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GAS–LIQUID CHROMATOGRAPHY OF NUCLEOSIDES

EFFECT OF SILYLATING REAGENTS AND SOLVENTS*

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SUMMARY

The aims of this investigation were to study the completeness of silylation of nucleosides with three different reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA), bis(trimethylsilyl)acetamide (BSA) and trimethylsilylimidazole (TMSI), and to investigate the effect of different solvents (acetonitrile, pyridine, dimethylformamide, chloroform, methylene chloride, hexane, benzene, and toluene) on quantitation of derivatization.

Closed-tube silylations of the nucleosides were performed with BSTFA, BSA, and TMSI, and for the most complete silylation, the optimal time, temperature, and molar excess of reagent were: for BSTFA, 150°-15 min and 225 molar excess; for TMSI, 60°-3 h and 1000 molar excess; and for BSA, 120°-2 h and 250 molar excess. Also, silylations of seven major and minor nucleosides were carried out using a 1000 molar excess of BSTFA, BSA, and TMSI at 25° with 5 min sonication, and at optimal silylation conditions as described above for the three reagents. The silylating strengths were determined by the increase in RWR (= weight response of nucleoside/weight response of pyrene) values, and are summarized for the amino group containing nucleosides silylated at room temperature as BSTFA > TMSI > BSA, and for silylation under optimal conditions as BSTFA > BSA > TMSI. The efficiency of silylation for the hydroxyl group-containing nucleosides silylated at room temperature was BSTFA > TMSI > BSA, and for silylation under optimal conditions BSTFA > TMSI = BSA.

Significantly lower RWR values were obtained for all the nucleosides when silylations were made at 25°-5 min, 1000 molar excess, sonication, and a comparison was made with silylation at 150°-15 min, 225 molar excess. Room temperature silylations using BSA and BSTFA also gave two peaks for cytidine at retention tempera-

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tures (RT) of 238° and 250° on a 4 w/w % OV-11 on 100–120 mesh Supelcoport 1 m × 4 mm I.D. glass column.

Solvents also had a pronounced effect on the trimethylsilylation of nucleosides. The RWR values obtained for TMS nucleosides in different solvents were compared and acetonitrile was found to be best. When the derivatized samples in certain solvents were further diluted and chromatographed an increase in $RWR_{N/P}$ value was observed. From this, it was concluded that the solubility was the primary factor for incomplete derivatization in these experiments. When the amount of water was above 10 ppt, all of the nucleosides showed a significant decrease in $RWR_{N/P}$ values. Cytidine gave two peaks (RT = 238° and RT = 250°) in the presence of 0.5 ppt water. The peak with an RT at 250° was used in the gas–liquid chromatographic analysis of cytidine in a mixture of nucleosides; thus the presence of 0.5 ppt of water has a significant effect on derivatization and stability of the cytidine derivatives. Also, silylation of nucleosides with BSTFA containing 1 % TMCS, and non-solvent silylations, showed no significant differences in $RWR_{N/P}$ values.

INTRODUCTION

Since the introduction of the trimethylsilyl (TMS) derivatives of the nucleic acid components by Hancock and Coleman[1], efforts have been made by several groups of researchers to use these derivatives for the quantitative gas–liquid chromatographic (GLC) analysis of the nucleosides. A one-step derivatization procedure is most attractive in the use of the TMS derivative, as almost all other derivatives are formed by two or more reaction steps.

The use of various silylating reagents in the derivatization and chromatography of purine and pyrimidine bases at the macro level was demonstrated by Gehrke and Ruyle[2]. Later, Gehrke and Lakings[3] demonstrated application of the same method for analyses at the micro level of each base (500 ng). Jacobson *et al.*[4] reported the quantitative analysis of the nucleosides using bis(trimethylsilyl)acetamide (BSA) as the silylating reagent, and, in 1968, Stalling *et al.*[5] reported on the synthesis of a new silylation reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA). BSTFA was shown to be a more powerful silylating reagent than BSA, and it and its byproduct, MSTFA, are more volatile than BSA and its byproduct MSA. Also, the chromatograms obtained with BSTFA contained fewer extraneous peaks than those obtained with other silylation reagents. The optimal silylation conditions for derivatization and chromatography of nucleosides using BSTFA were reported earlier by Gehrke and Patel[6]. A recent paper by Lakings *et al.*[7] describes the determination of TMS methylated nucleic acid bases in urine by GLC.

