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SEPARATION CHARACTERISTICS OF ALKYLATED GUANINES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behavior of thirteen alkylated guanines on normal-phase silica gel and amino columns and on reversed-phase ODS and phenyl columns was studied. The larger the alkyl substituent at the same position of guanine the weaker was the retention in the normal-phase chromatographic system and the greater the retention during reversed-phase chromatography. O^6-Derivatives possess the lowest polarity in each set of isomers. An amino column was found to be of highest efficiency in terms of separation of the set of ethylguanine isomers and of benzylguanines studied. A phenyl column provided the best resolution of methylated guanines.

INTRODUCTION

The alkylation of DNA bases, especially guanine, has been considered to be of great significance in chemical carcinogenesis and mutagenesis[1–3]. A variety of chromatographic techniques have, therefore, been proposed for the reliable and rapid analysis of the modified nucleobases formed *in vitro* and *in vivo*. Ion-exchange chromatography[4–8] and reversed-phase chromatography (including ion-pair chromatography)[8–15] were commonly employed. In a reversed-phase chromatographic method, a phenyl column was shown to give a more satisfactory separation of common nucleobases and of methylated purines and pyrimidines[16,17]. Normal-phase unmodified silica was also found useful for the separation of polar compounds such as nucleobases and nucleosides[18–20]. These methods were limited to unmodified nucleobases and nucleosides and to methylated or ethylated nucleobases. This paper addresses the lack of comprehensive chromatographic data for alkylated guanines by evaluating the separation of a series of alkylated guanines under both normal-phase and reversed-phase chromatographic conditions. Emphasis was placed on the separation of the isomers having identical alkyl substituents at various nucleophilic sites on the guanine (*i.e.*, an isomer set) and, therefore, having direct application for site-specificity studies of DNA alkylation.

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EXPERIMENTAL

Materials

Guanine (Gua), 6-chloroguanine(6-ClG), 1-methylguanine (1-MG), N^2-methylguanine (N^2-MG), 3-methylguanine (3-MG), and 7-methylguanine (7-MG) were purchased from Sigma. O^6-Methylguanine (O^6-MG)[21], O^6-ethylguanine (O^6-EG)[21], O^6-allylguanine (O^6-AG)[22], O^6-benzylguanine (O^6-BG)[23,24], 7-ethylguanine (7-EG)[25], 7-allylguanine (7-AG)[22], 7-benzylguanine (7-BG)[26], N^2-ethylguanine (N^2-EG)[27], and N^2-benzylguanine (N^2-BG)[27] were synthesized in this laboratory following published methods and identified by ultraviolet and mass spectrometry. From commercial sources were obtained analytical grade chemicals and solvents.

Apparatus

The high-performance liquid chromatography (HPLC) measurements were carried out on a Perkin-Elmer Series 2 liquid chromatograph equipped with an LC-75 spectrophotometric detector at ambient temperature (usually 23°C). The wavelength selected for all measurements was 254 nm. Separations were examined using a Silica A column (Perkin-Elmer, 10 μm, 25 cm × 0.26 cm I.D.), a Chromasil NH_2 column (American Scientific Products, 10 μm, 25 cm × 0.46 cm I.D.), an ODS column (Alltech, 10 μm, 25 cm × 0.46 cm I.D.) and a μBondapak phenyl column (Waters Assoc., 10 μm, 30 cm × 0.39 cm I.D.). Data were taken with a Hewlett-Packard 3390A integrator. A Graphic Controls PHM 7900 pH meter with a Corning combination electrode was used to measure pH values.

Procedure

Each individual guanine derivative was dissolved in a solvent mixture consisting of 1 volume of 0.1 *M* hydrochloric acid and 5 volumes of methanol at a concentration of approximately 20–100 μg/ml. Mixtures were generated by combinations of individual standard solutions. These abbreviations were used for sets of alkylated guanine isomers: set 1 for a solution of 1-MG, N^2-MG, 3-MG, O^6-MG (containing a small amount of 6-ClG, a residue from the synthesis of O^6-MG), 7-MG, and Gua; set 2 for N^2-EG, O^6-EG, 7-EG and Gua; set 3 for N^2-BG, O^6-BG, 7-BG and Gua and set 4 for O^6-AG (containing 6-ClG), 7-AG and Gua. All solutions were stored in a freezer. For the measurement of the retention time (t_R), a 0.1–0.5 μg amount of each compound in about 5 μl was applied to the HPLC system. The solvent peak was measured as the time of non-retarded solute (t_0). The mobile phases were prepared daily and degassed by ultrasound prior to use. Buffers were prepared weekly and kept in refrigerated storage. The pH of the buffer was adjusted as follows to the desired value with the addition of an appropriate acid or base: 5% orthophosphoric acid or 5% ammonium hydroxide for ammonium phosphate buffer solutions; 97% formic acid for ammonium formate buffers and 5% sulfuric acid or 5% sodium hydroxide for sodium octanesulfonate solutions.

