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DETERMINATION OF TRIMETHYLSILYL METHYLATED NUCLEIC ACID BASES IN URINE BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A method for the determination of the urinary excretion level of methylated nucleic acid bases by gas–liquid chromatography (GLC) has been developed. A clean-up procedure prior to GLC analysis consisted of hydrolysis, filtration, charcoal adsorption, and anion exchange. Studies to determine optimum derivatization conditions for conversion of the methylated bases to their trimethylsilyl derivatives and to evaluate GLC parameters and columns to obtain the best separation were conducted. Application of the method was shown by determining the excretion levels of methylated bases in urine of normal controls and of patients with various types of malignancy.

INTRODUCTION

Biomedical interest in the methylated nucleic acid bases has been stimulated by reports of elevated levels of the methylated bases in malignant tumors[1–9], compared with normal control tissues[1,2,4,5,10]. These methylated bases, which are found as minor constituents of transfer ribonucleic acid (tRNA)[11–15] and to a lesser extent in ribosomal RNA[16], result from the modification of tRNA by specific tRNA methylases at the enzymatic level[17–20]. Abnormally high levels of tRNA methylase activity has been noted by Borek[12] in studies of thirty different malignant tumors compared with their corresponding normal tissues.

Although methylated nucleic acid bases have been shown to be normal excre-

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tion products in human urine as a result of the metabolic degradation of tRNA[15]. Borek[12] noted elevated excretion levels for methylated bases by patients with malignant tumors. Weissmann *et al.*[9], Adams *et al.*[1], Fink *et al.*[4,5], and Park *et al.*[10], have shown that increased methylated base excretion occurs in patients with acute leukemia.

To establish if a relationship between the levels of methylated bases in urine and neoplastic growth exists and to determine if the measurement of these compounds might serve as either a diagnostic method or for monitoring the effects of treatments, an accurate, sensitive, and convenient method of analysis is necessary. Methods for the analysis of methylated bases in urine have been developed by Weissmann *et al.*[21], Adams *et al.*[1], and by Fink *et al.*[5] and consist of ion-exchange chromatography for sample cleanup, paper chromatography for separation, and ultraviolet (UV) spectroscopy for detection. A number of methylated bases in normal and cancer-patient urine have been detected and semi-quantitatively analyzed using these methods; however, these procedures lacked specificity and sensitivity, and were too time consuming for extensive clinical studies. High-pressure anion-exchange chromatography has been used by Waalkes *et al.*[22] for the analysis of minor nucleosides and bases in urine and gave quantitative results, but the method does not lend itself to the frequent rapid analyses for clinical studies.

Gas–liquid chromatographic (GLC) methods for the analysis of the major nucleic acid bases have been reported by Lakings and Gehrke[23,24], and the analysis of minor nucleosides by GLC has been developed by Chang *et al.*[25]. GLC, because of its sensitivity, specificity, and speed of analysis, presents a quantitative analytical tool with the ability of rapid, frequent analyses necessary for a study of the excretion levels of the methylated bases in urine. This investigation describes the development of such a procedure

EXPERIMENTAL

Apparatus

A Model 402 biochemical gas chromatograph (F & M. Division of Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with dual electrometers, dual hydrogen flame detectors, and an Electronix 16 dual pen strip chart recorder was used in this study.

For elevated temperature reactions, a sand-bath with a variable temperature control ($\pm 2°$) was used.

Standard calibration solutions of the methylated bases, prepared from stock solutions, and samples were dried on a hot plate (75°) under a stream of pure nitrogen gas.

Glass ion-exchange columns (Fisher and Porter, Warminster, Pa., U.S.A.) used for sample cleanup were 5 × 150 mm with a 50-ml reservoir, 9 × 150 mm, and 15 × 150 mm.

