Journal of Chromatography, 116 (1976) 83–92

CHROM. 8697

HIGH-RESOLUTION LIQUID CHROMATOGRAPHIC ANALYSIS OF METHYLATED PURINE AND PYRIMIDINE BASES IN TRANSFER RNA

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(Received August 12th, 1975)

SUMMARY

Methylated and major purine and pyrimidine bases were separated and quantified by high-resolution liquid chromatography after hydrolyzing transfer ribonucleic acids (tRNAs). Separation was accomplished by eluting the hydrolyzed samples from an anion-exchange column with a concentration gradient of ammonium acetate at pH 9.2. Isolated samples of tRNA were hydrolyzed to the free bases with a trifluoroacetic acid–formic acid mixture at 200°. Detection limits of 100–200 ng/ml were measured for the methylated bases; analytical data are reported for ten methylated bases plus the four major bases of calf liver and rat liver tRNA.

INTRODUCTION

Major purine and pyrimidine bases, nucleosides, and nucleotides have been measured using a variety of analytical techniques including gas–liquid chromatography (GLC)[1–5], high-performance liquid chromatography (HPLC)[6–11], and thin-layer or paper chromatography[12–14]. Reliable results on the composition of the major purine and pyrimidine bases in nucleic acids have been obtained with many of these methods reported[1–4,6,11–14]. However, only the method by Randerath *et al.*[13–15] provides the necessary separation of the major and minor bases when a quantitative analysis is required for a minor nucleic acid constituent, such as a methylated base in tRNA. Certain minor nucleic acid constituents cannot be separated from the major

* Operated for the Energy Research and Development Administration by Union Carbide Corporation.

bases by GLC, and the derivatizing agent can produce extraneous peaks on the chromatogram[16]. The HPLC methods were developed to provide rapid analysis of nucleic acid composition[6,7,10,11,17], and not for the separation and analysis of minor and methylated bases present in specific ribonucleic acids. Recent results by Sen and Ghosh[9] indicate that HPLC can be used to separate some major nucleosides from minor methylated nucleosides in tRNA, although quantification was not described.

Using radioactive labeling and two-dimensional paper chromatography, Munns *et al.*[12] and Randerath *et al.*[13–15] have developed methods for the analysis of methylated nucleic acid components, although not all the major and methylated components were completely separated. Radioactive methionine was used to label the methylated bases in the method by Munns *et al.*[12], providing results for *in vitro* studies; however, for *in vivo* experiments, dilution of the radioactive label makes the method impractical. Randerath *et al.*[13–15] developed a post-hydrolysis labeling technique which allows the analysis of any isolated tRNA. However, the analysis time is long, and sample manipulations require special care if satisfactory results are to be obtained.

The presence of methylated purine and pyrimidine bases in tRNA has been known and studied for some time. The methylation of the major bases by specific tRNA methylases occurs on the intact macromolecule. Increased tRNA methylases activity has been found in a variety of different tumors as summarized by Borek[18]. Reports on the increased urinary excretion of certain methylated nucleic acid derivatives by cancer patients[19–23] support the evidence for increased methylation of tRNA in cancer cells. Previous investigations by Berquist and Mathews[24] and particularly Viale *et al.*[25] have shown that tRNAs from tumor cells contain increased amounts of methylated bases compared to their normal tissue counterpart. However, other studies[17] have indicated little if any differences in methylated base content between tRNA in normal and related tumor tissues. Further analyses are needed, particularly of individual isoaccepting tRNAs, since minor variations may not be detected in a total population of tRNAs.

A reliable and practical method for the quantitative analysis of the methylated bases present in tRNA would provide further information on the extent of modification in the tRNA of neoplastic cells. This in turn would aid in understanding the effects of the increased tRNA methylase activity found in tumor cells and the greater urinary excretion of certain methylated nucleosides and bases by patients with malignant disease. This report describes a quantitative method applied to tRNA for the analysis of specific methylated bases that have been detected in the urine from patients with cancer.

MATERIAL AND METHODS

Apparatus

A Mark II UV Analyzer (Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A.) equipped with a 150 × 0.455 cm I.D. column containing Aminex 27 (Bio-Rad Labs., Richmond, Calif., U.S.A.) anion-exchange resin, a two-chamber gradient generating system[26] and a programmable temperature control system was used for this study. The eluate from the chromatographic column was monitored at 254 and 280 nm by a duoMonitor (Laboratory Data Control, Riviera Beach, Fla., U.S.A.),

and a Servo/Riter II (Texas Instruments, Houston. Tex., U.S.A.) dual-pen recorder was used to record the chromatograms.

