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HIGH-RESOLUTION GAS CHROMATOGRAPHY OF METHYLATED RIBONUCLEOSIDES AND HYPERMODIFIED ADENOSINES

EVALUATION OF TRIMETHYLSILYL DERIVATIZATION AND SPLIT AND SPLITLESS OPERATION MODES

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SUMMARY

Methylated ribonucleosides and hypermodified adenosines were trimethylsilylated on chromatographed by high-resolution gas chromatography on a fused-silica capillary column operated in split and splitless modes. Evaluation of micro-silylation (50-μl volume) of methylated ribonucleosides showed that N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and pyridine at 150°C gave greater yields than silylation with either BSTFA alone or BSTFA and pyridine at 75°C. N-Methyl-N-trimethylsilyltrifluoroacetamide gave lower yields of derivatives of N^6-substituted adenosines, such as N^6-methyladenosine, relative to those obtained with BSTFA. Methylated ribonucleosides generally gave sharp, symmetrical peaks on the SE-54 column operated in the split mode; however, the compounds were not as well resolved as the cytokinin-active hypermodified adenosines on the relatively non-polar SE-54 stationary phase. The splitless operation mode employing a cold trapping procedure (40°C initial temperature) yielded sharp peaks and nanogram quantities of N^6-methyladenosine were detectable. Most hypermodified adenosines separated well from other compounds, although several peaks of unknown composition eluted in the same chromatographic region as the methylated ribonucleosides when the cold trapping splitless technique was used.

INTRODUCTION

A large number of structural modifications of ribonucleosides are found in tRNA from plant, animal and microbial sources and in biological fluids[1–3]. These can

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range from simple methylation of a base or sugar moiety to more complex derivatizations as exemplified by the modified isopentyladenosines termed hypermodified ribonucleosides. The latter compounds function as cytokinins or plant cell division factors[2,3]. It was reported that changes in ribonucleoside methylation in tRNA in animals were associated with cellular differentiation processes and the development of tumorous or autonomous tissues[4,5]. Recent evidence has also indicated the presence of altered patterns of ribonucleoside methylation[6] and an additional cytokinin-active hypermodified adenosine[7] in tRNA of hormone-autonomous plant tissues. As part of a study of changes in ribonucleoside structural modifications in tRNA we are interested in methods of separation of these compounds.

Several investigations have dealt with methods of preparation and conditions for gas chromatographic (GC) separation of trimethylsilyl (TMS) derivatives of the parent nucleic acid bases and their corresponding ribonucleosides[8–15]. Lakings *et al.*[16] studied the efficiency of silylation and separation of several methylated bases. Hattox and McCloskey[17] studied chromatographic behavior of TMS derivatives of naturally occurring and synthetic modified ribonucleosides on two packed GC columns and Chang *et al.*[18] developed a GC method for quantitative analyses of two methylated ribonucleosides.

Cytokinin-active bases and ribonucleosides were silylated and separated on packed GC columns[19–21]. Claeys *et al.*[22] used capillary GC to resolve some permethylated cytokinin bases, and we recently reported[23] separation of cytokinin hypermodified bases and ribonucleosides by fused-silica capillary GC operated in split mode.

In the present investigation we studied reagents and reaction conditions for the preparation of TMS derivatives of ribonucleosides and chromatography of these compounds on high-resolution fused-silica capillary GC in split and splitless operation modes.

EXPERIMENTAL

Chemicals

trans-Ribosylzeatin (tZR), N^6-(Δ^2-isopentenyl)adenosine (IPA), phloretin (Ph), and methylated ribonucleosides including 1-methyladenosine (m^1A), N^6-methyladenosine (m^6A), N^6,N^6-dimethyladenosine (m^6_2A), 3-methylcytidine (m^3C), N^2-methylguanosine (m^2G), 1-methylinosine (m^1I), and 5-methyluridine (m^5U) were purchased from Sigma (St. Louis, MO, U.S.A.). The remaining cytokinin ribonucleosides were generously provided by Dr. J. Corse (USDA, ARS, Albany, CA, U.S.A.). N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA), BSTFA containing 1 % trimethylchlorosilane (TMCS), and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Pierce (Rockford, IL, U.S.A.). Pyridine and acetonitrile were obtained from Regis (Chicago, IL, U.S.A.). Methylene chloride and P_2O_5 were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Preparation of modified ribonucleoside standards

Stock solutions used for evaluations of relative weight response (RWR) under various silylation conditions were prepared by dissolving 1 mg of each methylated ribonucleoside in 2 ml distilled water, except N^2-methylguanosine, which was dis-

solved in water adjusted to pH 1 with 1 *N* hydrochloric acid. The concentration of each solution was confirmed by calculations based on the UV absorbance at the λ_{max} for each compound and the published absorptivities[24].

