

Detection of the acrolein-derived cyclic DNA adduct by a quantitative ^{32}P -postlabeling/solid-phase extraction/HPLC method: Blocking its artifact formation with glutathione

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Abstract

Acrolein (Acr), a hazardous air pollutant, reacts readily with deoxyguanosine (dG) in DNA to produce cyclic 1, N^2 -propanodeoxyguanosine adducts (Acr-dG). Studies demonstrate that these adducts are detected *in vivo* and may play a role in mutagenesis and carcinogenesis. In the study described here, a quantitative ^{32}P -postlabeling/solid-phase extraction/HPLC method was developed by optimizing the solid-phase extraction and the ^{32}P -postlabeling conditions for analysis of Acr-dG in DNA samples with a detection limit of 0.1 fmol. It was found that Acr-dG can form as an artifact during the assay. Evidence obtained from mass spectrometry indicates that the Acr in water used in the assay is a likely source of artifact formation of Acr-dG. The formation of Acr-dG as an artifact can be effectively blocked by adding glutathione (GSH) to the DNA sample to be analyzed. In addition, Acr-dG was detected as a contaminant in the commercial dG and dT 3'-monophosphate samples. Finally, this method was used to detect Acr-dG in calf thymus and human colon HT29 cell DNA with an excellent linear quantitative relationship.

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Keywords: Acrolein; Deoxyguanosine; Cyclic DNA adduct; Glutathione; ^{32}P -postlabeling; High-performance liquid chromatography; Solid-phase extraction; Mass spectrometry

Acrolein (Acr)¹ is a ubiquitous pollutant in urban air [1,2]. Incomplete combustion of gasoline and diesel fuels is known to emit aldehydes [3,4]. Acr is present in automobile exhaust at concentrations as high as 30 $\mu\text{g}/\text{L}$ [5]. Combustion of alternative fuels containing alcohol or ether produces even more aldehydes than combustion of conventional fuel [6]. Cigarette smoke is another important source of Acr, with concentrations up to 300 $\mu\text{g}/\text{L}$ in mainstream

smoke [7,8]. High-temperature cooking of oils generates Acr as a major product [9]. Because of widespread exposure and potential harmful effects to humans, Acr is one of the most studied pollutants.

In addition to the environmental exposure, Acr is also an oxidative product of polyunsaturated fatty acids [10]. Acr is important not only because of its ubiquity and abundance, but also because of its toxicity as an irritant to the respiratory system and potential role in causing cancer. It has been demonstrated that Acr is mutagenic and carcinogenic [1]. These effects could be attributed to its modification of DNA bases forming promutagenic cyclic adducts. As a major reaction, Acr reacts with deoxyguanosine (dG) in DNA to produce two pairs of diastereomeric adducts (Scheme 1A): (6*R/S*)-3-(2'-deoxyribos-1'-yl)-5,6,7,8-tetrahydro-6-hydroxypyrimido[1,2- α]purine-10(3H)one

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¹ Abbreviations used: Acr, acrolein; GSH, glutathione; T4 PNK, T4 polynucleotide kinase; SPE, solid-phase extraction; TOF, time of flight; dH₂O, deionized water; ESI, electrospray ionization; bp, bisphosphate; CID, collision-induced dissociation; CAD, collision-activated dissociation.

preexisting contaminant in dG and dT 3'-monophosphates from commercial sources. We obtained evidence that a trace amount of Acr in the water used in the assay is likely responsible for the artifact formation. The artifact formation of Acr-dG that interferes with the assay can be effectively prevented by adding glutathione (GSH) to the DNA samples to be analyzed.

Materials and methods

Chemicals

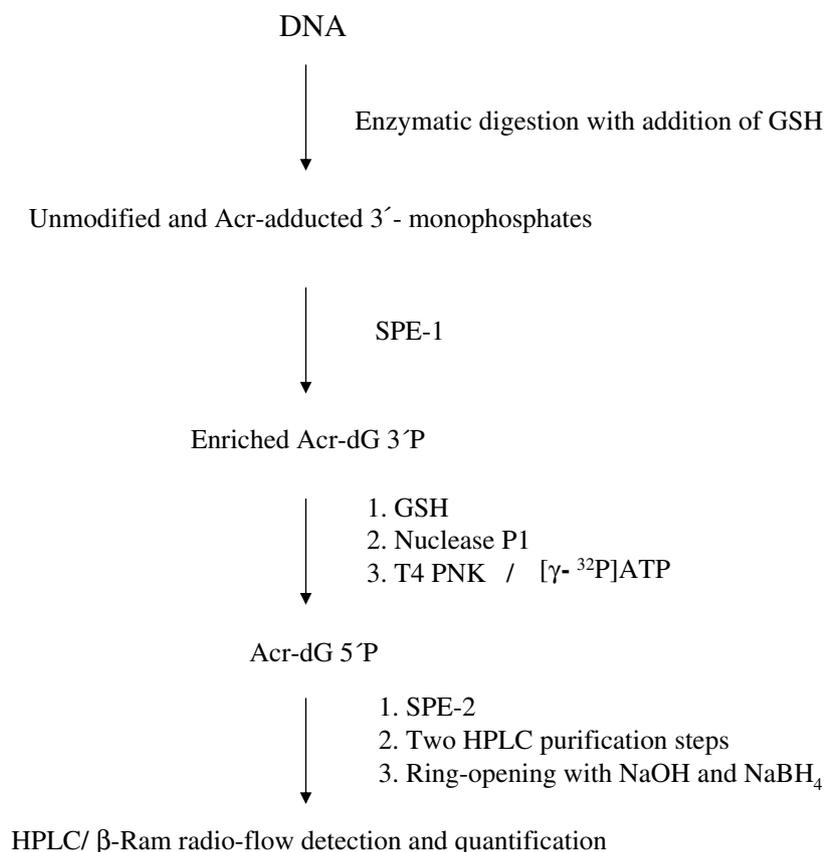
Acr, micrococcal nuclease, dG, dG 3'-monophosphate, 2-deoxyadenosine 3'-monophosphate, 2-deoxycytidine 3'-monophosphate, and thymidine 3'-monophosphate were obtained from Sigma–Aldrich Company (St. Louis, MO, USA), Acr-dG 3'- or 5'-monophosphate was prepared as previously described, and the identities of these standards were established by their UV spectra and mass spectroscopy [13]. Spleen phosphodiesterase was from Worthington Biochemical (Lakewood, NJ, USA), nuclease P1 was from Yamasa Shoyu Company (Choshi, Japan), and [γ -³²P]ATP and T4 polynucleotide kinase (T4 PNK) were from Amersham (Piscataway, NJ, USA). GSH, calf thymus DNA, and other reagents were from Sigma–Aldrich and Fisher Chemical (Fair Lawn, NJ, USA).

