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QUANTITATIVE STRUCTURE–RETENTION RELATIONSHIPS FOR PURINE COMPOUNDS ON REVERSED-PHASE PACKINGS

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SUMMARY

The capacity factors of 86 purine compounds (bases, nucleosides, 2′-deoxynucleosides, 3′, 5′-cyclic nucleotides and 5′-nucleotides) were determined on four different reversed-phase packings with an aqueous phosphate buffer as eluent. Using purine as the parent structure, the effects of substituents on retention behavior were quantified. The tabulated group contribution values allow the retention of many purine compounds to be predicted under similar chromatographic conditions; for example, a linear regression analysis of predicted and observed retention for 36 randomly chosen compounds on a μBondapak-C_{18} column gave a correlation coefficient of 0.9999 and a standard error of 0.01830. In addition, the group contribution terms, derived from the different stationary phases, were compared to determine if retention on one packing could be used to predict retention on another. Furthermore, the elucidation of purine structures through the use of structure–retention relationships was examined.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) on microparticulate, chemically bonded, silica packings is well suited for the separation of purine compounds in complex biological matrices[1–7]. As the popularity of RP-HPLC for purine separations increased, so did the number of studies on the effects of various chromatographic parameters on retention[7–12]. While such studies have greatly facilitated the optimization of purine separations, the relationships between purine structure and retention have been mainly qualitative[7,11–13].

Since chromatographic retention is a function of solute structure, the analysis of retention data should permit the elucidation of structural components of unknown compounds. The assignment of retention factors for various substituents was suggested by Martin[14] and validated by Bate-Smith and Westall[15]. Horváth and coworkers[16,17] and others[18–21] have determined the quantitative structure–retention relationships (QSRRs) for a variety of compounds and demonstrated the value of the functional group contribution terms for both the prediction of retention and structural elucidation.

The QSRRs are based on linear free-energy relationships and assume that the

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difference between the retention of a substituted compound and its parent molecule is due to the sum of independent functional group contributions[17,19]. The substituent effect on purine retention in its logarithmic form, T_j, can be expressed by

$$T_j = \ln k'_i - \ln k'_p \quad (1)$$

where k'_p and k'_i are the capacity factors of the purine parent and any substituted purine compound that differs in structure by substituent j. Hence, the capacity factor of any given purine compound (PC) may be obtained from

$$\ln k'_{pc} = \ln k'_p + \sum_{j=1}^{N} T_j \quad (2)$$

where N is the total number of substituents on PC. The T_j values are expected to be constant for the functional groups located at the same position on the different purine compounds. However, it is important to note that some groups may affect certain molecular changes, depending on the presence and/or absence of other substituents and, therefore, require additional assignment of group contribution terms.

In our study, the QSRRs for purine compounds (bases, nucleosides, 2′-deoxy-nucleosides, 3′,5′-cyclic nucleotides, and 5′-nucleotides) on four commercially available reversed-phase packings were established through the simultaneous solution of a set of linear equations[17]. We will demonstrate that RP-HPLC retention can be predicted from purine structure. In addition, we have examined the use of the QSRRs in structural elucidation[20,21] of purine compounds under fixed chromatographic conditions.

EXPERIMENTAL

Instrumentation

An isocratic liquid chromatographic system, equipped with an M 6000A pump (Waters Assoc., Milford, M.A., U.S.A.) a constant-temperature (±0.1°C) column compartment (DuPont, Wilmington, DE, U.S.A.) housing a Model 7125 sample injector (50-μl loop, Rheodyne, Berkely, CA, U.S.A.), and an M 440 dual-wavelength detector (Waters Assoc.), was used to obtain retention data. Retention times were determined with a Hewlett-Packard (Avondale, PA, U.S.A.) HP 3380 A integrator; data thus obtained were verified by manual measurements from chromatograms of purines, detected by their UV absorbance at 280 and 254 nm and traced with a two-pen recorder (Houston Instruments, Austin, TX, U.S.A.).

