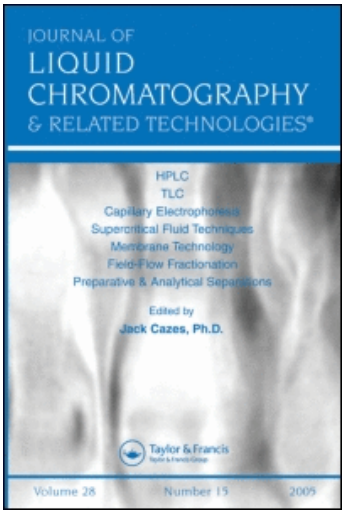

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Solvent Composition-Capacity Factor Dependencies of Iodoamino Acids

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SOLVENT COMPOSITION - CAPACITY FACTOR DEPENDENCIES OF IODOAMINO ACIDS.*

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ABSTRACT

The retention behaviour of several iodoamino acids on μBondapak C_{18} columns has been investigated as the volume fraction, ψ_s, of the organic solvent modifier was varied over the range $0.08 < \psi_s < 0.8$ under low pH, low ionic strength conditions. Bimodal plots of the logarithmic capacity factor, log k', versus ψ_s were observed with selectivity reversals from a reversed phase to a polar phase elution mode occurring at ψ_s ca 0.6 for acetonitrile-based eluents. The calculated slope parameters (s-values) of the iodoamino acids were similar to those found with other low molecular weight solutes but were considerably smaller than s-values observed with polypeptides or proteins of comparable capacity factor characteristics.

INTRODUCTION

Over the past several years, the relationship between mobile phase composition and retention behaviour for peptides and proteins separated on alkylsilicas has attracted increasing

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attention. In particular, studies[1-4] on the influence of the organic solvent modifier on the chromatographic properties of these ionogenic substances have been undertaken not only to validate predictions based on solvophobic theory[5] but also to permit practical interconversions between isocratic and gradient elution data (and vice versa). These investigations, which have allowed improved criteria to be established for resolution optimisation, have also demonstrated that in general peptides and proteins do not show linear dependencies of their logarithmic capacity factors, log k', on the volume fraction, ψ_s, of the organic solvent modifier. Instead, curvilinear and even bimodal concave plots are commonly observed. It is also clear that the slope of the tangent (s-value) of the plots obtained from these measurements depends on the molecular characteristics of the solute as well as the choice of chromatographic conditions. With well defined elution conditions and a specified hydrocarbonaceous stationary phase, the slopes of the plots of log k' versus ψ_s for a series of polypeptides[3,4], as well as neutral solutes such as polystyrenes[6], tend to increase with molecular size. However, molecular size per se is known[1,2] not to be the dominant parameter in controlling the retention of polypeptides and related ionogenic solutes to alkylsilicas but rather the interfacial hydrophobic contact area established between the solute and the hydrocarbonaceous ligand.

Many low molecular weight amino acid derivatives, such as the non-polar dansyl amino acids and phenylthiohydantoin amino acid derivatives, exhibit comparable retention with alkylsilicas in terms of their capacity factors (and thus comparable hydrophobic contact areas) to that shown by larger peptides. However, the retention behaviour of amino acid derivatives is generally more predictable and the overall chromatographic performance in terms of resolution and peak shape is usually superior to that observed with larger molecules. Mobile phase composition is known to play

a important role in the expression of these differences which are believed to arise from more complex secondary equilibrium and kinetic processes. The present study examines one aspect of these differences, namely the solvent composition-capacity factor dependencies using as model compounds several hydrophobic iodoamino acid derivatives. The study reveals that in common with other low molecular weight neutral or acidic organic compounds, the s-values of iodoamino acids are significantly smaller than those observed for polypeptides. Further, elution order reversals typified by polar phase selectivity are observed at high organic solvent content.

MATERIALS AND METHODS

Reagents: Acetonitrile was purchased from Waters Assoc. (Aus.) Pty. Ltd. Sodium sulphate, orthophosphoric acid, sulphuric acid and triethylamine were all AnalaR grade reagents from BDH (Poole, Great Britain) or May & Baker (Dagenham, Great Britain). Water was quartz distilled and deionised using a Milli-Q system (Millipore, Bedford, MA.). The iodoamino acid derivatives were obtained from Henning (Berlin, G.F.R.). Stock solutions of the iodoaminoacid derivatives were prepared by dissolving the compounds in 1% methanolic NH_4OH at a concentration of ca 10 mg/ml.

Apparatus: All chromatographic data were collected using a Waters Assoc. (Milford, M.A.) HPLC system which consisted of a M6000A solvent delivery unit, a U6K universal liquid chromatographic injector, and a M440 UV absorbance detector coupled to a M720 data module. Sample injections were made with SGE model 50A syringes (SGE, Melbourne, Aus.). The pH measurements were performed with a Radiometer PH M-64 meter equipped with a combination glass electrode. The μBondapak C_{18} columns were purchased from Waters Assoc. (Aus.).

Chromatographic Procedures

A flow rate of 1.0 ml/min was used for this investigation. The solvent reservoirs, precolumn delivery systems and columns were maintained at 20°. Bulk solvents and appropriate isocratic mobile phases were prepared and degassed by sonication as reported previously[3]. The μBondapak C_{18} columns were equilibrated to new mobile phase conditions for at least 30 mins. Sample sizes varied between 1 μg and 5 μg of iodoamino acid and were within the linear range of the adsorption isotherms. The capacity factors for isocratic retention experiments were calculated by established methods with all data points representing an average of triplicate measurements. The precision of the measurements was generally 2%. The s-values for the various solutes were calculated from the curve tangent or by linear regression analysis of the isocratic retention data using a Hewlett-Packard-97 calculator. The ionic strength and pH of the eluents were selected on the basis of previous experience[3,4] with peptides and other ionogenic solutes to minimised changes in retention as a consequence of changes in ionisation state and/or extent of solvation.

