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Note

Improved method for determination of 5-methylcytosine by ion-pair reversed-phase high-performance liquid chromatography

L. CITTI*, P. G. GERVASI, G. TURCHI and L. MARIANI
Institute of Mutagenesis and Differentiation of CNR, Via Svezia 10, 56100 Pisa (Italy)
and
M. DURANTE
Institute of Genetics, University of Pisa, Via Matteotti 1/A, 56100 Pisa (Italy)
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Increasing interest in the 5-methylcytosine content of the DNA of prokaryotic and eukaryotic cells has resulted from reports indicating that it may be involved in the control of DNA replication, gene control and DNA repair[1–4]. High-performance liquid chromatography (HPLC) has been extensively employed in the determination of 5-methylcytosine using mainly strong cation-exchange columns[5]. In addition, "ion-suppression" and "ion-pair" techniques have greatly increased interest in reversed-phase columns, which have advantages over other HPLC modes[6,7]. Recently a method has been described to separate 5-methylcytosine and other DNA bases using an ion-pair reversed-phase technique[8]. The present paper describes an improvement of this method for the 5-methylcytosine determination.

EXPERIMENTAL

Materials

Sodium hexanesulphonate (SHS) was obtained from Fluka (Buchs, Switzerland); standard bases of DNA and 5-methylcytosine were purchased from various commercial sources (Boehringer, Mannheim, G.F.R.; Fluka, Buchs, Switzerland; Sigma, MO, U.S.A.). All other reagents were of analytical-reagent grade. Freshly distilled water was employed. Buffers and samples to be used in the HPLC analysis were filtered immediately before use.

Cellulose filters (0.45 μm) were purchased from Sartorius (Göttingen, G.F.R.).

A pre-packed Hybar column (250 × 4 mm I.D.) containing reversed-phase LiChrosorb RP-8 (7-μm particles) was obtained from Merck (Darmstadt, G.F.R.).

Apparatus

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 3 liquid chromatograph equipped with an LC-75 UV–visible spectrophotometric detector with a programmable autocontroller, and an M-2-calculating integrator was employed.

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DNA isolation and hydrolysis

DNA was extracted as described previously[9]. Purification of the extracts was carried out by two ethidium bromide preparative ultracentrifugations. To the DNA solutions, caesium chloride (final density 1.5873 g/ml) and ethidium bromide (200 μ/ml) were added. Ultracentrifugation was performed in a Beckman 65 Ti rotor at 50,000 rpm for 24 h. The ethidium bromide–DNA complex was collected; after the removal of ethidium bromide with isoamyl alcohol, the DNA was recovered by ethanol precipitation.

Hydrolysis of DNA (10 μg) was performed in a sealed glass vial with 100 μl of 88% formic acid at 175°C for 30 min. The hydrolysates were then lyophilized and stored at 4°C. Samples for HPLC analysis were prepared by dissolving the lyophilized residues in 0.1 *N* hydrochloric acid; the resulting solutions were neutralized at about pH 5 with 1 *N* potassium hydroxide solution and filtered through a 0.45-μm cellulose filter.

HPLC conditions

Samples were loaded on the C_8 reversed-phase column equilibrated at 1 ml/min (about 85 atm. pressure) with a mixture of 1% of 70% methanol and 99% of 20 m*M* potassium phosphate buffer containing 5 m*M* SHS, pH 5.4.

Chromatographic runs were performed isocratically at the starting conditions for 12 min, then with a linear gradient up to 20% of eluent in 13 min. The column was re-equilibrated for at least 15 min (about 5 column volumes) between two different runs. Using this procedure, the retention times were reproducible.

The molar percentages of the bases were determined from the areas of the peaks in the elution pattern compared with the areas on calibration graphs obtained using pure standards.

