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Analysis of DNA adducts of acetaldehyde by liquid chromatography–mass spectrometry

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Abstract

A highly sensitive method using liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) was developed for the analysis of DNA adducts of acetaldehyde (AA). AA, which is the primary oxidative metabolite of ethanol, is considered to possess carcinogenic activity. AA reacts with the exocyclic amino group of guanine in DNA to form N^2-ethylguanine (Et-Gua) and 1,N^2-propanoguanine (Pr-Gua) adducts. With the present method, such adducts were detected as the base forms from DNA chains using depurination in the pretreatment process. In our measurement with LC–ESI-MS, the limits of detection (LODs) of the Et-Gua and Pr-Gua adducts of the base forms were $3.0 \cdot 10^{-10}$ and $1.0 \cdot 10^{-9}$ *M*, respectively, and the LODs are about two orders of magnitude lower than those of the nucleoside forms. Calf thymus DNA samples treated with AA and $NaBH_3CN$ were analyzed by this method. Et-Gua was clearly detected and, in the absence of $NaBH_3CN$, Pr-Gua was detected predominantly. Furthermore, the method was also applied to study whether or not these two adducts are formed in DNA of cultured HL-60 cells during exposure to AA for 24 h. Pr-Gua was clearly detected and traces of Et-Gua were also detected in the DNA of the cells. Although the sensitivity of this method is lower by at least one order of magnitude than the ^{32}P-postlabeling assay, currently the most sensitive method, our method does not involve complex enzymatic reactions for the postlabeling and the use of troublesome radioactive materials. Furthermore, it enables structural identification of guanine adducts. The present method would be a useful tool for studies of Et-Gua and Pr-Gua adducts in connection with carcinogenesis.

Keywords: Acetaldehyde; Aldehydes; DNA; Ethylguanine; Propanoguanine; Guanine

1. Introduction

Acetaldehyde (AA) is a mutagen and carcinogen which is ubiquitous in the human environment [1,2]. It is formed endogenously as a primary product during the metabolic oxidation of ethanol by alcohol dehydrogenases in the liver. It is also present in many foods, alcohol beverages, tobacco smoke [2] and automobile exhaust gases [1]. Additionally, AA is primarily used as a substrate for the manufacture of acetic acid. Therefore, a large amount is produced and many workers are exposed to it during handling [1]. It has been shown to induce mutations, sister chromatid exchanges in bone marrow cells of rodents and in cultured human lymphocytes [1,2]. Inhalation of AA promotes tumors of the respiratory tract, particularly adenocarcinomas and squamous cell carcinomas of the nasal mucosa in rats and produces laryngeal carcinomas in hamsters [1,2]. On the basis of the available experimental evidence, AA is considered carcinogenic to experimental animals and, most likely, carcinogenic to humans.

AA is also a highly reactive electrophile and reacts

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with the exocyclic amino group of guanine (Gua) to form an unstable Schiff base, which can be stabilized by reduction or other reaction under physiological conditions. The N^2-ethylguanine (Et-Gua) adduct is considered one of the most stable DNA adducts of AA [3–9]. This adduct has been detected in granulocyte and lymphocyte DNA of alcoholic patients and in cultured human buccal cells exposed to AA followed by treatment with $NaBH_4$ as a reductant [6,7]. These phenomena suggest that AA is mutagenic, induced by alcohol consumption. There is the possibility that this adduct may generate G→C transversion mutations in vivo [8,9]. Et-Gua is considered to be the main DNA adduct of AA, and extensive research has been reported on this adduct [3–9]. However, recently, the 1,N^2-propanoguanine (Pr-Gua) adduct has been reported to be another DNA adduct of AA [10]. Although the genotoxicity of Pr-Gua is not established at this moment, a detailed study of the Pr-Gua adduct as well as that of Et-Gua is required.

The detection of DNA adducts has become a popular approach for studying chemical mutagenesis and carcinogenesis. The ^{32}P-postlabeling assay is one of the most sensitive methods for the detection of DNA adducts [11–14] and the assay has been one of the most useful and often the last solution in the analysis of real samples. However, it lacks the ability to provide structural information. It requires complex enzymatic reactions and the efficiency of the reactions has to be considered. In addition, many investigators cannot use this method because it requires troublesome handling of radioactive materials. On the other hand, mass spectrometry (MS) is a powerful tool for structural identification and offers excellent limits of detection in many cases. When gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis is used in conjunction with MS, we can detect many kinds of DNA adducts accurately with structural information from MS measurements. Recently, Arimoto-Kobayashi et al. detected alkylguanines such as O^6-methylguanine and N^7-methylguanine in calf thymus DNA following exposure of *N*-nitrosamine to UV irradiation using HPLC with MS and fluorescence detection [15].