A sand-bath with a variable temperature control (±2°) was used for hydrolysis of the transfer RNA samples.

Reaction vials described by Gehrke and Lakings[27] were made from Pyrex 9826 culture tubes (Corning, Corning, N.Y., U.S.A.).

Reagents

The major and methylated bases were obtained from the following sources. Calbiochem (Los Angeles, Calif., U.S.A.): adenine (Ade), cytosine (Cyt), guanine (Gua), uracil (Ura), 6-methyladenine (6-MeAde), 6-dimethyladenine (6-Me_2Ade), 5-methylcytosine (5-MeCyt), pseudouridine (Ψ), and thymine (Thy): Cyclo Chem. (Los Angeles, Calif., U.S.A.): 2-methyladenine (2-MeAde), 3-methylcytosine (3-MeCyt), 1-methylguanine (1-MeGua), 7-methylguanine (7-MeGua), 2-methylguanine (2-MeGua), 2-dimethylguanine (2-Me_2Gua), and 1-methylhypoxanthine (1-MeHypo); and Sigma (St. Louis, Mo., U.S.A.): 1-methyladenine (1-MeAde).

Calf liver tRNA was purchased from Sigma, rat liver tRNA was obtained from General Biochemicals (Chagrin Falls, Ohio, U.S.A.).

Formic acid (97–100%) and trifluoroacetic acid (TFA) were obtained from Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.). Acetic acid and ammonium hydroxide used to prepare buffers were from J. T. Baker (Phillipsburg, N.J., U.S.A.).

The anion-exchange resin used for separation of the purine and pyrimidine bases was Aminex A-27 (a 12–15 μm particle size strong base anion-exchange resin of 8% nominal crosslinking) and was purchased from Bio-Rad Labs.

Sample preparation

Approximately 2.0 mg of tRNA was dissolved in 2.0 ml of water, and 400-μl and 75-μl aliquots were placed in a culture tube with a PTFE-lined screw cap. Hydrolysis of the tRNAs to the free bases was accomplished using the method of Lakings and Gehrke[3]. By this procedure, 400 μl of a 1:1 trifluoroacetic acid–formic acid mixture were added to the sample, the vial tightly sealed, and the contents heated at 200° for 1.5 h. The hydrolyzing agents were removed by volatilizing at 60° under a stream of nitrogen, the residual sample was dissolved in 1.0 ml of 0.1 *N* NaOH and placed on the liquid chromatograph.

RESULTS

Separation studies on the major and methylated bases

Initial data indicated that the best separation of the major and methylated bases would be accomplished by gradient elution at a basic pH. Singhal and Cohn[10] reported on the anion-exchange chromatographic parameters for the separation of nucleosides and showed partial base separation under these conditions (0.2 *M* ammonium acetate, pH 9.7). Using these conditions as a starting point, optimal conditions were sought for separation of the four major bases (Ade, Cyt, Gua, and Ura) and twelve methylated bases (1-MeAde, 2-MeAde, 6-MeAde, 6-Me_2Ade, 3-MeCyt, 5-MeCyt, 1-MeGua, 2-MeGua, 7-MeGua, 2-Me_2Gua, 1-MeHypo, and Thy) and to include pseudouridine at a major to minor base ratio of 10 to 1. Chromatographic