When changing the eluents, the silica gel and amino columns were washed with methylene chloride–methanol (2:1, v/v) and the ODS column and the phenyl column were eluted with water–methanol (1:1, v/v), then equilibrated for 1 h with the selected mobile phase. After each day's set of experiments, the columns were washed with the solvents indicated above.

RESULTS AND DISCUSSION

The retention behavior of the alkylated guanines on four normal and reversed-phase columns was investigated. The capacity factors, k', were measured and calculated as a function of the mobile phase composition and pH. Optimum chromato-

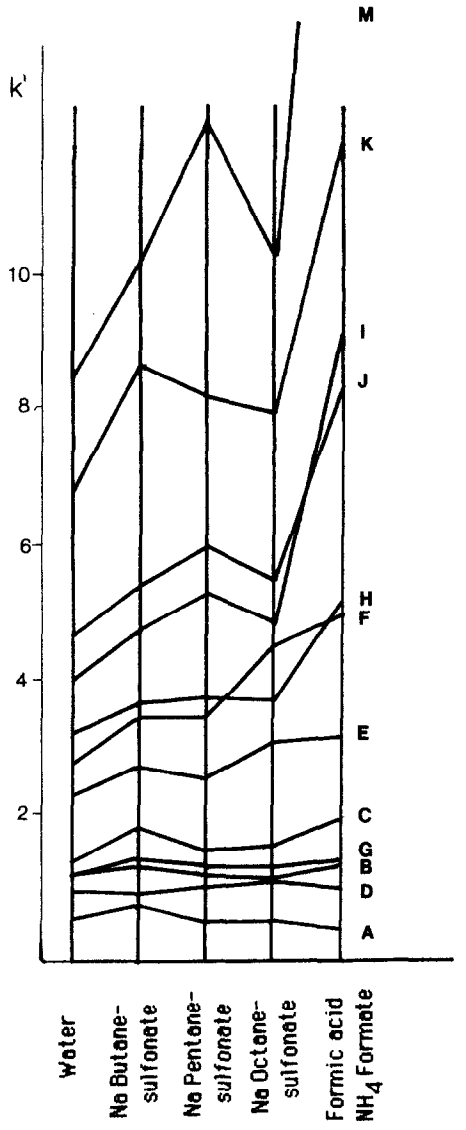

Fig. 1. Effect of the type of buffer solution on the capacity factor (k') of alkylated guanines on a silica gel column. Mobile phase: methylene chloride–methanol–water or buffer solution (90:9:1, v/v/v) containing 0.02 *M* of salt indicated (pH 3.1). A = O^6-BG; B = 7-BG; C = N^2-BG; D = O^6-EG; E = 7-EG; F = N^2-EG; G = O^6-MG; H = 7-MG; I = N^2-MG; J = 1-MG; K = 3-MG; L = 6-ClG; M = Gua; S = solvent.

graphic conditions were established for the separation of each set of alkylated guanine isomers.

Normal-phase HPLC

Preliminary evaluation revealed that the addition of water (*ca.* 1%) to the eluent of a suitable proportion of methanol and methylene chloride resulted in an improvement in peak width but not in the reduction of peak tailing for the separation of methylated guanines on silica gel and amino columns. Several ion-pairing agents and buffer systems were tested and the peak symmetry and column efficiency were improved. However, ion-pairing agents were not found to be advantageous in either selectivity or efficiency over a formic acid–ammonium formate buffer. A representative selection of chromatographic data is shown in Fig. 1. For both silica gel and amino columns, mixtures of methylene chloride, methanol and aqueous ammonium formate were selected as the mobile phases.

