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SEPARATION OF METHYLATED BASES OF RIBONUCLEIC ACID BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The separation of fourteen methylated purine and pyrimidine bases that have been reported to occur in transfer RNA was investigated by thin-layer chromatography using two solvent systems and five adsorbants containing various percentages of Silica Gel GF and Avicel microcrystalline cellulose. By increasing the amount of cellulose in the adsorbant, disproportionate decreases in the R_F values of the methylated bases were observed in both solvent systems. From these data a two-dimensional thin-layer chromatographic system has been developed that satisfactorily resolves a mixture of the fourteen methylated bases employed in this investigation.

INTRODUCTION

In recent years considerable effort has been directed towards determining the methylated base patterns of various RNA populations and/or the corresponding methylase activities associated with cells or tissues undergoing changes in growth and differentiation[1–3]. While column and paper chromatographic techniques are available for separating and estimating methylated base constituents[4–6], these techniques are tedious and time-consuming, and hence limit the progress of such studies. Although BJORK AND SVENSSON[7] and GRIPPO *et al.*[8] have developed thin-layer systems that resolve a number of methylated bases, only methylated purines were resolved in the former case, and resolution of N[2]-methylguanine and N[2]-dimethylguanine was not achieved in the latter. More recently, RANDERATH[9] has developed a thin-layer system capable of separating the methylated components of transfer RNA, however, this procedure involves the preparation of derivatives of methylated nucleosides prior to chromatography.

The present report describes a rapid and reliable two-dimensional thin-layer chromatographic system that separates a mixture of fourteen methylated bases reported to be contained in various transfer RNA preparations[3–5,9].

METHODS AND MATERIALS

The sources of the four non-methylated and fourteen methylated standard bases used in this investigation, are presented in Table I. Chromatographic analysis of these bases, initially dissolved in water and aqueous ethanol and developed in the thin-layer systems listed in Tables I and II, revealed the presence of a single UV-absorbing component.

Acetonitrile, ethyl acetate, 1-butanol, 2-propanol, methanol, formic acid (88%) and ammonium hydroxide (28–30%) were of analytical grade, purchased from Mallinckrodt and used without further purification. The formic acid and ammonium hydroxide solvent systems employed in this investigation and described in the legends of Tables I and II were modifications of systems used by TOCKSTEIN[10] and RANDERATH[9], respectively. Silica Gel GF_{254} and Avicel microcrystalline cellulose were purchased from Brinkmann, and only the latter was washed with versene buffer as described by GRIPPO *et al.*[8], the Silica Gel GF was used without modification.

Glass plates (20 × 20 cm) were coated with Silica Gel GF alone or with mixtures of Silica Gel GF and cellulose. Adsorbants (60-g batches) containing 100, 67, 50, and 33% Silica Gel GF were prepared by adding 60, 40, 30 and 20 g of Silica Gel GF to the appropriate amounts of cellulose. To these mixtures were added 140, 145, 150, and 155 ml of distilled water, respectively, with the resultant slurries being shaken

TABLE I

SOURCE AND R_F VALUES OF BASES CHROMATOGRAPHED ON COMPOSITE SILICA GEL GF_{254}–AVICEL MICROCRYSTALLINE CELLULOSE ADSORBANTS

Solvent system: ethyl acetate–methanol–water–formic acid (100:25:20:1). Source: S = Sigma Chemical Co., St. Louis, Mo.; C = Cyclo Chemical Co., Los Angeles, Calif.; B = Burroughs Wellcome & Co., Tuckahoe, N.Y.; H = Dr. R. HALL, McMaster University, Hamilton, Ontario, Canada.