The acceptance of the TMS derivatives in analytical GLC has brought about the development and introduction of new silylation reagents and also modifications of existing silylation mixtures. A reagent mixture of catalytic amounts of trimethylchlorosilane (TMCS) in BSA has advantages for certain classes of compounds[8,9], and the Regis Chemical Company[10] has suggested a similar use of 1 % TMCS with BSTFA. This is explainable by the fact that as TMCS reacts with a replaceable hydrogen, HCl is formed, which has a catalytic effect on the silylation reaction. In this study, three different silylating reagents, BSTFA, BSA, and trimethylsilylimidazole (TMSI) were

investigated to determine the completeness of silylation of nucleosides. Silylations were also carried out using BSTFA containing 1% TMCS.

Gehrke and Leimer[11] reported on the quantitative silylation of the twenty protein and a number of non-protein amino acids in different solvents. It was observed that the number of chromatographic peaks for the TMS derivatives of glycine, arginine, glutamic acid, etc., were determined by the polarity of the solvent. The effect of different solvents—acetonitrile, pyridine, dimethylformamide, chloroform, methylene chloride, hexane, benzene, and toluene—on silylation of nucleosides was investigated to achieve improvement in quantitation of the derivatization, and the effect of water on derivatization with silylation reagents was investigated to ascertain the need for anhydrous conditions.

EXPERIMENTAL

Reagents and materials

Acetonitrile, hexane, benzene, toluene, and chloroform were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.) and were of "nanograde" purity-spectral grade. Pyridine was analytical reagent grade and was freshly distilled over potassium hydroxide before use. BSTFA, BSA, and TMSI were from Regis (Chicago, Ill., U.S.A.) and were stored refrigerated. The liquid phase OV-11 and the solid support 100–120 mesh Supelcoport were obtained from Supelco (Bellefonte, Pa., U.S.A.). The nucleosides were obtained from Mann Labs. (New York, N.Y., U.S.A.) and were "Mann Assayed".

The apparatus and glassware, instrumental and chromatographic conditions, internal standard method of calculation, and preparation of the chromatographic columns were the same as reported earlier[6].

Optimal concentration for derivatization reagent

The nucleoside standard containing 1 μmole each of uridine, adenosine, guanosine, and cytidine per ml water was used. 0.56 ml TMSI (4004 μmoles per 4 μmoles nucleosides to give 1000 molar excess), 0.5 ml acetonitrile (containing 250 μg pyrene), and 0.4 ml acetonitrile were added and the samples were then silylated in a closed tube at 60°-3 h, 90°-2 h, and 120° and 150°-15 min, and chromatographed. The derivatizations were also carried out with 0.1 ml BSA (500 μmoles/4 nucleosides to yield 125 molar excess), 0.5 ml acetonitrile (containing 250 μg pyrene), and 0.4 ml acetonitrile at 120° and 150° for 15 min, 1 and 2 h.

The optimal molar excess for each silylation reagent was also determined using 1 μmole of thymidine or guanosine.

The analytical reagents from Regis contained 3.75 mmoles/ml of BSTFA, 5.00 mmoles/ml of BSA, and 7.15 mmoles/ml of TMSI, respectively. The following dilutions of these reagents were made before derivatization to obtain 2000 μmoles/ml for each: (a) 8 ml BSTFA + 7 ml acetonitrile, (b) 6 ml BSA + 9 ml acetonitrile, and (c) 4 ml TMSI + 10.3 ml acetonitrile.

To the dried thymidine or guanosine standard containing 1 μmole of nucleoside, 0.5 ml of acetonitrile (containing 250 μg pyrene) and 0.1, 0.25, 0.5, 1.0, and 2.0 ml amounts of the silylating reagent corresponding to the 200, 500, 1000, 2000, and 4000 molar excess were added. The final volume was adjusted to 2.5 ml with acetonitrile.

The silylation times and temperatures followed for BSTFA, BSA, and TMSI were 150°-15 min, 120°-2 h, and 60°-3 h, respectively. Five microliters of TMS nucleoside standard were chromatographed on a 4 w/w% OV-11 on 100–120 mesh Supelcoport 1 m × 4 mm I.D. glass column.

Silylation with different reagents: BSTFA, BSA, TMSI

Two nucleoside standards, *viz.* (a) uridine, inosine, xanthosine, and guanosine, and (b) thymidine, inosine, adenosine, and cytidine, containing 1 μmole of each nucleoside/ml water were used.

