Short communication

High-performance liquid chromatographic method with electrochemical detection for the analysis of O^6-methylguanine

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Abstract

An improved system consisting of a combination of high-performance liquid chromatographic methods with electro-chemical detection for the separation and analysis of the DNA adduct O^6-methylguanine (O6MG) has been developed. This adduct is produced by the interaction of methylating agents with DNA and induces mispairing in the DNA of the target cells. A good separation of modified from unmodified bases is first achieved with an HPLC system using a Partisil 10 SCX column and a salt gradient. A second HPLC step with electrochemical detection and a C18 column is used for farther separation and quantitation of O^6-methylguanine. This method shows a linear response up to 15 pg of O6MG tested. The lowest amount detected was 0.5 pg of O6MG and is highly reproducible. This method is useful to study DNA damage as a product of cellular metabolism and its effects on the process of carcinogenesis.   © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Many exogenous chemicals undergo activation and other modifications inside of the cell leading to products that can react with different macromolecules in the cellular environment. DNA is a frequent target for these activated metabolites and the production of adducts can lead to dramatic changes in cellular functions [1–4]. Some chemical agents such as dimethyl sulfate, N-methyl-N′-nitro-N-nitroso-guanidine, dimethylhydrazine and others of a similar nature can modify DNA producing methylations in various positions of each of the purine and pyrimidine bases [5–8]. Nevertheless it is well documented that not all of the adducts produced have the same potential to alter DNA in a permanent way. A particular modification of guanine in position 6 resulting in the production of O^6-methylguanine (O6MG) has been shown to induce mispairing and is associated with malignancy [9–12]. For this reason the study of this adduct is important in the understanding of DNA damage by methylating agents [13–18]. For our research it was necessary to develop a method for detection and quantitation of O6MG compatible with the use of tissue culture cells exposed to chemical carcinogens.

A number of methods that utilize fluorescence,

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radioactivity, and electrochemical detection for the identification of O6MG have been reported [19–21]. The best results are obtained with the use of an electrochemical detector. Electrochemical detection has been shown to be a powerful tool because of its good selectivity, simplicity and sensitivity. The main difficulty arises when it is necessary to purify this adduct from a biological sample in which normal DNA bases are present in high excess (a 10^6 to 1 ratio) making it difficult to obtain a sufficient quantity of pure O6MG for analysis. We report here a method using HPLC with an electrochemical detection system for the separation and quantitation of O^6-methylguanine from DNA of cells treated with methylating agent.

2. Experimental

2.1. Chemicals and reagents

Standard O^6-methylguanine and 1,2-dimethyl-hydrazine were obtained from Sigma (St. Louis, MO, USA). Potassium phosphate was obtained from J.T. Baker (Phillipsburg, NJ, USA), ammonium formate from Fisher Scientific (FairLawn, NJ, USA) and HPLC grade methanol from Burdick & Jackson (Muskegon, MI, USA). The highest quality reagents were used for electrochemical detection.

2.2. Cell treatment and DNA extraction

Human colon cells LS174T (ATCC, Rockville, MA, USA) were grown according to the instructions of the supplier (37°C and 5% CO_2 in MEM media). Fresh dimethylhydrazine solution was added to 100 mm petri-dishes containing cells (about 70% confluence) to a final concentration of 20 μM and incubated for 24 hours. After removing the media from each dish of cells, 3.0 ml of lysis solution were added (0.1 M NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0 and 0.5% SDS). The detached cells in solution were pooled in a plastic tube and shaken for 20 min at room temperature. Then, 1 volume of phenol/chloroform was added and the mixture was centrifuged at 10,000×g for 30 min at 4.0°C. The supernatant fraction was recovered and an equal volume of isopropanol was added. DNA was

extracted with a glass rod and washed twice with 75 and 100% ethanol respectively. Total DNA was resuspended in 2.0 ml of TE buffer and 15 μl of RNAse A solution was added (10 mg/ml), and 20 μl of proteinase K was added (10 mg/ml). After incubation at 42°C for 3 hours DNA was extracted with phenol/chloroform and washed with ethanol. The extraction was repeated again as described above.

2.3. Preparation of samples

Extracted DNA from cells treated with di-methylhydrazine was hydrolyzed using 1.0 ml of formic acid (pH 2.0) at 85°C for 60 min for every 200 μg of DNA. The volume of the solution was reduced to 200-400 μl under N_2 flow at 70°C prior to being injected into the HPLC. This method was tested with up to 800 μg of hydrolyzed DNA in 200 μl of sample volume and the separation under these conditions remain unchanged.

