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Note

Preparation of trimethylsilyl derivatives of ribonucleosides for gas chromatography

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Unfortunately, ribonucleosides cannot be analysed directly by gas chromatography (GC), and various derivatives have been studied, including methyl[1], acetyl and isopropylidene[2] and the trimethylsilyl (TMS)[3,4] derivatives. TMS derivatives gave better results than the others.

Silylation is a one-step derivatization procedure, whereas other derivatives are formed in two or more reaction steps. Gehrke and Patel[5] compared the silylation strengths of N,O-bis(trimethylsilyl)acetamide(BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-trimethylsilylimidazole (TMSI), and found that for nucleosides BSTFA was superior to BSA and TMSI. They further studied the reaction conditions for the quantitative derivatization of nucleosides with BSTFA[6], and found that the optimal conditions were 150°C for 15 min using closed-tube silylation with a 225 *M* excess of BSTFA and acetonitrile or pyridine as solvent[7].

In this work, a new silylating reagent, trimethylsilyl-N,N-dimethylcarbaminate (DMSIC), was compared with BSTFA used according to the method of Gehrke and Patel[7].

EXPERIMENTAL

Analytical-reagent grade pyridine was obtained from Merck (Darmstadt, F.R.G.) and chromatographically pure acetonitrile from Pierce (Rockford, IL, U.S.A.). BSTFA was purchased from Pierce and kept in a refrigerator at 4°C. α-Pseudouridine ($\alpha\psi$), inosine (Ino), 6-methyladenosine (m^6Ade), N^2,N^2-dimethylguanosine (m^2_2Gua) and 1-methylinosine (m^1 Ino) were obtained from Sigma (St. Louis, MO, U.S.A.), β-pseudouridine ($\beta\psi$) from Calbiochem (San Diego, CA, U.S.A.) and adenosine (Ade) from N. B. Co. (Cleveland, OH, U.S.A.). *n*-Octacosane was obtained from Applied Science Labs. (State College, PA, U.S.A.) and was used as the internal standard (I.S.). DMSIC was produced by the Department of General and Inorganic Chemistry of the Eötvös Lóránd University (Budapest, Hungary)[8,9].

A 2.8 m × 3 mm I.D. glass column was packed with 2% (w/w) OV-17 on

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TABLE I

EFFECT OF ACETONITRILE AND PYRIDINE ON SILYLATION WITH DMSIC

Derivatization: 60°C, 30 min, 7.5 molar excess of DMSIC. For detailed derivatization and chromatographic conditions, see Experimental. RWR = Relative weight response; S.D. = standard deviation; R.S.D. = relative standard deviation (%).

Nucleoside	*No solvent*			*Acetonitrile*			*Pyridine*		
	Av. RWR	*S.D.*	*R.S.D.*	*Av. RWR*	*S.D.*	*R.S.D.*	*Av. RWR*	*S.D.*	*R.S.D.*
β-Pseudouridine	0.86	0.025	1.59	0.83	0.041	4.2	0.77	0.022	3.8
6-Methyladenosine	0.82	0.034	2.2	0.86	0.038	4.0	0.74	0.019	2.5
1-Methylinosine	0.79	0.016	1.1	0.75	0.033	4.5	0.68	0.040	6.3