Silylation at 25° with 1000 molar excess of reagent for 5 min with sonication. To 1-ml aliquots of the standards, dried with nitrogen gas, 0.5 ml acetonitrile containing 250 μg pyrene was added. A 1000 molar excess per nucleoside of reagent was used by addition of 1.05 ml BSTFA, 0.8 ml BSA, and 0.56 ml TMSI (undiluted). The samples were made to a final volume of 2.5 ml with acetonitrile, then sonicated for 5 min, and 5 μl were chromatographed on a 4 w/w% OV-11 on 100–120 mesh Supelcoport 1 m × 4 mm I.D. glass column.

Derivatization at optimal reaction conditions for each reagent. One milliliter of nucleoside standard solutions (a) and (b) was dried and 0.5 ml of acetonitrile containing 250 μg of pyrene was added. A molar excess of 225, 500, and 1000 for BSTFA, BSA, and TMSI was used by adding 0.24, 0.40, and 0.56 ml of undiluted reagents, respectively. The final volume was brought to 2.5 ml with acetonitrile, then silylated as follows with: BSTFA, 225 molar excess, 150°-15 min; BSA, 500 molar excess, 120°-2 h; TMSI, 1000 molar excess, 60°-3 h.

Effect of various solvents and water on silylation with BSTFA

A variety of silylating solvents—acetonitrile, pyridine, dimethylformamide, methylene chloride, chloroform, benzene, toluene, and hexane—were studied. All of the solvents were freshly distilled and stored dry. Two milliliters of the stock nucleoside solutions described earlier were pipetted and dried. To this, 0.5 ml BSTFA and 0.5 ml of solvent containing 500 μg pyrene as internal standard (IS) were added. Closed tube silylations were made at 150°-15 min, then chromatographed on a 4 w/w% OV-11 on 100–120 mesh Supelcoport 1 m × 4 mm I.D. glass column.

In addition, experiments were made on the effect of water on the derivatization of the nucleosides. To 10 ml of anhydrous acetonitrile, water was added at levels of 1, 2, 10, 20, 30, and 40 ppt. The two nucleoside standard solutions containing 250 μg of each nucleoside mixture/ml were used. Two milliliters of each standard solution were used. To each sample, 0.5 ml of BSTFA, 0.5 ml of acetonitrile containing 500 μg pyrene, and 0.5 ml of acetonitrile containing the water added to give a final concentration of 0.5, 1, 5, 10, 15, and 20 ppt water in the silylation vials. After silylating at 150°-15 min, a 5-μl sample was chromatographed. The RWR* values for the nucleoside (pyrene) were plotted against water concentration in ppt. Also, silylations were carried out in pure BSTFA, with acetonitrile–BSTFA (3:1), and with acetonitrile–BSTFA (3:1) containing 1% TMCS as a silylation catalyst.

* $RWR_{N/P}$ = weight response of nucleoside/weight response of pyrene.

RESULTS AND DISCUSSION

The time, temperature, and molar excess for optimal silylation reaction conditions reported in the literature for BSA[4] are 120°-2 h, 100 molar excess and for TMSI[11] are 60°-3 h, 1000 molar excess. In our experiments on silylation of nucleosides with BSTFA, the following conditions gave the best results: a closed-tube reaction in an oil bath at 150°-15 min, with a 225 molar excess of BSTFA. Non-reproducible results were observed if a sand bath or other heating device was used. This was probably due to different rates of heat transfer from metal to glass or air to glass as compared to liquid to glass. The derivatization tube was only 1/4 full and was immersed in an oil bath to the level of liquid in the glass tube. This allowed a reflux of solvent.

The four major ribonucleosides—uridine, adenosine, guanosine, and cytidine—were silylated using a 1000 molar excess of TMSI at 60°-3 h, 90°-2 h, 120°-15 min, and 150°-15 min, and with a 125 molar excess of BSA at 120° and 150° for 15 min, 1 h, and 2 h, respectively. The $RWR_{N/P}$ values obtained at various reaction times and temperatures were compared with each other and the most complete silylation was obtained at 60°-3 h, using TMSI, and at 120°-2 h, using BSA. These reaction conditions were then selected for further experimental work.

