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Note

Measurement of methylated DNA purines by reversed-phase high-performance liquid chromatography and fluorimetric detection

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DNA covalent binding is considered one of the critical events in chemically-induced mutagenesis and carcinogenesis[1]. Studies on the mode of action of alkylating agents have stimulated the development of analytical techniques for the measurement of specific DNA adducts in target tissues.

Mainly two column liquid chromatographic methods have emerged in recent years for the quantitative analysis of chemically-modified bases. One involves the use of reversed-phase high-performance liquid chromatography (RP-HPLC) followed by the monitoring of ultraviolet absorption, whereas the other is based on HPLC by cation-exchange columns and peak detection by fluorescence spectrometry[2–10]. Whereas some reversed-phase systems are time-consuming and require the use of complicated gradients or two columns[2,5], others have been simplified so that separation of normal and modified bases is achieved under isocratic conditions[3,4]. However, the detection method based on ultraviolet absorption spectrometry does not provide the adequate sensitivity for the optical determination of adducts at picomol levels, which is desirable in chemical carcinogenesis studies, and radiolabelled carcinogens must be used to overcome this problem.

The introduction by Herron and Shank[6] of fluorimetric detection allowed a 60-fold increase in sensitivity thus offering the advantage that methylated bases could be measured by an entirely optical system, without requiring the use of radiolabelled carcinogens and liquid scintillation counting. Compared to the ion-exchange columns used in those studies, however, reversed-phase columns are easier to equilibrate and offer a higher degree of reproducibility in resolution and retention volumes[11].

An analytical technique is described here, in which reversed-phase separation and fluorimetric detection are combined together. This method was succesfully applied to the measurement of methylated purines in the DNA of rat liver and colon after 1,2-dimethylhydrazine[12].

MATERIALS AND METHODS

Authentic markers for HPLC included adenine, 3-methyladenine, guanine, 7-

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methylguanine (7MeG) (Sigma, Poole, U.K.) and 06-methylguanine (06MeG) (kindly supplied by Dr. P. F. Swann, Middlesex Hospital Medical School, London, U.K.). Calf thymus DNA was from Sigma and 1,2-dimethylhydrazine · 2HCl (DMH) was from Aldrich (Gillingham, U.K.). All other reagents were of analytical- or HPLC-grade and were purchased either from Sigma or BDH (Poole, U.K.). The markers were dissolved in 0.1 *M* hydrochloric acid whereas calf-thymus DNA was hydrolysed in 0.1 *M* hydrochloric acid at 70°C for 40 min in order to release free purines. DNA from the livers of rats treated with DMH (20 mg/kg, subcutaneously) was isolated by phenol extraction according to the modified Kirby procedure[13], purified as previously described[12] and hydrolysed in a volume of 0.1 *M* hydrochloric acid to obtain a concentration of 5–6 mg DNA/ml. Approximately 100 μg of DNA were injected onto the column. All samples were filtered through 0.45 μm Millipore filters before use.

The chromatographic system consisted of a Waters model 6000A pump, a Rheodyne injection valve with a 20 μl loop and a Waters μBondapak C_{18} column, 30 cm x 3.9 mm I.D. (10 μm). A pre-column with the same packing was also used.

The detection apparatus included a Perkin Elmer LS-4 fluorescence spectrometer equipped with a 4 μl flow cell. Peaks were integrated by a Perkin Elmer LCI-100 computing integrator.

The purines were eluted with 10 m*M* ammonium formate (pH 3.8) containing 10% of methanol at a flow-rate of 1 ml/min at room temperature and the fluorescence was monitored at 286 nm excitation and 366 nm emission wavelength.