Effect of methanol content. The influence of methanol was studied (Table I) and k' was found to decrease with increasing methanol content in the mobile phase. However, the dependence of log k' on the methanol content shows a non-linear relationship. Brugman *et al.*[18], noticed similar phenomena with nucleobases on a silica gel column and attributed it to the involvement of other distribution processes than adsorption. As can be seen from Table I there was a minimal influence of methanol content on the selectivity with the exception of the N^2-derivatives. The

TABLE I

EFFECT OF METHANOL CONTENT IN THE MOBILE PHASE ON THE CAPACITY FACTORS OF ALKYLATED GUANINES

Mobile phase: methylene chloride–methanol–0.05 *M* solution of ammonium formate and formic acid, pH 3.2.

	Silica gel column			*Amino column*		
	93:6:0.66	*90:9:0.66*	*85:14:0.66*	*84:14:2*	*80:18:2*	*73:25:2*
O^6-BG	1.16	0.82	0.50	0.20	0.22	0.19
7-BG	3.15	1.97	1.01	0.54	0.51	0.42
N^2-BG	4.04	2.25	0.94	1.42	1.27	1.15
O^6-AG	1.73	1.08	0.66	0.37	0.36	0.21
7-AG	5.10	2.78	1.25	1.06	0.79	0.54
O^6-EG	1.97	1.25	0.73	0.45	0.39	0.28
7-EG	6.20	3.26	1.73	1.09	0.93	0.61
N^2-EG	8.55	4.08	1.46	2.74	2.29	1.63
O^6-MG	2.63	1.52	0.87	0.66	0.56	0.36
7-MG	9.34	4.51	2.38	1.77	1.32	0.85
N^2-MG	13.1	5.70	2.40	4.28	3.15	2.01
1-MG	13.6	6.70	3.08	3.07	2.00	1.00
3-MG	22.5	10.1	4.53	3.36	2.22	1.23
6-ClG	2.47	1.34	–	0.86	0.69	–
Gua	31.6	12.1	4.75	8.56	6.34	3.08

TABLE II

EFFECT OF WATER CONTENT IN THE MOBILE PHASE ON THE CAPACITY FACTORS OF ALKYLATED GUANINES

Mobile phase: methylene chloride–methanol–0.05 *M* solution of ammonium formate and formic acid, pH 3.2.

	Silica gel column			*Amino column*			
	90:9:0.33	*90:9:0.66*	*90:9:1*	*80:18:0.6*	*80:18:1*	*80:18:1.5*	*80:18:2*
O^6-BG	0.91	0.82	0.64	0.23	0.24	0.20	0.22
7-BG	1.98	1.97	1.45	0.74	0.71	0.62	0.49
N^2-BG	2.37	2.25	2.14	2.86	2.43	1.78	1.35
O^6-AG	1.20	1.08	0.92	0.36	0.35	0.33	0.35
7-AG	3.11	2.78	2.38	1.03	0.92	0.87	0.81
O^6-EG	1.36	1.25	1.08	0.39	0.39	0.34	0.40
7-EG	3.39	3.26	3.09	1.12	1.19	1.01	1.01
N^2-EG	4.33	4.08	5.14	4.17	3.16	2.65	2.25
O^6-MG	1.67	1.52	1.34	0.45	0.46	0.49	0.51
7-MG	5.57	4.51	4.24	1.42	1.38	1.30	1.24
N^2-MG	6.49	5.70	6.26	4.39	3.81	3.44	3.10
1-MG	7.73	6.70	6.72	2.05	1.94	1.88	1.85
3-MG	11.8	10.1	10.2	2.30	2.18	2.10	1.91
6-ClG	1.30	1.34	1.38	–	–	–	–
Gua	19.6	12.1	18.9	8.47	7.82	5.96	5.53

retention order of 7-BG/N^2-BG and 7-EG/N^2-EG on the silica gel column was reversed when the proportion of methanol was increased to 14:85.

Influence of water content. Table II presents the effect of the proportion of aqueous buffer solution in the mobile phase on k'. When the amount of buffer solution was increased on the silica gel column, the k' values decreased for all compounds. When the aqueous buffer content was increased further, k' values increased again for N^2-EG, N^2-MG and Gua. A similar phenomenon was noticed earlier with nucleobases and nucleosides and was attributed to the transition from a distribution process (adsorption) to a liquid–liquid partition process when changing the water content[18]. For most compounds, k' steadily decreased on an amino column when aqueous buffer content increased. No inflection was seen. Due to the difficulty in measuring small changes in k', this tendency was not observable for a few compounds with low k' values.

