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Note

Improved fluorometric method for quantitative high-performance liquid chromatographic analysis of methylated guanine derivatives in DNA

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The initiation of cancer by chemical carcinogens such as the N-nitrosoalkylating agents is thought to be related to their capacity to damage cellular DNA[1–4]. Factors which may be critical in the genesis of cancer by such compounds include the specific positions which are alkylated in DNA bases, the relative proportions of the lesions and the persistence of alkylated products in DNA following carcinogen exposure[3,5,6]. Determination of picomole quantities of carcinogen-DNA adducts is thus quite important in studies designed to examine mechanisms operative in carcinogenesis induced by alkylating agents or the effect of potential modifiers of this process.

The most rapid and accurate method routinely available for the quantitation of small amounts of methylated purines in DNA is high-performance liquid chromatography (HPLC), in which reversed-phase or cation-exchange techniques are utilized[7–9]. As compared with the cation-exchange procedure developed by Herron and Shank[7], a reversed-phase system developed for separation and quantitation of methylated bases is relatively complicated and time-consuming, requiring an intermediate sample extraction and two separate chromatographic steps[8]. Superior resolution of normal and methylated bases is not obtained and, when ultraviolet absorbance spectroscopic detection is used, the latter procedure is not as sensitive. In fact, fluorescence detection is not compatible with the latter procedure because the predominantly organic, neutral mobile phase affects molecular fluorescence and fluorometer response[8].

However, in our laboratory, difficulties have been encountered in attempts to obtain adequately reproducible results with the procedure developed by Herron and Shank[7]. In most cases, inadequate separation of normal and methylated bases was observed. Thus, an improved procedure for separating and quantitating methylated purines was developed and applied for the analysis of hydrolysates of *in vitro* alkylated calf thymus DNA, and liver DNA derived from rats treated with radioinert

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N-methyl-N-nitrosurea (NMU), a direct-acting alkylating agent. As reported herein, the method utilizes cation-exchange HPLC and fluorescence detection. At the same level of sensitivity previously reported[7], better resolution of normal and methylated bases is obtained.

EXPERIMENTAL

Instrumentation

A Waters HPLC system comprised of a Model M720 solvent delivery system equipped with Model M6000A and Model M45 pumps, a WISP 710B automatic sample injector and Model M730 data reduction and documentation module was used. Fluorescence was monitored using a Gilson Medical Electronics (Middleton, WI, U.S.A.) Model 121 on-line fluorescence detector equipped with a 280-nm wavelength excitation power source, lamp and filter. The colored-glass emission filter was from Corning, with a 350–470 nm passband.

Column

The column used (200 × 4.0 mm I.D.) was packed with silica-based strong cation-exchange resin (Nucleosil 5-SA, 5.0 μm particle size) by the manufacturer (Macherey-Nagel), and purchased from Rainin Instrument (Woburn, MA, U.S.A.). Throughout the study, a Waters inline filter and disk were used to protect the column from particulate matter. The filter and disk were periodically cleared by sonication in 6.0 *M* nitric acid and rinsing in filtered HPLC grade water.

Chemicals and standards

Calf thymus DNA, NMU, bovine pancreatic RNase, 7-methylguanine, adenine and guanine were purchased from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) and ammonium dihydrogen phosphate (analytical reagent grade) were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). O^6-Methylguanine was supplied through the courtesy of Dr. A. E. Pegg. All other reagents were analytical reagent grade and purchased from Sigma.

Preparation of standards and alkylated DNA

All standard bases were prepared by dissolution in 0.1 *M* hydrochloric acid, quantitated by absorption spectroscopy and diluted to the desired concentrations in 0.1 *M* hydrochloric acid. They were subsequently stored at 4°C and remained stable for weeks. Calf thymus DNA (3.0 mg/ml) was alkylated with radioinert NMU (12 m*M* in 80 m*M* sodium phosphate buffer, pH 7.4) by incubation at 37°C for 60 min. Following incubation, alkylated DNA was precipitated by the addition of 3–4 volumes of absolute ethanol (0–4°C) and washed twice with additional aliquots of cold ethanol. As described previously[10], the DNA was hydrolyzed with 0.1 *M* hydrochloric acid at 37°C.