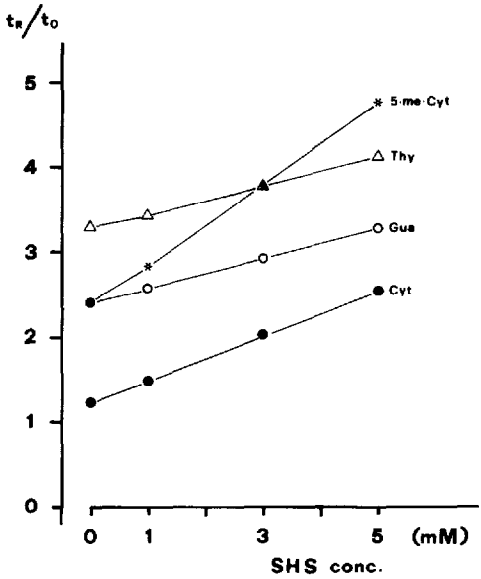

Fig. 1. Variation of retention times of four isocratically eluted bases as a function of SHS concentration. The dead time (t_0) value estimated for these chromatographic conditions was 2.4 min.

TABLE I

RELATIONSHIP BETWEEN $\Delta t_R/t_0$ AND pK_a VALUES FOR DNA BASES

t_0 = dead time.

Base	$\Delta t_R/t_0$	pK_a*
5-Methylcytosine	2.33	4.6
Cytosine	1.29	4.45
Guanine	0.87	3.2
Thymine	0.80	–

* See ref. 10.

RESULTS AND DISCUSSION

The ion-suppression technique, in our reversed-phase column, was unable to separate the five bases of DNA; in fact, in the pH range 4.8–6.5, the retention times (t_R) of guanine and 5-methylcytosine were similar, and 5-methylcytosine gave a very broad tailing peak under more acidic conditions. In contrast, the ion-pairing technique was very effective with the C_8 column. Using 20 mM phosphate buffer at a

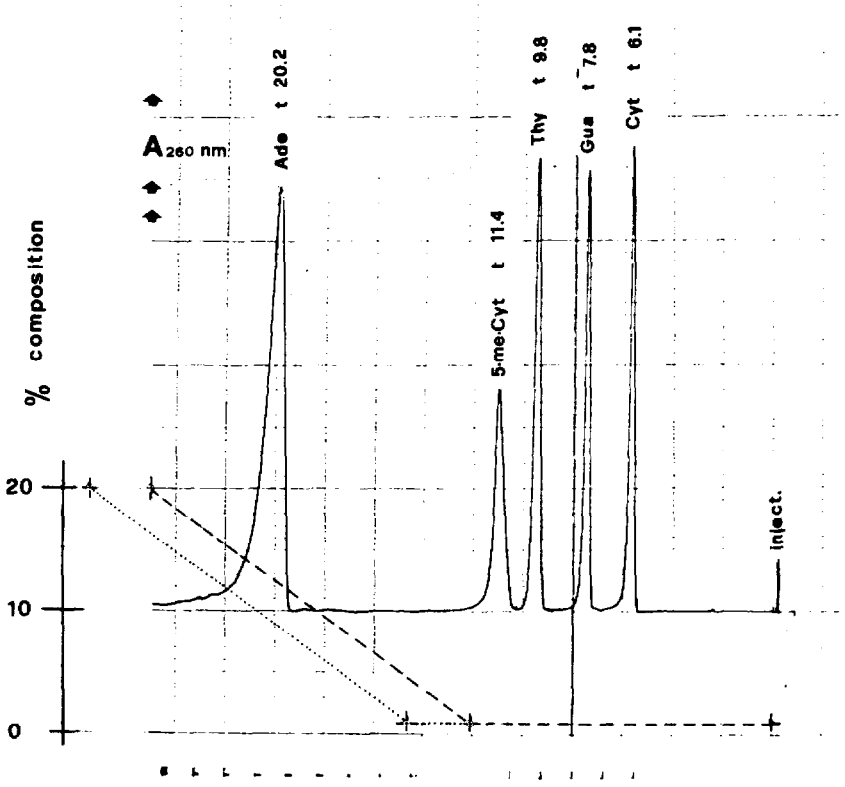

Fig. 2. Chromatographic pattern of standard bases monitored at 260 nm. The run is from right to left. Retention times (t) are in minutes. The dashed line represents the inlet percentage composition of dilute methanol in the eluent and the dotted line represents the outlet composition.