Reagents

The methylated purine and pyrimidine bases (MeB) were obtained from Cyclo (Los Angeles, Calif., U.S.A.): 1-methyladenine (1-MeAde), 2-methyladenine (2-

MeAde), 1-methylhypoxanthine (1-MeHypo). N^2,N^2-dimethylguanine (N^2,N^2-DiMeGua), N^2-methylguanine (N^2-MeGua), 1-methylguanine (1-MeGua), and 7-methylguanine (7-MeGua); from Mann Research Labs. (New York, N.Y., U.S.A.): thymine (Thy), 5-methylcytosine (5-MeCyt), N^6,N^6-dimethyladenine (N^6,N^6-DiMeAde), and N^6-methyladenine (N^6-MeAde); and from Sigma (St. Louis, Mo., U.S.A.): N^6-(Δ^2-isopentenyl)adenine (N^6-Δ^2-IsoAde). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Regis (Chicago, Ill., U.S.A.) in PTFE-lined screw-cap vials. Acetonitrile and dichloroethane were of "nanograde" purity and were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.) as were formic acid, acetic acid, and pyridine. The charcoal (80–100 mesh, Cat. No. 926043) was obtained from Regis. The AG 1 X 2 anion-exchange resin was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.) The water was prepared by passing distilled water through a water demineralizer (Ion Exchange Products, Chicago, Ill., U.S.A.). The other reagents and materials used were of the highest purity available.

Chromatographic conditions

The chromatographic column and instrumental settings described earlier[26] were used in the initial part of this study. Also, the optimum silylating conditions and solvent developed for the major bases[26] were used. Initial studies showed that the methylated bases were silylated by BSTFA and were chromatographically stable. However, some MeB gave multiple peaks with the BSTFA–acetonitrile mixture, and complete separation was not obtained on the 1.0 m × 4 mm I.D. column (10% SE-30 on Supelcoport, 100–120 mesh).

To determine the optimum silylating conditions for the MeB that would give single reproducible peaks, an evaluation of acetonitrile, dichloroethane, hexane, and benzene as the silylation solvent at temperatures of 100° and 150° and for 15 and 30 min was conducted. To obtain the best separation of the MeB, the following columns were studied: 3, 5, and 10% SE-30; 3 and 5% OV-3; and 3 and 5% OV-7 on 100–120 mesh Supelcoport.

Cleanup studies

Previous reports[1,10] have shown that the excretion level of methylated bases in urine was quite low, *i.e.* 1–10 mg per 24-h urine volume. Because of this low excretion level and the presence of many urinary compounds that interfered with the GLC analysis of the MeB, a cleanup step was required. Many methods for sample cleanup were available and included liquid–liquid extraction, column chromatography, anion and cation exchange, and adsorption chromatography. Each of these methods was evaluated independently and in combination with the other procedures to develop a total cleanup method for the analysis of the MeB in urine that gave good recovery results and yet removed most of the interfering compounds from the urine sample.

Analysis of normal and cancer-patient urine

Normal 24-h urine specimens collected without preservative but refrigerated at all times were obtained from laboratory personnel and from the National Cancer Institute, NIH. Cancer-patient 24-h urine specimens were obtained from the National Cancer Institute, NIH. Aliquot samples were frozen and stored at −20° or lower. The normal and cancer subjects were maintained on a regular diet with restricted protein.

Beverages containing caffeine or theophylline were excluded the day before and during the collection period.

Calculation of the excretion levels of the methylated bases were as follows:

$$\mu\text{g MeB per aliquot} = \frac{H_{\text{MeB}}}{H_{\text{I.S.}}} \times \frac{\mu\text{g I.S.}}{RWR_{\text{MeB/I.S.}}}$$

$$RWR_{\text{MeB/I S.}} = \frac{H_{\text{MeB}}}{H_{\text{I S.}}} \times \frac{\mu\text{g I.S.}}{\mu\text{g MeB}}$$

where H_{MeB} and $H_{\text{I.S.}}$ = height of peak of methylated base and internal standard, respectively, and $RWR_{\text{MeB/I.S.}}$ = relative weight response of the methylated base to an internal standard.

To calculate the mg per 24-h urine volume excretion level of the methylated bases:

$$\text{mg MeB per 24-h urine} = \frac{\mu\text{g MeB per aliquot}}{\text{ml aliquot}} \times \frac{\text{24-h urine volume (ml)}}{1000\ (\mu\text{g/mg})}$$

The recovery of the MeB added to urine was determined by analyzing an aliquot of urine and calculating the milligram amount per 24-h urine of each methylated base. Another aliquot of the same sample was then "spiked" with a known amount of the MeB and analyzed.

