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SEPARATION OF THE METHYLATED NUCLEOSIDE CONSTITUENTS OF RIBONUCLEIC ACID BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

In an attempt to separate a number of methylated nucleoside constituents reportedly present in various mammalian RNA preparations, a comparison of the migration of selected nucleoside standards was assessed via thin-layer chromatographic techniques with respect to three solvent systems and an adsorbent of varying proportions of silica gel and microcrystalline cellulose. The results of these studies revealed that a mixture of seventeen methylated constituents could be separated following two-dimensional development on an adsorbent containing 60% cellulose and 40% silica gel. Constituents separated included the 2′-O-methylated derivatives of adenosine, guanosine, cytidine and uridine, 1-, N^6- and N^6,N^6-methyladenosines, 1-methylinosine, 1-, 7-, N^2- and N^2,N^2-methylguanosines, 3-, 4- and 5-methylcytidines and 3- and 5-methyluridines. In addition, all of the above constituents were separated from their respective non-methylated nucleosides and bases.

INTRODUCTION

In recent years numerous investigators utilizing a variety of chromatographic techniques have attempted to resolve the methylated constituents reportedly present in tRNA, rRNA and mRNA[1–12]. Although varying degrees of success have been obtained, in many instances complete chromatographic resolution of the constituents under consideration was not achieved[3,10,12]. While more recent methods have been developed which resolve either methylated bases or 2′-O-methylribose constituents[1,9,11], no single thin-layer chromatographic (TLC) procedure is available for separating both types of methylated derivatives. Since mRNA, rRNA and tRNA all contain significant quantities of both base- and ribose-methylated nucleosides[12–14], it seems obvious that a simple, rapid chromatographic method which separates all types of methylated constituents simultaneously would be desirable.

Employing a similar strategy as that used for separating the methylated bases of tRNA[15], we describe here a two-dimensional TLC procedure which completely resolves seventeen methylated nucleoside constituents present in mammalian RNA.

MATERIALS AND METHODS

The sources of nucleoside and base standards employed in this investigation are listed in Table I. Analysis of these constituents via the chromatographic system described herein revealed the presence of a single UV-absorbing component. Stock

TABLE I

SOURCES AND R_F VALUES OF NUCLEOSIDES AND BASES CHROMATOGRAPHED ON A COMPOSITE ADSORBENT CONTAINING 60% CELLULOSE AND 40% SILICA GEL GF

Solvent systems: I, ethyl acetate–methanol–water–88% formic acid (100:25:20:1); II, acetonitrile–ethyl acetate–2-propanol–1-butanol–58% ammonium hydroxide–water (40:30:20:10:22:5); III, ethyl acetate–methanol–0.8 *M* boric acid (100:25:20). Sources: S = Sigma, St. Louis, Mo., U.S.A.; C = Cyclo, Los Angeles, Calif., U.S.A.; R = Dr. Morris Robins, University of Alberta, Alberta, Canada; F = Dr. Jack Fox, Sloan Kettering Inst., New York, N.Y., U.S.A.; P = P-L Biochemicals, Milwaukee, Wisc., U.S.A.

Standard constituent	*Source*	R_F *value*		
		Solvent system		
		I	*II*	*III*
Nucleosides:				
Adenosine (A)	S	0.45 (1.07)*	0.51 (1.34)	0.26
1-CH_3-A	S	0.09 (0.21)	0.45 (1.18)	0.00
N^6-CH_3-A	S	0.54 (1.29)	0.61 (1.61)	0.48
N^6,N^6-diCH_3-A	S	0.61 (1.45)	0.69 (1.82)	0.59
2′-O-CH_3-A	P	0.64 (1.53)	0.65 (1.71)	0.59
1-CH_3-Inosine	S, C	0.34 (0.81)	0.48 (1.26)	0.13
Guanosine (G)	S	0.22 (0.52)	0.14 (0.37)	0.12
1-CH_3-G	S, C	0.29 (0.69)	0.41 (1.08)	0.13
7-CH_3-G	S, C	0.11 (0.26)	0.14 (0.37)	0.00
N^2-CH_3-G	S	0.29 (0.69)	0.21 (0.54)	0.11
N^2,N^2-diCH_3-G	S	0.32 (0.76)	0.24 (0.63)	0.11
2′-O-CH_3-G	R	0.40 (0.95)	0.26 (0.68)	0.41
3′-O-CH_3-G	R	0.39 (0.94)	0.24 (0.63)	0.40
Cytidine (C)	S	0.21 (0.50)	0.30 (0.79)	0.11
3-CH_3-C	S	0.15 (0.38)	0.55 (1.45)	0.00
4-CH_3-C	F	0.34 (0.56)	0.42 (1.11)	0.11
5-CH_3-C	S	0.22 (0.52)	0.34 (0.89)	0.12
2′-O-CH_3-C	P	0.30 (0.71)	0.47 (1.24)	0.35
Uridine (U)	S	0.49 (1.17)	0.21 (0.55)	0.31
3-CH_3-U	S	0.63 (1.50)	0.62 (1.63)	0.45
5-CH_3-U	S	0.56 (1.33)	0.37 (0.97)	0.38
2′-O-CH_3-U	P	0.62 (1.48)	0.38 (1.00)	0.63
Bases:				
Adenine	S	0.42 (1.00)	0.38 (1.00)	0.45
Guanine	S	0.07 (0.17)	0.08 (0.21)	0.06
Cytosine	S	0.21 (0.50)	0.41 (1.08)	0.22
Uracil	S	0.65 (1.55)	0.42 (1.11)	0.60