Columns

The effects of the purine substituents on retention were determined with four different commercially available reversed-phase columns, which are referred to as columns A–D. Column A (300 × 3.9 mm I.D.) was packed with irregularly shaped 10-μm polymeric octadecylsilica (μBondapak C_{18}, Waters Assoc.). Columns B–D (250 × 4.6 mm I.D.) contained 6-μm spherical monomeric packings and were obtained from DuPont Instruments; column B was a Zorbax-ODS (octadecylsilica);

column C was a Zorbax C_8 (octylsilica); and column D was a Zorbax-TMS (trimethylsilica). Data on the physical characteristics of these columns can be obtained from the manufacturers.

Chromatographic conditions

The eluent was 0.02 *M* KH_2PO_4 (HPLC grade, Fischer Scientific, Fair Lawn, NJ, U.S.A.), pH adjusted from 2.0 to 7.5 with orthophosphoric acid or potassium hydroxide. It was degassed by sonication and purged with helium. The flow-rate was 0.5–3.5 ml/min and the temperature was 35°C, unless otherwise noted. The void times (t_0) were determined by the methods of Berendsen *et al.*[22] and Neidhart *et al.*[23].

The compounds chromatographed were of the highest purity available from various sources; the majority of compounds were from Sigma (St. Louis, MO, U.S.A.) and Vega Biochemicals (Tuscon, AZ, U.S.A.). Stock solutions were prepared in the mobile phase at concentrations *ca.* 100μ *M*.

Computational methods

The average capacity factors were determined from a minimum of at least five serial injections. The natural logarithms of these values were used.

Two computational methods were used in the determination of the functional group contribution terms. One method employed the FORTRAN program Linsys[24] to solve the linear equations simultaneously by coefficient matrix inversion. The input of the program includes the ln k' values for 24 purine compounds* as the observed constants and the square coefficient matrix of the indicator variables. The indicator variables were set to unity if a certain substituent was present, and to zero otherwise. To validate the use of a small data set in the T_j determinations, a second method was used with a modified POLREG program[24]. It is based on a multiple regression technique, followed by statistical analysis through the use of the *F*-statistic, as demonstrated by Chen and Horváth[17]. The constant matrix was the ln k' for a group of 32 compounds, which corresponds to a coefficient matrix of eleven substituent effects, each represented at least three times.

RESULTS AND DISCUSSION

QSRRs of purine compounds on reversed-phase packings

The purine structure showing the locations of substituents is shown in Fig. 1.

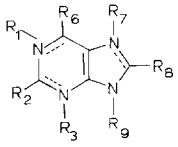

Fig. 1. Purine structure, indicating substituent locations.

* The 24 compounds used in the Linsysprogram are 1-methylhypoxanthine, xanthine, guanine, 2-methyladenine, 2-methylguanosine (N_2-methylguanosine), 2,2-dimethylguanosine (N_2,^{2}-dimethylguanosine), 3-methylxanthine, hypoxanthine, adenine, 6-methylpurine, 6-methyladenine (6-methylaminopurine), 6-dimethyladenine, 6-thiopurine (6-mercaptopurine), 1-methyladenine, 7-methylguanine, 7-methylguanosine, uric acid, inosine, 2′-deoxyinosine, 3′,5′-cyclic inosine monophosphate, AMP, ADP, ATP and purine.

TABLE I
CALCULATED T_j VALUES

Position	*Substituent*	*Column*			
		A	*B*	*C*	*D*
R_1	Methyl	0.890	2.34	1.23	0.892
R_2	Oxo	0.220	−0.294	0.0520	−0.663
	Amino	0.110	−0.477	0.0160	0.440
	Methyl	0.820	0.724	1.10	0.690
	Methylamino	1.18	1.32	1.03	0.933
	Dimethylamino	2.45	3.42	2.09	1.97
R_3	Methyl	1.70	2.17	1.39	0.875
R_6	Oxo	−0.750	−1.91	−0.954	−0.795
	Amino	0.320	−0.127	0.264	0.257
	Methyl	1.14	1.26	1.18	0.777
	Methylamino	2.20	3.02	1.23	1.42
	Dimethylamino	3.41	4.68	2.19	2.62
	Thio*	1.28	1.48	−0.538	3.20
	Imino**	2.03	−4.04	−1.33	0.595
R_7	Methyl (base)	0.790	0.656	1.44	1.07
	Methyl (N′-side)§	−0.670	1.78	0.460	0.460
R_8	Oxo	−0.802	−1.99	−1.10	−1.10
R_9***	Ribo	1.19	1.02	0.870	0.109
	d-Ribo	1.31	1.45	1.31	0.561
	c-Ribo	1.56	0.940	0.680	0.584
	mp-Ribo	−0.740	−1.89	1.47	−1.92
	dp-Ribo	−1.24	−2.72	−3.51	3.10
	tp-Ribo	2.74	−5.22	−3.73	3.36
	Purine	1.92	2.54	2.15	0.933