RESULTS AND DISCUSSION

Chromatographic properties of the methylated bases

The determination of the optimum reaction conditions for converting the MeB to their volatile trimethylsilyl (TMS) derivatives consisted of experiments on silylation solvents and various reaction times and temperatures. Of the solvents evaluated, only dichloroethane and acetonitrile gave good symmetrical peaks for most of the MeB when silylated with BSTFA at 150° for 15 min. Double peaks for some MeB were obtained with acetonitrile as solvent; and dichloroethane, which gave single peaks for all the bases except 5-MeCyt, showed incomplete silylation. One base, 1-MeHypo, did not give a good chromatographic peak under any of the experimental conditions and was therefore not included in further studies. However, dichloroethane–acetonitrile (1:1) as solvent gave good single peaks for all the MeB except 5-MeCyt, which was a doublet.

The reproducibility of silylation and stability of the derivatives were determined by analyzing standard mixtures of the MeB at the optimum silylating conditions (BSTFA–acetonitrile–dichloroethane mixture, 2:1:1, v/v, heated in a closed tube at 150° for 15 min). Four independent samples were used to determine the reproducibility: the average relative molar response ($RMR_{\text{MeB/I S}}$), standard deviation (S.D.), and retention times are presented in Table I. One of the samples used in the reproducibility study was analyzed on the next three days to determine the stability of the derivatives, and these data are presented in Table II.

TABLE I

REPRODUCIBILITY OF DERIVATIZATION AND CHROMATOGRAPHY OF THE TMS METHYLATED BASES

Detector flame ionization detector. Silylation with BSTFA in dichloroethane–acetonitrile (2:1:1, v/v) at 150° for 15 min. Retention times on a 10% SE-30 on Supelcoport (100–120 mesh) column; initial temperature, 90°; programmed at a rate of 7.5°/min. $RMR_{MeB/I.S.}$: four independent determinations, relative molar response to acenaphthene as internal standard; $RMR_{MeB/I.S.}$ = [area MeB/moles MeB]/[area I.S./moles I.S.]. Standard deviation calculated from four independent determinations; average relative S.D. = 3.8%.

Compound	*Retention time*	$RMR_{MeB/I.S.}$	*S.D.*
N^6-MeAde	13.2	0.58	0.024
N^6N^6-DiMeAde	13.0	0.64	0.029
N^6-Δ^2-IsoAde	18.4	0.86	0.025
2-MeAde	16.4	0.57	0 008
N^2-MeGua	17 2	0.58	0.017
N^2,N^2-DiMeGua	18.0	0.83	0 020
7-MeGua	19.6	0 39	0.032
5-MeCyt	9.6–12.0	0.73*	0.024
Thy	6.8	0.63	0.025

* Two peaks were obtained for 5-MeCyt, each was integrated independently then added to obtain total area for *RMR* calculation.

In a later study on the optimum silylation of the five MeB (N^6,N^6-DiMeAde, N^6-MeAde, N^2-MeGua. N^2,N^2-DiMeGua, and 7-MeGua), it was found that the BSTFA–acetonitrile (1:1) mixture heated in a closed vial at 150° for 15 min gave single, reproducible peaks. The $RMR_{MeB/I.S.}$ values for these five bases were the same as shown in Table I. Further, the greater solubility of the MeB and the ease of handling a single solvent made acetonitrile the solvent of choice.

Once reproducible chromatographic peaks had been obtained for the TMS MeB, experiments were conducted to obtain a GLC column that would separate these

TABLE II

STABILITY OF TMS METHYLATED BASES

Samples used in reproducibility study. Detector, flame ionization detector. Samples silylated with BSTFA in dichloroethane–acetonitrile (2:1:1, v/v) at 150° for 15 min. $RMR_{MeB/I.S.}$ = relative molar response to acenaphthene as internal standard; $RMR_{MeB/I.S.}$ = [area MeB/moles MeB]/[area I.S./moles I.S.]. Silylated samples left at room temperature in a closed reaction vial between injections.

Compound	$RMR_{MeB/I.S.}$			
	After 1 day	*After 2 days*	*After 3 days*	*After 4 days*
N^6-MeAde	0.56	0.57	0.59	0.56
N^6N^6-DiMeAde	0.67	0.65	0.65	0.66
N^6-Δ^2-IsoAde	0.90	0.89	0.85	0.87
2-MeAde	0.56	0.57	0.59	0.60
N^2-MeGua	0.59	0.58	0.56	0.58
N^2,N^2-DiMeGua	0.84	0.84	0.81	0.78
7-MeGua	0.43	0.39	0.43	0.41
5-MeCyt	0.73	0.71	0.75	0.70
Thy	0.60	0.62	0.64	0.62