2.4. Chromatographic conditions

A volume of sample between 100-200 μl was injected into the HPLC system (Waters, model 515 with gradient and UV detector model 2487 set at 280 inn; Milford, MA, USA) containing a Whatman Partisil 10 SCX column (250 × 4.6 mm. 10 μm particule size, Clifton, NJ, USA). Solution A (12% methanol pH 3.0) and solution B (200 mM ammonium formate pH3.0; 12% methanol) were used for the gradient. A constant 1.5 ml/min flow and 5% of solution B were the initial conditions. From time 0 mm to 3.0 mm a gradual increase up to 7% B was applied. The gradual increase of solution B follows a hyperbolic curve with minimum change at the beginning and higher change rate at the end (curve # 7 of the gradient elution model). These conditions were maintained until 13 min of running time had elapsed. Then the gradient was returned to the initial conditions (curve # 6) in 1.0 min. The collected fraction (1.5 ml) was filtered though a 0.2 μm filter (Whatman, Clifton, NJ, USA), evaporated to dryness at 70°C under N_2 then resuspended in 0.5 ml of formic acid (5.0 M) and dried again under the same conditions to reduce the salt concentration. The sample was resuspended in distilled water. A second

HPLC (ESA, Inc. model 582; Chelmsford, MA, USA) consisting of solvent delivery system with a pulse dampener and a electrochemical detector
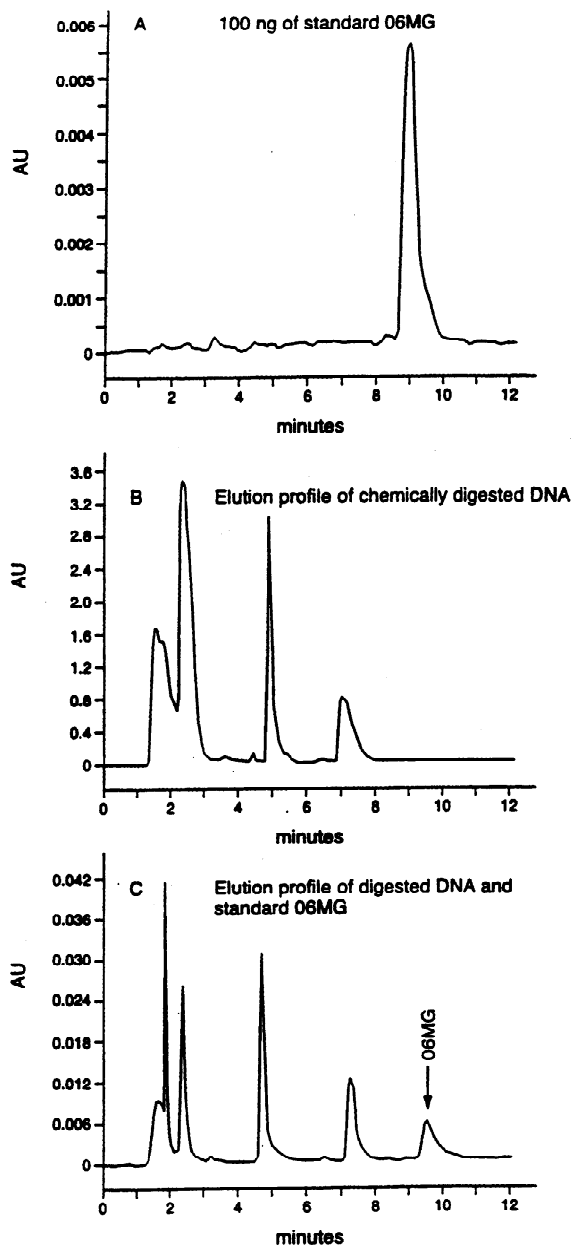

Fig. 1. HPLC chromatogram using UV detector and a salt gradient. Fig. 1a shows a chromatogram of 100 ng of standard O^6-methylguanine. The elution profile of chemically digested DNA is shown in panel b. A mixture of standard O6MG and digested DNA is shown in panel c.

(ESA, Inc. model Coulochem II and a guard cell model 5020; analytical cell model 5011) was used in the final step. A recently developed *hydrophilic* C18 column was used (AQUA C18, 5 μC18, 200Å, 150×4.6 mm, Phenomenex, Torrance, CA, USA). Isocratic conditions and 0.5 ml/min of mobile phase flow (50 mM Potassium Phosphate pH 5.0; 6% methanol) were used during the process. Voltages of +500 mV and +600 mV were applied to channel A and B respectively, of the analytical cell and +850 mV to the guard cell.