* Substituent is in the thione form (Lactam).
** Represents protonated form.
*** Ribo = D-ribofuranosyl; d-Ribo = 2′-deoxyribofuranosyl; c-Ribo = 3′-5′-cyclic monophosphate; mp-Ribo = 5′-monophosphate; dp-Ribo = 5′-diphosphate; tp-Ribo = 5′-triophosphate.
§ See text for explanation.

The T_j values for the different substituents on the purine molecule on 4 different columns were calculated from the simultaneous solution of 24 linear equations (Table I). These values apply for aqueous phosphate buffer eluents at pH 5.7 and are for groups commonly found on the purines of biological interest. For unusual purines, additional terms are necessary to establish the QSRRs precisely.

When using the tabulated T_j values, one must be aware of changes in the underlying structure and the degree of ionization that may result from the substituents. Thus, a compound which has only two functional groups may require more than two T_j terms for an accurate prediction of its retention. In cases such as these, the T_j terms may represent a combination of structural changes. For example, prior to making a substitution on the 7 position, the purine molecule undergoes a prototropic shift from carbon-9 to carbon-7 to accomodate the methyl group (Fig. 2). Therefore, the T_j term for substituting a methyl for a hydrogen at the 7-position encompasses both the prototropic shift and the methyl substitution. In Table I, there is also an additional term for the 7-methyl when R_9 is occupied (*e.g.*, ribosyl) which

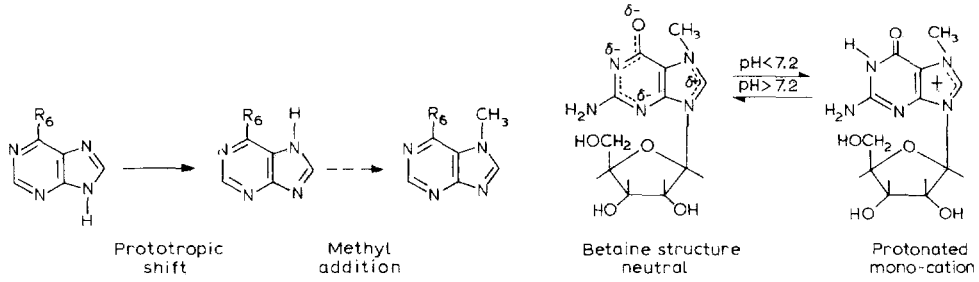

Fig. 2. Prototropic shift, prior to methylation in the 7-position.

Fig. 3. Effect of pH on charge formation in a 7-methyl-substituted purine nucleoside, 7-methylguanosine.

represents the combined effects of substituting a methyl group at the 7-position and charge formation (Fig. 3). Two terms are given for the 6-amino group, since the imino form predominates when there is a methyl in the R_1-position; however, at pH 5.7 1-methyladenine will be protonated (pK_a 7.2) and the tabulated value for the 6-imino group represents its charged form (Fig. 4). Likewise, the T_j value for 8-oxo is applicable only to the uric acids that are charged at pH 5.7.