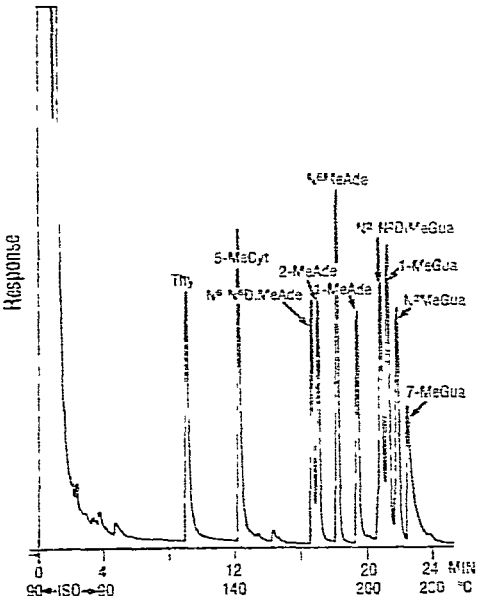

Fig. 1. GLC analysis of methylated bases in a standard mixture. Column: 5% OV-3 on 100–120 mesh Supelcoport, 1.0 m × 4 mm I.D. Sample: 100 μg of each MeB Silylation with 200 μl BSTFA and 200 μl acetonitrile at 150° for 15 min. Chromatographic conditions: volume injected, 4 μl; initial temperature, 90°, initial hold, 4 min; program rate, 7.5°/min, final temperature, 260°; attenuation, 1.3×10^{-9} A.f.s.

compounds. A baseline separation of all the commercially available MeB was not considered essential in the early stages of this research as the MeB composition of urine had not previously been well defined. Of the three liquid phases investigated, only OV-3 gave separation of all the MeB, but not baseline separation. The best chromatograms were obtained with a 1.0 m × 4 mm I.D. glass column packed with 5% OV-3 on 100–120 mesh Supelcoport. A typical chromatogram of ten MeB derivatives is shown in Fig. 1.

Cleanup method development

Each of the cleanup methods evaluated, including liquid–liquid extraction, column chromatography, cation and anion exchange and adsorption chromatography, provided partial cleanup of urine for methylated base analysis. Liquid–liquid extractions removed many urinary compounds from the MeB, but urinary salts remained in the aqueous phase with the MeB. No organic solvents were found that would extract the MeB quantitatively from the urine samples. The MeB were not adsorbed by any of the column chromatographic materials evaluated and were thus separated from urinary compounds that were retained by the adsorbent. However, urinary salts also were not adsorbed and thus not separated from the MeB. Cation- and anion-exchange chromatography (CIE and AIE, respectively) did retain the MeB under proper conditions, but the high level of salts present in urine prevented the use of CIE and AIE as an initial cleanup step. Adsorption chromatography proved to be the most powerful cleanup method for MeB analysis in urine. The MeB were strongly

adsorbed to charcoal, and the urinary salts and many organic compounds were removed from the sample by washing the charcoal with dilute acid or base and organic solvents. The cleanup method which provided the best sample cleanup with the lowest loss of MeB is presented below.

Cleanup method

Hydrolysis. (1) Place 4.0 ml of urine in a 16 × 75 mm Pyrex culture tube and make to 1 *N* in HCl by adding 1.0 ml of 5 *N* HCl. (2) Seal the tube tightly with a PTFE-lined screw cap and heat at 110° for 4.0 h. (3) Cool the sample and add 1.0 ml of 4.0 *N* KOH to partially neutralize the excess HCl.

Filtration. (1) Place the hydrolyzed sample in a 10-ml syringe with a 0.5-μm Solvinert filter (Millipore, Bedford, Mass., U.S.A.) and pass the sample slowly through the filter which retains the particulate matter present after sample hydrolysis. (2) Wash the hydrolysis tube and syringe with two 1-ml washes of 0.1 *N* HCl and add the washes to the previous filtrate.

Charcoal adsorption cleanup. (1) Place the filtered sample directly on a 70 × 5 mm charcoal column (80–100 mesh) and pass it through the column at a flow-rate (*ca.* 0.2 ml/min) slow enough to allow complete adsorption of the methylated bases. (2) Wash the charcoal column with 60 ml of de-ionized water, 30 ml of pyridine–95% ethanol (1:1), 50 ml of de-ionized water, and 25 ml of acetic acid at a flow-rate of *ca.* 1.0 ml/min. (3) Elute the methylated bases from the charcoal with 40 ml of formic acid–acetic acid (1:1) at a flow-rate of *ca.* 0.5 ml/min. (4) Dry the eluate on a hot plate (75°) under a stream of pure nitrogen gas.