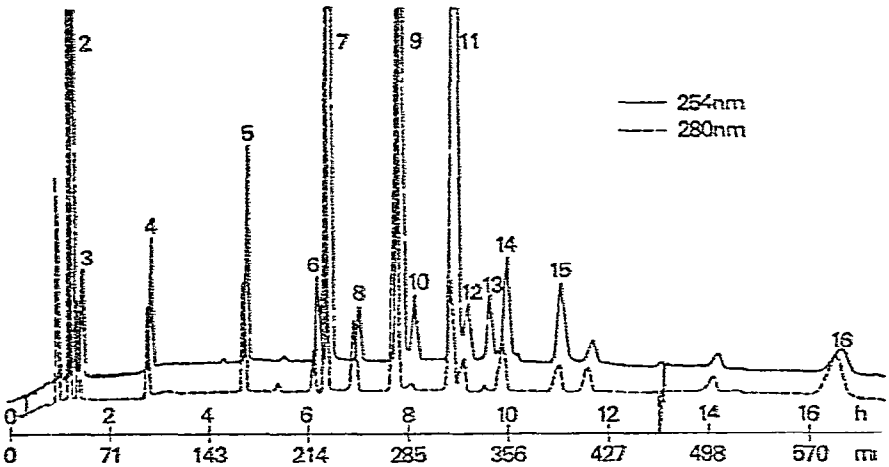

Fig 1. Standard mixture of bases and methylated bases at a 10:1 ratio. Buffer of first chamber: 0.015 *M* ammonium acetate (pH 9.2), of second chamber: 1.2 *M* ammonium acetate (pH 9.2). Temperature, ambient for the first 1.75 h, then programmed to 60° in 1.5 h, and maintained at 60° for the remainder of the analysis. Flow-rate: 35.6 ml/h (3.65 ml/cm²·min). UV output: 0.04 O.D. unit full scale. 1 = 3-MeCyt; 2 = Cyt, 3 = 5-MeCyt, 4 = 1-MeAde; 5 = 1-MeGua; 6 = Thy, 7 = Ura; 8 = 7-MeGua; 9 = Gua; 10 = 2-MeAde; 11 = Ade; 12 = 2-MeGua; 13 = 1-MeHypo; 14 = 2-Me_2Gua; 15 = 6-MeAde; 16 = 6-Me_2Ade

TABLE I

METHYLATED AND MAJOR BASE STANDARD VALUES

Compound	*Retention volume** *(ml)*	*R.S.D.***	*Peak height** *(cm)*	*R.S.D.***
3-MeCyt	31.1	4.7	9.57***	6.6
Cyt	39.8	8.3	19.95	7.5
5-MeCyt	47.1	5.3	5.38	6.8
1-MeAde	97.2	4.8	7.34	4.9
1-MeGua	165.7	6.5	9.90	6.9
ψ	190.5	7.3	2.33	9.9
Thy	214.8	7.0	4.42	5.3
Ura	229.8	6.9	11.57	9.8
7-MeGua	244.6	6.1	3.58***	5.3
Gua	255.0	5.7	7.10	8.9
2-MeAde	268.6	9.4	3.18	5.9
Ade	300.0	7.0	9.71	5.3
2-MeGua	310.9	5.5	2.74	8.6
1-MeHypo	314.7	5.6	3.08	8.1
2-Me_2Gua	328.3	5.7	4.90	7.9
6-MeAde	380.8	6.0	2.88	7.3
6-Me_2Ade	619.1	5.2	1.85***	9.2

* Average of six independent analyses.

** Relative standard deviation.

*** 280-nm scale used for quantitation for 3-MeCyt, 7-MeGua, and 6-Me_2Ade.

conditions which yielded the best separation using the "Autograd" system[26] utilized a 0.015 *M* ammonium acetate (pH 9.2) in the first chamber and a 1.2 *M* ammonium acetate buffer (9.2) in the second chamber. The temperature was maintained at ambient room temperature for the first 1.75 h, then programmed to 60° in 1.5 h, and maintained at 60° for the remainder of the analysis. The flow-rate through the column was maintained between 34 and 36 ml/h (3.5–3.7 ml/cm^2·min). A typical chromatogram of the major and methylated bases at a 10:1 ratio is shown in Fig. 1. Table I gives the average elution volume of each base for six independent analyses and the relative standard deviation of the elution volume for each base.

Quantitation of the major and methylated bases separated by HPLC

The minimum detectable level (MDL) and the linearity of response over a range of concentration were evaluated since quantitative analysis of the methylated bases in tRNA was the primary goal. Standard stock solutions of four bases were analyzed at concentrations of 1–5 μg/ml for the methylated bases and 5–40 μg/ml for the major bases. Graphs of peak area *versus* concentration and peak height *versus* concentration were constructed and found to be linear for each compound over the concentration range of interest. Peak height and peak area gave similar results. Measurement of the width at half peak height was found to be difficult and a possible source of error in calculating peak area due to the narrow width of some peaks. Consequently, the peak height was chosen for quantitative calculation. Figs. 2 and 3 present the peak height data for the four major bases and for four selected methylated bases, respectively.

