QUANTITATIVE GAS-LIQUID CHROMATOGRAPHY OF TMS NUCLEIC ACID CONSTITUENTS[1,2]

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Before gas-liquid chromatographic analysis of the purine and pyrimidine bases, nucleosides, or nucleotides can be accomplished, these compounds must first be converted to suitable volatile derivatives. Among compounds which have been investigated are various combinations of acetyl, methyl, or isopropylidene derivatives of the nucleosides (Miles and Fales, 1962), the N-methyl derivatives of the purine and pyrimidine bases (MacGee, 1964), and the trimethylsilyl (TMS) derivatives of the nucleosides (Hancock and Coleman, 1965). More recent work evaluated the TMS derivatives of adenosine and adenosine containing compounds (Hancock, 1966), and the TMS derivatives of purine and pyrimidine bases, nucleosides, and nucleotides (Hashizume and Sasaki, 1966).

The TMS derivatives of the nucleic acid constituents appear to be the most suitable since the problem of multiple derivatives is minimal. These derivatives have been formed using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in

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basic solvent systems. Bis(trimethylsilyl)acetamide (BSA) has been shown to be an extremely potent silylating reagent for compounds with replaceable hydrogens (Klebe, et al., 1966). However, the use of BSA to form TMS derivatives of nucleic acid constituents has not been evaluated. The major aim of this investigation was to develop a quantitative method for the analysis of nucleic acid constituents, especially the purine and pyrimidine bases. The reaction conditions necessary for quantitative and reproducible preparation of TMS derivatives using BSA are herein described. Relative molar responses, retention temperatures, and chromatographic conditions are also presented.

EXPERIMENTAL

Silylation Procedure. Preparation of TMS Derivatives with BSA.

The purine or pyrimidine base, nucleoside, nucleotide, or mixture ($\leq$ 5 mg) was weighed into a 15 x 75 mm screw top culture tube containing an 8 x 12 mm teflon covered magnetic stirring bar. BSA (up to 2.0 ml, 100 mole excess) and acetonitrile (3 times the volume of BSA used) were added to the sample in a glove box filled with dry nitrogen, and the tube was tightly capped with a teflon lined cap. The sample tube was then placed to a depth of ca. 1/2 inch in a magnetically stirred oil bath at 150° C for 45 minutes. This oil bath was located behind a safety shield in a fume hood. The sample was cooled to room temperature prior to gas chromatographic analysis. The 3:1 v/v ratio of acetonitrile:BSA was necessary for complete miscibility of BSA.

RESULTS AND DISCUSSION

Retention Temperature and Relative Molar Response

The molar response of the hydrogen flame detector to the

five main purine and pyrimidine bases and other bases, nucleosides, and nucleotides was determined relative to phenanthrene. These data are presented in Tables I and II. Both cytosine and 5-methyl cytosine exhibited two chromatographic peaks using the above experimental conditions. However, these conditions were found to be necessary for the reproducible formation of the guanine TMS derivative. Further evaluation of the two peaks for cytosine showed the peak areas to be a function of silylation

TABLE I. RELATIVE MOLAR RESPONSE OF THE TMS DERIVATIVE OF PURINE AND PYRIMIDINE BASES USING A FID[a]

Compound	Retention Temperature[b], °C	RMR[c]	S.D.[d]
Uracil	120	0.67	0.010
Thymine	130	0.68	0.012
Purine	140	0.38	
Cytosine	148; 161	0.54[e]	
5-Methyl cytosine	152; 165	0.45[f]	
Hypoxanthine	176	0.58	
Adenine	185	0.60	0.004
Xanthine	202	0.71	
Guanine	211	0.72	0.006
Phenanthrene, IS.	172	1.00	

[a] FID - Flame Ionization Detector

[b] Chromatographic Conditions are described in Experimental Section.

[c] RMR determined from at least two independent determinations. RMR = Molar Response relative to Phenanthrene

$$RMR = \frac{\text{Molar Response of Bases}}{\text{Molar Response of Phenanthrene}}$$

[d] Standard deviation calculated from 4 or more independent determinations.

[e] Room temperature silylation for 30 minutes.

[f] Response based on first eluted peak.