In previous work, we developed a capillary electrophoresis system equipped with an amperometric detection cell for the detection of damaged deoxyguanosines such as N^2-ethyl-2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine [16–18]. This amperometric method enables us to detect guanine and guanine adducts selectively because of their lower oxidation potentials compared with other bases and their adducts. Unfortunately, the sensitivity of this system is inadequate to detect damaged guanosines in the DNA of real organisms. On the other hand, we employed LC–electrospray ionization (ESI) MS to detect the guanine adducts of nucleoside forms in our preliminary experiment, but no marked improvement of sensitivity could be achieved compared with the previous method, perhaps because of difficulty in the ionization of nucleosides (data not shown). Referring to the work of Kobayashi et al. mentioned above [15], however, MS detection of DNA adducts as base forms seemed to markedly improve sensitivity. In this study, we developed an LC–ESI-MS method for the detection of DNA adducts of AA as base forms. The present method was applied to the detection of Et-Gua and Pr-Gua adducts formed in calf thymus DNA and to study whether the two adducts form in DNA of cultured HL-60 cells exposed to AA.

2. Experimental

2.1. Reagents and chemicals

Adenine (Ade), guanine (Gua), calf thymus DNA and sodium cyanoborohydride ($NaBH_3CN$) were obtained from Nacalai Tesque (Kyoto, Japan). AA and ammonium acetate were obtained from Merck (Schuchardt, Germany) and Kishida (Osaka, Japan), respectively. The cell culture medium RPMI-1640, fetal bovine serum and penicillin-streptomycin were obtained from Sigma–Aldrich Japan (Tokyo, Japan). Isogen, a reagent used for DNA extraction, was obtained from Nippon Gene (Tokyo, Japan). Et-Gua and Pr-Gua were synthesized as reported previously [10,19]. All other chemicals were of analytical grade.

2.2. HPLC separation procedure

HPLC separations were performed on an HP1100 series LC–mass-selective detection system (Hewlett-Packard, USA) fitted with an electrospray interface

operating in the positive ion mode. UV absorbance of the eluate was monitored at 254 nm. All analyses were performed at 30 °C using a C_{18} column (Hypersil ODS, 5 μm; 4.0×125 mm; Hewlett-Packard). The injection volume was fixed at 20 μL. Isocratic separations were achieved using 10 m*M* ammonium acetate and 15% methanol as eluent at a flow-rate of 500 μL/min. Conditions of the ESI source of the mass spectrometer were optimized using an optimizing program to obtain the highest signal intensity and were as follows: voltage, 2.1 kV; capillary temperature, 350 °C; using the above eluent. The selective-ion monitoring (SIM) mode was used to detect Et-Gua (*m*/*z* 180.1) and Pr-Gua (*m*/*z* 222.1).

2.3. Analysis of Et-Gua and Pr-Gua adducts in calf thymus DNA by LC–MS

Calf thymus DNA (1.0 mg) and AA (0.10 mL) were dissolved in 100 m*M* phosphate buffer (pH 8.0) in a total volume of 1.0 mL and the solution was incubated for 48 h in a chamber maintained at 37 °C. The DNA was then dialyzed against distilled water for 4 h and the mixture was hydrolyzed in 0.10 *M* HCl at 70 °C for 1 h to release Ade, Gua, Et-Gua and Pr-Gua. Only purine bases, namely those related to Ade and Gua, are released with this treatment (depurination) [15].

2.4. Cell cultures

HL-60 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 50 units/mL penicillin G and 20 μg/mL streptomycin sulfate at 37 °C in a humidified environment containing 5% CO_2. The cells were suspended at $1.5 \cdot 10^6$ cells/15 mL/100 mm dish and then incubated for 48 h. After incubation of the cells, AA diluted with RPMI-1640 medium was supplemented to the growth medium at a concentration of 50 m*M* and the cells were cultured continuously for 24 h. After incubation of the cells with AA, the cells were collected as pellets by centrifugation and used for DNA extraction with Isogen reagent. The DNA thus obtained was dissolved in distilled water and the concentration determined spectrophotometrically. 1 AU at a wavelength of 260 nm corresponds to 0.050 mg/mL of double-stranded DNA. The control DNA sample was also prepared from cells cultured in the same manner, but without AA.

