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SEPARATION OF ALKYLATED GUANINES, ADENINES, URACILS AND CYTOSINES BY THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

A one-dimensional thin-layer chromatographic (TLC) method using a mixture of two solvents and commercially available silica gel plates to separate mixtures of alkylated guanines, adenines, uracils and cytosines is presented. R_F values for the bases and the solvent systems used are listed. The addition of approximately 1 ml of ammonium hydroxide to the solvent has been found to prevent streaking and results in non-distorted developed spots. A mixture of 19 adenine and uracil bases was resolved on silica gel plates employing two-dimensional TLC. Chloroform–methanol (90:10) was used for the first dimension and chloroform–propanol (90:30) for the second.

INTRODUCTION

Alkylating agents have been observed to produce profound biological effects such as in tumorigenicity in experimental animals. Correlated with this has been the observation of alkylation of cellular nucleic acids. Consequently, much recent research has been directed towards determining methylated purine and pyrimidine base patterns of nucleic acids in animals exposed to carcinogenic alkylating agents. Paper and column chromatographic techniques have been used for separating and estimating methylated base constituents[1–3], but these methods are generally tedious and time consuming[4]. Although thin-layer chromatography (TLC) has been employed for the separation of a number of methylated nucleic acid bases, these systems either resolved only certain methylated purines[5] or gave unsatisfactory results[6]. Randerath[7] developed a system whereby components of tRNA were separated after the preparation of derivatives of the nucleotides. Munns *et al.*[4] separated methylated bases of RNA by two-dimensional TLC employing a mixture of four solvents for the first dimension, a mixture of six solvents for the second dimension, and custom-made plates of a mixture of silica gel and cellulose.

This study describes a TLC method that uses a mixture of only two solvents

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and commercially available silica gel plates to separate methylated guanines, adenines, uracils, and cytosines. Observations on the separation of methylated purines and pyrimidines in different aliphatic alcohol systems will be discussed, and the interactions of methylated bases within each base class and their effect on the separation of individual bases will be presented. The addition of approximately 1 ml of ammonium hydroxide to the solvent system has been found to prevent streaking and results in

TABLE I

LIST OF METHYLATED BASES AND THEIR ABBREVIATIONS AS USED IN THIS STUDY

Abbreviations of suppliers: S = Sigma, St. Louis, Mo., U.S.A.; CC = Cyclo Chem., Los Angeles, Calif., U.S.A.; CPL = Chem. Procure Lab., College Point, N.Y., U.S.A.; A = Aldrich, Milwaukee, Wisc., U.S.A.; CF = Chemicals Fabrik; S-M = Schwartz-Mann, Orangeburg, N.Y., U.S.A.; P9 = Project 9, FCRC, Frederick, Md., U.S.A.

Group	*Compound*	*Abbreviation*	*Solvent*	*Supplier*
A	Adenine	A	Methanol	S
	1-Methyl adenine	1-MeA		S
	N-6-Methyl adenine	N^6-MeA		S
	9-Methyl adenine	9-MeA		CC
	N-6-Dimethyl adenine	N^6,N^6-diMeA		CC
	9-Ethyl adenine	9-EtA		CC
	N-6-Dimethyl-9-ethyl adenine	N^6,N^6-diMe-9-EtA		CC
	3-Methyl adenine	3-MeA		CPL
	7-Methyl adenine	7-MeA		CPL
	6-Methoxy purine	6-Me-O-P		A
B	Uracil	U	Methanol	S
	3-Methyl uracil	3-MeU		S
	5-Methyl uracil	5-MeU		S
	6-Methyl uracil	6-MeU		S
	1-Methyl uracil	1-MeU		CC
	1-Ethyl uracil	1-EtU		CC
	1,3-Dimethyl uracil	1,3-diMeU		CC
	1,5-Dimethyl uracil	1,5-diMeU		CC
	1-Ethyl-5-methyl uracil	1-Et-5-MeU		CC
	5,6-Dimethyl uracil	5,6-diMeU		CF
C	Cytosine	C	Methanol	S
	1-Methyl cytosine	1-MeC		S
	3-Methyl cytosine	3-MeC		CC
	1,6-Dimethyl cytosine	1,6-diMeC		CC
	9-Ethyl cytosine	9-EtC		CC
	5-Methyl cytosine	5-MeC		CF
D	9-Ethyl guanine	9-EtG	Ethanol–water (1:1), heating*	CC
	N-2-Methyl guanine	N^2-MeG		CC
	1-Methyl guanine	1-MeG		CF
	3-Methyl guanine	3-MeG		CF
	9-Methyl guanine	9-MeG		CF
	Guanine	G		SM
	6-O-Methyl guanine	6-O-MeG		P9
	7-Methyl guanine	7-MeG		S