Experiments were conducted at the optimal derivatization conditions for BSA (120°-2 h), BSTFA (150°-15 min), and TMSI (60°-3 h) using 200, 500, 1000, 2000, and 4000 molar excess of each reagent per nucleoside thymidine (pyrimidine) and guanosine (purine). The most complete silylation was achieved with a 225 molar excess of BSTFA, a 500 molar excess of BSA, and a 1000 molar excess of TMSI. As observed earlier, a decomposition of thymidine occurred on use of a higher molar excess of BSTFA and BSA. About 20–30% breakdown of thymidine occurred when a molar excess of 4000 with BSA and BSTFA was used.

TABLE I

COMPARATIVE SILYLATION WITH DIFFERENT REAGENTS AT ROOM TEMPERATURE

Silylation conditions: BSTFA, BSA, and TMSI all have 1000 molar excess, room temperature, 5 min sonication. Each value is a single determination on an independent sample.

Nucleoside	*RT (°C)*	*BSTFA*		*BSA*		*% Decrease*	*TMSI*		*% Decrease*
		RWR	*Av.*	*RWR*	*Av.*		*RWR*	*Av.*	
Thymidine	214	0.25	0.26	0.14	0.15	42	0.24	0.24	8
		0.26		0.15			0.23		
Uridine	218	0.67	0.67	0.61	0.61	9	0.63	0.64	4
		0.67		0.60			0.65		
Inosine	224	0.69	0.69	0.61	0.61	11	0.66	0.66	5
		0.68		0.61			0.65		
Adenosine	228	0.83	0.82	0.64	0.64	22	0.44	0.44	46
		0.81		0.64			0.43		
Xanthosine	234	0.62	0.62	0.55	0.56	10	0.59	0.59	5
		0.62		0.56			0.59		
Guanosine	239	0.38	0.39	0.27	0.28	28	0.20	0.21	46
		0.39		0.28			0.21		
Cytidine	$C_1 + C_2$	0.20	0.20	0.15	0.15	25	0.11	0.11	45
	(238 + 250)	0.19		0.15			0.11		

TABLE II

% DECREASE IN RWR VALUES COMPARED TO BSTFA

Nucleoside	*% Decrease in $RWR_{N/IS}$ to BSTFA*	
	BSA	*TMSI*
Containing amino groups: adenosine, guanosine, cytidine	25	45
Containing hydroxy groups: uridine, inosine, xanthosine	10	5
2'-Deoxynucleoside: thymidine	42	8

In other experiments, comparative silylations of seven major and minor nucleosides were carried out using a 1000 molar excess of BSTFA, BSA, and TMSI at room temperature (25°) with 5-min sonication. The $RWR_{N/P}$ values for silylation with BSA and TMSI were compared with those for BSTFA, as shown in Table I. The per cent decreases in RWR values are summarized in Table II.

Also, a comparison was made on silylation of the nucleosides at the best reaction conditions for each of the three reagents BSTFA, BSA, and TMSI (Table III). Table IV shows the average per cent decrease in the $RWR_{N/P}$ values for BSA (120°-2

TABLE III

COMPARATIVE SILYLATION WITH DIFFERENT REAGENTS AT OPTIMAL CONDITIONS

Silylation conditions: BSTFA, 225 molar excess, 150°-15 min; BSA, 500 molar excess, 120°-2 h; TMSI, 1000 molar excess, 60°-3 h. Each value is a single determination on an independent sample. For chromatographic conditions, see Experimental.

Nucleoside	*RT (°C)*	*BSTFA*		*BSA*		*% Decrease*	*TMSI*		*% Decrease*
		RWR	*Av*	*RWR*	*Av.*		*RWR*	*Av.*	
Thymidine	214	0.48	0.48	0.36	0.35	27.1	0.36	0 35	27
		0 47		0.34			0.35		
		0.49		0.35			0.35		
Uridine	218	0.78	0.79	0.74	0.75	5.1	0.75	0.75	5
		0.80		0.75			0.76		
		0.79		0.75			0.75		
Inosine	224	0.81	0.81	0.76	0.76	6.2	0.76	0.76	6
		0.82		0.75			0.76		
		0.81		0.76			0.77		
Adenosine	228	1.01	1.01	0.96	0.96	4.9	0.78	0.78	22.8
		0.99		0.95			0.76		
		1.02		0.96			0.78		
Xanthosine	234	0.79	0.80	0.73	0.74	7.5	0.73	0.74	7.5
		0.80		0.74			0.74		
		0.80		0.74			0.76		
Guanosine	239	0.68	0.68	0.64	0.64	5.9	0.51	0.51	25
		0.67		0.63			0.52		
		0.70		0.64			0.51		
Cytidine	250	0.36	0.37	0.35	0.36	2.7	0.28	0.28	24
		0.37		0.36			0.28		
		0.37		0.36			0.29		