Effect of pH. The effect of pH of the buffer in the mobile phase on k' was studied over the pH range 2.6–4.1 on both silica gel and amino columns. As expected for basic compounds such as amino purines and amino pyrimidines[20] in normal-phase chromatography, the k' increased with increasing pH for most of the guanine derivatives investigated. However, this effect was weak and on both columns retention was not very sensitive to pH. An exception was the exocyclic amino alkylated N^2-isomers where the k' was more pH dependent (Fig. 2, minimum observed at pH 3.2) on a silica gel column.

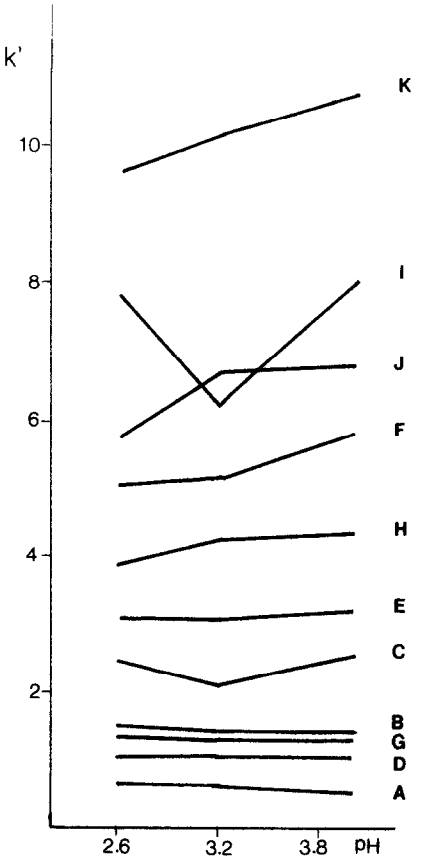

Fig. 2. Effect of pH values of the buffer solution in the mobile phase on the k' of alkylated guanines on a silica gel column. Mobile phase: methylene chloride–methanol–buffer solution (90:9:1, v/v/v) containing 0.05 *M* of sodium formate and formic acid. Key as in Fig. 1.

Using a normal-phase silica gel or amino column, all four sets of isomers were well separated under appropriate conditions. In terms of column efficiencies and peak shapes, an amino column showed improved performance. The height equivalent to a theoretical plate (HETP) was 0.026 mm at a flow-rate of 1.0 ml/min for an amino column (25 cm × 0.46 cm I.D.) while the HETP of the silica gel column was 0.06 mm at the same flow-rate. A typical chromatogram of a separation for set 3 on an amino column is shown in Fig. 3. As the alkyl chain length increased in alkylated guanine analogues of the same class (*i.e.* a different alkyl substituent at the same position of guanine) the capacity factor decreased. Within each set of guanine derivatives, O^6-isomers had the lowest retention.

Reversed-phase HPLC

On an ODS column, an ion-pairing technique was employed for better selectivity and column efficiency. An earlier report[17] using a phenyl column confirmed our preliminary experiments which showed that with a mixture of ammonium phos-

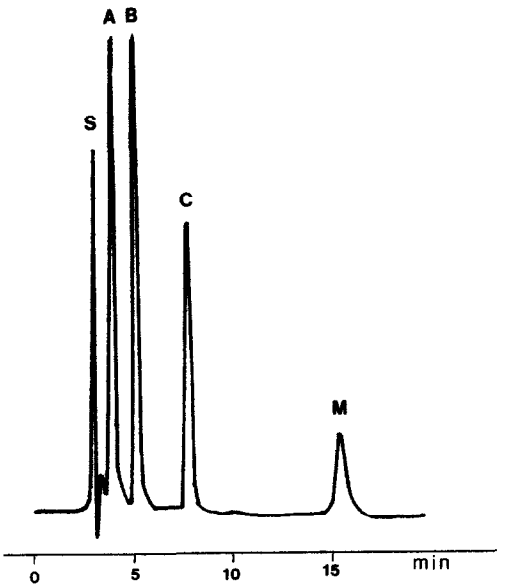

Fig. 3. Chromatogram of set 4 on an NH_2 column. Mobile phase: methylene chloride–methanol–0.05 *M* of ammonium formate + formic acid, pH 2.6 (80:18:1.5, v/v/v), flow-rate: 1 ml/min, after 6.5 min changed to 2 ml/min. Key as in Fig. 1.