3. Results and discussion

3.1. HPLC separation of Ade, Gua, Et-Gua and Pr-Gua, and their MS detection

Fig. 1 shows a schematic diagram of the formation of the two DNA adducts of AA. AA reacts with the exocyclic amino group of Gua to form an unstable Schiff base, namely N^2-ethylidineguanine, which can be stabilized by reduction and converted into Et-Gua. Pr-Gua is generated by reaction with two molecules of AA [10].

As a condition of LC separation prior to MS analysis, ammonium acetate was added to the eluent to improve the efficiency of ionization in the ESI process. This modifier also seemed to contribute to an improvement in the separation of Ade and Gua. The present method involves pretreatment of DNA samples with hydrochloric acid to cut off only the purine bases hydrolytically from the DNA chains. Thus, it was necessary to separate Gua, Ade, Et-Gua and Pr-Gua completely in this work. Fig. 2 shows the reversed-phase (RP) HPLC separation of an authentic standard mixture of Ade, Gua, Et-Gua and Pr-Gua. They were completely separated within 8 min (see Experimental section for HPLC conditions).

Under the optimized conditions, the limits of detection (LODs) of Et-Gua and Pr-Gua were $3.0 \cdot 10^{-10}$ and $1.0 \cdot 10^{-9}$ *M*, respectively, with a signal-to-noise (*S*/*N*) ratio of 3 (equivalent to 6.0 and 20 fmol loaded on the column, respectively). Calibration curves relating to the peak areas of SIM signals were constructed using authentic standards and the curves exhibited good linearity in the range of concentrations from 5.0 n*M* to 1.0 μ*M*. The correlation coefficients for Et-Gua and Pr-Gua were 0.997 and 0.999, respectively. Quantification of the guanine adducts in DNA was based on these calibration curves.

As mentioned above, the two guanine adducts were detected as the base forms obtained by depurination of DNA samples, because a much higher sensitivity for the base forms in ESI-MS detection

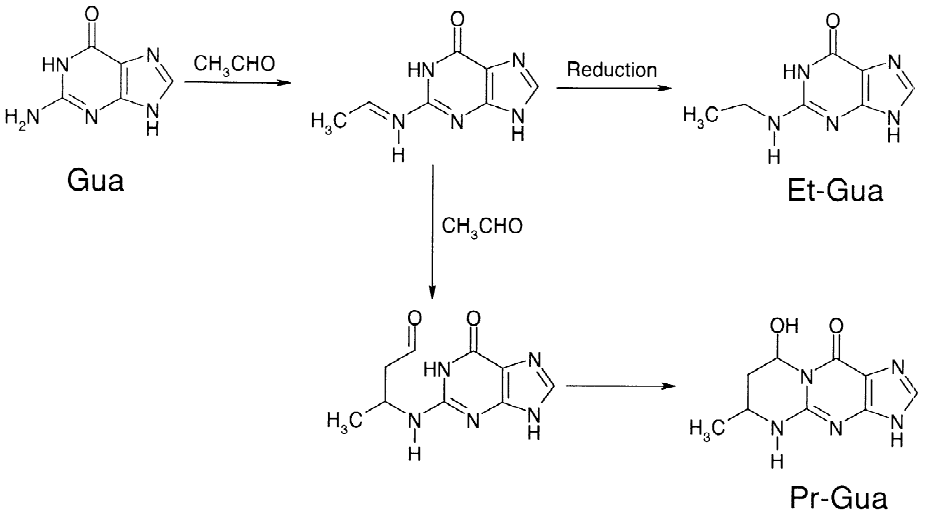

Fig. 1. Formation of Et-Gua and Pr-Gua in the reaction of Gua with acetaldehyde.

was expected than for the nucleoside forms [14]. In our measurements, the detection limit of the Et-Gua adduct of the base form was more than 100-fold lower than that of the 2′-deoxynucleoside form (data not shown). This remarkable improvement in sensitivity would make the present LC–ESI-MS method useful in biological studies.