TABLE IV

AVERAGE % DECREASE OF RWR VALUES COMPARED TO BSTFA

Nucleoside	*% Decrease in $RWR_{N/IS}$ to BSTFA*	
	BSA	*TMSI*
Containing amino groups: adenosine, guanosine, cytidine	5	24
Containing hydroxy groups: uridine, inosine, xanthosine	6	6
2′-Deoxynucleoside: thymidine	27	27

h, and 500 molar excess) and TMSI (60°-3 h, and 1000 molar excess) as compared to BSTFA (150°-15 min, and 225 molar excess).

The silylating strength for these three reagents was determined from the average per cent decrease in RWR values given in these experiments. The strength of the reagents for the amino group containing nucleosides silylated at room temperature was BSTFA > BSA > TMSI. For the hydroxy group containing nucleosides silylated at room temperature BSTFA > TMSI > BSA and for silylation under optimal conditions BSTFA > TMSI = BSA. The experimental data are given in Tables I and III. When the nucleosides were silylated with BSTFA at 25°-5 min, sonication, and 1000 molar excess of reagent (Table I) significantly lower $RWR_{N/P}$ values were obtained as compared to silylation with BSTFA at optimal reaction conditions at 150°-15 min, 225 molar excess (Table III). The average per cent decreases in RWR values were:

Amino group containing nucleosides	—adenosine	19%
	guanosine	43%
	cytidine	46%
Hydroxy group containing nucleosides	—uridine	15%
	inosine	15%
	xanthosine	22%

Guanosine and cytidine, both amino group containing nucleosides, showed a large decrease in RWR value on silylation at room temperature, indicating incomplete silylation of the amino group. When cytidine was silylated at room temperature with BSTFA and BSA, two peaks were obtained at retention temperatures of 238° and 250° on a 4 w/w% OV-11 on 100–120 mesh Supelcoport 1 m × 4 mm I.D. glass column. The RWR values obtained for these peaks using BSTFA, BSA, and TMSI are given in Table V.

TABLE V

RWR VALUES FOR CYTIDINE

Reagent	*RWR values*		
	RT = 238°	*RT = 250°*	*Total*
BSTFA	0.11	0.09	0.20
BSA	0.11	0.04	0.15
TMSI	0.11	0.00	0.11