2.5. Calibration and quantitation

A calibration curve was prepared for O^6-methylguanine under the HPLC conditions described. Standards containing 0, 2, 4, 10, and 15 pg were used. Measurements of the area under the curve were made using three computer programs (Adobe Photoshop, Adobe Illustrator and Image 1.59) with the Scanner UMAX model 630 connected to a Macintosh computer.

3. Results and discussion

3.1. Optimization of the HPLC for separation

For the use of large amounts of sample (>0.5 g digested DNA) it was necessary to devise conditions that would allow the modified guanine (O^6-methyl-
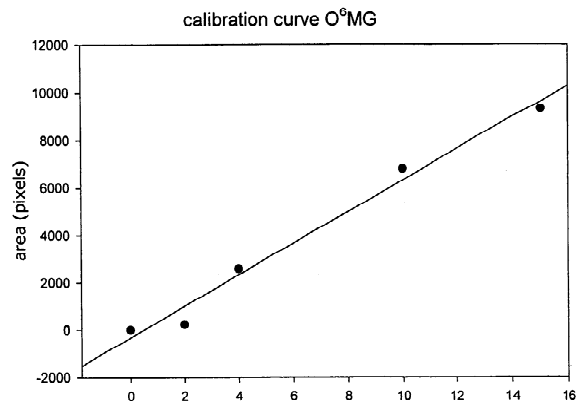

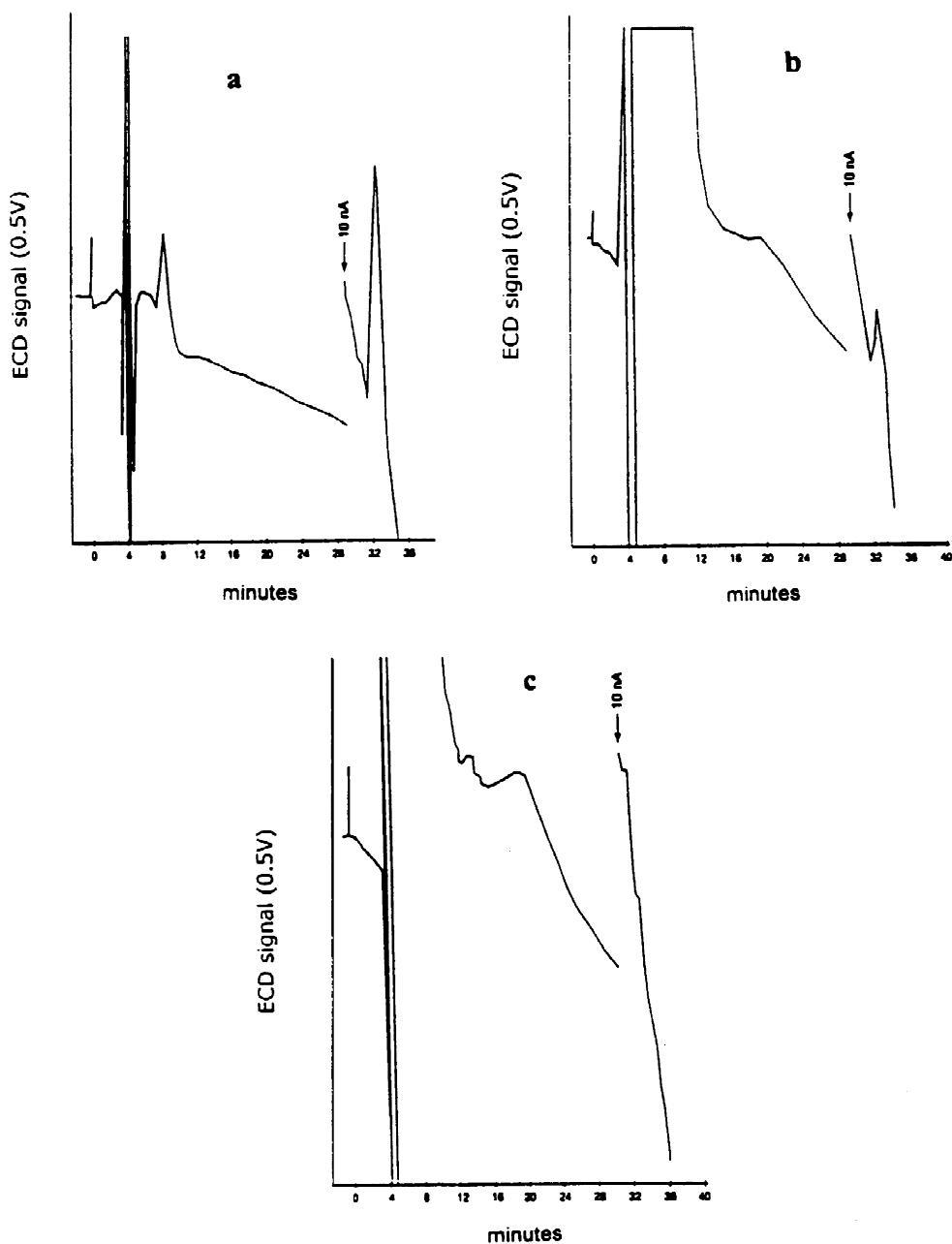Fig. 2. Calibration curve of O^6-methylguanine using 2.0, 4.0, 10.0 and 15.0 pg of standard.

