procedure (*46*), the DHR−3′-monophosphate deoxyribonucleoside adducts formed from enzymatic digestion of DNA were fortified with the phase-transfer agent tetrabutylammonium chloride and then extracted with *n*-butanol four times. The adducts collected from the *n*-butanol fraction were ^{32}P-postlabeled followed by HPLC analysis (data not shown). On the basis of three trials, the yields of [^{32}P]DHR−3′,5′-deoxyribonucleoside bisphosphate adducts were erratic and much lower than those determined by nuclease P1 enrichment. Therefore, this approach was found to be less satisfactory than nuclease P1 enrichment.

(3) HPLC Separation of the [^{32}P]DHR−3′,5′-Deoxyribonucleoside Bisphosphate Adducts. After the (i) synthetically prepared DHR−3′-dGMP adducts, (ii) combined synthetically prepared DHR−3′-dGMP and DHR−3′-dAMP adducts, and (iii) DNA from the reaction of DHR and calf thymus DNA were ^{32}P-postlabeled under the optimal conditions described above, the resulting [α-^{32}P]DHR−3′,5′-deoxyribonucleoside bisphosphate adducts were separated by HPLC. After a number of trials using different HPLC columns, solvent systems, and elution profiles (data not shown), an optimal separation condition for separation of the DHR−3′,5′-bisphosphate adducts was developed. The developed HPLC profile for separation of the two epimeric DHR−3′,5′-dG-bisphosphate adducts is shown in Figure 12A. The optimal HPLC conditions for separation of the mixture of DHR−3′,5′-dG-bisphosphate and DHR−3′,5′-dA-bisphosphate adducts are like those shown in Figure 12D. Similarly, the

optimal HPLC conditions for separation of the DNA adducts from the reaction of DHR and calf thymus DNA are the same as shown in Figure 11D.

(4) Evaluation of Intra- and Interexperimental Reproducibility of ^{32}P-Postlabeling/HPLC Results. On the basis of our experience in employing or developing a ^{32}P-postlabeling/HPLC methodology for detection of carcinogen-modified DNA adducts (*49*−*60*), we first attempted to establish reliable conditions for validating interexperimental reproducibility. However, the DNA enzyme digestion products (e.g., DHR−3′-dGMP) and/or ^{32}P-postlabeling products (e.g., DHR−3′,5′-dG-bisphosphate) are highly unstable to the DNA digestion enzymes, incubation media, and experimental conditions. As shown in Figure 11, the yield of ^{32}P-postlabeling is dependent on the amount of MN and SPD. It was also found that the storage of DNA, the use of [γ-^{32}P]ATP from different batches or the same batch but on a different day, and re-preparation of the buffer media can all result in poor interexperimental reproducibility (data not shown). Consequently, we decided to develop a ^{32}P-postlabeling/HPLC methodology that allowed us to detect and quantify DNA adducts from a large number of samples (up to 30 samples) on the same day.

Thus, intraexperimental reproducibility was determined using the synthetic DHR−3′-dGMP adduct standards and DNA from the reaction of DHR with calf thymus DNA for ^{32}P-postlabeling/HPLC analysis. DNA samples in triplicate were enzymatically digested, adduct enriched (by nuclease P1), and ^{32}P-postlabeled at the same time (on the same day). The levels of modification of the DNA from the reaction of DHR with calf thymus DNA were 615.6, 577.4, and 578.0 adducts/10^8 nucleotides (SD = 590.4 ± 21.6, 4% of relative standard deviation). Among these adducts, the levels of the two enantioneric DHR−3′,5′-dG-bisphosphate adducts I (P4) and II (P6) were 33.7 ± 2.1 and 23.9 ± 1.2 adducts/10^8 nucleotides, respectively.

(5) Analysis of [^{32}P]DHR−5′-dGMP Adducts by HPLC. Attempts were made to enzymatically 3′-dephosphorylate [^{32}P]DHR−3′,5′-dG-bisphosphate adducts to the corresponding [^{32}P]DHR−5′-dGMP adducts followed by HPLC analysis. On the basis of comparison of the HPLC retention time of the synthetic DHR−5′-dGMP standard, no DHR−5′-dGMP adduct was detected (data not shown).

(6) ^{32}P-Postlabeling/TLC Analysis of DHR−3′-dGMP Adducts. After ^{32}P-postlabeling of the DHR−3′-dGMP adducts as described above, the resulting [^{32}P]-DHR−3′,5′-dG-bisphosphate adducts were separated using TLC on PEI-cellulose plates (*45*, *46*). Comparison of the autoradiography results with those from controls indicated no adducts were detected (data not shown).