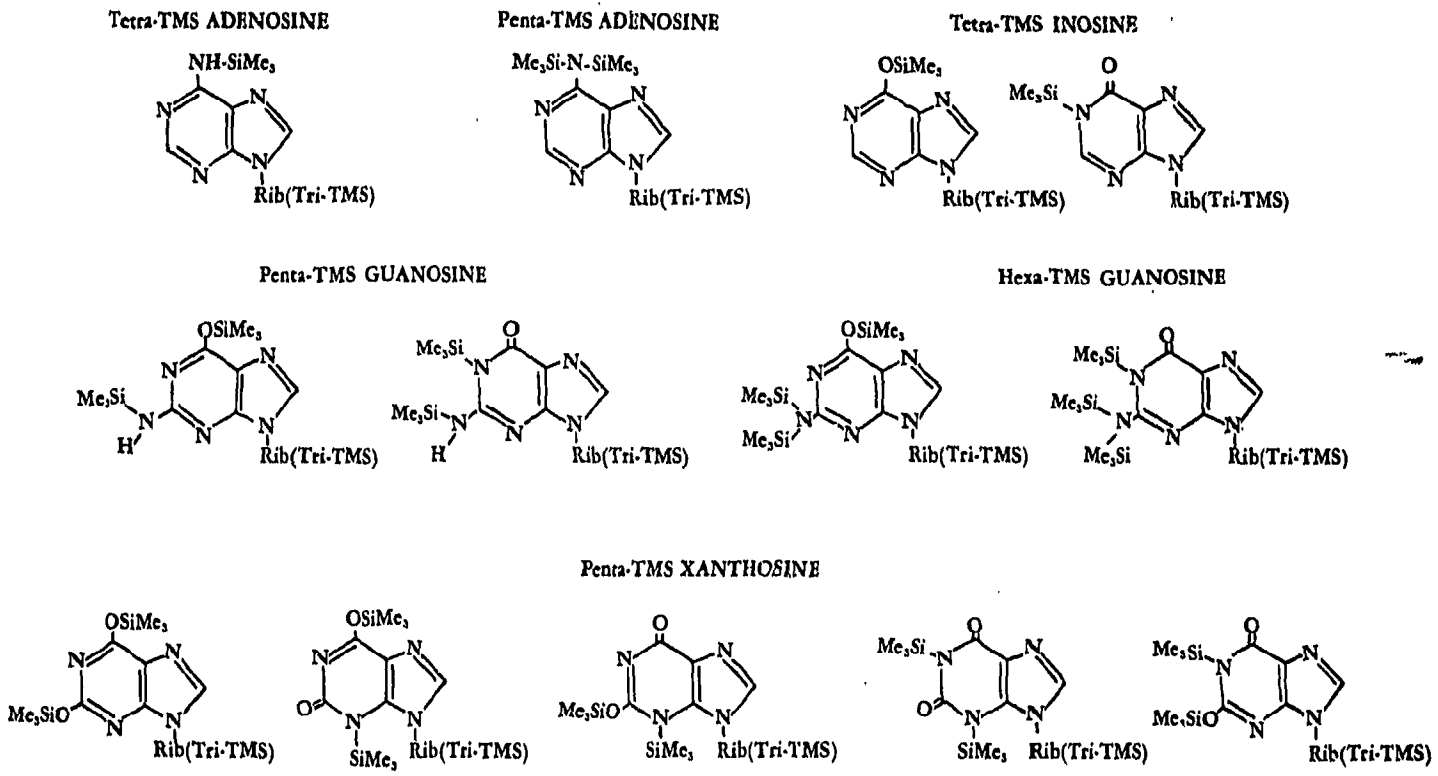

Fig. 1. Silylated purine nucleosides. Rib(Tri-TMS) = 2′,3′,5′-tri-O-TMS-β-D-ribofuranosyl group.

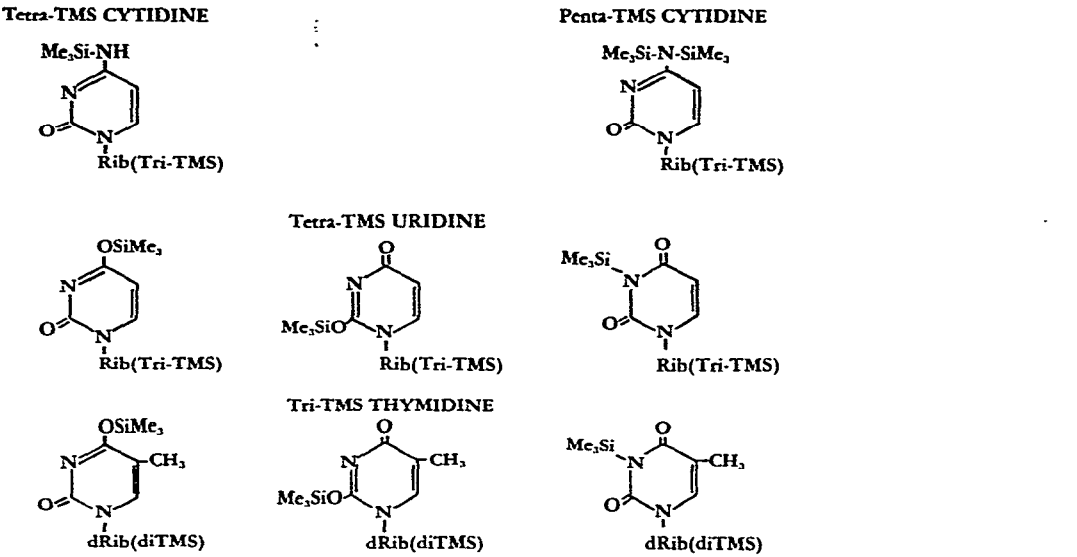

Fig. 2. Silylated pyrimidine nucleosides. Rib(Tri-TMS) = 2′,3′,5′-tri-O-TMS-β-D-ribofuranosyl group; dRib(diTMS) = 3′,5′-di-O-TMS-β-D-deoxyribofuranosyl group.