* The solvents used for group D are indicated except for the case of guanine where ethanol–water–formic acid (50:50:5) was employed.

non-distorted developed spots. This leads to better separations and more reliable visual or densitometric quantitation.

MATERIALS AND METHODS

Apparatus

Standard glass tanks were used for plate development. A viewing cabinet with long (366 nm) and short (254 nm) ultraviolet (UV) lamps (Brinkmann, Westbury, N.Y., U.S.A.) was used to locate the spots on the plate.

Reagents

All solvents used were glass-distilled (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). The reagents were analytical grade. Sources of the free and alkylated purine and pyrimidine bases are listed in Table I. Drummond micropipettes were used for spotting the samples on silica gel plates (Brinkmann, EM silica gel 60 F-254).

Procedure

Solutions of adenine, uracil, and cytosine bases were made in methanol; those of alkylated guanines bases were made in hot ethanol–water (1:1); solutions of guanine were made in ethanol–water–formic acid (50:50:5). (Guanine does not dissociate in dilute formic acid[4].)

RESULTS AND DISCUSSION

The purine and pyrimidine bases and the abbreviations used in this study are listed in Table I. The bases were divided (Table I) into four groups: adenines (A), uracils (B), cytosines (C), and guanines (D). Each group was spotted separately on a

TABLE II

R_F VALUES OF METHYLATED AND ETHYLATED ADENINES IN VARIOUS SOLVENTS

Solvents used are as follows: α = chloroform–methanol–ammonia (90:10:1); β = chloroform–ethanol–ammonia (90:20:1); γ = chloroform–1-propanol–ammonia (90:30:1); δ = chloroform–2-propanol–ammonia (90:30:1); λ = chloroform–methanol (90:10); μ = chloroform–methanol (90:10) + ammonia (vapour).

Base	$R_F \times 100$					
	α	β	γ	δ	λ	μ
Adenine	19	33	18	25	24	15
1-MeA	3	4	0	0	0	4
3-MeA	26	36	14	18	16	37
7-MeA	9	13	3	5	7	12
9-MeA	46	59	40	44	46	61
N^6-MeA	28	50	39	44	47	28
N^6-diMeA	52	70	71	71	64	52
9-EtA	56	70	58	60	58	70
N^6,N^6-diMe-9-EtA	92	93	93	88	90	94
6-Me-O-P	37	59	50	55	59	27

TABLE III

R_F VALUES OF METHYLATED URACILS

For solvent systems α, β, γ and δ see Table II.

Base	$R_F \times 100$			
	α	β	γ	δ
Uracil	19	28	28	32
1-MeU	48	63	57	61
3-MeU	45	65	69	72
5-MeU	25	44	50	57
6-MeU	22	40	44	47
1,3-diMeU	82	91	86	87
5,6-diMeU	29	54	60	63
1,5-diMeU	59	79	77	80
1-Et-5-MeU	69	89	89	90

TLC plate and developed in an appropriate solvent system. The R_F values of the four groups in the different aliphatic alcohol systems used are listed in Tables II–V.