Discussion

Pyrrolizidine alkaloids are a class of genotoxic naturally occurring phytochemicals, a number of which have been found to induce tumors in experimental animals (*2*, *3*, *6*, *10*, *61*−*75*). Pyrrolizidine alkaloids are biologically inert and require metabolism to exert their tumorigenicity. However, the mechanisms that lead to tumorigenicity are not clear. No pyrrolizidine alkaloid-derived DNA adducts have been identified in vivo or in vitro, and no established methodologies are available for their detection. In this paper, we report the development of a ^{32}P-postlabeling/HPLC method for detection of DHR-derived DNA adducts and utilization of this method for detection and quantification of these adducts from in vitro and in vivo samples.

^{32}P-postlabeling methodology is a highly sensitive method for detecting and quantifying carcinogen-modified DNA adducts (*45*−*59*). Because ^{32}P-postlabeling is conducted on a small scale, the products, [^{32}P]-3′,5′-bisphosphate of carcinogen-modified 2′-deoxyribonucleosides, are always formed in an extremely small quantity, and thus, they are usually not characterized. In our study, however, the postlabeling products, DHR−3′,5′-dG-bisphosphate adducts, were characterized by mass spectrometry and confirmed by co-hromatography with synthetic DHR−3′,5′-dG-bisphosphate standards.

Although determination of recovery between different trials performed at different times can be normalized on the basis of a concentration−response relationship (curve), possible variation in enzyme activity and experimental conditions can result in quantitative deviations between trials. Thus, without ^{32}P-postlabeling of a cold authentic standard of known quantity in parallel with the tested samples, quantitative comparison of ^{32}P-postlabeling products obtained from different trials is not reliable. For our study, in each experiment, external standards, a pair of epimeric DHR−3′-dGMP synthetic adducts at a known level that closely matched the range of the modification level of the biological DNA samples, were analyzed in parallel with the biological samples.

The necine base of DHR and other dehydropyrrolizidines contain a pyrrole moiety that facilitates polymerization under acidic conditions (*6*). Similarly, the DHR-derived DNA adducts, including DHR−3′-dGMP, DHR−3′-dAMP, DHR−5′-dGMP, and DHR−3′,5′-dG-bisphosphate, are all unstable. We have found that 25% of the DHR−3′-dGMP adducts decomposed on storage at room temperature for 48 h, and that more than 50% of these adducts decomposed on storage in buffer at pH <7.0 and 37 °C for 48 h. This instability seriously handicapped the development of ^{32}P-postlabeling conditions. Because of adduct decomposition in the presence of MN and SPD, it was necessary to use quantities of MN and SPD for digestion of DNA that were much smaller than those conventionally employed for ^{32}P-postlabeling of other types of carcinogenic chemicals. The optimal conditions that were developed included the use of shorter incubation times in both DNA enzyme digestion (reduced from the commonly used 4 h to 20 min) and nuclease P1 enrichment (reduced from 40 to 20 min).

Presumably because of the high lability of the products under the conditions used for enzymatic digestion, satisfactory absolute interexperimental reproducbility could not be obtained. However, optimal conditions were developed that allowed us to detect and quantify DHR-modified DNA adducts with a good intraexperimental reproducibility. With this approach, a total of 30 DNA samples could be analyzed at the same time. As such, the quantity of the ^{32}P-postlabeling products obtained from different trials could be reliably compared.

In addition to the two DHR−3′-dGMP adducts, another six chromatographic peaks are detected in calf thymus DNA from the reaction with DHR. These six chromatographic peaks were not observed in control samples, but were also formed in liver DNA of rats treated with riddelliine. Due to the lack of synthetic standards and insufficient quantities for structural determination, their identities were not determined. As shown in Figure 11,

the total quantity of these six adducts was much higher than those of the two DHR−3′-dGMP adducts, with the later accounting for less than 10% of the total DNA adducts.

DHR has been found as a metabolite commonly formed from metabolism of a number of pyrrolizidine alkaloids in vitro and in vivo, including retrorsine (*13*, *28*), monocrotaline (*13*, *28*−*31*), senecionine (*13*, *32*, *33*), and indicine (*13*). As described in the following paper, these eight DHR-derived DNA adducts have been observed in livers of rats fed riddelliine. Thus, these DNA adducts may also be formed from rats, and probably other mammals, fed other retronecine-derived tumorigenic pyrrolizidine alkaloids, such as monocrotaline, retrorsine, senecionine, and indicine. As such, these eight DHR-derived adducts may be able to serve as biomarkers of retronecine-derived pyrrolizidine alkaloid-associated carcinogenesis. Consequently, our development of sensitive and reliable ^{32}P-postlabeling/HPLC methodology for detecting and quantifying DHR-derived DNA adducts will facilitate the risk assessment of human exposure to pyrrolizidine alkaloids.

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