HPLC systems

HPLC analysis was performed using four systems: System 1 is a Shimadzu HPLC system with a SPD-M10A VP photodiode array detector (Kyoto, Japan) using a C18 reverse-phase column (Gemini, 5 μ , 110 Å, 5 μ m, 250 \times 4.6 mm) from Phenomenex (Torrance, CA, USA). The solvent systems used were: (A) 5 mM sodium citrate (pH 6.9) and (B) methanol/water 50:50 with 0 \rightarrow 30% B for 40 min at 0.6 mL/min. System 2 is an Agilent 1100 HPLC system with a G1315B photodiode array detector (Palo Alto, CA, USA) using a C18 reverse-phase column (Gemini, 5 μ , 110 Å, 5 μ m, 250 \times 4.6 mm). The solvent systems were: (A) 50 mM triethylamine phosphate (pH 6.4) and (B) methanol/water 50:50 with 0 \rightarrow 40% B for 40 min at 0.6 mL/min. System 3 is a Waters HPLC system with dual UV for online radioactivity monitoring; a β -Ram radio-flow detector (IN/US Systems, Inc., Fairfield, NJ, USA) was used with mixing scintillation cocktail at a flow rate of 0.6 mL/min using a C18 reverse-phase column (Gemini, 5 μ , 110 Å, 5 μ m, 250 \times 4.6 mm). The solvent systems were: (A) 5 mM sodium citrate (pH 5) and (B) methanol/water 50:50 with 0 \rightarrow 50% B in 50 min at 0.6 mL/min. System 4 is a Waters HPLC system with dual UV for online radioactivity monitoring by a β -Ram radio-flow detector (IN/US Systems, Inc.), used with mixing scintillation cocktail at a flow rate of 0.6 mL/min using a SAX ion-exchange column (Phenosphere, 5 μ , 80 Å, 5 μ m, 250 \times 4.6 mm) from Phenomenex; the solvent system was 100% (NH₄)₂HPO₄ (pH 6) at 0.6 mL/min.

³²P-postlabeling/SPE/HPLC method

The method is outlined in Scheme 2. One to one hundred micrograms of DNA was incubated at 37 °C for 3.5 h with 100 μ L digestion mixture containing 0.2 unit/ μ g micrococcal nuclease, 0.001 unit/ μ g spleen phosphodiesterase, 5 mM CaCl₂, and 15 mM sodium succinate (pH 6.0) in the presence of 0.5 mM GSH. After digestion, a small portion of the digest (5 μ L) was used to quantify dG 3'P using HPLC System 1. The remaining solution (95 μ L) was used for analysis of Acr-dG. The SPE column (C18, 200 mg, 1-mL volume; Varian, Harbor City, CA, USA) was preconditioned with 2 mL 100% methanol followed by 1 mL deionized water (dH₂O) and 1 mL of 2% methanol in 5 mM ammonium formate, pH 3.5. After the sample was loaded onto the SPE column (SPE-1), it was washed with 1.7 mL of 5 mM ammonium formate (pH 3.5) containing 2% methanol to remove most of the unmodified nucleotides, and Acr-dG 3'P was eluted with 0.7 mL of 30% methanol in water and collected in 1.5-mL Eppendorf tubes containing 70 nmol of GSH for a final 0.1 mM solution. The eluted adduct fraction was dried in a SpeedVac at room temperature overnight. Nuclease P1 (40 μ L mixture containing 10 units nuclease P1, 0.05 mM zinc chloride, 30 mM sodium acetate, pH 5.0) was added to hydrolyze residual unmodified nucleotides in the collected fraction. The mixture was vortexed and incubated at 37 °C for 1 h, followed by drying in a SpeedVac at room temperature for 1 h. The Acr-dG 3'P was then converted to ³²P-labeled Acr-dG 5'P by adding 3 μ L of T4 PNK (30 unit/ μ L, pH 7.6), 3 μ L of T4 PNK dilution buffer, 4 μ L of T4 PNK 10 \times buffer, 1 μ L of [γ -³²P]ATP (10 μ Ci/ μ L), and 29 μ L dH₂O at 37 °C for 45 min. After labeling, the ³²P-labeled Acr-dG 5'P was separated from the rest of the mixture by SPE-2 as follows: dH₂O (60 μ L) was added to the ³²P-labeled mixture and loaded onto a preconditioned SPE column (2 mL 100% methanol, 1 mL dH₂O, and 1 mL 5 mM sodium citrate, pH 6). The column was then washed with 1.5 mL sodium citrate (5 mM, pH 6) and eluted with 1 mL 15% methanol in sodium citrate (5 mM, pH 6). The adduct fraction from the SPE-2 step was then dried in a SpeedVac at room temperature for 1 h. It was spiked with the Acr-dG 5'P as UV marker and purified with reverse-phase HPLC System 1, followed by HPLC System 2. After the collected fraction containing Acr-dG 5'P was dried to 200 μ L, it was treated with 20 μ L 10 M NaOH and 5 mg of sodium borohydride crystals at room temperature for 10 min to yield the ring-opened derivative (Scheme 1B) and then neutralized with 20 μ L of 3.3 M H₃PO₄. For final analysis of the ³²P-labeled Acr-dG 5'P, HPLC System 3 was used. For the analysis of each set of samples, a standard (Acr-dG 3'P, 10 fmol), a negative control (unmodified nucleotides: dG, dC, dA, and dT, each 25 nmol), and a water blank were included.