The R_F values of adenine and 9 adenine bases developed in various chloroform–alcohol solvent systems [chloroform–methanol (90:10), chloroform–ethanol (90:20), chloroform–1-propanol (90:30), and chloroform–2-propanol (90:30)] are listed in Table II. Approximately 1% ammonia was added to these solvent systems to prevent streaking. The effect of ammonia on streaking and separation of the bases will be discussed later.

Fig. 1 is a graphic representation of the relative R_F values of the adenine bases with respect to 9-MeA, showing that the most complete separation of the adenines occurs in the methanol solvent system. This figure also shows that the separation of the adenines is a compromise between the bases present and the solvent mixture used. Circled R_F pairs in the figure indicate that the two spots did not resolve completely in that solvent system. The propanol solvent systems did not completely resolve because of some degree of spot streaking (probably a result of lowered solubility of ammonium hydroxide in propanol). Streaking was not observed for the methanol and ethanol systems. It is possible that certain pairs of bases may be resolved better in one solvent than another. For example, 3-MeA and N^6-MeA have a ΔR_F value of

TABLE IV

R_F VALUES OF METHYLATED AND ETHYLATED CYTOSINES

ε = Chloroform–methanol–ammonia (90:30:1); ζ = chloroform–ethanol–ammonia (90:55:1); η = chloroform–1-propanol–ammonia (90:60:1); θ = chloroform–2-propanol–ammonia (90:60:1).

Base	$R_F \times 100$			
	ε	ζ	η	θ
Cytosine	20	16	7	6
1-MeC	45	38	14	12
3-MeC	31	23	8	6
5-MeC	32	28	11	9
1,6-diMeC	52	49	19	18
9-EtC	57	54	22	21

TABLE V

R_F VALUES OF METHYLATED AND ETHYLATED GUANINES

For ε, ζ, η, θ see Table IV; ι = chloroform–methanol (90:30); κ = chloroform–methanol (90:30) + ammonia (vapour).

Base	$R_F \times 100$					
	ε	ζ	η	θ	ι	κ
Guanine	15	12	3	3	St*	12
1-MeG	38	25	9	8	33	37
3-MeG	28	15	4	4	23	21
7-MeG	50	35	13	15	43	46
9-MeG	40	25	8	8	32	29
9-EtG	47	36	12	13	47	39
N^6-MeG	36	26	8	8	St*	28
O^6-MeG	76	66	46	46	70	72

* St = streaking.