TABLE III

EFFECT OF METHANOL CONTENT IN THE MOBILE PHASE ON THE CAPACITY FACTORS OF ALKYLATED GUANINES

	*ODS column**				*Phenyl column***			
	20:80	*30:70*	*40:60*	*50:50*	*4:96*	*10:90*	*20:80*	*35:65*
O^6-BG	–	>15	14.2	4.01	>17	>17	16.7	5.51
7-BG	–	10.5	3.44	1.39	>17	>17	14.4	2.76
N^2-BG	–	15.6	4.89	1.87	>17	16.6	7.84	2.67
O^6-AG	–	10.6	3.72	1.19	7.97	5.10	2.81	1.04
7-AG	–	2.39	0.99	0.36	5.13	3.40	1.51	0.54
O^6-EG	–	8.15	2.87	1.16	5.06	3.07	1.69	0.76
7-EG	–	4.84	1.65	0.85	1.46	0.86	0.39	0.11
N^2-EG	7.94	2.31	0.99	0.44	2.39	1.90	1.01	0.44
O^6-MG	5.57	3.69	1.39	0.48	2.40	1.60	0.91	–
7-MG	2.88	1.31	0.55	–	1.38	0.99	0.51	–
N^2-MG	2.29	0.99	0.43	–	1.37	0.88	0.51	–
1-MG	2.69	1.14	0.53	–	1.03	0.67	0.38	–
3-MG	4.99	2.33	1.02	–	0.75	0.37	0.18	–
6-ClG	–	1.07	0.57	0.26	3.29	2.20	1.34	0.44
Gua	–	–	–	–	0.46	0.30	0.18	–

* Mobile phase: methanol–0.01 *M* solution of ammonium phosphate + 0.005 *M* solution of sodium octanesulfonate, pH 4.0.

** Mobile phase: methanol–0.01 *M* solution of ammonium phosphate, pH 3.3.

phate buffer and methanol as the mobile phase, adequate separation of methylated guanines could be achieved. Further improvements were not obtained with the use of ion-pairing agents.

Influence of methanol content. The effect of methanol content on k' was investigated and the results shown in Table III. As expected, all alkylated guanines studied exhibited a significant decrease in k' with an increase in the proportion of methanol. Except for few compounds (*e.g.* N^2-MG, 7-EG and 7-BG), the selectivity was maintained with varying methanol content on an ODS or a phenyl column. However, due to apparently different separation mechanisms involved, for the compounds in the same set, the retention on ODS or phenyl columns were not in the same order.

Effect of pH. The retention behavior of the alkylated guanines with varying pH of the mobile phase was studied (Tables IV and V). In accordance with the prediction of retention behavior of organic bases in reversed-phase ion-pairing chromatography, the capacity factor values on an ODS column for all guanine derivatives decreased markedly with increasing pH. On a phenyl column without ion-pairing agent, k' values increased with increasing pH of the mobile phase (in the pH range 3.0–4.8). The dependence of selectivity on pH was observed to be significant. As was reported earlier for guanine in reversed-phase LC[28], for several alkylated guanines, k' values were also observed to have inflection points at an intermediate pH value.

Effect of buffer concentration. The effect on k' resulting from changes in phosphate buffer concentrations in the phenyl column system and the effect of sodium octanesulfonate concentration on the ODS column was found to be minimal. A

TABLE IV

EFFECT OF pH VALUES OF THE MOBILE PHASE ON THE CAPACITY FACTORS OF ALKYLATED GUANINES ON AN ODS COLUMN

Mobile phase: methanol–0.01 *M* solution of ammonium phosphate + 0.005 *M* sodium octanesulfonate. A, 3:7; B, 4:6.

	pH (mobile phase A)			*pH (mobile phase B)*		
	4.0	*5.2*	*6.2*	*3.1*	*4.0*	*5.3*
O^6-BG	>15	>15	>15	19.8	14.2	9.6
7-BG	10.5	7.61	6.42	6.23	3.44	2.92
N^2-BG	15.6	9.91	9.77	9.73	4.89	3.74
O^6-AG	10.6	4.83	4.81	6.20	3.72	2.00
7-AG	2.39	1.44	1.36	1.81	0.99	0.56
O^6-EG	8.15	3.50	2.82	4.75	2.87	1.51
7-EG	4.84	–	–	–	1.65	–
N^2-EG	2.31	1.31	1.24	1.91	0.99	0.48
O^6-MG	3.69	1.54	1.97	2.50	1.39	0.73
7-MG	1.31	0.53	0.51	1.06	0.55	0.26
N^2-MG	0.99	0.51	0.52	0.71	0.43	0.18
1-MG	1.14	0.54	0.38	1.03	0.53	0.15
3-MG	2.33	0.64	0.38	1.50	1.02	0.17
6-ClG	1.07	1.04	1.08	0.63	0.57	0.49
Gua	–	–	–	0.36	0.18	0