Scheme 2. Outline of the quantitative ³²P-postlabeling/SPE/HPLC method.

Removing preexisting Acr-dG 3'P in negative control samples

To remove Acr-dG 3'P in the negative control samples, SPE-1 (see above) was used to separate Acr-dG 3'P from the unmodified nucleotides. The unmodified nucleotides were present in the washing fraction (1.7 mL of 5 mM ammonium formate containing 2% methanol), and Acr-dG 3'P was in the elution fraction (0.7 mL 30% methanol in water). To confirm that the washing fractions contained only the unmodified nucleotides and were free of Acr-dG 3'P, they were dried, dissolved in 100 μ L dH₂O, and then analyzed by the method described above.

Blocking artifact formation of Acr-dG 3'P with GSH

To illustrate the effect of adding GSH on Acr-dG formation, a reaction mixture of 0.5 mM dG with different concentrations of GSH (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM) and 0.5 mM Acr in phosphate buffer, pH 7.4, was incubated for 18 h at 37 °C. The effect of adding GSH on Acr-dG 3'P artifact formation in the assay with DNA (10 μ g) and dG 3'P (25 nmol) samples was investigated. GSH was added to the samples at the DNA digestion step and again at the elution (SPE-1) step. DNA digestion mixture (100 μ L), containing different amounts of GSH (0, 0.5, 1, 2 and 4 mM), and the SPE-1 eluting fraction (700 μ L),

containing 0, 0.1, and 0.2 mM GSH, were used. Samples were then analyzed by the method described above. To determine whether the addition of GSH affects ³²P-postlabeling efficiency, samples containing 5 fmol of Acr-dG 3'P were labeled in the presence of different concentrations of GSH (0, 0.1, and 1 mM).

Validation of the assay with Acr-dG standard and DNA samples

The method was validated with Acr-dG standard, calf thymus DNA, and DNA from human colon cancer cells (HT29 cell line). The detection limit, linearity, and recovery were determined. Acr-dG standard of different quantities (1, 5, 10, 20, and 100 fmol) were analyzed. To detect Acr-dG 3'P in DNA, different amounts of calf thymus DNA (1, 5, 10, 25, and 50 μ g) and DNA isolated from human colon HT29 cells (25, 50, 65, 85, and 100 μ g) were analyzed. To determine intraassay variability, each sample was analyzed at least in triplicate.

Mass spectrometry

Electrospray ionization mass spectrometry (ESI MS) and ESI MS/MS were carried out using a QSTAR Elite mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with a NanoSpray II source

head and syringe pump for direct infusion experiments. Experiments were performed using the positive ion mode with ion source gas pressure 1 psi, ion spray voltage 2.20 kV, and solvent flow rate 1 μ L/min. All samples were dissolved in 1:1 mixture of acetonitrile and water, containing 1% formic acid used as a standard mobile phase for all experiments. For both TOF-MS and MS-MS scans, the mass range from 50 to 500 m/z was monitored.

i. Preparation of Acr-GSH conjugate standard for MS

The standard of Acr-GSH conjugate was prepared by mixing 1 mmol of Acr with 1.05 mmol of GSH in 10 mL of water. After 2 h, the sample was sequentially diluted 100,000 times by pipetting 10 μ L of reaction mixture into 1 mL of water, and then by taking 10 μ L of the resultant solution to 1 mL of water. Before analysis by mass spectrometry, 100 μ L of this mixture was diluted with 900 μ L of a 1:1 mixture of acetonitrile and water containing 1% formic acid. The TOF-MS spectrum was scanned from m/z 50 to 500 over 1 s, and then the scan was repeated 60 times (1 min total time). For the MS-MS experiment, product scan ion mode was used to search the fragmentation products of ion with m/z 364.1 Da. Collision energy (CE) was adjusted to 20 eV, and collision gas (CAD) to 5 (arbitrary units). The spectrum was scanned from m/z 50 to 500 over 1 s, and then the scan was repeated 60 times (1 min total time).