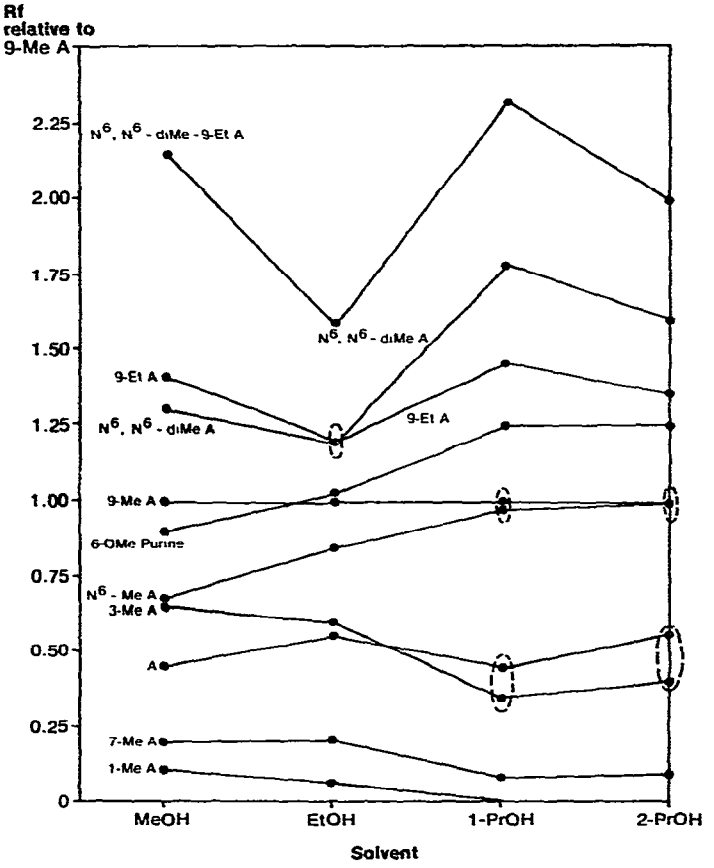

Fig. 1. A plot of relative R_F values of adenine bases with respect to 9-MeA using α, β, γ and δ solvent systems. Dashed circles indicate incomplete separation of spots.

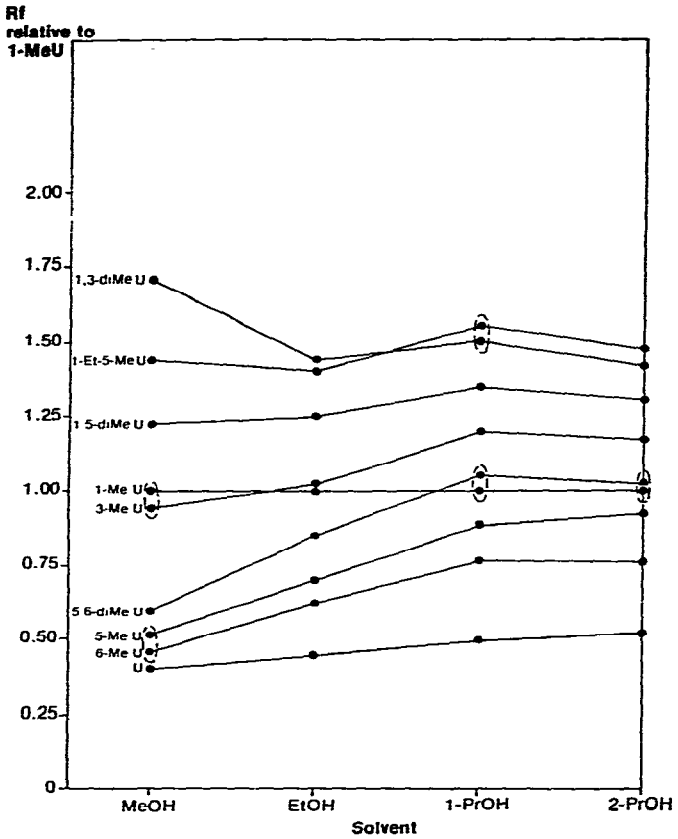

Fig. 2. A plot of relative R_F values of uracil bases with respect to 1-Me using α, β, γ and δ solvent systems. Dashed circles indicate incomplete separation of spots.

0.02 in chloroform–methanol, 0.14 in chloroform–ethanol, 0.25 in chloroform–1-propanol and 0.26 in chloroform–2-propanol. While the separation of this pair is better in the latter three solvent systems, it is clear from Fig. 1 that other pairs did not resolve completely (N^6, N^6-diMeA and 9-EtA in the ethanol system and 9-MeA and N^6MeA in the propanol systems). Although ΔR_F for 3-MeA and N^6-MeA is 0.26 in chloroform–2-propanol, the spots are not resolved completely due to streaking and diffusion.

Table III lists the R_F values of uracil and eight methyluracils in the four alcoholic systems used for the adenine bases (Table II). A graphic representation of the relative R_F values of the uracils with respect to 1-MeU is given in Fig. 2. The figure shows that the best resolution was obtained when the ethanol system was utilized. As mentioned earlier, an analyst may find one alcoholic system more useful than another for the bases he wishes to resolve. A mixture of 19 adenine and uracil bases (groups A and B) has also been resolved on silica gel plates employing two-dimensional TLC. Chloroform–methanol (90:10) was used for the first dimension and chloroform–1-propanol (90:30) for the second. This separation is shown in Fig. 3.