* Values in parentheses represent R_F values relative to the absolute R_F value for adenine in solvent systems I and II.

solutions of these standards were prepared with 10 m*M* ammonium formate (pH 4.5) to a final concentration of 0.2–1.0 mg/ml and stored at −20°.

Chromatographic equipment and accessories included a Desaga spreader (Brinkmann, Westbury, N.Y., U.S.A.), glass plates (20 × 20 cm), silica gel GF (Sigma, St. Louis, Mo., U.S.A.), Avicel microcrystalline cellulose (Brinkmann) and various solvents (Mallinckrodt, St. Louis, Mo., U.S.A., and/or Fisher Scientific, Pittsburgh, Pa., U.S.A.) listed in Table I. Both solvents (analytical grade) and adsorbents were used without further purification.

Thin-layer plates providing maximum resolution of a mixture of standard constituents (totaling 26) were prepared by mixing 30 g of cellulose and 20 g of silica gel with 125 ml of distilled water. The resultant mixture was then shaken vigorously for 30–60 sec, deaerated and transferred to a Desaga spreader which coated five plates with adsorbent to a thickness of 0.4 mm. Coated plates were air-dried at room temperature overnight prior to their storage in a desiccator.

In determining absolute R_F values, 0.5–10.0 μg of each standard constituent were applied approximately 2 cm from the bottom edge of the thin-layer plates with calibrated micropipettes and following an appropriate drying period (approximately 15 min), the plates were transferred to pre-equilibrated tanks containing 100 ml of one of the solvent systems described in Table I. Development was conducted at room temperature and continued until the solvent front had ascended a distance of approximately 16 cm (development time was 60–80 min for all solvent systems). After the plates had been withdrawn and following a brief drying period, the location of each nucleoside or base was detected with the aid of a UV-lamp.

For two-dimensional chromatography, a 10 m*M* ammonium formate solution (pH 4.5) containing equal quantities of each standard nucleoside and base (1 μg per 15 μl) was prepared and 15–30 μl of this solution were applied to the corner of the plates, 2–3 cm from each edge. These plates were developed as described above with the exception that development in both dimensions was conducted in a continuous manner. This was accomplished by absorbing the solvent from the top of the plate into coarse filter-paper which was clamped across the entire top edge of the plate and hanging over the front (1–2 cm) and back (8–10 cm). Continuous development in both dimensions was conducted for approximately 150 min with an intermediate drying period of 60 min between changes of solvent systems. For optimal results the migration of each standard was monitored by momentarily replacing the lid of the tank with a UV-lamp.

RESULTS AND DISCUSSION

The absolute and relative R_F values of the nucleoside standards following their chromatography in three different solvent systems are presented in Table I. Although the absolute R_F values varied approximately 15% as assessed by repeated chromatographic analyses, the relative R_F values (listed in parentheses) remained quite constant (variation less than 5%). Also included are the R_F values of four non-methylated base standards; not shown are the chromatographic results obtained for various nucleotide standards which in all instances possessed R_F values of zero (*i.e.*, remained at the origin). Inspection of these data with regard to solvent systems I and II revealed that the uridine and adenosine derivatives possessed the largest R_F values in system I