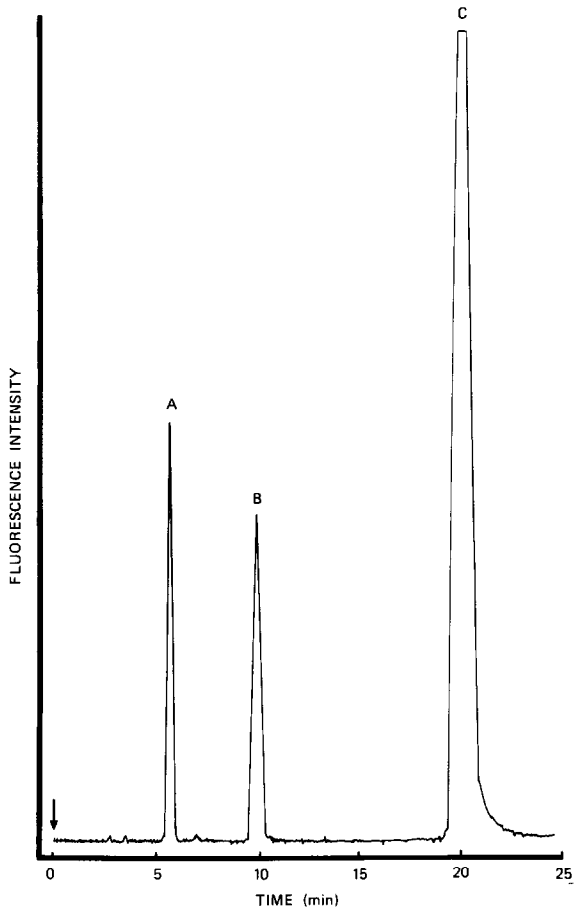
Calibration curves (concentration *versus* peak area) were made with known concentrations of guanine, 7MeG and 06MeG. An external standard method was used for the integration of unknown peaks.

RESULTS AND DISCUSSION

Under the chromatographic conditions used in this study, 3-methyladenine eluted at 4 min, guanine at 5.5–6.1 min, adenine at 7.6–8.3 min, 7MeG at 9.6–10.4 min, and 06MeG at 19–20 min. In this way five bases of different polarity could be eluted in less than 25 min under isocratic conditions. The retention times remained constant after repeated use of the same column over a long period of time, or with different types of C_{18} reversed-phase-columns, such as Spherisorb ODS or Novapak C_{18}. In order to optimise reproducibility of the results, great care had to be taken in the preparation of the mobile phase. In fact, variations in pH (0.1–0.3 units) or in the amount of methanol (1–10%) resulted in a loss of resolution between guanine and adenine or adenine and 7MeG.

Excellent resolution was obtained between guanine, 7MeG and 06MeG as shown in Fig. 1. The standard mixture contained 2.4 nmol of guanine, 1.9 nmol of 7MeG and about 1 nmol of 06MeG. The larger peak corresponding to 06MeG was due to the higher fluorescence of this molecule as compared to the other purines. Although a lower pH increased the fluorescence response of 06MeG, it failed to resolve guanine from adenine.

Limits of detection were 40 pmol for 7MeG and 4 pmol for 06MeG, which are in agreement with those published in a previous work[6]. The fluorescence response was linear for guanine from 0.6 to 60 nmol, for 7MeG from 40 pmol to 4 nmol and for 06MeG from 4 to 700 pmol. This linearity in the fluorescence response together with



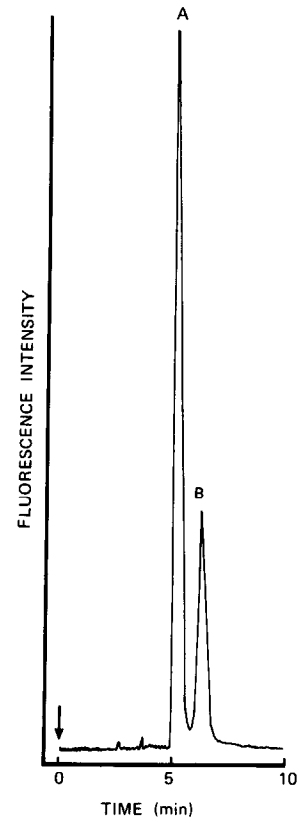


Fig. 1. Elution profile of a standard mixture containing guanine (A), 7MeG (B) and 06MeG (C). Column: 30 cm x 3.9 mm I.D., μBondapak C_{18}. Mobile phase consisted of 10 m*M* ammonium formate (pH 3.8) containing 10% methanol at a flow-rate of 1 ml/min. The peaks were monitored by fluorescence detection with the excitation wavelength set at 286 nm and the emission at 366 nm.