Stock solutions of methylated ribonucleosides for long term storage in a freezer were made at the same concentration as above using 75% ethanol as solvent. Cytokinin-active hypermodified adenosines were dissolved in 95% ethanol at a concentration of 0.5 μg/μl.

Micro-silylation of methylated ribonucleosides

The following method was used for determination of RWR values and other chromatographic purposes: 50 μl of methylated ribonucleoside stock solution and 50 μl of IPA solution were concentrated to near dryness under a stream of nitrogen in a 1-ml screw-cap reaction vial. The sample was azeotroped with methylene chloride and then dried over P_2O_5 at 10^{-2} Torr overnight. A 50-μl volume of silylating reagent or 10 μl of solvent plus 40 μl of silylating reagent was added. Silylating reagents were either BSTFA, BSTFA containing TMCS (99:1, v/v) or MSTFA. The solvent was pyridine or acetonitrile. Vials were sealed with PTFE-lined screw caps and heated in an oil bath at 75 or 150°C for 30 min. Cytokinin-active hypermodified adenosines were silylated as described previously[23].

Determinations of RWR values for methylated ribonucleosides

RWR values for methylated ribonucleosides were determined on a Varian Aerograph Series 2100 gas chromatograph equipped with a Hewlett-Packard Model 3380 A integrator. A 0.5–1.0-μg sample of each compound plus IPA internal standard was silylated as described above and chromatographed on a 1.8 m × 2 mm I.D. glass column packed with 3% SP-2250 coated on 100–120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Nitrogen was used as carrier gas at a flow-rate of 30 ml/min. The injector temperature was 270°C, the flame ionization detector (FID) temperature was 280°C, and the column oven temperature was varied depending upon compound separation requirements. RWR values were calculated as follows:

$$\text{RWR} = \frac{\text{Peak area of silylated ribonucleoside}}{\text{Weight of silylated ribonucleoside}} \Big/ \frac{\text{Peak area of silylated IPA int. std.}}{\text{Weight of silylated IPA int. std.}}$$

If a methylated ribonucleoside yielded a shoulder or a secondary peak in addition to the major peak when chromatographed on the packed column, the area of the minor peak was included in the calculation of the RWR value. Earlier work on parent ribonucleosides showed that doublet peaks occurred that were composed of two silylated species of a given ribonucleoside[11,15].

Capillary GC of methylated ribonucleosides in split mode

Silyl derivatives of methylated ribonucleosides were chromatographed by high-resolution GC on a 30 m × 0.25 mm I.D. SE-54 (94% methyl, 5% phenyl, and 1% vinyl silicone) fused silica capillary column (J&W Scientific, Rancho Cordova, CA, U.S.A.) operated in the split mode. A microprocessor-controlled Hewlett-Packard 5880A gas chromatograph was used with inlet at 270°C, FID at 280°C, and the

column oven temperature-programmed from 180 to 265°C at 4°C/min after a 2-min initial hold. Helium was used as the carrier and makeup gas; the split ratio was 60:1 and the carrier linear velocity through the column was 31 cm/sec.

Capillary GC of modified ribonucleosides in splitless mode

For splitless operation mode the Hewlett-Packard gas chromatograph containing the SE-54 fused-silica capillary column was used with the inlet temperature at 270°C and FID at 280°C. The inlet purge flow was 1.2 ml/min and the carrier gas linear velocity was 31 cm/sec. The sample was injected with a purge inactivation time of 40 sec and an initial column oven temperature of 40°C. This procedure cold-trapped the silylation reagent (BSTFA) and the organic solvent containing the ribonucleoside sample. After 2 min, the oven was temperature-programmed at 10°C/min to 265°C and then maintained at 265°C for 60 min.

Mass spectrometry (MS)

The numbers of silyl moieties per molecule (molecular weights) of silylated hypermodified ribonucleosides were determined by electron impact using a Hewlett-Packard Model 5985 GC–MS instrument operated at 70 eV or by chemical ionization with a Finnegan Model 3300-6100 GC–MS instrument at 150 eV using methane as reagent gas.

RESULTS AND DISCUSSION

Conditions for silylation

Effects of temperature and solvent composition on the micro-silylation reaction of some methylated ribonucleosides with BSTFA are shown in Table I. Generally, compounds were silylated at 75°C in BSTFA with or without the inclusion of pyridine in the reaction mixture. However, increasing the reaction temperature to 150°C noticeably increased the yield of derivatives in several instances. The presence of pyridine in the reaction mixture at 150°C also improved the yield in several cases.

