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Isocratic hydrophobic interaction chromatography of dansyl amino acids

Correlation of hydrophobicity and retention parameters

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ABSTRACT

Hydrophobic interaction chromatography of the dansyl derivatives of 20 common amino acids has been carried out, and the dependence of log k' (k' = capacity factor) on the ammonium sulfate concentration in the mobile phase determined. Linear relationships above 0.2 M ammonium sulfate are found. For the amino acid derivatives studied, the slopes of the equation relating retention (log k') to salt concentration (M_s) in the mobile phase (log $k' = mM_s + b$) correlate well with other measures of amino acid hydrophobicity.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is commonly utilized for protein separations[1–4]. However, the influence of analyte structure on retention in HIC is not yet well understood. Although structure–retention relationships have been discussed extensively in reversed-phase studies[5–10], there have been few reports on similar studies in HIC[11,12].

Because it should be easier to examine the effect of the mobile phase and of solute structure on retention in HIC if small molecules are employed, we have undertaken a study of dansyl amino acid derivatives under HIC conditions that are conventionally applied for protein separations. Retention parameters are reported as a function of ammonium sulfate concentration in the mobile phase. The relationship between retention parameters in HIC and a solute's contact area with the stationary phase are discussed.

EXPERIMENTAL

Materials

A kit of dansyl amino acids was obtained from Mann Research Labs. (New

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York, NY, U.S.A.). Dansyl amide was obtained from Sigma (St. Louis, MO, U.S.A.). Ammonium sulfate (ultrapure grade) was obtained from Schwarz-Mann Biotech (Cleveland, OH, U.S.A.). All other reagents were of A.C.S. analytical-reagent grade.

Methods

The chromatographic system consisted of two Waters Model M6000A pumps, a Rheodyne Model 7125 injection valve, a 150 × 4.6 mm I.D. SynChropak propyl hydrophobic interaction column (SynChrom, Lafayette, IN, U.S.A.) with 6.5-μm particle packing, and a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator. The column dead time was determined by water injection.

Mobile phases were prepared with high-purity HPLC-grade water obtained in-house with a Millipore (Bedford, MA, U.S.A.) Milli-Q water purification system as follows: Mobile phase A: 2.1 *M* ammonium sulfate, 0.02 *M* potassium phosphate monobasic, adjusted to pH 6.0 with a sodium hydroxide solution. Mobile phase B: 0.02 *M* potassium phosphate monobasic, adjusted to pH 6.0 with a sodium hydroxide solution.

The composition of the mobile phase was controlled by a Waters Model 660 solvent programmer. Stock solutions of each dansyl amino acid were prepared using high-purity HPLC-grade water (unbuffered) at a concentration *ca.* 1.0 mg/ml. Mobile phases and stock solutions were filtered through a Millipore HA (0.45 μm) filter and stored at 4°C when not in use. A 20-μl injection loop was used for all injections (approximately 20 μg per injection). The dansyl amino acids were detected at 254 nm using a Waters Lambda-Max Model 480 spectrophotometer. The flow-rate was 2.0 ml/min throughout the study. The chromatographic column was maintained at 30.0 ± 0.2°C with a circulating water jacket.

RESULTS AND DISCUSSION

In isocratic HIC, the log of the capacity factor, k', is linearly related to the salt concentration (M_s) in the mobile phase[13].

$$\log k' = \log k'_0 + mM_s \tag{1}$$

This equation is derived from the linear relationship of the log of the retention factor to the surface tension of the mobile phase[14] and from the linear relationship between surface tension and salt concentration[15]. The Setschenow equation for the salting-out of non-polar compounds from aqueous solution is of a similar form[16].

Melander and Horváth[13] have discussed the linear relationship in HIC between k' and M_s. However, at low salt concentrations, deviations from linearity are observed due to electrostatic effects. Above a certain salt concentration (which is dependent on the solute), concomitant ionic shielding suppresses the electrostatic interactions, and $\log k'$ then becomes a linear function of M_s. They propose that the slope of eqn. 1, m, is dependent on the non-polar contact area between the solute and the stationary phase or the molecular contact area upon binding.

Eqn. 1 is analogous to the equation in reversed-phase chromatography (RPC) relating $\log k'$ to the organic volume fraction in the mobile phase, φ.

$$\log k' = \log k'_0 + S\varphi \tag{2}$$

Analogous to the m values in HIC, S values are presumed to reflect the magnitude of the non-polar contact area between the solute and the hydrocarbon ligands of the stationary phase. S values for polypeptides are usually larger than those for small solutes[17]. For example, Terabe *et al.*[18] have studied the RPC of peptides and found that the larger the peptide molecule, the steeper the slope in eqn. 2. These findings also support a direct relationship of the slope to the non-polar contact area of the solute with the stationary phase.