The quantitative analysis of the base composition of a tRNA was performed by using a simple proportion as the peak heights of the bases were linear over a wide concentration range.

$$\mu\text{g base in tRNA} = \mu\text{g base standard} \times \frac{\text{peak height base in tRNA}}{\text{peak height base standard}}$$

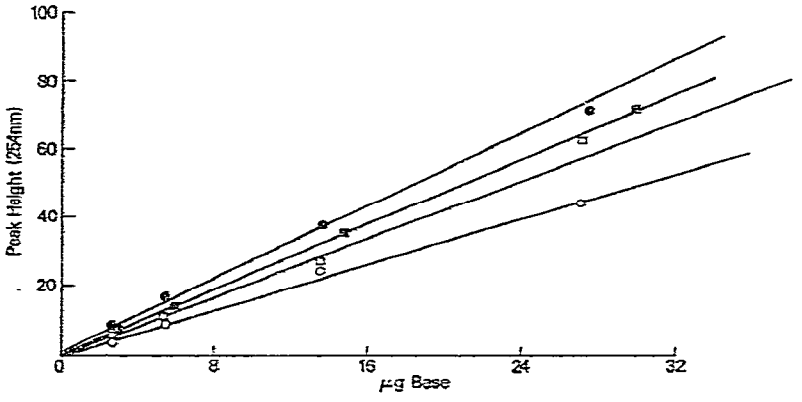

Fig. 2. Standard curves for major bases. Each point represents an average of three independent analyses. Chromatographic conditions were as given in Fig. 1. ●, Cyt; ■, Ura; □, Ade; ○, Gua.

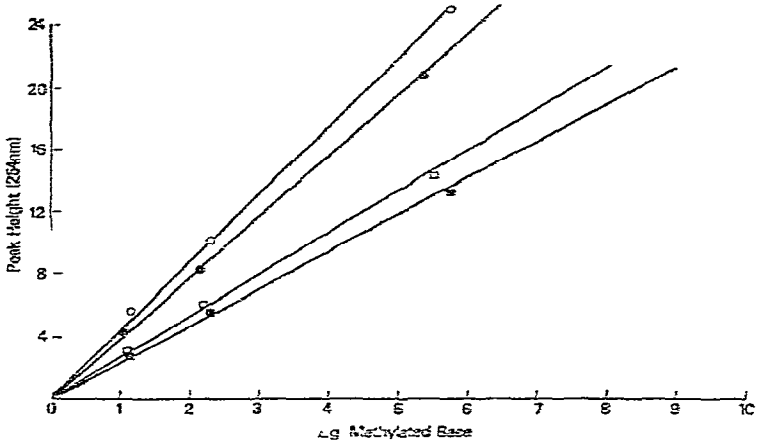

Fig. 3. Standard curves for four methylated bases. Each point represents an average of three independent analyses. Chromatographic conditions were as given in Fig. 1. Other methylated bases gave similar graphs. ○, Thy, ●, 6-MeAde, □, 2-Me_2Gua, ■, 7-MeGua.

Six independent analyses of a standard solution of methylated and major bases were made. The average peak height and relative standard deviation are presented in Table I.

The MDL for the methylated bases was determined by analyzing successively lower concentrations of selected methylated bases until a signal to noise ratio of 3:1 was obtained. The MDL for the methylated bases using this analytical system was found to be between 0.1 and 0.2 μg/ml.

Hydrolysis evaluation

The stability of the major bases during hydrolysis with a formic acid–TFA mixture at 200° for 1.5 h had been determined previously[3]. No loss or destruction of the major bases was found. To determine the effect of this hydrolysis method on the methylated bases, a standard mixture containing the twelve methylated bases and pseudouridine at a concentration of 2.5 μg/ml were carried through the entire hydrolysis and analytical procedure. Only two of the compounds were found to be affected by the hydrolysis conditions. Pseudouridine was completely degraded with no UV absorbing peaks appearing on the chromatogram, and about 15% of 1-MeAde partially isomerized to 6-MeAde. This change had been demonstrated previously by Munns *et al.*[12]. As a consequence, the pseudouridine level in tRNA could not be determined by this method; and measured amounts of 1-MeAde and 6-MeAde were combined and reported as one value. Table II shows the good stability of the methylated bases when carried through the hydrolysis step and analysis by liquid chromatography, with the exception of a small loss of 1-MeAde.