ii. Trapping Acr in water by GSH and detection by MS

Ten milliliters of a 10 μ M solution of GSH in water was allowed to stand in a closed vial for 2 h, and then the solution was evaporated using a Savant SpeedVac concentrator. The residue was reconstituted in 10 mL of water and was again left in a closed vial at room temperature for 2 h; the sample was then evaporated again. The procedure was repeated eight more times, and a total of 100 mL of water was used for trapping Acr in water by GSH. After the last cycle, the sample was dried and dissolved in 500 μ L of a 1:1 mixture of acetonitrile and water containing 1% formic acid. The TOF-MS spectrum was scanned from m/z 50 to 500 over 1 s, and then the scan was repeated 120 times (2 min total time). For the MS-MS experiment, the product scan ion mode was used to search the fragmentation products of ion with m/z 364.1 Da. Collision energy (CE) was adjusted to 20 eV, and collision gas (CAD) to 5 (arbitrary units). The spectrum was scanned in multiple channel averaging mode from m/z 50 to 500 over 1 s, and then the scan was repeated 120 times (2 min total time).

Results and discussion

A quantitative ³²P-postlabeling/SPE/HPLC assay for Acr-dG

Previously, we reported a SPE/HPLC-based ³²P-postlabeling assay to detect different cyclic 1,*N*²-propa-nodeoxyguanosine adducts [24]. Although it is highly

sensitive, capable of detecting 0.5 fmol in 80 μ g DNA (9 adducts/10⁹ dG), it suffers from significant assay variability. This problem is due largely to the poor separation of Acr-dG adducts from the unmodified nucleotide 3'-monophosphates before labeling, resulting in inefficient labeling and low recovery. The assay variability limited its application as a dosimeter for risk assessment studies in tissues from rodents and humans. It is therefore important to develop an assay with better quantitative characteristics.

In the study described here, we developed an assay with more efficient recovery and quantification by three approaches. First, we optimized the SPE-1 conditions for separation of the 3'-monophosphates of Acr-dG from the unmodified nucleotides before labeling. Second, we developed a ³²P-postlabeling condition that yields, instead of the 3',5'-bisphosphates, the 5'-monophosphates of Acr-dG, for better HPLC separation after labeling. Finally, we added GSH to the DNA digestion mixture and the fraction eluted from SPE-1 to prevent artifact formation of Acr-dG adducts.

With the previous method, the adduct levels in DNA were underestimated, because the recovery of adducts in DNA samples was invariably lower than that of a positive control sample containing only adduct standards without the unmodified nucleotides. It has been shown that even subnanomole quantities of the unmodified nucleotides can interfere with the ³²P-labeling of adducts [24]. Therefore, SPE-1 is an important step to remove the unmodified nucleotides in DNA digest before labeling. To develop optimal SPE-1 separation, several buffers, such as sodium citrate and ammonium formate with different concentrations and pH, were tested as washing solvents using the synthetic UV standards of Acr-dG. The results in Table 1 indicate that 5 mM ammonium formate (pH 3.5) containing 2% methanol provided the best separation. The best elution solvent was 30% methanol in H₂O, because inorganic salts in buffer could interfere with labeling efficiency. For example, using 1 mL of 5 mM sodium citrate would result in a labeling mixture with 125 mM concentration of the salt after reducing its volume to 40 μ L. At this concentration, the labeling efficiency was found to be 32 times less than that in only water.

To enrich the adduct in the eluted fraction after SPE-1 for ³²P-labeling, nuclease P1 was used to hydrolyze the residual unmodified deoxynucleoside 3'-monophosphates to the deoxynucleosides. In the previous assay, Acr-dG 3',5'-bisphosphate (3',5'-bP) was analyzed as the final product. However, because of its polarity, it is poorly separated from ATP by SPE-2 after labeling. In the current assay, the labeled 3',5'-bP of Acr-dG was converted to Acr-dG 5'P as the final product. The large polarity difference between Acr-dG 5'P and ATP presents a significant advantage for purification before HPLC analysis. In addition to catalyzing the transfer of *P*_i from ATP to the 5'-hydroxyl terminus of nucleoside 3'-monophosphates, T4 PNK is also known to catalyze the removal of 3'-phosphoryl groups [25]. Therefore, reaction of T4 PNK with

Table 1
Percentage recovery of the unmodified and Acr-dG (synthetic UV standard) in the SPE-1 step using different buffers

Buffer	Fraction (buffer volume)	% Recovery		
		dG	dA	Ac-dG
5 mM sodium citrate, pH 7.2	First wash (1 mL)	69	0	0
	Second wash (2 mL with 5% MeOH)	31	83	26
	Final elution (1 mL with 5% MeOH)	0	9	69
5 mM ammonium formate, pH 6	First Wash (2 mL)	100	74	0
	Second wash (0.25 mL)	0	21	5
	Final elution (1.25 mL with 5% MeOH)	0	5	95
5 mM ammonium formate pH 3.5	First wash (1 mL with 2% MeOH)	87	0	0
	Second wash (0.7 mL with 2% MeOH)	13	100	0
	Final elution (0.7 mL dH ₂ O with 30% MeOH)	0	0	100

[γ -³²P]ATP not only can add 5-phosphate to Acr-dG 3'P, yielding Acr-dG 3',5'-bP, but can also remove the 3-phosphate from Acr-dG 3'P and Acr-dG 3',5'P to form Acr-dG and Acr-dG 5'P, respectively. Taking advantage of these activities, we developed an optimal condition that allows the conversion of Acr-dG 3',5'P to its 5'-monophosphate as the final product. Several conditions were tested with varying amounts of T4 PNK and times for labeling. It was determined that 90 units of T4 PNK for 45 min at 37 °C gave the best yield of Acr-dG 5'P. We used an additional ion-exchange HPLC system (System 4) to confirm the identity of the radioactive peak by comigration with the synthetic UV standard. Because the ring-opening reaction is unique to γ -OH-Acr-dG, the comigration of its ring-opened derivative with the synthetic UV standard provided unequivocal structural confirmation. In Fig. 1 are typical HPLC chromatograms obtained from the analysis of a standard and a DNA sample with the detection of the ring-opened Acr-dG 5'P.