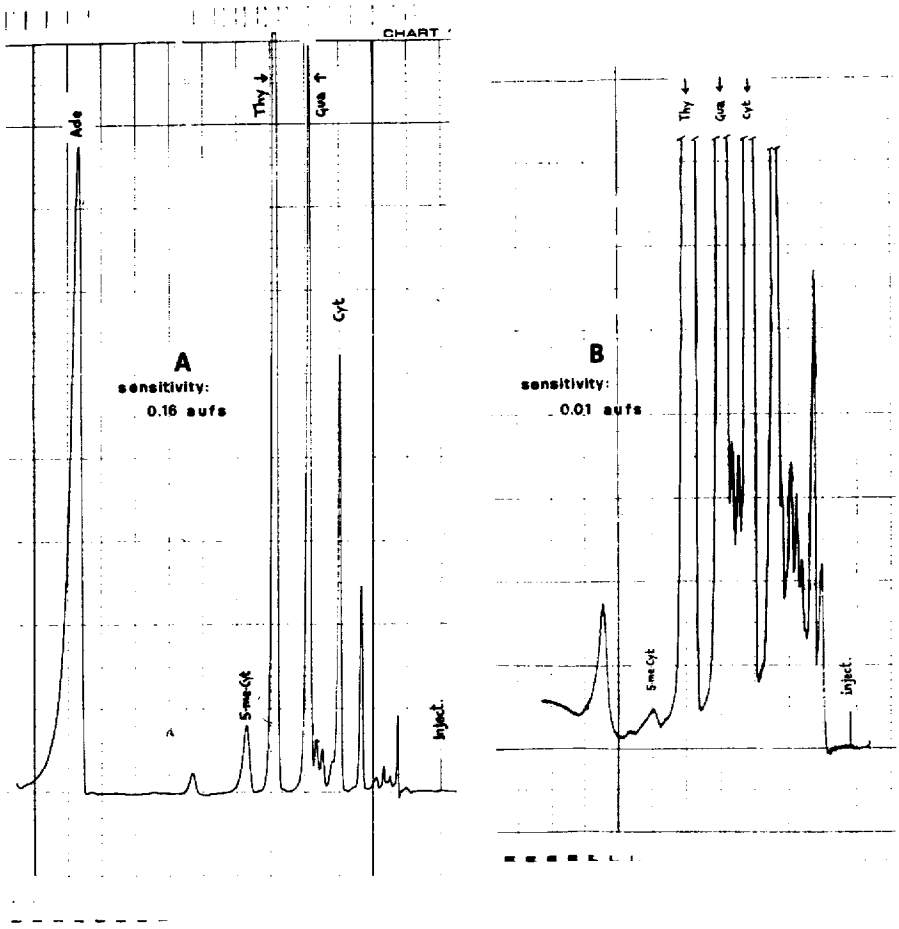

Fig. 3. Chromatographic runs on authentic DNA hydrolysates. (A) Analysis of a mixture from about 11.4 μg of *Nicotiana* DNA; (B) analysis of a mixture from about 6.4 μg of V79 cells DNA.

fixed pH of 5.4, we obtained an almost linear dependency between the retention times of four bases and the ion-pairing reagent concentration, as shown in Fig. 1. The main effect was for 5-methylcytosine and cytosine according to their pK_a values. Table I shows the relationship between the $\Delta t_R/t_0$ values (calculated between 5 and 0 m*M* SHS) and the pK_a values of the bases.

Using a 5 m*M* concentration of SHS ion-pair reagent, we obtained the separation shown in Fig. 2. The linear gradient step is necessary to decrease the retention time and increase the sharpness of the adenine peak.

The method was tested with various DNA samples. The 5-methylcytosine contents obtained for DNA extracted from the amphidiploid hybrid *Nicotiana glauca* × *Nicotiana langsdorffii* and from V79 Chinese hamster cells were 33.47 ± 0.5% and 1.42 ± 0.2% of the total cytosine, respectively. The chromatograms are depicted in Fig. 3.

The sensitivity of the method was high. We obtained good resolutions for very

small samples of down to about $4 \cdot 10^{-11}$ mol of 5-methylcytosine injected (about 1.28 μg of DNA hydrolysate from V79 Chinese hamster cells).

In conclusion, this method improves the original technique described by Ehrlich and Ehrlich[8] (which needs two different chromatographic runs), giving in the same chromatographic run a high resolution of all of the DNA bases tested. Moreover, the retention times are short enough, the separation of cytosine and guanine is better and the starting chromatographic conditions are quickly restored between two different runs.

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