Anion-exchange cleanup. (1) Dissolve the dried sample from the charcoal column in 2.0 ml of 0.1 *N* KOH and place it on a 70 × 9 mm AG 1 X 2 anion-exchange resin (acetate form) at a flow-rate of *ca.* 0.2 ml/min. (2) Wash the sample container with two 2-ml rinses of de-ionized water and place them on the resin after the sample is completely on the resin. (3) Wash the column with 100 ml of de-ionized water at a flow-rate of *ca.* 1.0 ml/min. (4) Elute the methylated bases from the resin with 40 ml of 0.02 *N* acetic acid at a flow-rate of *ca.* 0.5 ml/min and collect the eluate in a 50-ml beaker. (5) Add the internal standard (uracil) in dilute acetic acid (*ca.* 50 μg/1.0 ml) to the eluate.

Sample drying and silylation. (1) Concentrate the eluate from the AIE column to approximately 2.0 ml on a hot plate (75°) under a stream of pure nitrogen gas, then transfer it to a 4.5-ml culture tube with a PTFE-lined screw cap. (2) Rinse the beaker with two 1-ml portions of 0.02 *N* acetic acid and add to the culture tube. (3) Evaporate the sample to dryness on a hot plate (75°) under a stream of pure nitrogen. (4) To ensure complete dryness, add *ca.* 1 ml of dichloromethane and evaporate to azeotropically remove any remaining traces of water. (5) Silylate the dried sample with 0.2 ml of BSTFA and 0.2 ml of acetonitrile heated in a closed tube at 150° for 15 min. (6) Analyze the silylated sample on a 1.0 m × 4 mm I.D. 5% OV-3 on 100–120 mesh Supelcoport column.

An initial hydrolysis of the urine was found to be necessary to hydrolyze methylated nucleosides present in urine to the free bases and to breakdown many large molecules which allowed for easier sample cleanup. Filtration of the hydrolyzed urine prior to placing the sample on the charcoal column prevented the particulate matter from plugging the charcoal column and interfering with the cleanup method.

Analysis of normal and cancer-patient urine

Previous reports using ion-exchange chromatography, paper chromatography, and UV spectroscopy have shown that MeB are excreted in normal and in cancer-patient urine. Weissmann *et al.*[9] have reported a number of methylated purines in normal urine including 1-MeHypo at 0.4 mg per 24 h, 7-MeGua at 6.5 mg per 24 h, 8-OH-7-MeGua at 1.6 mg per 24 h, and N^2-MeGua at 0.5 mg per 24 h. Park *et al.*[10] reported MeB excretion levels for normal humans: 7-MeGua at 5.4 mg per 24 h, 8-OH-7-MeGua at 1.0 mg per 24 h, and N^2-MeGua at 0.4 mg per 24 h; and for leukemia patients: 7-MeGua at 8 mg per 24 h, 8-OH-7-MeGua at 4 mg per 24 h, and N^2-MeGua in quantities too small for quantitation. These data indicate an increase in the average excretion of MeB by leukemia patients as compared to normal humans.

Fink *et al.*[4,5] were able to detect 1-MeAde, N^6-MeAde, and various methylated nucleosides using a procedure similar to that of Weissmann. Also, Fink reported that the methylated nucleosides were excreted at higher levels than the free bases. Mirvish *et al.*[27] also found MeB in normal human urine at levels similar to those reported earlier. A study by Heirwegh *et al.*[6], and a later study by Waalkes *et al.*[28] showed that caffeinated beverages and high-purine diets did not affect the excretion of the MeB, but did increase total purine excretion.

The methylated base excretion levels of normal humans were determined by analyzing ten normal urines by the cleanup and GLC method outlined earlier. The excretion values obtained for the five bases detected (N^6,N^6-DiMeAde, N^6-MeAde, N^2,N^2-DiMeGua, N^2-MeGua, and 7-MeGua) are presented in Table III. The average excretion and standard deviation of each base for the ten normals were calculated and ranged from 5.3 ± 2.4 mg per 24 h for N^6-MeAde to 1.1 ± 1.0 mg per 24 h for N^2-MeGua. These normal excretion values agree quite well with earlier reported values for these bases. A typical chromatogram for a normal urine sample is shown in Fig. 2.