TABLE I

SOLVENT AND TEMPERATURE EFFECTS ON THE SILYLATION OF SOME METHYLATED RIBONUCLEOSIDES

Values, expressed as FID response (RWR)*

Ribonucleoside	*75°C*		*150°C*	
	BSTFA	*BSTFA–pyridine*	*BSTFA*	*BSTFA–pyridine*
1-Methyladenosine	0.44	0.67	0.84	1.07
N^6-Methyladenosie	1.15	1.10	1.04	1.18
N^6,N^6-Dimethyladenosine	1.53	1.47	1.60	1.66
3-Methylcytidine	0.82	0.82	0.89	1.05
N^2-Methylguanosine	0.51	0.54	0.55	0.59
3-Methyluridine	1.36	1.42	1.59	1.80
5-Methyluridine	0.79	0.76	0.94	0.91

* Relative to internal standard isopentenyladenosine. Each value is mean of two determinations.

Preliminary trials with acetonitrile–BSTFA or pyridine–BSTFA containing 1% TMCS gave results similar to those noted above. Among the compounds tested, methylated derivatives of adenosine and uridine frequently silylated more readily than methylated derivatives of cytidine or guanosine. Examples of compounds which silylated poorly under the micro-reaction conditions are 5-methylcytidine and 1-methylguanosine which yielded weak or nondetectable responses when chromatographed on the SE-54 capillary column. Chang *et al.*[18] reported a high yield for two methylated ribonucleosides, N^2,N^2-dimethylguanosine and 1-methylinosine using a relatively large volume (0.5 ml) of BSTFA and solvent for the silylation reaction.

Silylation of some modified ribonucleosides was also evaluated with MSTFA, also a strong silyl donor. Several compounds gave approximately equal yields of derivatives in both BSTFA and MSTFA at 150°C. However, N^6-substituted adenosines, such as N^6-methyladenosine gave a greater yield (2.8-fold) in BSTFA than in MSTFA. In contrast, when a relatively large amount of guanosine (125 μg) was treated with MSTFA, a 1.8-fold yield increase over that with BSTFA was obtained. A shift in the retention time of the guanosine product with MSTFA indicated the formation of a different silyl derivative.

Capillary GC in split mode

Results of chromatography of several representative silylated methylated ribonucleosides, prepared with BSTFA, on the SE-54 fused silica capillary column are shown in Fig. 1. Each compound (0.75 μg) was injected at a split ratio of 60:1. Most compounds gave sharp, symmetrical peaks in the split mode. However, the first emerging peak which corresponded to 3-methylcytidine was broader than the others. This may have resulted from the incomplete resolution of more than one silylated derivative of this ribonucleoside. The peak corresponding to N^2-methylguanosine was sharp and symmetrical, but the response was relatively low. This probably resulted from a less efficient silylation reaction for this compound. Tests with parent

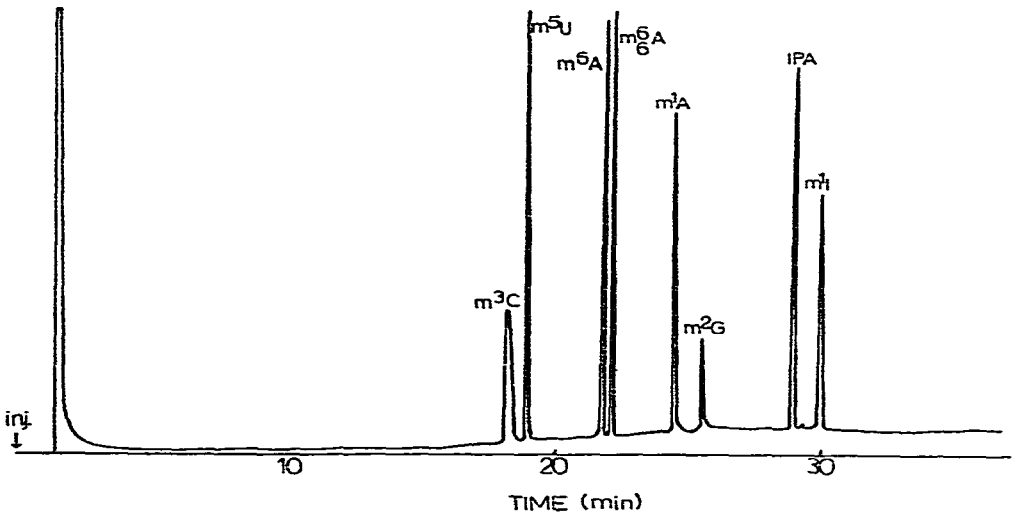


Fig. 1. Chromatogram of BSTFA-prepared silylated modified ribonucleosides on SE-54 fused-silica capillary column using split mode (60:1).

ribonucleosides showed that these compounds overlapped with some methylated ribonucleosides, for example, adenosine eluted with N^6-methyladenosine on the SE-54 column under the split condition. A more polar stationary phase may be useful for separation of these compounds in the split mode conditions. Earlier results[23] showed that hypermodified (cytokinin-active) adenosines were obtained longer and separated better on the SE-54 column; these compounds were structurally similar and relatively non-polar (*e.g.*, geometrical isomers of isopentenyl adenosine derivatives).