It should be mentioned that the diastereomers of the Pr-Gua adduct as the bases could not be separated by the present separation system. Therefore, the

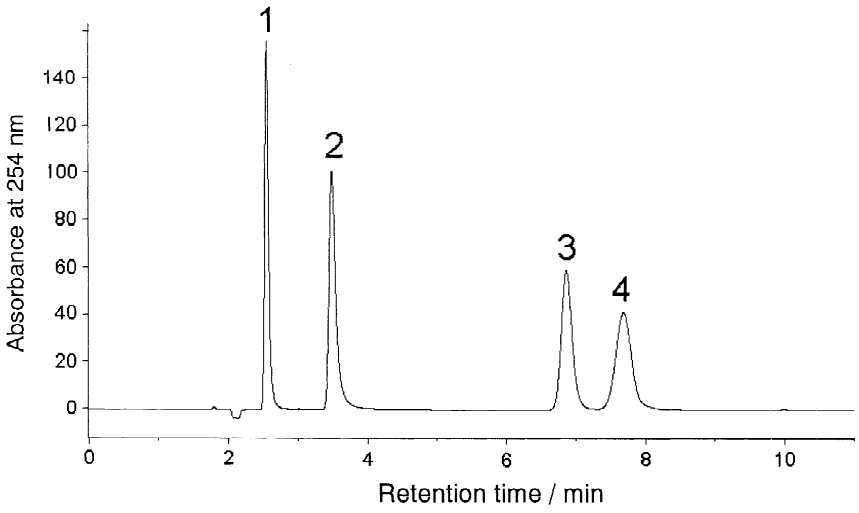

Fig. 2. Representative reversed-phase HPLC profile of the standard solution of Ade, Gua, Et-Gua and Pr-Gua ($1.0 \cdot 10^{-4}$ *M* of each analyte). Conditions: eluant, 10 m*M* ammonium acetate and 15% methanol; column, Hypersil ODS (5 μm; 125×4.0 mm); flow-rate, 500 μL/min; detection wavelength, 254 nm; column temperature, 30 °C. Peaks: 1=Gua, 2=Ade, 3=Et-Gua, 4=Pr-Gua.

adduct was detected and quantified as the total amount of diastereomers in this study.

3.2. Analysis of Et-Gua and Pr-Gua adducts in calf thymus DNA

Calf thymus DNA was treated in vitro with AA in the absence or presence of $NaBH_3CN$ as reductant. After dialyzing against distilled water, the DNA samples were hydrolyzed and analyzed by LC–ESI-MS. Fig. 3 shows the HPLC separation of hydrolyzed calf thymus DNA samples reacted with 1.8 *M* AA for 48 h in the absence of $NaBH_3CN$. The levels of Pr-Gua adducts found in calf thymus DNA samples were 28.4 ± 6.4 adducts/10^5 normal Gua ($n=3$). The Pr-Gua adduct was first identified in reactions of DNA with crotonaldehyde [20,21]. The Pr-Gua adduct has been detected in oral tissue of heavy smokers by ^{32}P-postlabelling assay and was attributed to crotonaldehyde in tobacco smoke [22]. Recently, Wang et al. reported that the Pr-Gua adduct was formed when 2′-deoxyguanosine and calf thymus DNA were each reacted with AA [10]. We should recognize Pr-Gua as a promising candidate as a DNA adduct of AA.

On the other hand, the Et-Gua adduct formed in calf thymus DNA samples with AA in the presence of 110 m*M* $NaBH_3CN$ for 0.5 h is shown in Fig. 4, and the level was 1381 ± 172 adducts/10^5 normal Gua ($n=3$).

3.3. Analysis of Et-Gua and Pr-Gua adducts in cultured HL-60 cell DNA

The present method was also applied to study whether or not the two guanine adducts are formed in DNA of cultured HL-60 cells during exposure to AA. Fig. 5 shows HPLC profiles of hydrolyzed DNA samples of cultured HL-60 cells exposed to 50 m*M* AA for 24 h. Pr-Gua was clearly detected and a trace of Et-Gua was also detected in this sample. The level of the Pr-Gua adduct detected in this sample was 2.75 adducts/10^5 Gua (Et-Gua could not be quantified). Bases, nucleosides, nucleotides and RNA in the cells were removed before hydrolysis, thus the detected damaged guanine would have arisen from the DNA of the cells [23,24]. In addition, neither