TABLE III

ANALYSIS OF METHYLATED BASES IN NORMAL HUMAN URINE

mg MeB per 24-h urine = mg MeB per ml urine × 24-h urine volume (ml); mg MeB per ml urine = [area MeB × mg I.S.]/[area I.S. × $RWR_{MeB/I.S.}$], $RWR_{MeB/I.S.}$ = [area MeB × mg I.S.]/[area I.S. × mg MeB].

Sample	*mg Methylated base per 24-h urine volume (ml)*				
	N^6,N^6-DiMeAde	*N^6-MeAde*	*N^2,N^2-DiMeGua*	*N^2-MeGua*	*7-MeGua*
1	trace	4.3	1.0	trace	5.4
2	1.4	2.8	0.5	trace	2.4
3	3.5	5.8	0.8	trace	3.1
4	3.5	5.8	0.8	3.5	7.1
5	trace	6.2	1.6	trace	6.1
6	2.1	5.6	impurity	trace	1.4
7	4.2	7.7	impurity	2.3	3.7
8	1.8	4.7	impurity	trace	3.7
9	4.4	10.6	2.0	3.3	7.7
10	3.5	5.8	1.2	1.1	5.2
Average ± S.D.	2.3 ± 1.0	5.8 ± 2.4	1.2 ± 0.5	1.1 ± 1.0	5.2 ± 2.1
Range	trace–4.4	2.8–10.6	0.5–2.0	trace–3.5	1.4–7.7

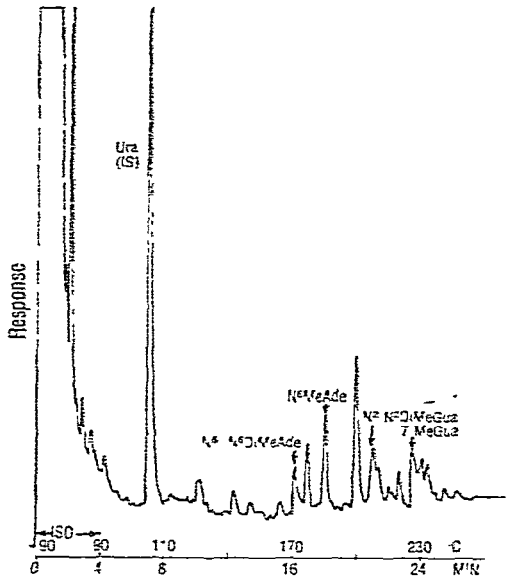

Fig. 2. GLC analysis of methylated bases in normal human urine Sample: 4.0 ml. Attenuation: 6.4×10^{-10} A.f.s. Cleanup by hydrolysis, filtration, charcoal, and AIE. For further conditions, see the legend to Fig. 1.

To determine the excretion level of the methylated bases from cancer patients, fourteen cancer-patient urines were analyzed. Five different types of cancer were evaluated: three colon cancer, three breast cancer, three melanoma, three lung cancer, one kidney cancer, and one rectal cancer. The MeB excretion levels of the cancer patients were generally higher than the levels excreted by normal humans. The

TABLE IV

ANALYSIS OF METHYLATED BASES IN CANCER-PATIENT HUMAN URINE

Calculation of mg MeB per 24-h urine volume· see Table III

Sample		*mg Methylated base per 24-h urine volume (ml)*				
		N^6,N^6-DiMeAde	*N^6-MeAde*	*N^2,N^2-DiMeGua*	*N^2-MeGua*	*7-MeGua*
Colon cancer	1	—	5.8	3.4	—	5.7
	2	5.9	10.7	3.7	5.2	12.9
	3	5.7	5.1	4.5	2.5	10 2
Breast cancer	1	—	5.0	4.0	—	4.9
	2	2.0	2.4	0.7	1.3	1.2
	3	9 6	6.6	4.3	2.5	8.8
Melanoma	1	—	14.8	5.2	—	9.8
	2	4.5	12.1	4.1	3.1	18.6
	3	5.5	5 3	1.0	1.0	9.5
Lung cancer	1	—	—	trace	—	trace
	2	1.4	2.3	1.6	1.7	6 2
	3	8.4	31 4	23.3	trace	9.1
Kidney cancer	1	—	24.6	3 1	—	—
Rectal cancer	1	—	10.0	7.4	—	6.2