Carcinogen treatment, DNA isolation and hydrolysis

NMU was dissolved in 0.9% sodium chloride (pH 5.0, glacial acetic acid) and used immediately. Groups of 50-day old ovariectomized, virgin female Sprague-Dawley rats (Harlan, Madison, WI, U.S.A.) individually received a 50 mg/kg body wt.

dose of NMU via the jugular vein under light ether anesthesia. Rats were killed by carbon dioxide asphyxiation 1–48 h after exposure to the carcinogen, and the livers were removed. DNA was isolated by the method of Margison *et al.*[11], and hydrolyzed[10] in 0.1 *M* hydrochloric acid at 37°C. Quantitation of DNA was by measurement of absorbance at 260 nm.

Chromatography

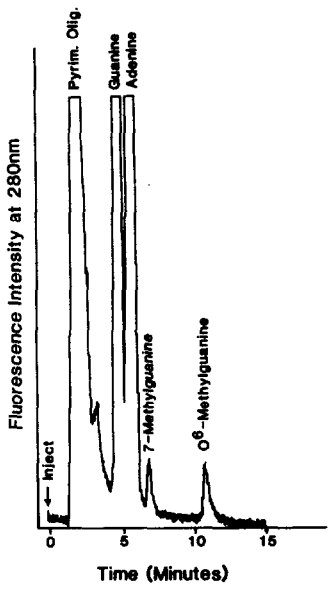
The buffer system consisted of ammonium dihydrogen phosphate (pH 2.5, hydrochloric acid), ranging from 0.4–0.9 *M*, depending on the efficiency of the individual columns used. With columns of higher theoretical plate count, more concentrated buffer was required for elution, compared to columns of lower plate counts. At lower buffer concentrations, the addition of methanol to 5% (v/v) concentration adequately sharpened individual peaks without significantly altering retention times. Samples were eluted at a flow-rate of 0.7–1.0 ml/min at ambient temperature. The column pressure was stable, usually between 2400–2500 p.s.i. Standard curves for the methylated bases were prepared by injecting known quantities dissolved in 0.1 *M* hydrochloric acid. Sample solutions of 50–150 μl containing 100–350 μg of hydrolyzed DNA were commonly injected, although occasionally it was necessary to inject more of a particular sample that was methylated to a lesser extent.

RESULTS AND DISCUSSION

Separation of the purine bases in the present system resulted in retention times of 4.3–4.9 min for guanine, 5.2–5.8 min for adenine, 6.9–7.1 min for 7-methylguanine and 10.6–11.1 min for O^6-methylguanine at 0.7 ml/min. As shown by a profile of alkylated calf thymus DNA hydrolysate (Fig. 1), resolution of the 7-methylguanine peak from the quantitatively much larger adenine peak is nearly complete. In this system, the 7-methylguanine peak is considered to be baseline resolved and fully integrable, although calculation of peak quantities may also be done by peak height measurements and external standardization.

To more thoroughly evaluate the utility of the HPLC system, a time-course experiment was performed in which rats were sacrificed at discrete intervals after NMU administration, and the alkylation of liver DNA was examined. Fig. 2 illustrates a typical chromatogram of liver DNA obtained from rats treated with NMU. Again, the resolution of the 7-methylguanine peak from the adenine peak is nearly complete and the O^6-methylguanine peak is suitable for electronic integration. As summarized in Table I, utilizing peak height measurements which were precise to 10–15%, we were able to detect proportionally accurate[3,4] amounts of methylated adducts in the picomole range, well above the limits of detection, which were about 10–12 pmol of 7-methylguanine, and 2–3 pmol of O^6-methylguanine. These limits of detection compare very favorably with those previously reported using a similar system[7] (about 40 pmol of 7-methylguanine and about 1 pmol of O^6-methylguanine), and are well below those obtainable using ultraviolet absorbance for spectroscopic detection[8].

Thus, it is apparent that utilization of a column containing 5-μm particles with the chromatographic conditions described herein yields superior resolution as compared with previous reports in which alkylated purines have been quantitated by



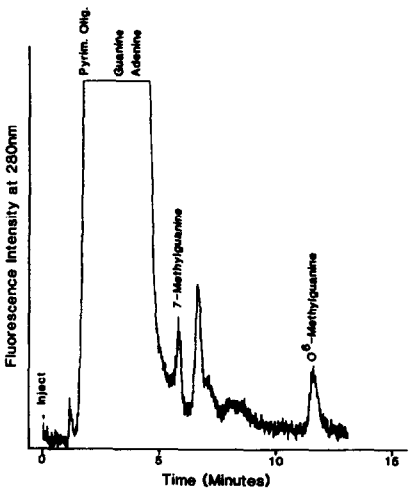


Fig. 1. Elution profile of calf thymus DNA hydrolysate alkylated *in vitro* with NMU. Column conditions: 0.4 *M* ammonium phosphate, 5% methanol, pH 2.5; flow-rate, 0.7 ml/min; ambient temperature. Pyrim. Olig. = pyrimidine oligonucleotide. (Redrawn from original.)