The degree of dissociation of the 3-methyl-substituted adenines must be taken into account. At pH 3–4 the tabulated 6-imino and 3-methyl T_j terms fit the observed ln k' of 3-methyladenine. However, at pH 7 the T_j values listed for 6-methylamino or 6-dimethylanimo, in addition to the 3-methyl, must be used to predict the retention of 3-methyl-6-methylamino purine or 3-methyl-6-dimethylaminopurine, respectively. Therefore, the pH of the eluent plays an important role in determining both the capacity factor and the T_j values for these compounds. However, certain relationships can be derived which permit the use of the tabulated T_j terms at one pH to predict the retention at another pH for any purine of which the pK_a is known.

Validity of calculated group contribution terms

While the use of a small data set to determine the group contribution terms in Table I may not be statistically sound, the constraints of sample availability, sample cost, and analysis time make it impractical to acquire ln k' data for a full complement of compounds to solve the T_j matrix in a statistical manner. To assess the reliability of T_j values determined from a small set of compounds, we therefore selected groups of 32 compounds in such a way that eleven substituent constants were indicated at least three times. The multiple regression technique was employed to solve the T_j matrix, and the statistical parameters are shown in Table II. A compar-

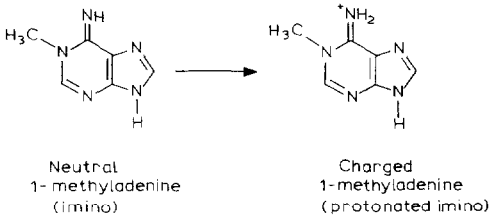

Fig. 4. Imino and protonated imino structures of 1-methyladenine.

TABLE II

STATISTICAL PARAMETERS WITH FOUR DIFFERENT COLUMNS

Statistical parameter	*Column*			
	A	*B*	*C*	*D*
Number of samples (n)	32	32	32	32
Number of substituents (m)	11	11	11	11
Correlation coefficient (r)	0.998	0.999	0.997	0.987
Coefficient of determination (r^2)	0.996	0.998	0.994	0.974
Standard error in estimate (s)	0.078	0.034	0.082	0.103
$F_{m,n-m}$ calculated*	475	953	316	71.5
$F_{m,n-m-1.0.005}$ tabulated	3.76	3.76	3.76	3.76

* $F_{m,n-m} = \frac{n-m}{n} \cdot \frac{r^2}{1-r^2}$

ison of the individual substituent terms with the corresponding values from Table I showed some numerical differences; however, based on the t-statistic (95% probability) the differences were determined to be insignificant. Thus, the method used to determine the T_j values in Table I with a small set of compounds is a justified means of establishing the QSRRs for the purines on the reversed-phase packings investigated.

Prediction of retention from group contribution terms

A comparison was made between the predicted and observed ln k' for each

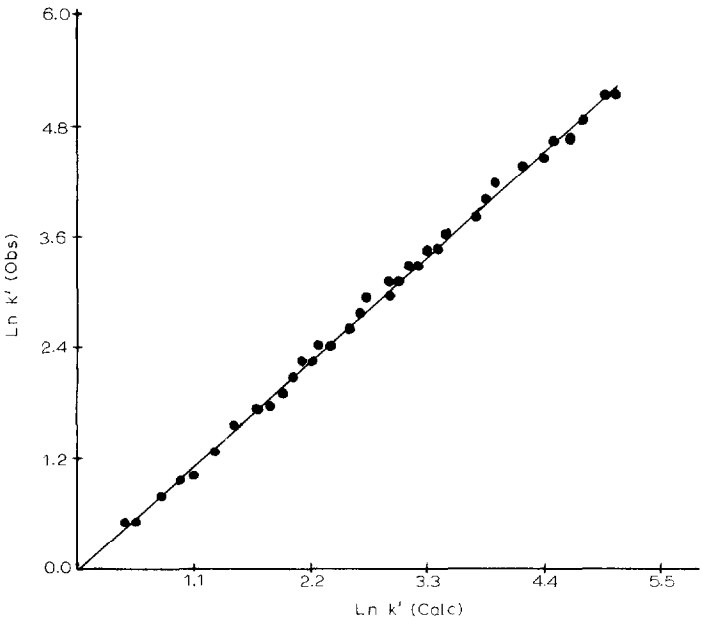

Fig. 5. Plot of predicted and observed capacity factors (k'). $y = 1.003\chi - 0.0429$, $r = 0.99986$ and $s = 0.01830$, where r is the correlation coefficient, and s is the standard deviation of the regression.

column with 62 compounds other than those used for the original solution of the T_j matrix. Excellent agreement was found for all the compounds chromatographed. Fig. 5 illustrates the correlation between the observed and predicted retention factors on the μBondapak C_{18} column for 36 randomly chosen compounds. Thus, with one chromatographic system, the retention of many compounds can be predicted fairly well from the tabulated T_j values and eqn. 2.