Capillary GC in splitless mode

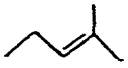
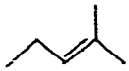
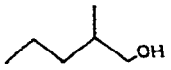
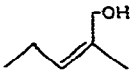
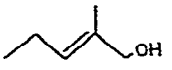
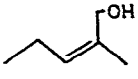
The effect of inlet-purge inactivation time on the peak area of *trans*-ribosylzeatin was measured. It was found that a purge inactivation time of at least 20 sec was needed for maximum recovery of the compound under the GC conditions developed for temperature-programmed analysis of the modified ribonucleosides.

Table II lists relative retention times and number of silyl moieties as determined by MS (from molecular weights) of some representative hypermodified adenosines on the SE-54 column operated in the splitless mode. Generally, these compounds separated well using the cold-trapping splitless technique, except *cis*-ribosylzeatin and methylthioisopentenyladenosine which overlapped. Fig. 2 shows a chromatogram of 20 ng each of N^6-methyladenosine and *trans*-ribosylzeatin and the internal standard phloretin which were silylated with BSTFA and then separated on

TABLE II

SEPARATION OF HYPERMODIFIED ADENOSINES (CYTOKININ ACTIVE) BY COLD TRAPPING TECHNIQUE IN SPLITLESS MODE

Column: SE-54 fused-silica capillary.

Compound	*Adenosine substituents*		*No. of TMS moieties per molecule*	*Relative retention time**
	N^6-Amino site	*C-2 site*		
Isopentenyladenosine		H	3	1.17
2-Methylthioisopentenyladenosine		CH_3S-	3	1.59
Dihydroribosylzeatin		H	4	1.43
cis-Ribosylzeatin		H	4	1.49
trans-Ribosylzeatin		H	4	1.58
cis-2-Methylthioribosylzeatin		CH_3S-	4	2.17

* Relative to internal standard phloretin.

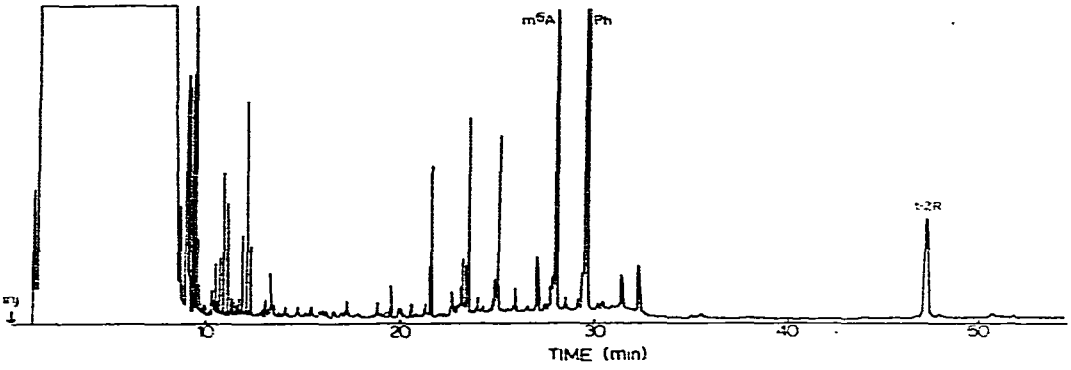


Fig. 2. Chromatogram of silylated derivatives of N^6-methyladenosine, *trans*-ribosylzeatin and the internal standard phloretin, 20 ng each, on SE-54 fused-silica capillary column using cold-trapping splitless technique.

the SE-54 column using the cold-trapping splitless technique. Results with N^6-methyladenosine as well as trials with other methylated ribonucleosides indicated that these compounds eluted in an area where interfering peaks of unknown composition appeared and therefore the method was not as useful for these compounds as for the hypermodified (cytokinin-active) adenosines. However, sharp, symmetrical peaks were obtained for both compounds and *trans*-ribosylzeatin separated well from all other peaks.

The relative FID response for silylated *trans*-ribosylzeatin in the splitless versus split mode of operation described above was 52-fold greater for the splitless operation. Injection of 2 ng of N^6-methyladenosine gave 25% of full scale deflection at maximum instrument sensitivity in the splitless mode.

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