Detection and quantification of Acr-dG in calf thymus DNA and DNA from human colon HT29 cells

The method was validated by generating a standard curve using different amounts of Acr-dG 3'P standard (Fig. 2A). The limit of detection in DNA samples was as low as 0.1 fmol. The average recovery of this assay was 13.6% (Table 2), approximately three times greater than that for the previously reported method. This method was then applied to detect and quantify Acr-dG in calf thymus DNA and DNA from human colon HT29 cells. The levels of Acr-dG in these samples were determined to be 227 ± 22 adducts/ 10^9 dG and 13.9 ± 0.9 adducts/ 10^9 dG, respectively, with less than 10% intraassay variability. Fig. 2B and C illustrate the linearity of the assay using different amounts of DNA (1–50 μ g for calf thymus and 25–100 μ g for HT29 cells). Because of the low levels of adduct in HT29 cells, we could not detect the adduct using less

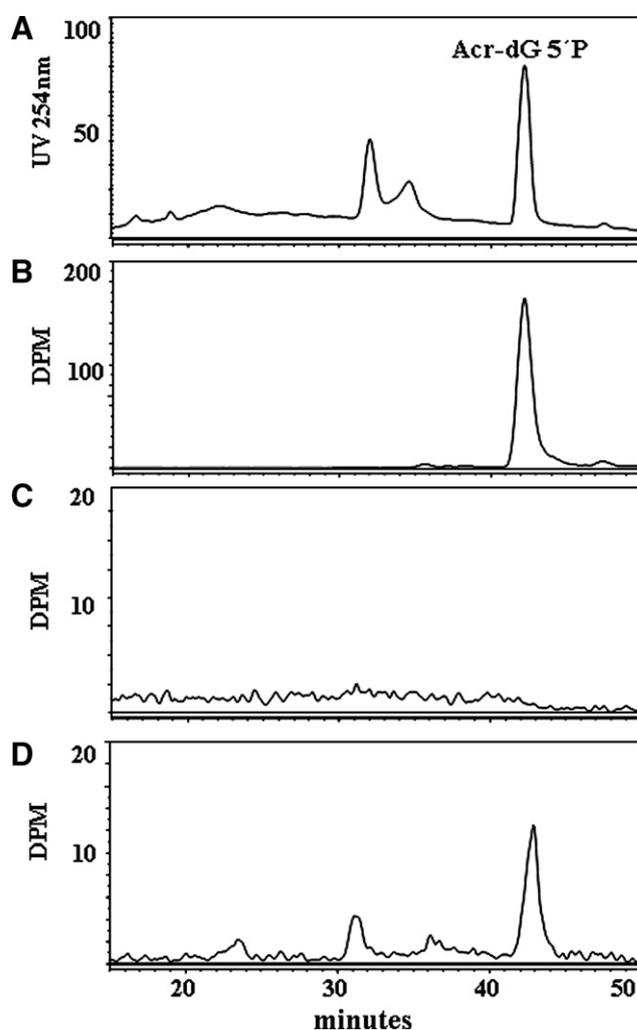


Fig. 1. Detection of the ring-opened Acr-dG 5'P in different samples by HPLC: (A) UV standard. (B) Radiolabeled adduct standard. (C) Blank (dH₂O) sample. (D) Radiolabeled adduct from calf thymus DNA.

than 25 μ g of DNA, whereas only 1 μ g calf thymus DNA was needed to detect Acr-dG.