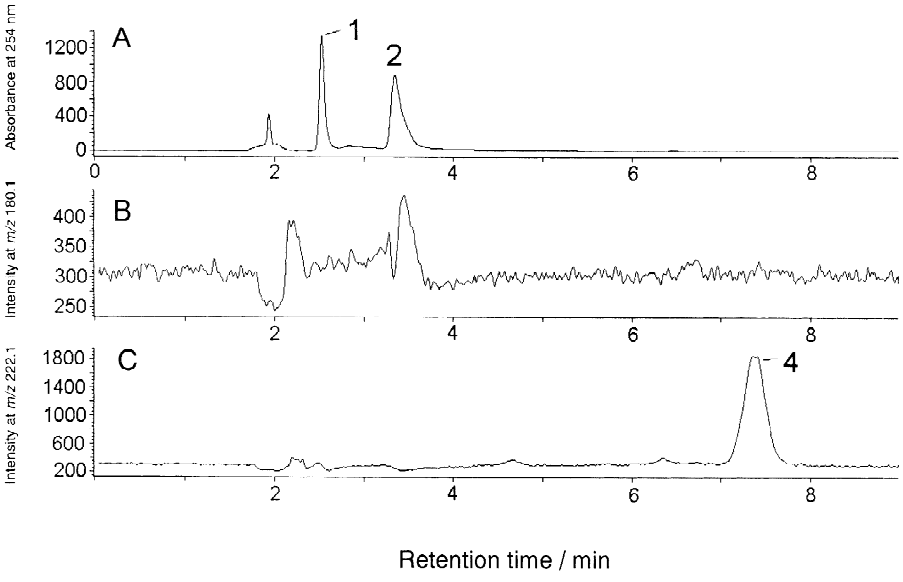

Fig. 3. Reversed-phase HPLC profile of the hydrolyzed calf thymus DNA sample exposed to 1.8 *M* acetaldehyde for 48 h in the absence of $NaBH_3CN$. (A) UV detection, (B, C) MS detection. Conditions: ESI-MS detection in the positive ion mode; voltage, 2.1 kV; capillary temperature, 350 °C; selective-ion monitoring, m/z 180.1 and 222.1. Other conditions as in Fig. 2.

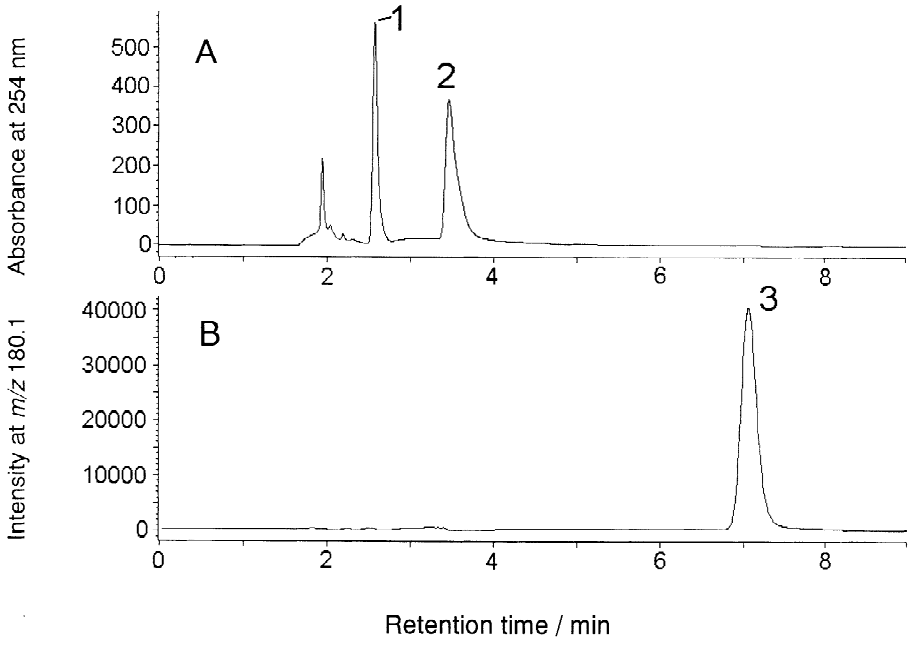

Fig. 4. Reversed-phase HPLC profiles of the hydrolyzed calf thymus DNA sample exposed to 1.8 *M* acetaldehyde for 0.5 h in the presence of 110 m*M* $NaBH_3CN$. (A) UV detection, (B) MS detection. Conditions: selective-ion monitoring, *m*/*z* 180.1. Other conditions as in Fig. 3.