Fig. 2. Elution of a DNA hydrolysate from calf-thymus. DNA was hydrolysed in 0.1 *M* hydrochloric acid at 70°C for 40 min. The two peaks correspond to guanine (A) and adenine (B). For chromatographic conditions see Fig. 1.

good resolution was particularly important for the quantitation of guanine and 06MeG in DNA hydrolysates from animal tissues. With such a system it was actually possible to detect and integrate very low amounts of 06MeG and 1000-fold larger amounts of guanine in the same hydrolysate without any further sample manipulation, re-chromatography or use of two different detection methods, as described by other workers[10,14]. Examples of elution profiles of hydrolysates either from calf-thymus DNA or rat liver DNA after DMH-treatment are given in Figs. 2 and 3 respectively. Normal and methylated bases were completely resolved and retention times were well in agreement with those of the corresponding authentic markers.

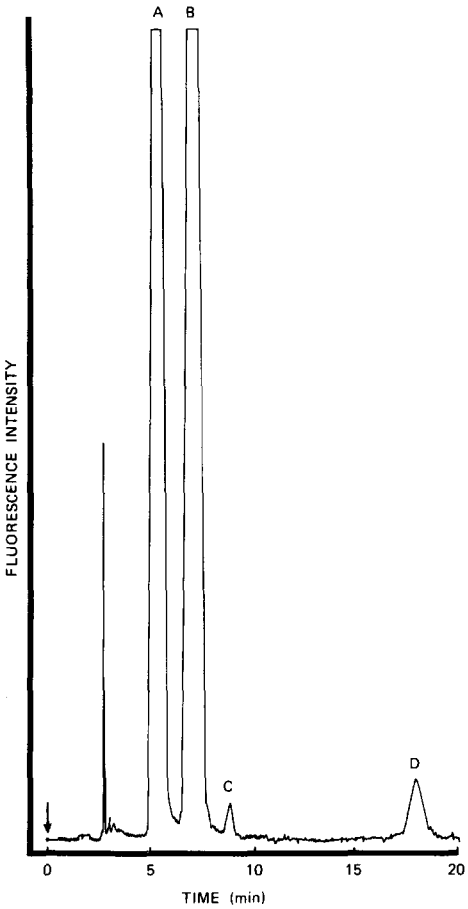


Fig. 3. Elution profile of a liver DNA hydrolysate from a rat treated with DMH (20 mg/kg, subcutaneously) and killed 6 h later. DNA was isolated by phenol extraction[13] and hydrolysed in 0.1 *M* hydrochloric acid at 70°C for 40 min. Peaks: (A) guanine; (B) adenine; (C) 7MeG; (D) 06MeG. Details on the chromatographic conditions are given in Fig. 1.

Peaks A and D in Fig. 3 represent 48 nmol of guanine and 49 pmol of 06MeG respectively. Linear regression analysis of peak area *versus* purine concentration in a series of DNA samples gave a correlation coefficient of 0.989 for guanine ($n=14$), 0.991 for 7MeG ($n=22$) and 0.998 for 06MeG ($n=32$). Because of their weaker polarity as compared to free purines, pyrimidine oligonucleotides did not elute within 25 min under the chromatographic conditions used here and were washed off the column after each run with 90–100% methanol.

In summary, the combination of reversed-phase chromatography and fluorimetric detection described here resulted in a rapid, efficient and sensitive analytical procedure for the determination of chemically-modified DNA purines.

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