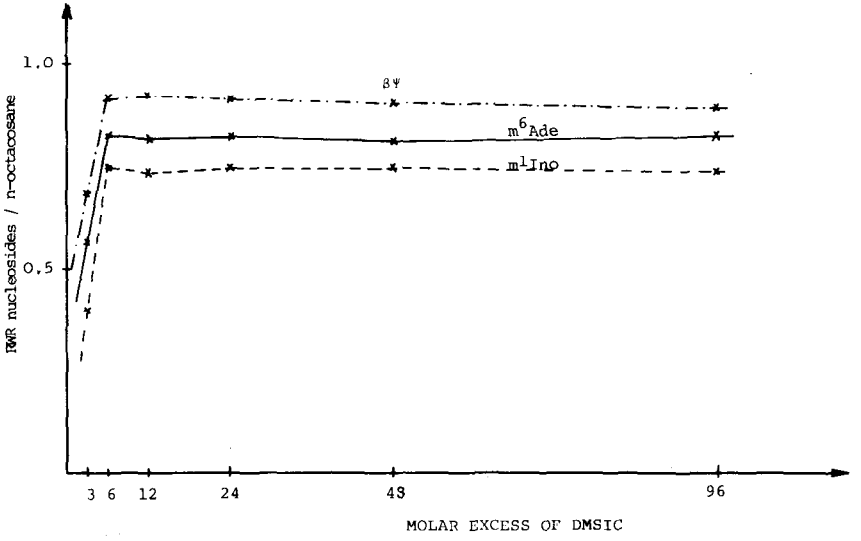

Fig. 1. Silylation of nucleosides as a function of molar excess of DMSIC.

80–100-mesh Chromosorb WPH (Chrompack, Middelburg, The Netherlands). Reacti-Vials with Mininert valves (Pierce) were used as silylation vessels.

Instrumental and chromatographic conditions

A Hewlett-Packard 5730A temperature-programmed-two-channel gas chromatograph equipped with two flame ionization detectors and a Hewlett-Packard 3380A integrator were used. The conditions were as follow: initial temperature, 210°C; programming rate, 5°C/min; final temperature, 310°C; carrier gas (nitrogen) flow-rate, 40 cm^3/min; air flow-rate, 150 cm^3/min; hydrogen flow-rate, 30 cm^3/min; injector temperature, 350°C.

The internal standard method was used to calculate the weight percentage concentration of each nucleoside. The relative weight response (RWR) values characterizing the TMS yields were calculated as

$$RWR_{NC_{28}} = \frac{area_N/grams_N}{area_{C_{28}}/grams_{C_{28}}}$$

where N = nucleoside and C_{28} = *n*-octacosane (I.S.).

In each instance five measurements were averaged and used for the calculations.

Preparation of TMS derivatives of ribonucleosides

Nucleoside standards and *n*-octacosane (I.S.) were dissolved in pyridine, the solution was measured into a reaction tube and the solvent was evaporated in a flow of nitrogen. A known excess of the silylating reagent was added to the dry material and the tube was closed securely, shaken for 1 min, then thermostated at a given temperature.

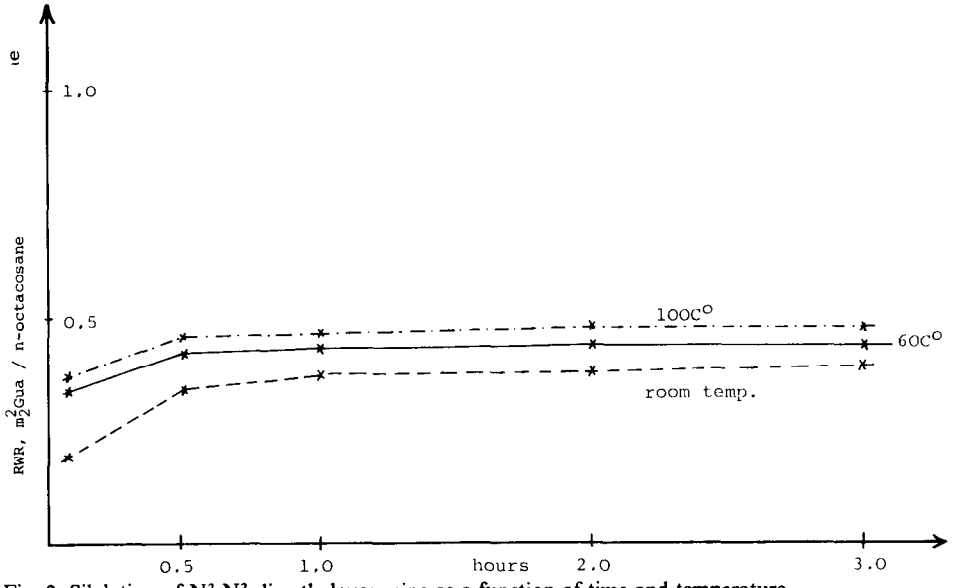

Fig. 2. Silylation of N^2,N^2-dimethylguanosine as a function of time and temperature.

Determination of optimal conditions

In order to determine the optimal conditions for maximal yield of the ribonucleoside TMS derivatives we studied (a) the effect of various solvents, (b) the effect of a molar excess of the reagent and (c) the effect of reaction temperature and time and (d) we compared our method with an accepted procedure.