TABLE V

COMPARISON OF EXCRETION LEVELS OF METHYLATED BASES FROM CANCER PATIENTS AND NORMAL SUBJECTS

mg MeB per 24-h urine = mg MeB per ml urine × 24-h volume urine (ml); mg MeB per ml urine = area MeB/area I.S. × mg I.S./$RWR_{MeB/I.S.}$

Compound	*Average and range of mg MeB per 24-h urine excreted*	
	*Cancer patients**	*Normals***
N^6,N^6-DiMeAde	3.1 (trace– 9.6)	2.3 (trace– 4.4)
N^6-MeAde	9.7 (trace–31.4)	5.8 (2.8–10.6)
N^2,N^2-DiMeGua	4.7 (trace–23.3)	1.2 (0 5– 2.0)
N^2-MeGua	1.2 (trace– 5.2)	1.1 (trace– 3.5)
7-MeGua	7.4 (trace–18.6)	5.2 (1.4– 7.7)
Average total MeB	5.2	3.1

* Average for 14 different cancer patients.
** Average for 10 different normals.

amounts of MeB per 24-h urine volume for the cancer patients are presented in Table IV. Table V gives a comparison of the average amount of MeB excreted by cancer patients and normal humans. Chromatograms from a colon cancer- and melanoma-patient urine are shown in Figs. 3 and 4. The peak eluting between N^6,N^6-DiMeAde and N^6-MeAde has the same retention temperature as adenine, and guanine elutes with the same retention temperature as the peak between N^2,N^2-DiMeGua and N^2-MeGua.

The levels of methylated bases observed in normal and the increased amounts

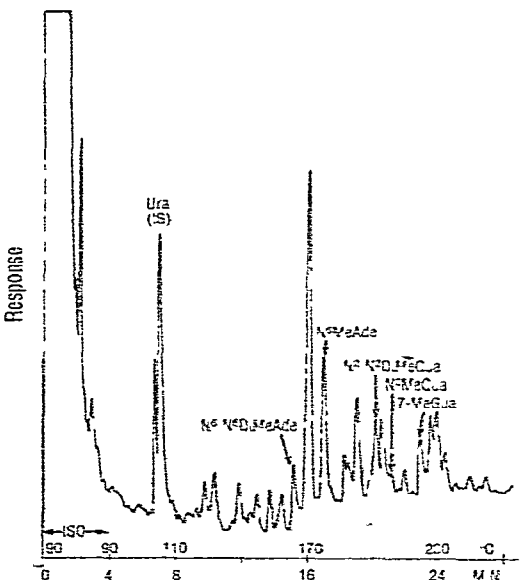

Fig. 3. GLC analysis of methylated bases in urine of a colon-cancer patient. Sample: 4.0 ml. Cleanup by hydrolysis, filtration, charcoal and AIE. For further conditions, see the legend to Fig. 1.

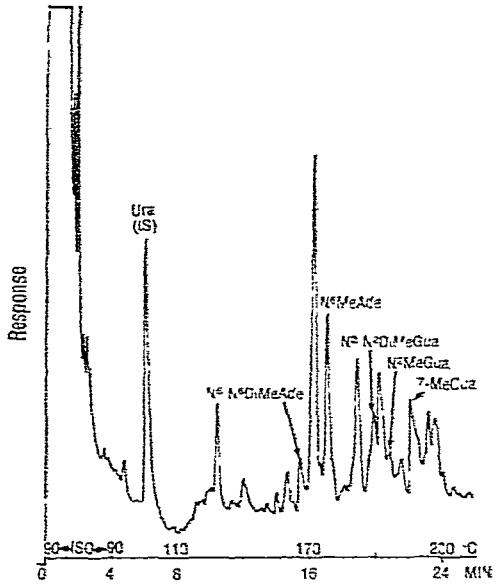

Fig. 4. GLC analysis of methylated bases in urine of a melanoma patient. Sample: 4.0 ml. Cleanup by hydrolysis, filtration, charcoal, and AIE. For further conditions, see the legend to Fig. 1.