Cytosine, a pyrimidine base, gives two chromatographic peaks on silylation[3]. Recent GC–MS studies[12] on TMS cytosine in our laboratory indicated that these two peaks corresponded to di-TMS cytosine and tri-TMS cytosine. Similarly, these two chromatographic peaks for cytidine could possibly represent the formation of tetra-TMS cytidine (RT = 238°) and penta-TMS cytidine (RT = 250°). A deviation from the optimal derivatization conditions always results in an increased RT = 238° peak with a corresponding lowering of the RT = 250° peak. Proposed structures for the various TMS nucleosides and deoxynucleosides and their different structural isomers are presented in Figs. 1 and 2.

Effect of solvents on silylation

The effect of various solvents on trimethylsilylation of nucleosides is presented in Table VI. The RWR values obtained for the TMS nucleosides in different solvents were compared to the RWR values with acetonitrile as solvent. From the differences in the values, a classification of solvents was made as follows:

(a) Acetonitrile and pyridine—excellent for both purine and pyrimidine nucleosides

(b) Dimethylformamide—excellent for purine nucleosides only

(c) Methylene chloride and chloroform—excellent for pyrimidine nucleosides only

(d) Benzene, toluene, and hexane —poor for purine and pyrimidine nucleosides

When solvents other than acetonitrile or pyridine were used, derivatization was incomplete. In dimethylformamide, chromatographic peaks for TMS thymidine and TMS cytidine were not obtained. Poor response for purine nucleosides was obtained when they were silylated in methylene chloride and chloroform. The silylation of nucleosides in benzene, toluene, and hexane gave very poor $RWR_{N/P}$ values for all

TABLE VI

EFFECT OF VARIOUS SOLVENTS ON SILYLATION WITH BSTFA

Derivatization: 150°-15 min, 225 molar excess BSTFA, 500 μg nucleoside/ml of solvent. For chromatographic conditions, see Experimental.

Nucleosides	$RWR_{N/P}$							
	Acetonitrile	*Pyridine*	*Dimethyl-formamide*	*Methylene chloride*	*Chloroform*	*Benzene*	*Toluene*	*Hexane*
Pyrimidines								
Thymidine	0.50	0.48	NP*	0 48	0.44	0.36	0.38	0.43
	0.51	0.47	NP	0.46	0.42	0.37	0.37	0.42
Uridine	0.78	0.75	0.67	0.76	0.74	0.32	0.33	0.56
	0.79	0.76	0.66	0.77	0.72	0.31	0.35	0.58
Cytidine	0.37	0.35	0.01	0.34	0.31	0.16	0.17	0.28
	0.36	0.34	NP	0.35	0.32	0.15	0.15	0.27
Purines								
Inosine	0.78	0.78	0.76	0.38	0.33	0.05	0.16	0.45
	0.77	0.78	0.77	0.36	0.32	0.06	0.18	0.43
Adenosine	1.01	0.97	0.99	0.58	0.51	0.54	0.56	0.85
	1.02	0.98	1.00	0.61	0.49	0.55	0.57	0.86
Xanthosine	0.78	0.73	0.78	0.38	0.34	0.13	0.19	0.40
	0.79	0.72	0.76	0.40	0.35	0.12	0.20	0.41
Guanosine	0.70	0.70	0.70	0.30	0.28	0.12	0.19	0.40
	0.71	0.71	0.69	0.28	0.27	0.13	0.17	0.41

* NP = No peak obtained.

nucleosides. From these observations, acetonitrile was selected as the silylating solvent for all experiments.

The above-mentioned derivatized sample in methylene chloride was further diluted to 5 ml and 5 μl were chromatographed. The diluted sample gave an increase in $RWR_{N/P}$ value for the purine nucleosides. When nucleoside standards containing 50 μg of each nucleoside/ml were derivatized at 150°-15 min, using 225 molar excess BSTFA, with either acetonitrile or methylene chloride, the RWR values obtained

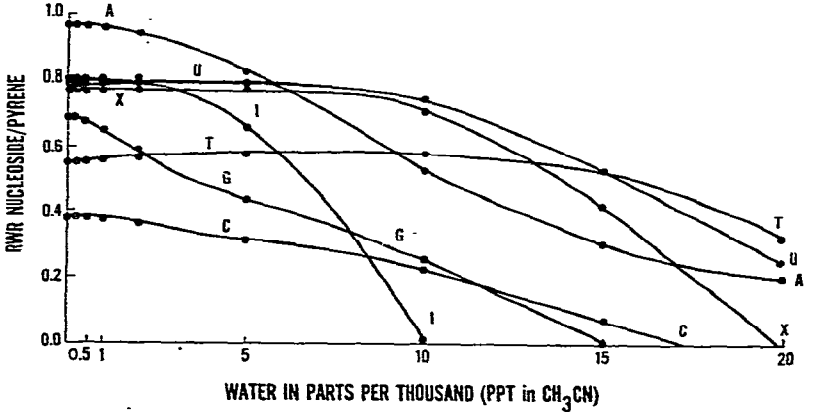

Fig. 3. Effect of water on silylation of nucleosides. A = Adenosine; C = cytidine; G = guanosine; I = inosine; T = thymidine; U = uridine; X = xanthosine.