Table IV lists the R_F values obtained for the cytosines in the following solvent

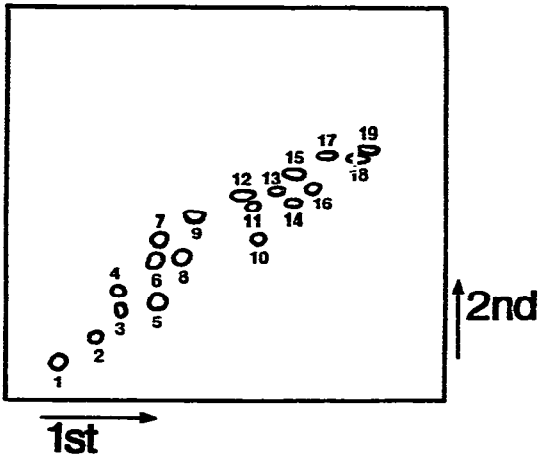

Fig. 3. Separation of alkylated adenine and uracil mixture in two-dimensional TLC. For conditions see text. 1 = 1-MeA, 2 = 7-MeA, 3 = A, 4 = U, 5 = 3-MeA, 6 = 6-MeU, 7 = 5-MeU, 8 = N^6-MeA, 9 = 5,6-diMeU, 10 = 9-MeA, 11 = 1-MeU, 12 = 3-MeU, 13 = 1-EtU, 14 = 9-EtA, 15 = N^6,N^6-diMeA, 16 = 1,5-diMeU, 17 = 1-Et-5-MeU, 18 = 1,3-diMeU, 19 = N^6,N^6-diMe-9-EtA.

systems: chloroform–methanol (90:30), chloroform–ethanol (90:30), chloroform–1-propanol (90:60) and chloroform–2-propanol (90:60). Approximately 1 % ammonia was added to each of these solvent mixtures to prevent streaking. The best resolution of the cytosines was obtained using the ethanol solvent system.

The R_F values of 8 guanines in the above 4 solvent systems are listed in Table V. In the propanol solvents, the guanines (unlike the adenines and uracils) did not migrate very far [this feature was also observed for the cytosines in the propanol solvents (Table IV)]. When the plates were developed to 14 cm in the propanol solvents, the guanines traveled in the order of 2 cm or less (with the exception of O^6-MeG).

The effect of ammonia on streaking of the bases

It was found that the presence of ammonia in the solvent mixture or in a small beaker placed at the bottom of the development tank prevents streaking and consequently enhances separation and capability for quantitation. Eight guanines were spotted on two plates. After drying, one plate was developed in a tank containing chloroform–methanol–ammonia (90:30:1) and the other plate was developed in a tank containing chloroform–methanol (90:30). The results shown in Fig. 4 clearly illustrate that the presence of ammonia in the tank eliminated the streaking of the bases on the plate, and resulted in uniform non-distorted spots. When the experiment was repeated for adenine, uracil, and cytosine bases, round non-tailing spots were obtained. This may be attributed to the prevention of the formation of ionized species of the bases on the plate. Any base on the acidic silica gel plate would behave according to the equation: $B + H^+ \rightarrow BH^+$. Because it is not chromatographed with the neutral base, this ionized form of the base will streak on the plate. The addition of a stronger base, ammonia, suppressed the formation of BH^+, according to $B + H^+ + NH_3 \rightarrow B + NH_4^+$. The use of ammonia clearly improves the chromatographic performance.