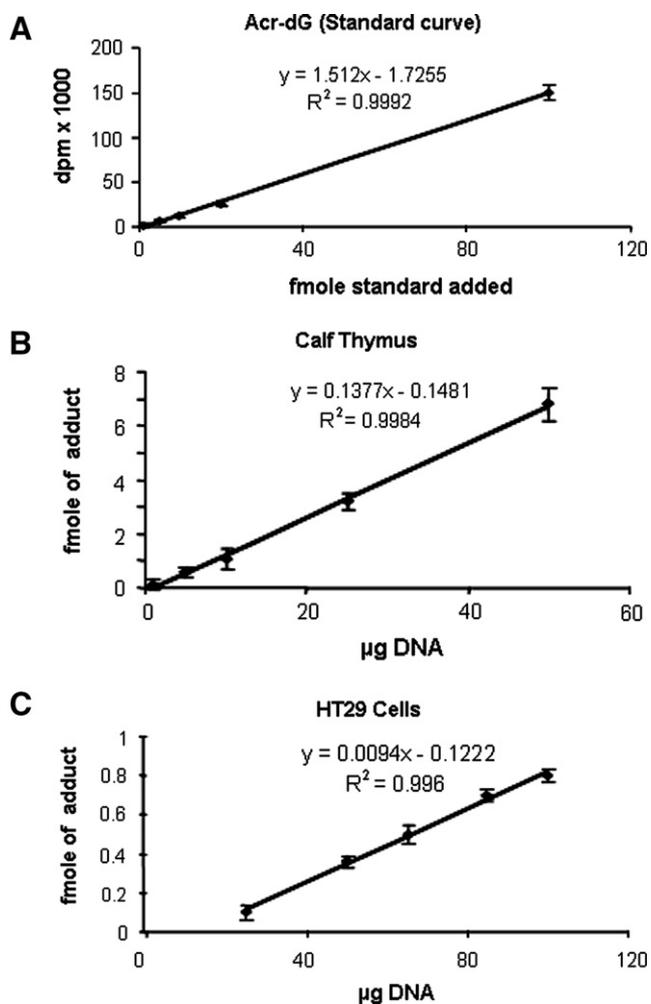


Fig. 2. (A) Standard curve obtained by using different amounts of Acr-G 3'P standard. (B, C) Linear relationships of Acr-dG detected in different amounts of calf thymus DNA (B) and DNA from HT29 cells (C). All data points were determined based on triplicate analyses.

Table 2
 Percentage recovery of different amounts of Acr-dG 3'P

Standard AdG 3'P (fmol)	% Recovery
100	17.7 ± 1.0
20	13.2 ± 1.2
10	12.3 ± 1.8
5	13.0 ± 0.6
1	11.6 ± 1.2
Average	13.6 ^a

^a n = 3, CV < 15%.

Detection of Acr-dG as an artifact in the assay and a preexisting contaminant in commercial dG 3'P and dT 3'P samples

During the development of the method, we made two unexpected observations. First, we identified Acr-dG 3'P as a contaminant in dG 3'P and dT 3'P from commercial sources. Second, we detected it as an artifact in two steps during the assay. To ensure that the Acr-dG we detected

was indeed originating from DNA, a sample consisting of all four unmodified deoxynucleoside 3'-monophosphates was included in each assay as a negative control (see Materials and Methods). Surprisingly, we detected 3.0 and 3.3 fmol of Acr-dG 3'P in 25 nmol of dG 3'P and dT 3'P, respectively. However, no Acr-dG 3'P was detected in dA 3'P, dC 3'P, and blank (H₂O) samples. Two possible sources could account for the detection of Acr-dG in these samples; one is that Acr-dG is formed during production of dG 3'P and dT 3'P as commercial products, and the other is that it is formed as an artifact in the assay. Acr can conjugate quantitatively with GSH, forming a stable 3-oxopropyl glutathione, a thioether [26]. As expected from this reaction, we found that addition of GSH (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM) can stoichiometrically block the formation of Acr-dG in the reaction of 0.5 mM Acr with 0.5 mM dG; thus, a complete block of Acr-dG formation was observed at 0.5 mM GSH (Fig. 3A). A trace amount of Acr could be present in the water used in the assay, as Acr is a ubiquitous environmental pollutant. To examine the latter possibility, we added GSH to the DNA digestion

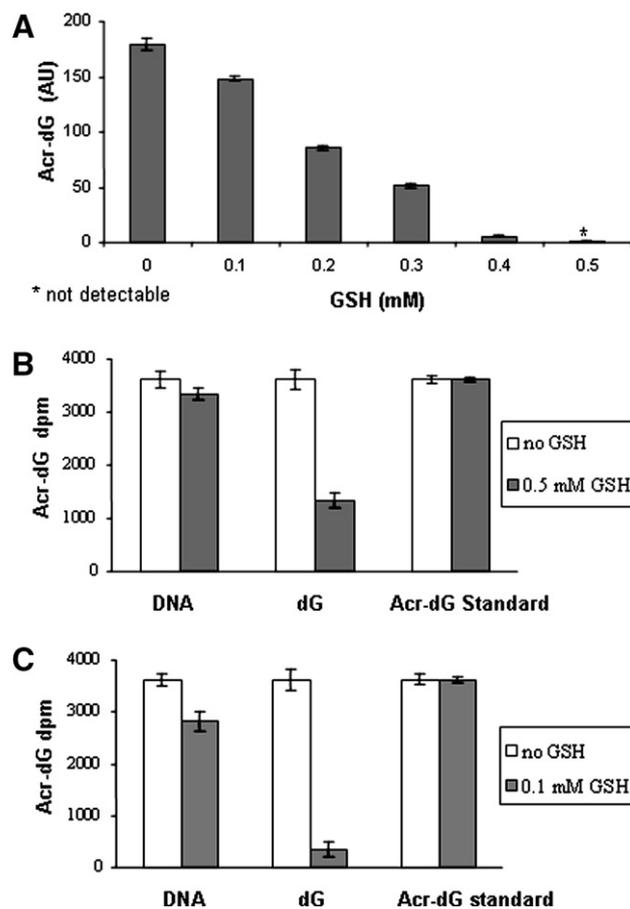


Fig. 3. (A) GSH blocked Acr-dG formation in the reaction of dG and Acr in a dose-dependent manner. (B) Acr-dG artifact formation was significantly suppressed by adding GSH (0.5 mM) to the enzyme mixture used for DNA digestion containing dG 3'P, but not with DNA or Acr-dG standard. (C) Adding GSH (0.1 mM) to the SPE-1 eluting fraction suppressed significantly the Acr-dG artifact formation with DNA and dG 3'P, but not with Acr-dG standard sample.