Several of the major and methylated nucleosides were also hydrolyzed and analyzed to determine whether complete hydrolysis to the free base had occurred. If the conditions for hydrolysis were carefully followed, total conversion of all the nucleosides to their corresponding base invariably resulted. However, incomplete hydrolysis occurred if the temperature fell below 200° or was maintained for less than

TABLE II

STABILITY OF THE METHYLATED BASES DURING HYDROLYSIS

Compound	*Peak height**		*% Recovery*
	*Unhydrolyzed**	*Hydrolyzed***	
3-MeCyt	22.8***	22 7***	99.6
5-MeCyt	17.8	17.5	98 4
1-MeAde	17.3	14.4	83.2
1-MeGua	21.4	21.2	99.1
Thy	11 6	11.2	96.6
7-MeGua	6.6***	6.2***	93.9
2-MeAde	7.9	7.6	96.2
2-MeGua	6.6	6.0	90.9
1-MeHypo	8.5	8.4	98.8
2-Me_2Gua	8.4	8.1	96.4
6-MeAde	21 3	20.9	98.1
6-Me_2Ade	5.8***	5 6***	96.6

* Peak height in cm measured at 254 nm.

** Each value represents an average of three independent analyses for unhydrolyzed and hydrolyzed peak heights.

*** Peak height measured at 280 nm for 3-MeCyt, 7-MeGua, and 6-Me_2Ade.

1.0 h. If the tRNA sample was incompletely degraded to its free bases, corresponding nucleosides and nucleotides could be detected. Absence of such peaks on the chromatograms provided a means whereby total hydrolysis of the tRNA sample could be verified.

TABLE III

COMPOSITION OF RAT LIVER AND CALF LIVER tRNA IN WEIGHT AND MOLE PERCENTAGES

Compound	*Rat liver tRNA*		*Calf liver tRNA*	
	*w/w %**	*mole %***	*w/w %**	*mole %***
3-MeCyt	0.04	0.14	0.02	0.05
Cyt	9.15	37.64	11.60	32.31
5-MeCyt	0.12	0.42	0.34	0.84
1-MeAde	0.03	0.10	0.05	0.11
1-MeGua	0.07	0.18	0.04	0.07
Thy	0.12	0 43	0.30	0.74
Ura	4.25	17.34	4.56	12.59
7-MeGua	0.06	0.15	0.05	0.09
Gua	7.75	23.38	17.64	36.13
Ade	5.55	18.71	7 32	16.78
2-MeGua	0.13	0 34	0 03	0.06
2-Me_2Gua	0.07	0.17	0.03	0.05
6-MeAde	0.33	0.99	0.09	0.19
6-MeAde + 1-MeAde***	0.45	1.09	0.14	0.30

* w/w % = weight methylated base/weight tRNA × 100.

** Mole % = moles methylated base/moles total bases × 100.

*** 6-MeAde and 1-MeAde were combined due to isomerization of 1-MeAde to 6-MeAde.

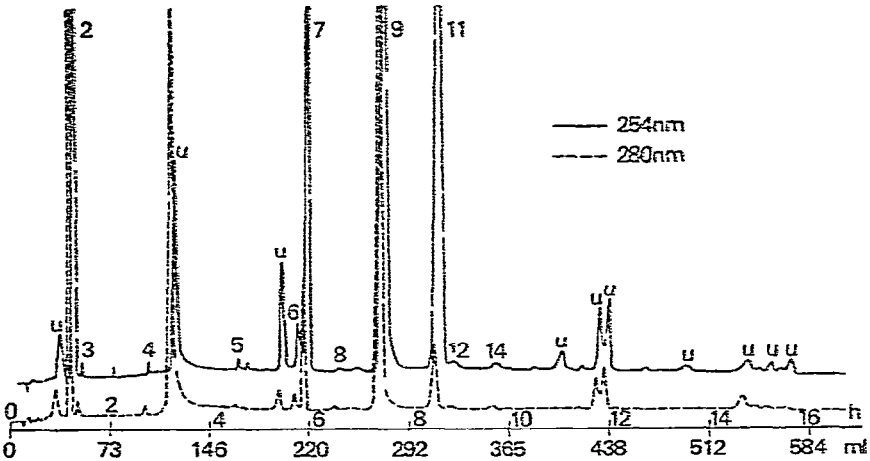

Fig 4 HPLC analysis of 0.5 mg calf liver tRNA. For analysis conditions and peak identification, refer to Fig. 1 Flow-rate: 36 5 ml/h (3.75 ml/cm²·mun). UV output: 0.08 O.D. unit full scale. U indicates unknown peaks on the chromatogram Hydrolysis conditions were as given in text.