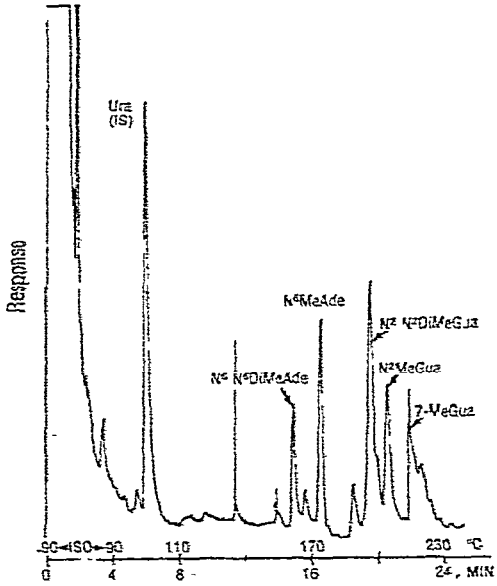

Fig. 5. GLC analysis of methylated bases in urine of a lung-cancer patient. Sample: 4.0 ml spiked with 50 μg of each MeB. Attenuation: 6.4×10^{-10} A.f.s. Cleanup by hydrolysis, filtration, charcoal, and AIE. For further conditions, see the legend to Fig. 1.

present in cancer patient urines may be due to the excretion of methylated nucleosides which are hydrolyzed to the free bases during the hydrolysis step in sample preparation. This is of particular importance for N^2,N^2-DiMeGua as analysis of the nucleoside, N^2,N^2-dimethylguanosine, has been reported in cancer patient and normal urine[22,25,28]. The corresponding nucleosides of the other methylated bases reported here have not been quantitatively analyzed in normal or cancer patient urine to date. Further research is necessary to determine whether the primary excretion product is the free base, nucleoside, or a combination of both.

The per cent recovery of the MeB through the analytical method was determined by analyzing "spiked" and "unspiked" aliquots of the same urine sample. A total of twelve recovery samples was analyzed, and the average per cent recoveries and ranges for the methylated bases were: N^6,N^6-DiMeAde, 49% (43–58%); N^6-MeAde, 53% (43–60%); N^2N^2-DiMeGua, 96% (87–109%); N^2-MeGua, 53% (43–65%); and 7-MeGua, 94% (90–109%); a chromatogram obtained from a lung cancer-patient urine plus the added MeB is shown in Fig. 5.

The low recovery obtained for the adenine derivatives and N^2-MeGua can be attributed to the pyridine–ethanol (95%) (1:1) wash of the charcoal column, which partially elutes these compounds. However, this wash was found to be essential in that it removed the methylated xanthines from the sample, and as stated earlier, xanthine and its methylated derivatives interfere with the chromatography of the methylated bases. With better control over the intake of caffeined beverages, this pyridine–ethanol (95%) wash would not be needed; and the recovery of the adenine derivatives and N^2-MeGua would be similar to that obtained for N^2,N^2-DiMeGua and 7-MeGua.

CONCLUSIONS

A GLC method for the analysis of methylated nucleic acid bases in human urine has been developed. The GLC and instrumental conditions of the methylated bases were first determined. The MeB were converted to their volatile TMS derivatives with BSTFA in acetonitrile as solvent by heating in a closed vial at 150° for 15 min. The derivatized bases were then analyzed on a chromatographic column of 5% OV-3 on Supelcoport (100–120 mesh).

Prior to the GLC analysis of MeB in urine, the sample must be cleaned to remove salts and interfering compounds. The cleanup method developed consisted of four steps: hydrolysis, filtration, charcoal adsorption, and AIE. The hydrolysis step converted any methylated nucleosides present to the free bases. The filtration step removed particulate matter present after hydrolysis which would interfere with further cleanup. Charcoal adsorption and AIE removed the salts and many other urinary compounds from the methylated bases and provided a relatively clean sample which could be analyzed by GLC.

Using this method, ten normal human and fourteen cancer-patient urines were analyzed. An increased excretion level for some methylated bases by cancer patients over normal humans was observed, and as noted in Table V, the overall average increase in excretion was about 70%. However, it is recognized that the number of samples analyzed was insufficient for definitive conclusions to be made. Whether this increased excretion is due to greater tRNA methylation by tRNA methylases and/or

secondary to the more rapid turnover of the tumor tissue tRNA for the cancer patients has not been ascertained. Future studies will determine whether excretion levels of methylated bases may be useful as a means for monitoring tumor growth or in evaluating the response of patients with malignancy to treatment.

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