Base	*Source*	*R_F value*				
		Silica gel–cellulose (%)				
		100:0	*67:33*	*50:50*	*33:67*	*0:100*
Adenine (A)	S	0.50	0.46	0.35	0.27	0.37
1-CH_3–A	S, B	0.10	0.09	0.07	0.05	0.08
2-CH_3–A	S, H	0.43	0.38	0.25	0.18	0.29
N^6-CH_3–A	S, B	0.55	0.57	0.45	0.45	0.60
N^6-diCH_3–A	S, B	0.63	0.67	0.55	0.58	0.74
1-CH_3–hypoxanthine	S	0.46	0.45	0.37	0.37	0.46
Guanine (G)	S	0.45	0.29	0.19	0.15	0.15
1-CH_3–G	S, B	0.45	0.36	0.24	0.22	0.25
7-CH_3–G	S, B	0.34	0.33	0.21	0.20	0.25
N^2-CH_3–G	S	0.50	0.43	0.28	0.28	0.33
N^2-diCH_3–G	S, B	0.47	0.45	0.30	0.33	0.42
Uracil (U)	S	0.67	0.64	0.42	0.49	0.54
3-CH_3–U	C	0.72	0.76	0.55	0.68	0.81
5-CH_3–U (thymine)	S	0.71	0.72	0.49	0.59	0.69
5-CH_2OH–U (unaltered)	C	0.61	0.55	0.31	0.32	0.35
5-CH_2OH–U (altered)	—	0.76	0.76	0.52	0.62	0.72
Cytosine (C)	S	0.24	0.19	0.12	0.09	0.14
3-CH_3–C	C	0.15	0.14	0.11	0.09	0.15
5-CH_3–C	S, B	0.22	0.20	0.12	0.10	0.17

TABLE II

R_F VALUES OF BASES CHROMATOGRAPHED ON COMPOSITE SILICA GEL GF_{254}–AVICEL MICROCRYSTALLINE CELLULOSE ADSORBANTS

Solvent system: acetonitrile–ethyl acetate–2-propanol–1-butanol–water–ammonium hydroxide (40:30:20:10:5:22).

Bases	*R_F value*				
	Silica gel–cellulose (%)				
	100:0	*67:33*	*50:50*	*33:67*	*0:100*
Adenine (A)	0.65	0.55	0.46 (0.35)[a]	0.28	0.32
1-CH_3–A	0.58	0.49	0.50 (0.07)	0.23	0.42
2-CH_3–A	0.68	0.57	0.59 (0.25)	0.33	0.46
N^6-CH_3–A	0.69	0.60	0.66 (0.45)	0.43	0.59
N^6-diCH_3–A	0.78	0.72	0.77 (0.55)	0.67	0.79
1-CH_3–hypoxanthine	0.61	0.49	0.49 (0.37)	0.28	0.44
Guanine (G)	0.38	0.34	0.22 (0.19)	0.15	0.15
1-CH_3–G	0.63	0.52	0.44 (0.24)	0.27	0.37
7-CH_3–G	0.52	0.44	0.32 (0.21)	0.24	0.23
N^2-CH_3–G	0.56	0.48	0.35 (0.28)	0.24	0.27
N^2-diCH_3–G	0.60	0.51	0.40 (0.30)	0.30	0.33
Uracil (U)	0.56	0.50	0.39 (0.42)	0.33	0.36
3-CH_3–U	0.74	0.74	0.68 (0.55)	0.70	0.76
5-CH_3–U (thymine)	0.67	0.62	0.57 (0.49)	0.51	0.60
5-CH_2OH–U (unaltered)	0.38	0.33	0.27 (0.31)	0.24	0.28
5-CH_2OH–U (altered)	0.20	0.15	0.14 (0.52)	0.24	0.16
Cytosine (C)	0.50	0.42	0.46 (0.12)	0.24	0.42
3-CH_3–C	0.59	0.54	0.63 (0.11)	0.36	0.64
5-CH_3–C	0.55	0.50	0.54 (0.12)	0.31	0.51

[a] R_F values in parentheses obtained with the formic acid solvent system listed in Table I.

vigorously for 30–60 sec and then spread onto five glass plates to a thickness of 0.5 mm as described by STAHL[11]. The plates were allowed to air-dry at room temperature for 60 min, heated an additional 60 min at 70–80°, and then stored in a desiccator at room temperature. The 100% cellulose layers were prepared by spreading a homogeneous slurry of 30 g of Avicel microcrystalline cellulose containing 100 ml of distilled water on plates to a thickness of 0.25 mm.