RESULTS

The effects of different solvents on the reaction are summarized in Table I. The reaction was completed in both the presence and absence of solvents. DMSIC dissolves all the nucleosides of different polarity, and consequently it is a suitable reaction medium. Therefore, experiments were carried out without additional solvent.

Results obtained by using different molar excesses of the reagent are shown in Fig. 1. Complete derivatization requires a 6–10-fold excess of the reagent. As the amount of biological samples available may be limited, the use of a 10–100-fold excess of the reagent is recommended in order, to be able to use manageable sample sizes.

Fig. 3. GC analysis of human urine. Sample, 4.0 μl.

TABLE II

EFFECTS OF TEMPERATURE AND TIME SILYLATION WITH DMSIC IN THE ABSENCE OF A SOLVENT

Silylation conditions: DMSIC, 6 molar excess, without solvent; (A) room temperature, 60 min; (B) 60(°C, 30 min; (C) 100°C, 30 min. Chromatography as described under Experimental. Abbreviations as in Table I.

Nucleoside	*Retention temperature (°C)*	*A*			*B*			*C*		
		Av. RWR	*S.D.*	*R.S.D.*	*Av. RWR*	*S.D.*	*R.S.D.*	*Av. RWR*	*S.D.*	*R.S.D.*
α-Pseudouridine	238	1.0	0.017	1.7	1.12	0.011	1.0	1.09	0.008	0.42
β-Pseudouridine	241	0.93	0.034	3.6	1.02	0.019	1.9	1.0	0.009	0.9
Inosine	255	0.36	0.016	4.4	0.42	0.014	3.3	0.4	0.022	5.5
6-Methyladenosine	259	0.75	0.033	4.4	0.77	0.016	2.0	0.8	0.024	3.0
N^2,N^2-Dimethylguanosine	271	0.34	0.023	6.7	0.43	0.015	3.5	0.47	0.008	1.7
1-Methylinosine	286	0.6	0.025	4.2	0.74	0.009	1.2	0.73	0.016	2.2

The example of m_2^2Gua (Fig. 2) shows the effect of temperature and time. The efficiency of silylation at room temperature was low. An increased temperature increased the yield of the derivatization reaction. Slight differences in yield were observed at 60 and 100°C, the latter being better. At lower temperatures the heat-induced decomposition of nucleosides can be avoided.

The amount of the reaction product did not change after 30 min. Consequently, TMS derivatives of the nucleosides were prepared in solvent-free medium at 60°C in 30 min (Table II). BSTFA and DMSIC reagents under optimal conditions both gave good results (Table III).

TABLE III

COMPARISON OF SILYLATION WITH BSTFA AND DMSIC REAGENTS UNDER OPTIMAL CONDITIONS

Silylation conditions: DMSIC, 6 molar excess, without solvent, 60°C, 30 min; BSTFA, 225 molar excess, acetonitrile, 150°C, 15 min. For chromatographic conditions, see Experimental. Abbreviations as in Table I.

Nucleoside	*DMSIC*			*BSTFA*		
	Av. RWR	*S.D.*	*R.S.D.*	*Av. RWR*	*S.D.*	*R.S.D.*
α-Pseudouridine	1.12	0.011	1.0	1.2	0.025	2.0
β-Pseudouridine	1.02	0.019	1.9	1.1	0.033	3.0
Inosine	0.42	0.014	3.3	0.37	0.015	4.1
6-Methyladenosine	0.77	0.016	2.0	0.8	0.016	2.0
N^2,N^2-Dimethylguanosine	0.43	0.015	3.5	0.35	0.017	4.8
1-Methylinosine	0.74	0.009	1.2	0.56	0.03	5.4

Fig. 3 demonstrates an example for the possible application of DMSIC as a silylating agent. The figure shows the elution profile of human urine nucleosides.

CONCLUSIONS

DMSIC is a very effective silylating reagent for ribonucleosides. The silylation reagent is a suitable solvent for the nucleosides and their TMS derivatives. Complete derivatization was achieved under mild reaction conditions, with a 15 molar excess of BMSIC at 60°C for 30 min without additional solvent.

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