It has been suggested that slope values in HIC are generally smaller than in RPC due to the weaker interaction of the solute with the stationary phase in HIC[19]. This is a difficult comparison to make, since not only are ammonium sulfate and its organic solvent counterpart likely to have different incremental solvent powers, but also the conventional concentration scales for HIC (molar or molal) and RPC (% organic component) are different. Moreover, stationary phases for RPC are more non-polar than those for HIC, both in the size and the packing density of the surface moieties. Finally, in contrast with HIC, RPC solvent systems usually denature proteins, thereby exposing a larger protein contact area. Any quantitative comparison of HIC and RPC retention parameters should take these differences into account. We will not pursue it further here.

Others have given interpretations of the slope and intercept values in eqn. 1. Zaslavsky *et al.*[20] discuss the relationship between the partition coefficient, K (which is linearly related to k') and ionic strength, as it relates to the measurement of the hydrophobicity of the amino acid side chains partitioning in a two-phase system. The slope (m) reflects the effect of ionic strength on the transfer of a given compound and the intercept (log k'_0) is the relative hydrophobicity of a substance at zero ionic strength. In another paper[21] they propose that the slope reflects the overall effect of ionic strength on a system of given ionic composition of all the ionogenic groups on the surface and that the intercept (log k'_0) is related to both the overall relative

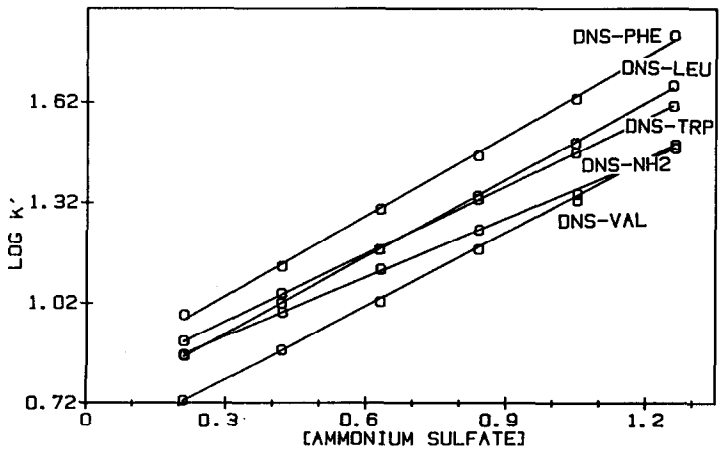

Fig. 1. Retention of dansylamide and dansylated amino acids on a SynChropak propyl column as a function of ammonium sulfate molar concentration. DNS-VAL = Dansyl valine; DNS-NH2 = dansyl amide; DNS-TRP = dansyl tryptophan; DNS-LEU = dansyl leucine; DNS-PHE = dansyl phenylalanine.

hydrophobicity of surface groups at zero ionic strength and the total number of equivalent CH_2 groups on the surface. Harnisch *et al.*[22] state that, in the RP-HPLC context, the slope is not only affected by solute–solvent and solute–stationary phase interactions, but also by solute size and structure.

For the dansyl amino acid derivatives, isocratic data were acquired with mobile phases ranging in concentration from 0.21 to 1.26 *M* ammonium sulfate. Fig. 1 gives an example of the plots of log *k versus* M_s for a few of the more non-polar dansyl amino acid derivatives (phenylalanine, tryptophan, valine and leucine), with dansyl amide included for comparison purposes.

From comparison of the retention order in isocratic elutions at 0.4 and at 1.0 *M* ammonium sulfate, it is evident that retention order changes with salt concentration. Therefore, it is misleading to infer the hydrophobicity of one solute relative to that of another from their retention order at any single salt concentration. Braumann *et al.*[23] have also pointed out that k' is not a good parameter to describe the hydrophobic nature of a solute since compounds with the same k' at a given mobile phase composition do not necessarily exhibit the same retention mechanism as inferred from the different values of slope (m in eqn. 1).

Table I gives the slope (m) and intercept (log k'_0) calculated from 0.21 to 1.26 *M* ammonium sulfate from eqn. 1. The dansyl derivative for lysine is in parentheses

TABLE I

LINEAR REGRESSION ANALYSIS OF $\log k' = \log k'_0 + mM_s$

Linear regression from 0.21 to 1.26 *M* ammonium sulfate, $n = 6$; correlation coefficient > 0.996.