For one-dimensional chromatography, 2–10-μg quantities of the standard bases were dissolved in either water, 50% aqueous ethanol or concentrated formic acid (1 mg/ml), and were applied approximately 2 cm from the bottom of the plates by means of calibrated pipettes. For two-dimensional chromatography, a formic acid solution containing equal quantities of each base (ranging from 2 to 10 μg and totaling 36 to 180 μg altogether) was applied to the corner of plates, 2–3 cm from each edge. All plates were developed in pre-equilibrated tanks at room temperature until the solvent front had ascended 17–19 cm. Upon completion of the first dimension, the plates were air-dried for 30–60 min and then heated for an additional 60 min at 70–80° prior to development in the second dimension

The development time for each solvent system was approximately 80 min although it varied somewhat with the composition of adsorbant. The bases were detected by means of a short-wave UV lamp (254 nm), and the area occupied by each was outlined by marking the plate with a pointed instrument (see Fig. 1).

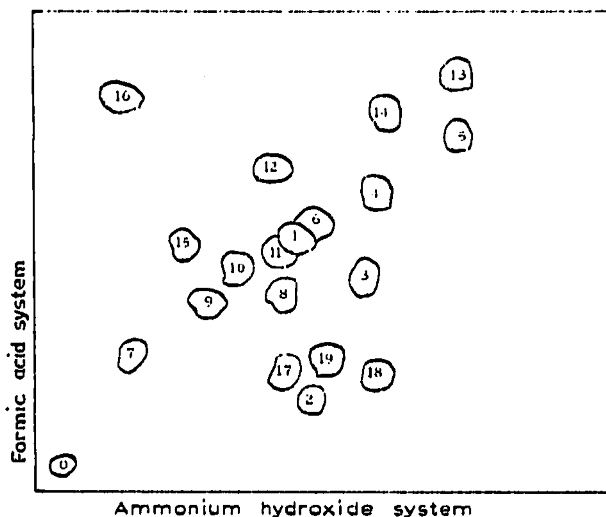

Fig. 1. Distribution of bases chromatographed in two dimensions on an adsorbant containing equal quantities of Silica Gel GF_{254} and Avicel microcrystalline cellulose. Solvent systems: from bottom to top, formic acid system (Table I); from left to right, ammonium hydroxide system (Table II). 0 = origin; 1 = adenine (A); 2 = 1-CH_3–A; 3 = 2-CH_3–A; 4 = N^6-CH_3–A; 5 = N^6-diCH_3–A; 6 = 1-CH_3–hypoxanthine; 7 = guanine (G); 8 = 1-CH_3–G; 9 = 7-CH_3–G; 10 = N^2-CH_3–G; 11 = N^2-diCH_3–G; 12 = uracil (U); 13 = 3-CH_3–U; 14 = 5-CH_3–U; 15 = 5-CH_2OH–U (unaltered); 16 = 5-CH_2OH–U (altered); 17 = cytosine (C); 18 = 3-CH_3–C; 19 = 5-CH_3–C.

RESULTS AND DISCSSION

Formic acid was employed as the primary solvent for preparing stock solutions of both purine and pyrimidine bases for several reasons. (1) Each base was soluble in formic acid to an extent of at least 2 mg/ml, in contrast to the inability of water or aqueous alcohols to dissolve appreciable quantities of guanine, as well as several of its methylated constituents. (2) It is of considerable importance that formic acid has been successfully employed in hydrolyzing nucleic acid preparations into their respective bases with minimal or no destruction, in contrast to the degradative effects of mineral acids[12]. However, in comparing duplicate samples of each standard base—one dissolved in water or 50% aqueous ethanol and the other in formic acid—on chromatograms developed in the solvent systems listed in Tables I and II, three of the eighteen bases, namely 5-hydroxymethyluracil, 1-methyladenine and N^6-methyladenine, contained additional UV-absorbing components when dissolved in concentrated formic acid. These additional products were apparently due to the action of formic acid, since only one UV-absorbing spot was detected for each base when dissolved in water or aqueous ethanol.