TABLE VII

EFFECT OF WATER ON DERIVATIZATION OF CYTIDINE

Silylation at 150°-15 min, 1000 molar excess. Each value is a single determination on an independent sample. For chromatographic conditions, see Experimental.

Water concentration (ppt)	*RWR*			
	RT = 238°		*RT = 250°*	
0.5	NP*		0.38	0.39
1.0	0.04	0.05	0.33	0.32
5.0	0.15	0.17	0.13	0.14
10.0	0.19	0.21	0.03	0.02
15.0	0.07	0.04	0.03	0.02
20.0	0.00	0.00	0.00	0.00

* NP = No peak obtained.

were within experimental error. Even though different silylating strengths of BSTFA can be obtained in various polarity solvents, in these experiments solubility of nucleosides was found to be the primary factor for incomplete derivatization.

The effect of water on silylation of nucleosides is shown in Fig. 3. At a water level of about 1 ppt, derivatization of adenosine, guanosine, and cytidine (all amino group containing nucleosides) and inosine was affected. When the concentration of water exceeded 10 ppt, nearly all of the nucleosides gave a significant decrease in $RWR_{N/P}$ values. Multiple peaks for inosine and cytidine were obtained. Hancock[13] reported as many as seven peaks for N-7-methylinosine in the presence of excess water, and Hashizume and Sasaki[14] reported the disappearance of the cytidine peak in the presence of water. All of the nucleosides showed chromatographic peaks corresponding to the respective bases, ribose, and other multiple degradation products when silylated in acetonitrile containing 20 ppt water. The effect of water on derivatization of cytidine is presented in Table VII. The peak obtained at the retention temperature of 250° is used in the GLC analysis of cytidine in a mixture of nucleosides. Thus the presence of water above 2–3 ppt has a significant effect on the derivatization or the stability of cytidine.

The silylation of nucleosides was carried out using BSTFA–acetonitrile (1:3) containing 1% TMCS (Table VIII). It was observed that 1% TMCS did not have any

TABLE VIII

RWR OF NUCLEOSIDES

Silylation at 150°-15 min, 1000 molar excess BSTFA. Each value is a single determination on an independent sample. For chromatographic conditions, see Experimental.

Nucleoside	*BSTFA*			*Av.*	*BSTFA–acetonitrile (1:3)*			*Av.*	*BSTFA–acetonitrile (1:3)*			*Av.*
Thymidine	0.20	0.20	0.21	0.20	0.27	0.26	0.26	0.26	0.28	0.29	0.28	0.28
Uridine	0.76	0.75	0.77	0.76	0.80	0.82	0.79	0.80	0.81	0.84	0.82	0.82
Adenosine	1.00	1.01	0.99	1.00	0.98	0.97	0.98	0.98	0.99	0.98	0.99	0.99
Guanosine	0.72	0.74	0.84	0.73	0.69	0.70	0.70	0.69	0.71	0.71	0.69	0.70
Cytidine	0.50	0.48	0.51	0.50	0.44	0.45	0.44	0.44	0.47	0.48	0.46	0.47

* BSTFA containing 1% TMCS.

catalytic effect on the silylation of nucleosides. The silylations were also carried out in the pure reagent. The non-solvent silylations were done because the amount of DNA and RNA from cells is in the microgram range and often only this amount of sample is available. As significant differences in $RWR_{N/P}$ values of nucleosides were not observed, it was concluded that such analyses were possible by non-solvent derivatization.

CONCLUSIONS

Silylation of nucleosides with BSTFA was found to be superior to that with TMSI and BSA. Acetonitrile was the best of various solvents tried for silylation with BSTFA and water, when present at a level of 10 ppt or greater, caused a significant decrease in the RWR values for the nucleosides. These derivatizations will be useful in the analysis of RNA and DNA hydrolysates and methylated nucleosides in biological fluids in support of research for bio-markers in cancer.

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