Fig. 3. HPLC chromatogram of isolated O^6-methylguanine using an electrochemical detector. The signal was recorded by a chart recorder. The arrow indicates change to a higher sensitivity (10 nAmp) prior to elution time of the standard. Panel A shows a chromatogram of the standard O6MG (10 pg); Panel B shows the chromatogram of digested DNA from cells treated with dimethylhydrazine; Panel C shows the chromatogram from untreated sample.

guanine) to elute without contamination with other bases. The new conditions are modifications (described below) of earlier techniques to separate DNA bases reported by other groups [19–21]. In our system using a flow rate of 1.5 ml/min of mobile phase increased efficiency of separation. Under these conditions O6MG elutes two minutes after the closest normal base peak. The use of initial conditions containing 5% (v/v) of solution B (described above), produces earlier elution of normal DNA bases, that begin to elute near the 2.0 min point after injection (Figs. 1, 2 and 3).

A very short gradient of ammonium formate (from 5% to 7%) of solution B as described above, nicely separates most of the peaks during the run (Fig. 1). Fig. 1a, 1b and 1c show the elution profiles of the standard O6MG, digested DNA alone and a mix of standard and digested DNA, respectively. Normal DNA bases appear before 7.0 min after injection. The adduct O6MG elutes last at the 9.0 minutes point. A slight shift to the right is observed in the elution time of the standard in the mixture. It is our experience that the time elapsed between the last two peaks (including the standard) is consistently reproduced using samples of different concentrations or even after the column has been used repeatedly.

3.2. HPLC with electrochemical detection

Fig. 2 shows the calibration curve for standard O6MG measured in the electrochemical detector using a low concentration range (0 to 15 pg total).

Using a 'screen mode' of operation voltages of +500 mV and +600 mV were applied to the analytical cell in channel A and B, respectively. This configuration ensures both a good signal for O6MG and a good baseline at the same time. The small difference in the voltage applied to the channels A and B (100 mV) increases selectivity because it operates just in the range in which the product of interest is electrochemically active. This reduces the possibility that other contaminants will change redox state resulting in additional signal to the detector. The potential (+850 mV) applied to the guard cell located before the injector, oxidizes any contaminant in the mobile phase improving the baseline.

The chromatogram for 10 pg of standard is shown in Fig. 3a. The arrow indicates the change in sensitivity from 100 nAmp. to 10 nAmp. in channel B of the analytical cell. This change is essential to visualize on the chart recorder the peak corresponding to a low amount of product (pg range). The low salt concentration in the samples, due to the short salt gradient used in the first HPLC, improves the quality of the baseline in the electrochemical detector. This detector is particularly sensitive to changes in the salt concentration as is observed in Fig. 3b and 3c. The residual salt increases the baseline during the first 16 min of the analysis but it is markedly lower at the time of O6MG elution.

4. Conclusions

A separation and quantitation system for O^6-methylguanine have been developed. The method described is suitable for the study of DNA damage produced by methylating agents on cells under tissue culture conditions. This system offers improvements in several aspects of the analysis of O6MG. The advantages of this method are summarized as follows: (a) Flow and gradient conditions in the first HPLC system increase the capability of the method to separate O6MG from other DNA bases; (b) The use of a short salt gradient during separation in the first HPLC system results in fractions containing low amounts of ammonium salt. This condition is the main determinant for the stability of the base line of the electrochemical detector in the second HPLC step; (c) The column used in the second HPLC system, a *hydrophilic* C18, increases the retention time of O6MG without reducing the sharpness of the peak. This change allows a significant amount of mobile phase to pass through the column eliminating contaminants that otherwise could co-elute with the fraction of interest.

Acknowledgements

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