Comparison of T_j values on different columns

Recent studies have focused on the effects of chromatographic packings on reversed-phase separations[25–31]. In general, the results of these studies suggest that chain length has no specific role in retention; the selectivities observed with different reversed phases are mainly due to the hydrocarbonaceous surface area and the accessability of residual silanols.

A plot of the T_j values for column A *versus* column B shows that these data are co-linear. The relationship between these two phases is given by

$$T_j(\mathrm{B}) = -0.639 + 1.63\ T_j(\mathrm{A}) \quad (3)$$

$n = 24$
$r = 0.987$
$s = 0.396$

where (A) and (B) denote the columns used, n is the number of terms, and s is the standard deviation. The correlation coefficient (r) of 0.987 implies very similar retention and selectivity. This is an interesting finding, since these packings have very different specifications. Furthermore, the correlation is improved to 0.999 when the terms for 1-methyl- and 9-ribo-3′,5′-cyclic monophosphate are excluded from the regression. No explanation for the deviation of the 1-methyl group is readily apparent; however, the spatial configuration of the cyclic-ribonucleotides may account for different interactions between the two stationary phases.

A comparison of the T_j values for columns B and C gives the following relationship:

$$T_j(\mathrm{C}) = 0.0488 + 0.600\ T_j(\mathrm{B}) \quad (4)$$

$n = 24$
$r = 0.902$
$s = 0.708$

As would be expected, the correlation of column A to C is commensurable to the above.

$$T_j(\mathrm{C}) = -0.339 + 0.982\ T_j(\mathrm{A}) \quad (5)$$

$n = 24$
$r = 0.893$
$s = 0.737$

However, the relationships between the T_j terms from columns C and D

$$T_j(\mathrm{D}) = 0.0449 + 0.786\ T_j(\mathrm{C}) \quad (6)$$

$n = 24$
$r = 0.810$
$s = 0.934$

and from columns B and D

$$T_j(\mathrm{D}) = 0.0663 + 0.535\ T_j(\mathrm{B}) \quad (7)$$

$n = 24$
$r = 0.829$
$s = 0.890$

show considerable scatter. From these relationships it becomes evident that T_j terms from an octadecylsilica packing can adequately estimate the retention on an octylsilica packing, as well as on octadecylsilica packings from other sources[17]. In addition, it is apparent that functional group retention increments on octadecylsilica or octylsilica will not always give a good estimate of retention on a trimethylsilica reversed-phase and *vice versa*. Furthermore, a deviation of group contribution terms from the calculated linearity between the reversed phases could provide a measure of the specificity and/or selectivity of these columns for some purine separations.

Elucidation of purine structure from retention data

Since the retention of a purine is a function of its structure and of the chromatographic conditions employed, it should be possible to use retention behavior as a means of structural elucidation. The foundation for predicting purine structures is laid by assigning T terms to the different functional groups at the various positions on the purine molecule. Once a library of group contribution terms for a large variety of purine substituents is established, a simple computational method can be used to extract a combination of all possible groups the sums of which fall within a certain range, set by the retardation factor of the unknown. Under constant chromatographic conditions, the retention factor of the unknown is likely to correspond to a lengthy list of possible structures; chromatographing the unknown on two or more different reversed phases (*e.g.* ODS, C_8 and/or TMS) and using the same type of computational method mentioned above should then dramatically reduce the list of possible structures.