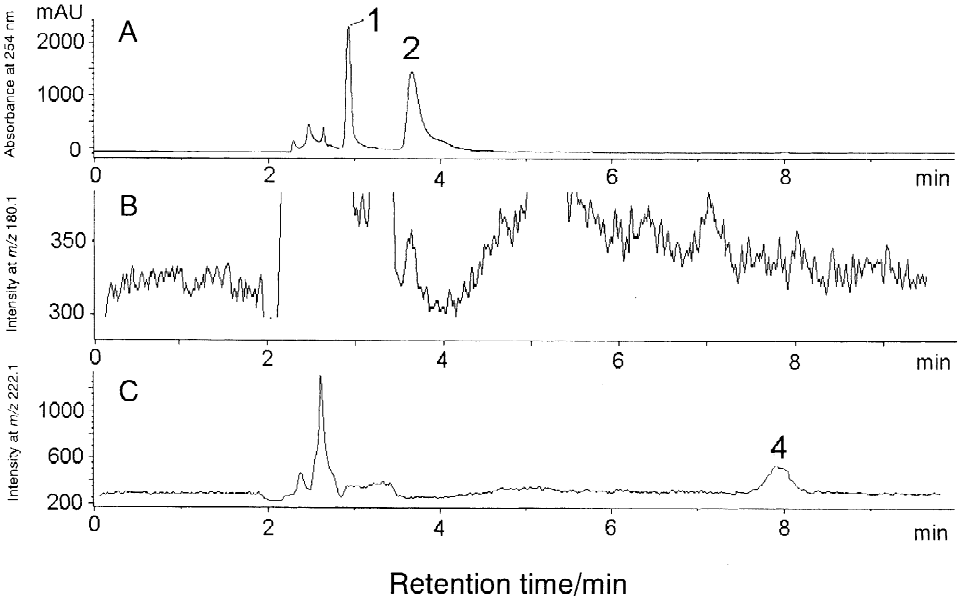

Fig. 5. Reversed-phase HPLC profiles of hydrolyzed samples of DNA isolated from HL-60 cells exposed to 50 m*M* acetaldehyde for 24 h. (A) UV detection, (B, C) MS detection. Conditions as in Fig. 3. Absorbance at 254 nm.

Et-Gua nor Pr-Gua was detected from the control DNA sample.

This is a direct observation of the formation of the two guanine adducts in DNA in the presence of AA under cellular conditions, and the Pr-Gua adduct was formed predominantly in this case. This result, however, may not reflect the practical situation of the two adducts present in human living cells because of the much higher concentration of AA in the medium compared with that in human tissues (i.e. 1–120 μM in blood 1 h after drinking [25]). Gua has to react with two molecules of AA in the formation of Pr-Gua. Dilution of AA should be disadvantageous for the formation of the Pr-Gua adduct taking into account the kinetics of the reaction. Thus, the Et-Gua adduct, which is formed with one molecule of AA, would be the main adduct at lower concentrations of AA. We should analyze the DNA of living cells cultured in the presence of lower concentrations of AA over extended periods to obtain information reflecting the practical situation in human tissues. Such a study using the present method is currently in progress.

4. Conclusion

We have successfully applied LC–ESI-MS combined with depurination of DNA as a pretreatment process for the highly sensitive detection of Et-Gua and Pr-Gua adducts in DNA. The limits of detection of Et-Gua and Pr-Gua adducts were $3.0 \cdot 10^{-10}$ and $1.0 \cdot 10^{-9}$ M, respectively. The sensitivity of the present method for Et-Gua is about 35-fold lower than that of the most sensitive ^{32}P-postlabeling assay [5]. However, the present method consists of simpler and safer procedures, and will enable us to perform more accurate analyses of DNA adducts compared with the ^{32}P-postlabeling assay. Of course, further improvement in sensitivity is required for this method and work is in progress. A small improvement in sensitivity will enable us to use the present method as a useful tool for studies of Et-Gua and Pr-Gua adducts in connection with carcinogenesis.

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