mixture and to the fraction eluted from SPE-1, because free dG 3'P that could react with Acr is present in the samples of these two steps during the assay. Our results demonstrated that addition of GSH decreased the amount of Acr-dG detected in the samples. The amount was reduced from 3.0 to 1.1 fmol on addition of 0.5 mM GSH to the samples containing dG 3'P. However, increasing the amount of GSH (1, 2, and 4 mM) in 25 nmol of dG 3'P did not further decrease the Acr-dG detected in the samples. These results indicate that Acr-dG is formed as an artifact in the assay and the artifact formation can be blocked by GSH. The observation that GSH did not completely block Acr-dG formation therefore supports that there is preexisting Acr-dG 3'P in the dG 3'P used in the negative control sample. To prepare a negative control sample free of Acr-dG contaminant, SPE-1 was used to remove Acr-dG 3'P from dG 3'P and dT 3'P samples as described above. Using the purified negative control samples, we detected, as expected, only 0.1 fmol of Acr-dG 3'P in 25 nmol of dT 3'P. However, the amount of Acr-dG detected in dG 3'P sample was increased to 7.5 fmol. These results further confirm that there was artifact formation of Acr-dG 3'P from dG 3'P during the assay steps.

Presence of trace amount of Acr in water used in the assay detected as Acr-GSH conjugate by mass spectrometry

A possible source of artifact formation of Acr-dG is the presence of trace amounts of Acr in the water prepared from the ion-exchange filtration system (Millipore) used in the assay. The presence of Acr in water was confirmed by trapping it with GSH and detecting the Acr-GSH conjugate by mass spectrometry. The mass chromatogram of the reaction

shows a peak with m/z 364.1192 Da, corresponding to the protonated $[M + H]^+$ Acr-GSH adduct (Fig. 4). This ion was not present either in water used for the reaction without GSH or in the mobile phase used for mass spectrometry.

The structure of the trapped Acr-GSH conjugate was further confirmed by a Collision induced dissociation (CID) MS-MS experiment. The fragmentation pattern matches that of the standard (Fig. 5A and B). Some of the characteristic fragments are as follows: The *b* series ions: b_1 , b_2 , and b_2° (b_2 after neutral loss of water) are clearly visible. In the *y* series, the y_2 ion is barely present in favor of the formation of y_2° . It was reported that this ion is formed by neutral loss of water during cyclization of y_2 fragment, which is a unique and predominant fragmentation mechanism of Acr-GSH conjugate [27]. However, this fragment can be also interpreted as an isobaric internal double backbone cleavage fragment $C(Acr)G$. Also, other fragments that are unique to Acr-GSH cysteinyl backbone cleavage, *g*, *h*, and *r*, were observed (Fig. 4A) [27]. We also noted the presence of the satellite ions z_2^+ and y_2^+ , which are derived from z_2 and y_2 fragments after additional cleavage of Acr moiety from the cysteine side chain. The fragmentation pattern for Acr-GSH in the reaction mixture shared several common ions with the fragments of the standard, although the mass chromatogram also showed a number of additional peaks that are probably derived from fragmentation of the interfering ion at m/z 363.2407 Da. The level of Acr in water was estimated based on the result from the ³²P-postlabeling assay. As described in previous sections, 7.5 fmol Acr (420.48 fg) was detected in approximately 1.7 ml of water. Therefore, the minimal concentration of Acr is calculated to be as low as 0.247×10^{-3} ppb in the water based on a quantitative reaction between Acr and dG 3'P.

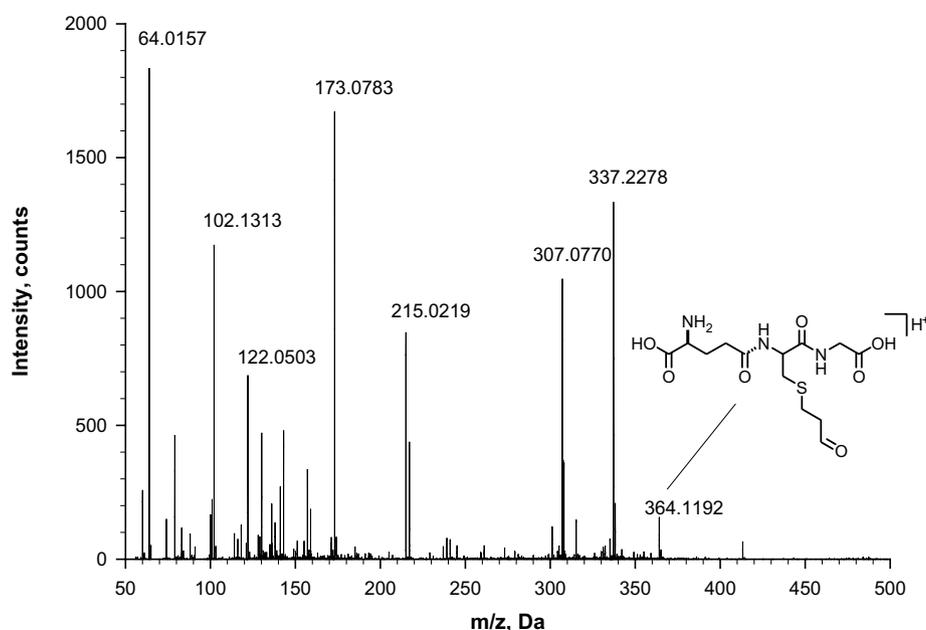


Fig. 4. TOF-MS mass chromatogram showing a molecular ion at m/z 364.1192 of the Acr-GSH in the reaction mixture of Acr trapped by GSH in the water used in our assay.