An example of the proposed scheme is given for a hypothetical unknown, 2,7-dimethyladenine ($m_{2,7}$-Ade). The ln k' values on two different reversed-phase columns are determined; ln k' 3.81 on the Zorbax-ODS and 2.93 on the Zorbax-TMS column. A search window of 3–5% variation in the computed ln k' on the two phases is chosen to account for any error in the observed values; however, 3% should be adequate to cover differences that may result from column aging or the use of physiological samples. The program then searches through the T_j input files to find the substituents that could give a ln k' of 3.81 on the ODS and 2.93 on the TMS stationary phases.

The proposed substituents in purine compounds which would correspond to the 3% to 5% range of the observed retardation factors are: 2-methyl, 6-amino and 7-methyl; 2-methyl, 6-methyl, 7-methyl and 9-ribo; 1-methyl, 2-methylamino, 6-thio, 8-oxo and 9-ribomonophosphate; 2-methylamino, 3-methyl, 6-oxo, 7-methyl and 9-ribo; 1-methyl, 2-amino, 3-methyl, 6-oxo, 7-methyl and 9-ribo. The actual identity of the unknown can then be readily ascertained from the list by either on-line or off-line characterization techniques[2,3].

This type of analysis would greatly simplify the procedures currently used in the identification of unknown purines, since it provides a starting point for choosing a method of identification. All the compounds listed above, except for $m_{2,7}$-Ade have a substituent at the R_9-position, which can be easily tested for through an off-line, pre-column, acid cleavage of the glycosidic bond. A negative acid hydrolysis reaction would, therefore, indicate $m_{2,7}$-Ade.

CONCLUSION

The quantitative relationships between the structures of purines and their reversed-phase retention, which previously were not available, were investigated. By determining the functional group increment terms, the rules set forth by Brown and Grushka[13], which predict elution orders, can now be viewed on quantitative terms where much of the generalizations are avoided. In addition, the results of this study illustrate how easily the retention of a great many purines can be predicted from the tabulated T_j terms. In turn, the T_j terms can serve as a diagnostic tool in assessing the selectivities of different reversed-phase packings. Conversely, the T_j values permit the elucidation of unknown purine structures and may guide the sequence of characterization methods toward unequivocal identifications. Thus, retention analysis, as illustrated herein, may be advantageous when standards are not readily available.

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APPENDIX

The compounds used in addition to those used in the Linsys program for the modification through the POLREG routine are as follows:

2-amino-6,8-dihydroxypurine (O_8-guanine), 2-amino-6-dimethyladenine, 2-dimethylaminoadenine, 1-methylguanine, 3-methylguanine, 2-methylguanine, 2,2-dimethylguanine, 7-methylisoguanine, isoguanine, 2-amino-6-mercapto-7-methylpurine (2-amino, 7-methyl, S^6-purine), 2-amino-6-mercaptopurine riboside, 6-amino-2-methylamino purine, 2-amino-6-methylpurine, 7-methyladenine, 2-aminopurine, 2-aminopurine riboside, 2,6-diamino-8-hydroxypurine, 1-methylxanthine, 7-meth-

ylxanthine, 1,3-dimethylxanthine (theophylline), 1,7-dimethylxanthine (paraxanthine), 1,3,7-trimethylxanthine (caffeine), 2-hydroxy-6-mercaptopurine, 2-hydroxy-6-methylpurine, 2-methylhypoxanthine, 3,7-dimethylxanthine, thiobromine, 7-methylhypoxanthine, 2-hydroxypurine, 6-mercapto-7-methylpurine, m_6-deoxyadenosine, m_1-deoxyadenosine, 6-methyladenosine, 1-methylguanosine, c-AMP, c-GMP, guanosine, adenosine, 1,3-dimethyluric acid, m_1-adenosine, GMP, IMP, 2'-deoxyguanosine, 2-deoxyadenosine, 2'-deoxy-3',5'-c-AMP, 2'-deoxy IMP, 2'-deoxy-3',5'-cGMP, 1,3,7-trimethyl uric acid, GDP, GTP, IDP, ITP, XMP, XDP, XTP, xanthosine, 1-methylpurine, 2-methylpurine, 3-methylpurine, 2,6-dimethylpurine and its riboside, 7-methylpurine, 1-methyl deoxyinosine.

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