TABLE II. RMR OF SELECTED NUCLEOSIDES AND NUCLEOTIDES

Compound	Retention Temperature, °C	RMR[a]	RMR Range
Thymidine	230		
Uridine	234	1.19	±0.10
Adenosine	260	1.60	±0.10
Guanosine	265	1.37	±0.09
Uridylic Acid	270	0.61	±0.05
Adenylic Acid	276	0.98	±0.03

[a] Calculated from at least two independent observations; samples heated for 45 minutes at 150° with a 100 mole excess of BSA.

time and temperature. The peak areas were inversely proportional to each other, thus indicating that two derivatives of cytosine exist in a state of equilibrium. At 150° C and with a 100 mole excess of BSA the relative peak area of the first eluted cytosine peak was constant if the samples were analyzed within a few hours after silylation. More extreme reaction conditions would not drive the reaction to only one product. However, a single peak for cytosine was obtained by conducting the reaction at room temperature, but this is then not sufficient for the silylation of the other bases, especially the purines. The precision of the described method for the basesis excellent (Table I).

Calibration curves for the five main bases (U, T, C, A, and G) were prepared and found to be linear over a sample range of 25 to 2,000 μg of each base. Excellent linearity and reproducibility were obtained with a relative standard deviation ranging from 1.1% for uracil to 3.1% for cytosine. The curve for cytosine was prepared using the first eluted peak.

The minimum detectable limit (MDL) was determined by making

serial dilutions of one of the samples used in preparing the calibration curves. The MDL (3:1 signal to noise ratio) was 3 to 5 nanograms or ca. 3×10^{-11} moles of each base injected.

The derivatives of the nucleosides and nucleotides were prepared using the derivatization method developed for the purine and pyrimidine bases. Single chromatographic peaks were obtained for the compounds investigated which contained ribose; however, when the same procedure was used for thymidine, a deoxyribose containing compound, two unresolved chromatographic peaks resulted. Further investigations are underway to delineate the optimum silylating conditions for the nucleosides and nucleotides.

Chromatographic and Instrumental Conditions

Figure 1 is typical of the chromatograms obtained from the TMS derivatives of the five main purine and pyrimidine bases and phenanthrene as the internal standard. This chromatogram repre-

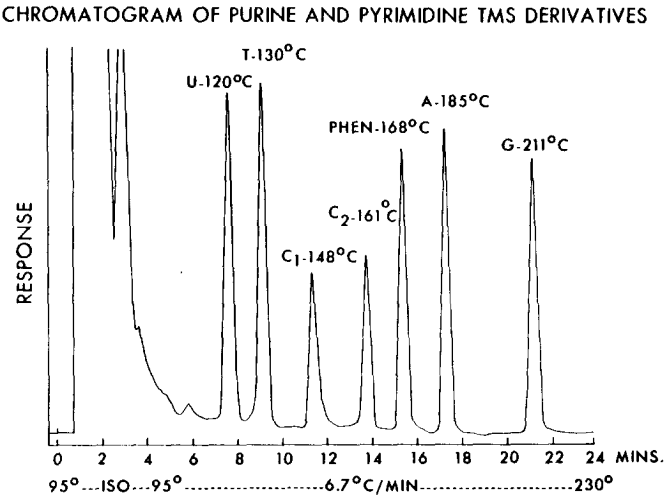


Fig. 1 4 w/w % SE-30 on 60/80 mesh, a.w. DMCS treated Chromosorb G 1 m x 4 mm, 5 µl injected. Each peak represents 1 µg of base.

sents 1 μg of each base injected. The chromatographic conditions were as follows:

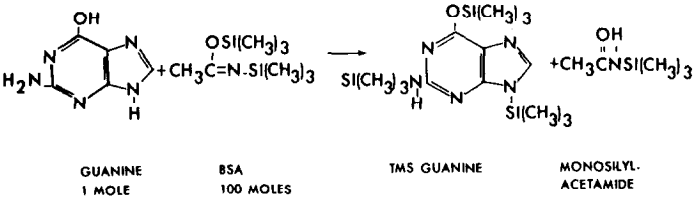
Instrument used: F and M Model 402 linear temperature programmed gas chromatograph equipped with two hydrogen flame ionization detectors.

Temperature program: Initial temperature 95° C, 4 minute initial program delay, subsequent effective program rate 6.7°C/min. to 240°C. Injection heater: 180°C.

Detector heater: 250°C. Carrier gas: 80 ml/min N_2.

Column: 4 w/w % SE-30 on 60/80 mesh, acid washed, DMCS treated Chromosorb G (vacuum dried at 100°C for 18 hours before coating), 1.00 m x 4 mm i.d. borosilicate-glass.

TRIMETHYLSILYLATION OF GUANINE



CLOSED TUBE, 150° C, 45 min. CH_3CN/BSA, 3:1 v/v

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