Dansyl derivative	*Slope (m)*	*Intercept (log k'_0)*
Phe	0.79388	0.80400
Ile	0.78844	0.81400
Leu	0.76394	0.70133
Tyr	0.74694	1.02930
Val	0.73374	0.57253
Met	0.73320	0.37960
Pro	0.67565	0.49040
Trp	0.67007	0.76533
Lys[a]	0.66299	0.22320
Ala	0.61524	0.21880
Amide	0.59932	0.74600
Thr	0.59565	0.12320
Cys	0.58912	0.71200
Gln	0.57565	0.07773
Gly	0.56136	0.42907
Ser	0.55510	0.16500
Glu	0.53544	−0.02453
Asn	0.52871	0.01773
His[b]	0.51605	0.25320
Arg	0.49578	−0.01573
Asp	0.46993	−0.03887

[a] Derivatized on ε-nitrogen, not on α-nitrogen.
[b] Partially ionized at pH 6.0.

because it is derivatized on the ε-nitrogen rather than on the α-nitrogen as are the other dansyl derivatives. Because of this, it should not be directly compared to the other dansyl derivatives. It also should be noted that dansyl histidine is expected to be partially ionized in the mobile phase (buffered at pH 6.0). The pK_2 of dansyl histidine, though not readily available, is probably a few tenths of a unit higher than that of histidine itself ($pK_2 = 6.0$), based on a simple electrostatic argument.

Slope values (m) of all the dansyl derivatives examined have been ranked in order from largest to smallest in Table I and show an interesting trend. The amino acids that are commonly thought of as hydrophobic (phenylalanine, isoleucine, leucine, valine, methionine and tryptophan) have large slope values, while the more hydrophilic amino acids (aspartic acid, glutamic acid, asparagine, glutamine and arganine) have small slope values. Thus, the slope value, reflecting the solute's non-polar contact area with the stationary phase, appears to correlate with that what is generally accepted to be the relative hydrophobicity (relative polarity) of the amino acid.

It has already been pointed out that the isocratic retention order changes for the dansyl amino acid derivatives at different salt concentrations. For this reason, the slope value (m), rather than retention time, is taken to be a better determinant of a solute's relative hydrophobicity. To set up a quantitative scale of hydrophobicity for the amino acids from the retention behavior of their derivatives, it is assumed that the dansyl group makes a constant contribution to the behavior of all the derivatives.

The relative order of the slopes (m) shown in Table I correlates well with other published scales of amino acid hydrophobic parameters. Many such scales have been published[24–31]. These relative scales of amino acid hydrophobicity are based largely on data from liquid–liquid partition experiments or statistical data based on the appearance of amino acid residues in the interior *versus* the exterior of proteins of known three-dimensional structure. An extensive review of many scales of amino acid hydrophobicity is given by Cornette *et al.*[32]. However, in order to directly compare published hydrophobic parameters with the slope values in Table I, all of the amino acid values within a particular scale have been reassigned values such that the most hydrophobic amino acid of the group is assigned a value of -10.0 and the most hydrophilic amino acid of the group is assigned a value of 10.0. The remaining amino acids in each group are scaled proportionally.

Table II shows the scaled values of the dansyl amino acids based on the slope values of Table I (Lys, the ε-derivative, is not used in the correlation or the t-tests even though it appears in Table I). For comparison purposes, the intercept data ($\log k'_0$) have been scaled proportionally, along with two published hydrophobicity scales. One is based on octanol–water partition data[24] and the other on surface tension data[25]. The correlation coefficients between the scaled slope data from this study and the other scales are listed as well as the results of a paired t-test (comparing an experimental scale with the slope values from this study) at the 98% confidence interval. The t-test is important because two particular scales may show a good correlation ($r \geq 0.75$) but fail the paired t-test because the differences between the values assigned for each amino acid may be significant between the two scales chosen to be compared. As can be seen from Table II, there appears to be good agreement between the slope values and the two independent scales of amino acid relative hydrophobicities. Other published scales have also been found to show a good correlation with the slope data given here and their correlation coefficients are given in Table III (all values have been scaled

TABLE II

CORRELATIONS BETWEEN HIC PARAMETERS (EQN. 1, TABLE I) AND HYDROPHOBICITY SCALES

All scales have been normalized to range from −10.0 to 10.0 (most hydrophobic to most hydrophilic). See text.