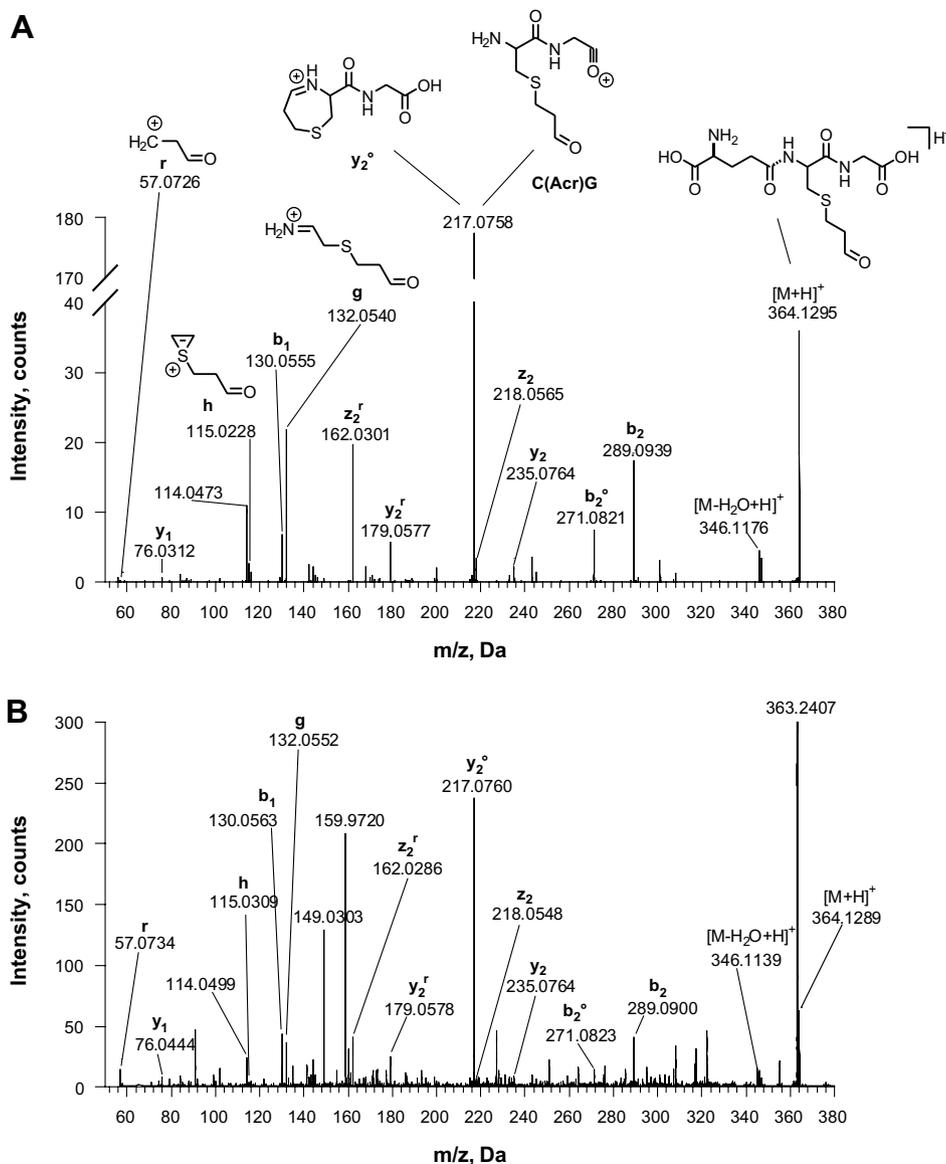


Fig. 5. CID mass chromatograms for Acr-GSH standard (A) and mixture from Acr trapping reaction (B).

Reducing the artifact formation of Acr-dG by GSH

To prevent the artifact formation of Acr-dG, GSH was added in two assay steps: first, before enzymatic digestion of DNA and then immediately after the SPE-1 purification step. To investigate the effect of adding GSH in these two steps, we compared the results with and without GSH. Fig. 3B illustrates that the formation of Acr-dG as an artifact was significantly suppressed by adding GSH (0.5 mM) to the enzyme mixture for DNA digestion containing dG 3'P, but not to DNA or Acr-dG standards. This indicates that the presence of free dG 3'P in the mixture is a prerequisite for Acr-dG formation. After SPE-1, GSH is washed out and the elution fraction does not contain GSH; however, a small amount of free dG 3'P may remain; therefore, more GSH was added. Fig. 3C shows that adding GSH (0.1 mM) blocked the formation of Acr-dG in both DNA

and dG 3'P samples, but not the standard of Acr-dG. The greater effect of GSH on artifact formation in the SPE-1 step than the digestion step may be explained by the fact that the water volume in the SPE-1 elution fraction (0.7 mL) is larger than the digestion mixture (0.1 mL). We found that GSH up to 1 mM does not affect ³²P-postlabeling efficiency. To ensure that the release of Acr is not from the SPE-1 column, we pretreated the SPE column with different amounts of GSH. The results indicated no significant change in artifact formation.

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