Fig. 2. Elution profile of rat liver DNA hydrolysate from rats treated with 50 mg/kg NMU and sacrificed 1 h post-carcinogen. Column conditions: 0.9 *M* ammonium phosphate, pH 2.5; flow-rate, 1.0 ml/min; ambient temperature. Pyrim Olig. = pyrimidine oligonucleotide. (Redrawn from original.)

TABLE I

QUANTITATION OF METHYLATED BASES IN RAT LIVER DNA FOLLOWING NMU ADMINISTRATION

Sixteen 50-day old ovariectomized, virgin female rats each received a single, 50 mg/kg body wt. dose of NMU via the jugular vein under light diethyl ether anesthesia. Groups of four rats were sacrified at the indicated times, the livers were removed and pooled, and DNA was isolated and hydrolyzed[10,11]. Samples of hydrolysates were separated and quantitated by cation-exchange HPLC as described in the Experimental section. Each value represents the mean of 2–3 determinations ± S.D.

Time of sacrifice post-NMU (h)	*7-methylguanine*		*O⁶-methylguanine*	
	pmol/mg DNA	*μmol/mol G*	*pmol/mg DNA*	*μmol/mol G*
1	509.3 ± 9.4	354.4 ± 6.6	49.6 ± 1.3	35.0 ± 0.9
8	395.4 ± 11.4	278.8 ± 8.0	36.6 ± 3.0	25.8 ± 2.2
24	360.2 ± 23.4	254.0 ± 16.4	17.8 ± 2.8	12.5 ± 2.0
48	290.2 ± 25.2	204.6 ± 17.7	14.1 ± 0.7	10.0 ± 0.5

cation-exchange HPLC. An additional advantage of the procedure is that sufficient resolution is obtained at ambient temperature. Although comparable separation has been achieved by other HPLC procedures[8], cation-exchange HPLC is less complicated and requires less time. However, as a consequence of using a high-capacity

cation-exchange resin, in contrast to the method of Herron and Shank, which utilizes a 10-μm particle size column of much lower capacity and a higher flow-rate[7], buffer of high ionic strength is required for elution of the samples and reduction of the retention times (especially of the O^6-methylguanine peak) to what may be considered reasonable for multiple analyses. As a result, it was compulsory to follow strict maintenance procedures for the HPLC system, including thorough flushing of the column and HPLC system with water followed by methanol after completion of a series of analyses, and periodic flushing of the system with 6.0 *M* nitric acid.

In summary, we report here an improved quantitative procedure for analysis of radioinert methylated purines by cation-exchange HPLC, using fluorescence detection. As compared with previous reports in the literature, the present method gives better resolution of methylated purines and is equally sensitive.

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REFERENCES

1 P. D. Lawley, *Mutat. Res.*, 23 (1974) 283.
2 P. D. Lawley, *ACS Monogr.*, 173 (1976) 83.
3 A. E. Pegg, *Adv. Cancer Res.*, 25 (1977) 195.
4 B. Singer and J. T. Kusmierek, *Annu. Rev. Biochem.*, 52 (1982) 655.
5 R. Goth and M. F. Rajewsky, *Proc. Nat. Acad. Sci. U.S.*, 71 (1974) 639.
6 B. Singer, *J. Nat. Cancer Inst.*, 62 (1979) 1329.
7 D. C. Herron and R. C. Shank, *Anal. Biochem.*, 100 (1979) 58.
8 J. F. Lawrence, R. Leduc and J. J. Ryan, *Anal. Biochem.*, 116 (1981) 433.
9 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 226 (1981) 267.
10 A. E. Pegg, W. Perry and R. A. Bennett, *Biochem. J.*, 197 (1981) 195.
11 G. P. Margison, J. M. Margison and R. Montesano, *Biochem. J.*, 165 (1977) 463.