In the last two instances, 1-methyladenine and N^6-methyladenine contained, in addition to their unaltered forms, products that were chromatographically identical to each other. Although a rearrangement of this type (1-methyladenine $\rightleftharpoons$ N^6-methyladenine) has previously been reported[8,9], it occurred under alkaline rather than the acid conditions described here. In regard to 5-hydroxymethyluracil, a second UV-absorbing spot was detected which differed chromatographically from

the other bases. The R_F values of this formic acid-generated by-product are listed in Tables I and II under the heading "5-CH_2OH-U(altered)".

Although the above data indicate that the use of formic acid is not suited for the hydrolysis and subsequent isolation and characterization of 5-hydroxymethyluracil from nucleic acid populations, it nevertheless may provide a potential means of liberating the majority of methylated bases contained in nucleic acid structures. The interconversion of 1-methyl- and N^6-methyladenine is of little consequence, since only the 1-methyladenine product has been reported to be present in mammalian RNAs[5].

Tables I and II contain information regarding the R_F values of each base with respect to both the solvent system and the composition of adsorbants employed. The relative positions of each base in these systems were reproducible in all cases; however, the absolute R_F values obtained from repeated analyses at different times varied to some degree. Trailing occurred with guanine (extensive) and N^2-methylguanine (moderate) on 100% Silica Gel GF layers but was significantly reduced upon increasing the proportion of cellulose in the adsorbant and was totally absent on 50, 67 and 100% cellulose plates. Considerable trailing was observed on 100% Silica Gel HF layers as well.

Inspection of Tables I and II reveals a number of differences in the rates of migration of each base that are attributed to both the type of solvent system and adsorbant employed. Generally, those bases possessing primary amine groups (adenines, guanines and cytosines) had lower R_F values in the formic acid solvent system (Table I) than in the ammonium hydroxide system (Table II) when chromatographed on adsorbants containing appreciable quantities of silica gel. These differences were, however, significantly reduced, absent, or even reversed when the guanine and adenine bases (with the exception of 1-methyl- and 2-methyladenine) were chromatographed on adsorbants containing 50 and 67% cellulose. In most instances the migration of each base in both solvent systems was depressed by increasing the proportion of cellulose. In the formic acid solvent system a sharp decrease in the R_F values of most bases occurred when the cellulose content of the adsorbant was increased from 33 to 50%. Although a similar decrease was observed in the ammonium hydroxide system, it occurred (in most cases) when the cellulose content was increased from 50 to 67%. This trend was abruptly reversed, however, when 100% cellulose plates were employed.

As shown by the data in Tables I and II, the migration of each base was affected to different degrees by changes in the composition of the adsorbant. This behavior was of particular value in selecting an appropriate adsorbant (or adsorbants) with which to separate mixtures of the methylated bases via two-dimensional chromatography. For our purposes, because of the number of bases and quantity of material required, an adsorbant containing equal amounts of silica gel and cellulose provided the best results. Thus, as illustrated in Fig. 1, a complete separation of the fourteen methylated bases was achieved by employing this adsorbant in a two-dimensional chromatographic system with the solvents listed in Tables I and II. Although the methylated bases were resolved from one another in the above system, complete separation of adenine from both N^2-dimethylguanine and 1-methylhypoxanthine was not attained. However, resolution of these bases can be achieved with the same solvent systems by using 100% cellulose plates.

Although 100% cellulose seems to be equal or slightly superior to a composite adsorbant with regard to separating methylated bases, the latter offers distinct advantages that neither silica gel or cellulose alone offers. Namely, the capacity of cellulose is limited, since layers greater than 0.25 mm tend to crack upon drying. However, when silica gel is mixed with cellulose, layer thickness, and hence capacity, can be increased at least two-fold. Furthermore, the use of silica gel increases the sensitivity of base detection. Even though most of the bases can be separated by two-dimensional development on 100% Silica Gel GF (not illustrated), N^2-dimethylguanine, 1-methylguanine and 1-methylhypoxanthine are not satisfactorily resolved from each other. Furthermore, other complications exist in the above system as a result of the trailing associated with guanine and N^2-methylguanine.

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