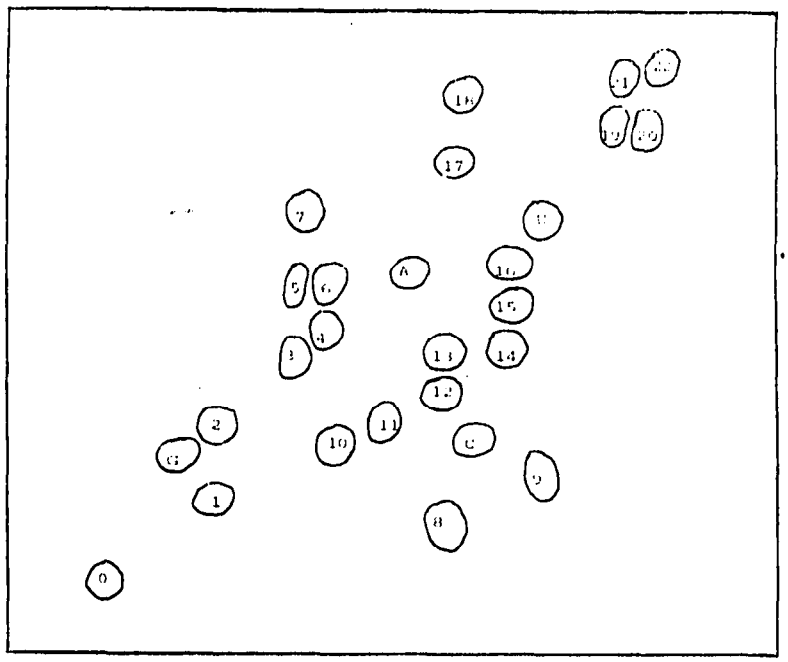

Fig. 1. Resultant distribution of the constituents listed in Table I following continuous chromatography on an adsorbent containing 60% cellulose and 40% silica gel. Solvent system I was from bottom to top and solvent system II from left to right. O = Origin; 1 = 7-methylguanosine; 2 = guanosine; 3 = N^2-methylguanosine; 4 = N^2,N^2-dimethylguanosine; 5 = 3′-O-methylguanosine; 6 = 2′-O-methylguanosine; 7 = uridine; 8 = 1-methyladenosine; 9 = 3-methylcytidine; 10 = cytidine; 11 = 5-methylcytidine; 12 = 4-methylcytidine; 13 = 1-methylguanosine; 14 = 2′-O-methylcytidine; 15 = 1-methylinosine; 16 = adenosine; 17 = 5-methyluridine; 18 = 2′-O-methyluridine; 19 = N^6-methyladenosine; 20 = 2′-O-methyladenosine; 21 = 3-methyluridine; 22 = N^6,N^6-dimethyladenosine; A = adenine; C = cytosine; G = guanine; U = uracil.

while in system II higher R_F values were associated with the adenosine and cytidine derivatives. These relationships are best depicted in chromatograms following two-dimensional development (see Fig. 1) and further demonstrate that the various methylated derivatives of guanosine, adenosine, cytidine and uridine occupy to a large extent discrete regions, or quadrants within the thin-layer plates (an exception was 1-methyladenosine).

Recently Al-Arif and Sporn[1] and Pike and Rottman[12] have demonstrated that a methyl group located at the 2′-O-position of nucleosides inhibited the formation of borate complexes normally associated with non-methylated or base-methylated nucleosides. Since borate–nucleoside complexes of the type described above have been shown to reduce dramatically the R_F values of those nucleosides not possessing a methyl group at the 2′-O- or 3′-O-position, it was of interest to examine the effects of borate in the TLC systems described herein. Thus as shown in Table I, replacement of formic acid with boric acid markedly reduced the R_F values of most base-methylated constituents. Although it was anticipated that the migration of 2′- and 3′-O-methylated nucleosides would not be affected (Table I), both N^6- and N^6,N^6-methyladenosine were also not markedly affected by borate. This latter result suggests that the position of the methyl group(s) of these compounds may be in close proximity with their 2′- and 3′-hydroxyl groups and thus inhibit the formation of the

borate–nucleoside complex. Although the presence of borate has been used successfully to resolve the ribose- and base-methylated nucleosides of RNA from one another[1,12], its use in the two-dimensional systems described below proved unsatisfactory as a result of the low R_F values associated with most of the base-methylated nucleosides.

Whereas inspection of the R_F values of the methylated nucleosides presented in Table I suggested that a mixture of these standards quite possibly could be resolved by two-dimensional chromatography in solvent systems I and II, analyses of such chromatograms revealed that four pairs of nucleosides were incompletely separated. These included (1) 2'-O- and 3'-O-methylguanosines, (2) N^2- and N^2,N^2-methylguanosines, (3) 1-methylinosine and 2'-O-methylcytidine and (4) 1-methylguanosine and 4-methylcytidine. Even though these nucleoside pairs possessed different R_F values, it was suspected that the relatively short development times (approximately 60–80 min in both directions) and hence migration distances were insufficient for their complete resolution. However, when the development time in both dimensions was extended to approximately 150 min by continuously absorbing the solvent from the top of the thin-layer plate, all 26 standard constituents were completely resolved from one another (Fig. 1). Thus by developing both dimensions in a continuous manner, individual differences in R_F values of the above nucleoside pairs were magnified to such an extent as to permit their complete resolution. Although either solvent system could be employed in the first dimension, repeated analyses indicated that for maximum separation it was preferable to employ system I first.

Of critical importance in the above TLC system were the relative percentages of microcrystalline cellulose (60%) and silica gel (40%) contained in the adsorbent. When the percentage of cellulose was decreased to 50% or below, certain nucleoside pairs (most notably the 2'- and 3'-O-methylguanosines and N^2- and N^2,N^2-methylguanosines) were incompletely resolved following two-dimensional continuous chromatography. Furthermore, trailing was observed with some of the guanosine constituents. Although plates possessing a higher content of cellulose (>60%) were satisfactory for separating the nucleoside standards employed, on many occasions the adsorbent would crack or chip from the plate to such a degree that chromatography was impossible.

At present we are investigating various enzymatic procedures to hydrolyze [methyl-^{14}C]-labeled RNA preparations (previously obtained from cells labeled with [methyl-^{14}C]methionine) to their respective [methyl-^{14}C]methylated nucleoside components for subsequent compositional analysis via the TLC method described herein.

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