TABLE V

EFFECT OF pH VALUES OF THE MOBILE PHASE ON THE CAPACITY FACTORS OF ALKYLATED GUANINES ON A PHENYL COLUMN

Mobile phase: methanol–0.01 *M* solution of ammonium phosphate. A, 10:90; B, 35:65.

	pH (mobile phase A)			*pH (mobile phase B)*		
	3.3	*4.1*	*4.8*	*3.0*	*3.3*	*3.8*
O^6-BG	–	–	–	3.81	5.51	5.57
7-BG	–	–	–	2.42	2.76	2.95
N^2-BG	–	–	–	2.40	2.67	2.95
O^6-AG	5.10	7.81	9.21	0.85	1.04	1.28
7-AG	3.40	4.21	3.99	0.46	0.54	0.60
O^6-EG	3.07	6.24	6.21	0.61	0.76	0.99
7-EG	0.86	1.25	1.10	0.06	0.11	0.12
N^2-EG	1.90	2.83	2.50	0.32	0.44	0.49
O^6-MG	1.60	2.88	2.88	–	–	–
7-MG	0.99	1.55	1.43	–	–	–
N^2-MG	0.88	1.27	1.12	–	–	–
1-MG	0.67	1.02	0.91	–	–	–
3-MG	0.37	0.71	0.76	–	–	–
6-ClG	2.20	2.35	2.16	0.45	0.44	0.42
Gua	0.30	0.43	0.37	–	–	–

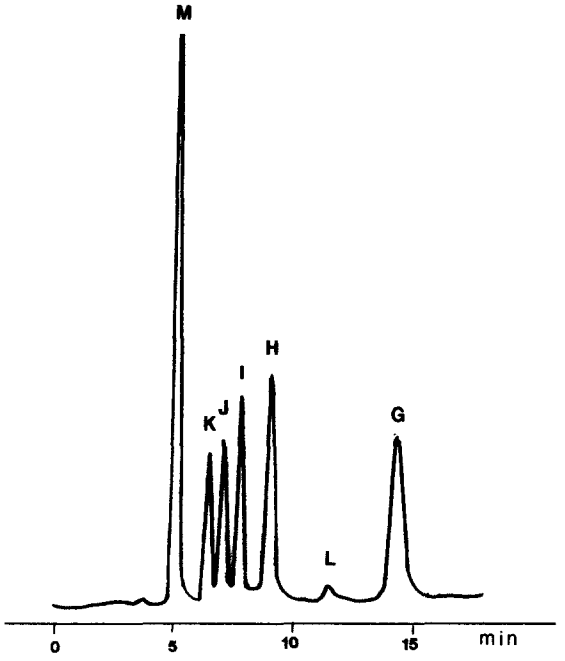

Fig. 4. Chromatogram of set 1 on a phenyl column. Mobile phase: methanol–0.01 *M* solution of ammonium phosphate (10:90, v/v), pH 4.8, flow-rate: 1 ml/min. Key as in Fig. 1.

similar result was reported in the HPLC study of 5-alkyluracils, where these compounds apparently did not form ion pairs in the pH range studied[12]. The peak widths of O^6-BG, N^2-BG, O^6-AG, O^6-EG and N^2-EG were narrowed slightly when the concentration of buffer or ion-pairing agent increased to 0.003 *M*.

Using an ODS column with ion-pairing techniques, set 2 and set 3 were adequately separated. However, in set 1, N^2-MG, 7-MG and (or) 1-MG were poorly resolved. The best separation for set 1 was achieved using a phenyl column. For example, the resolution (*R*) between 1-MG and N^2-MG is 2.22 and between N^2-MG and 7-MG is 2.95 (Fig. 4). 6-ClG, which was always overlapped with O^6-MG, was observed under these chromatographic conditions. In contrast with normal-phase chromatography described above, analogues with a longer alkyl substituent at the same position had stronger retention in a reversed-phase system. O^6-Derivatives always possessed the highest k' value in each set of alkylated guanine isomers.

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