Analysis of selected tRNA samples

The amounts of the various methylated bases present in calf liver and rat liver tRNA were determined by the methods described earlier. A 2.0-mg sample of tRNA was dissolved in 2.0 ml water. Duplicate aliquot samples of 0.5 and 0.07 ml for calf liver tRNA (*ca.* 0.5 mg and 70 μg tRNA) and 0.25 and 0.05 ml for rat liver tRNA (*ca.* 0.25 mg and 50 μg tRNA) were analyzed Analytical data for ten methylated and four major bases were determined and these data are presented in Table III.

Figs. 4 and 5 are chromatograms for the HPLC analysis of methylated bases

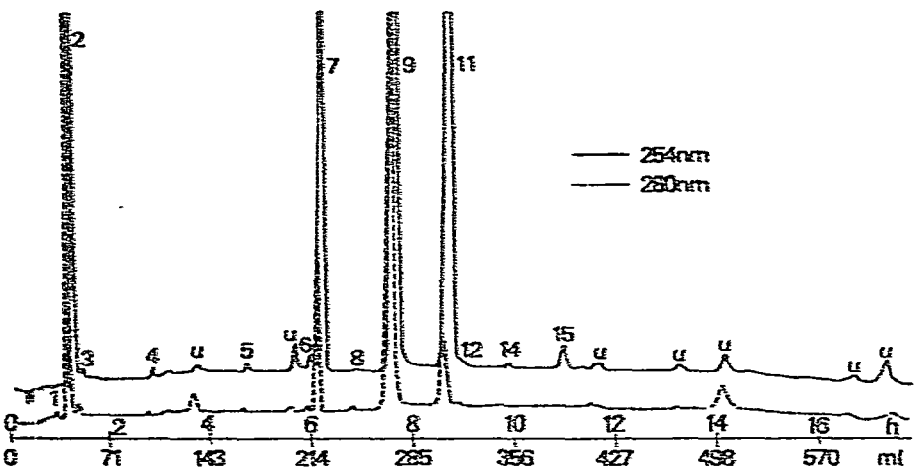

Fig. 5 HPLC analysis of 0.25 mg rat liver tRNA. For analysis conditions and peak identification, refer to Fig. 1. Flow-rate: 36 5 ml/h (3 75 ml/cm² min). UV output: 0.04 O D. unit full scale. U indicates unknown peaks on the chromatogram. Hydrolysis conditions were as given in text.

in calf liver and rat liver tRNA, respectively, and show the presence of a number of unidentified minor bases.

DISCUSSION

The quantitative analysis of the major and methylated base content of tRNAs found in normal and related tumor tissue provides information as to structural modifications and may provide clues as to possible functional or behavioral differences observed between normal and cancer cells. Since these macromolecules serve important roles in cell growth and its regulation, further study and understanding of the modified base content would be of distinct value. The method, as described in this report, had direct application for the separation and determination of the major and methylated purine and pyrimidine bases in tRNA. This procedure may be applicable to the quantitative measurement of those methylated purines and pyrimidines found as catabolic end products in the urine of normal subjects and cancer patients. No attempt was made to identify or measure the content of those structurally modified bases having substituents other than methyl groups. Further studies are necessary to determine if the analysis of other modified degradation products of nucleic acids could be performed using this procedure. This seems feasible with minor modifications in either the hydrolysis step or the liquid chromatographic separation.

The sensitivity of the method, found to be 100–200 ng methylated base per milliliter, could be improved by using a detector cell with a longer path length than the 3-mm path length used in this study. The analysis of the major and methylated bases on the same chromatogram is possible using an electronic integrator or a second recorder with a variable range. The analysis time may be shortened by changing the eluant concentrations, the column length, or the flow-rate. These parameters were not fully evaluated because the procedure as developed gave the required separations and the analysis time was not a limiting factor. Application of the method has been demonstrated using both rat and calf liver tRNA and has been shown to be sensitive and to provide reproducible results. Further studies are in progress to compare analytical results for tRNAs from tumor tissue with those from tRNAs obtained from normal tissue.

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