Amino acid	*Slope (m)*	*Intercept (log k'_0)*	*Octanol–water*[a]	*Surface tension*[b]
Phe	−10.0	−5.8	−7.2	−9.0
Ile	−9.7	−6.0	−7.2	−8.5
Leu	−8.2	−3.9	−6.6	−10.0
Tyr	−7.1	−10.0	−2.1	−8.3
Val	−6.3	−1.4	−3.7	−3.1
Met	−6.3	2.2	−3.7	−2.4
Pro	−2.7	0.1	−0.6	1.3
Trp	−2.4	−5.1	−10.0	−6.6
Ala	1.0	5.2	1.9	7.2
Thr	2.2	7.0	2.2	4.8
Cys	2.6	−4.1	−5.6	5.3
Gln	3.5	7.8	5.2	10.0
Gly	4.4	1.2	3.8	8.8
Ser	4.7	6.2	4.0	5.8
Glu	6.0	9.7	7.7	6.5
Asn	6.4	8.9	7.5	9.4
His	7.2	4.5	3.0	7.9
Arg	8.4	9.6	10.0	7.9
Asp	10.0	10.0	8.5	7.2
Correlation coefficient	1.00	0.827	0.853	0.917
Paired *t*-test (98% C.I.)[d]		Do not reject[c]	Do not reject	Do not reject

[a] Ref. 24.
[b] Ref. 25.
[c] There is no significant difference between the paired values of the amino acids between the scale in question and the slope values.
[d] C.I. = Confidence interval.

proportionally as before for direct comparison). The high correlation between this retention-based scale and independently derived scales for hydrophobicity supports the assumption that the non-amino acid structural components of the dansyl derivatives contribute additively and independently to their chromatographic retention.

The slope values are used instead of the intercept values for the comparison to the other scales of relative amino acid hydrophobicity because the slope values for a particular dansyl amino acid did not vary significantly with chromatographic column usage, but the intercept values slowly decreased to lower values with continued column use. In other words, column degradation did not change the solute's contact area with the stationary phase, but did change the strength of the interaction systematically at all ammonium sulfate concentrations, including the zero intercept. Because of this, slope values are considered here to be more reliable than intercept values in comparing our data to parameters obtained by other investigators.

TABLE III

CORRELATION OF LITERATURE SCALES WITH SLOPE SCALE (TABLES I AND II)

Scale type	*Ref.*	*Correlation coefficient*
Octanol–water distribution	24	0.853
ΔG_{CH_2} from solution to air–aqueous interface	25	0.917
ΔG_{CH_2} from dilute aqueous solution to the vapor phase	26	0.680
Based on previously published tables	27	0.794
Combination of previously published tables	28	0.779
Average surrounding hydrophobicity	29	0.791
Fractional aqueous solvent exposure in proteins	30	0.718
Optimal matching hydrophobicities	31	0.926

Braumann *et al.*[23] have recommended the use of the intercept (the RPC capacity factor at 100% aqueous mobile phase) as a better measure of hydrophobicity than the use of the capacity factor (retention order), but go on to state that the slope as well as the intercept is largely dependent on the hydrophobic surface area of the solute. Minick *et al.*[33] argue, that in RPC the slope is a more reliable measure of the hydrophobic properties of a solute than the intercept.

Even with the small systematic error in intercept, a good correlation exists between the slope values (m) and intercept values ($\log k'_0$) in this study (Table II, $n = 19$, $r = 0.827$). This suggests that the slope and the intercept are dependent variables, both reflecting similar molecular properties of the solute.

However, the effect of salt on retention is not the only parameter to be considered in determining a solute's retention in HIC. In an HIC study of the retention of lysozymes isolated from related bird spcies, Fausnaugh and Regnier[11] find that pH affects the intercept of $\log k'$ *versus* molality plots, but not the slope. This is taken to indicate that the contact surface area is not altered, but the ionization state of the amino acids in the contact surface area modifies the intercept (the strength of the association of the solute with the stationary phase). Similarly, our studies on the hydrophobic interaction chromatography of small molecules[12] showed that while adenosine and adenosine 5'-monophosphate (AMP) appear to have parallel plots of $\log k'$ *versus* salt concentration in the mobile phase, AMP has a much lower intercept ($\log k'_0$). Heinitz *et al.*[34], in a study of the retention characteristics of proteins in HIC and ion-exchange chromatography, also find that electrostatic interactions appear to influence retention at the high ionic strengths used in HIC.

Therefore, it is apparent that both hydrophobicity and hydrophilicity affect HIC retention. A theoretical framework that can adequately express the combined influence of both a solute's hydrophobic and hydrophilic structural components on chromatographic retention remains to be developed.

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