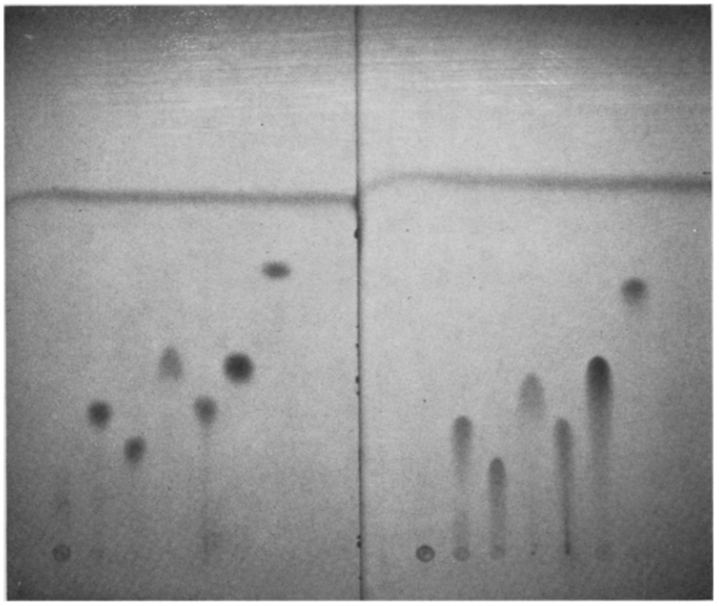

Fig. 4. Effect of ammonia on streaking of guanines. Left, with ammonia; right, without ammonia.

Effect of ammonia on the separation of the bases

The concomitant effect of ammonia on the separation of the adenines, uracils, cytosines, and guanines was also examined. The four groups of bases listed in Table I were each spotted on three different plates. The plates for the guanines and cytosines were then developed in three tanks containing the same solvent system, chloroform–methanol (90:30), and for the adenines and uracils chloroform–methanol (90:10) was used. Approximately 1 % ammonia was added to the second tank. A 5-ml beaker containing ammonia was placed in the bottom of the third tank. No ammonia was added to the first tank. The results showed that the best separation was achieved in the chloroform–methanol–ammonia (90:30:1) tank. (See Tables II and V for the adenines and the guanines respectively; uracils and cytosines behaved similarly). Separation in the ammonia vapor tank was better than in the tank without ammonia, and the R_F values were higher in the ammonia vapor tank than in the other two tanks. Hence, the use of ammonia as part of the solvent system or as vapor enhances the selectivity of separation of the bases.

For the adenines, guanines, cytosines, and uracils it was observed that for certain pairs of bases separation was better when they were spotted together (or with the whole group) than when they are spotted separately. Examples of such pairs are N^6-MeA and 6-Me-O-P; or 5,6-diMeU and 5-MeU. This phenomenon may be hypothesized as follows.

In describing adsorption chromatography as an equilibrium process, the term K_0 is used where K_0 is defined as $[X]_a/[X]_u$, the ratio of concentrations of solute X in the adsorbed phase, a, and unadsorbed phase, u (ref. 8). When two solutes are chromatographed together, both solutes compete for the same active sites on the adsorbent, *i.e.*, $X + Y + 2A \rightarrow X \cdot A + Y \cdot A$, where A are the adsorbent sites

and Y is a second solute. When the more highly adsorbed solute approaches saturation of the available adsorbent sites, the other solute will generally experience a shift to a larger $[X]_u$ and a smaller K_0 value. The K_0 value can be related to the distance traveled by a compound by the equation[8] $R'_M = \log K_0 + \log (W/V^0)$, where W is total weight of adsorbent in an adsorption system and V^0 is the bed void volume equal to the volume of solvent in the adsorbent bed, and $R'_M = \log [(1/\xi R_F) - 1]$ where ξ is the solvent concentration gradient parameter in TLC which can be determined experimentally. Thus, if K_0 is decreased (as explained above), R'_M is decreased and an increased R_F is observed for the solute having the competitively lower energy of adsorption. This displacement is exhibited as a slight increase in ΔR_F for such base pairs when chromatographed together rather than separately.

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