RESULTS AND DISCUSSION

Under chromatographic conditions where the extent of ionisation, solvation and buffer ion interactions for a group of polar, ionogenic solutes remains essentially constant, the dependency between the logarithmic capacity factor, log k', and the volume fraction, ψ_s, of the organic solvent modifier for solutes separated by reversal phase-HPLC may by approximated[3,4] by a linear relationship of the form

$$\log k'_i = \log k'_{i,w} - s_i\psi \qquad (1)$$

where $k'_{i,w}$ is the capacity factor of the solute in a neat water eluent, i.e. at $\psi = 0$, and s_i is the slope parameter for the

solute. The magnitude of the s-value depends on the molecular characteristics of the organic modifier, the stationary phase surface, and the solute itself. Generally, over the range of k'-values of interest in reversed phase HPLC, i.e. $0 < \log k' < 1.3$, reasonable linearity in the plots of logk' versus ψ_s is observed[7,8] for neutral and anionic solutes. However, the dependency of logk' on ψ_s over the ψ_s range corresponding to acceptable solute solubilities, i.e. usually over the range $0<\psi_s<0.8$, in the reversed phase HPLC of polypeptides and related ionised solutes is believed in general to be a polynomial which can be approximated[8,9] over a more limited range of solvent compositions to a quadratic relationship of the form

$$\log k' = A + B\psi_s + C\psi_s^2 \qquad (2)$$

As is evident from Figs. 1a,b the iodoamino acids (compounds 1-6, Table) also exhibit non-linear dependencies of their logk's on ψ_s over the range $0.08<\psi_s<0.80$. In these studies, two primary eluents were chosen to permit the evaluation of the dependency of logk' on ψ_s in the absence and presence of a fixed concentration of a cationic modifier (in this case a trialkylammonium salt) with both the ionic strength and pH held essentially constant. Data obtained with the primary eluent composed of aqueous 4mM sodium sulphate - 15mM orthophosphoric acid - 8% acetonitrile (pH 2.2) (elution system 1) as the volume fraction of acetonitrile is varied up to $\psi_s = 0.8$ are summarised in Fig. 1a. The corresponding experiments with the primary eluent composed of aqueous 4mM sulphuric acid - 15mM orthophosphoric acid - 15mM triethylamine - 8% acetonitrile (pH 2.2) (elution system 2) are summarised in Fig. 1b.

Several salient features are evident from these Figures. Firstly, with all the iodoamino acids examined the plots of logk' versus ψ_s pass through minima. Secondly, at high acetonitrile content in the mobile phase selectivity reversals are observed for

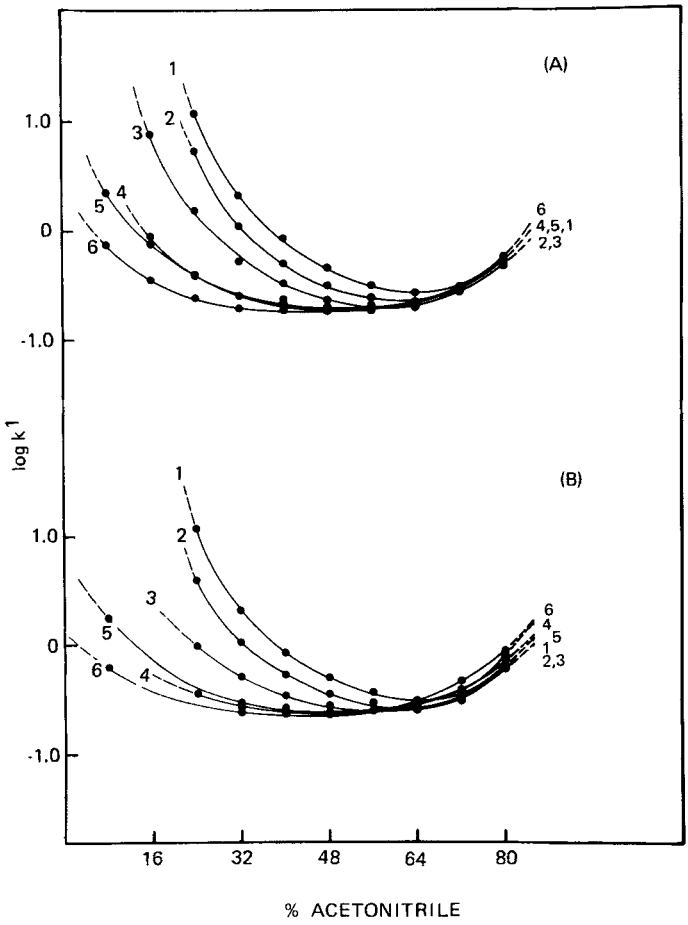

FIGURE: Plots of the logarithmic capacity factor, logk', for several iodoamino acids against the volume fraction, ψ_S, of the organic solvent in water-acetonitrile isocratic mobile phases. Conditions: column, μBondapak C_{18}; flow rate, 1.0 ml/min.; primary mobile phases (a) water - 4mM sodium sulphate - 15 mM orthophosphoric acid, pH 2.2, and (b) water - 4 mM sulphuric acid - 15 mM orthophosphoric acid - 15 mM triethylamine with the acetonitrile content adjusted over the ψ_S range 0.08 - 0.80. The compound key is listed in the Table.

all the iodoamino acids with both elution systems. Triethylamine and related alkylamines have been previously employed with low pH mobile phases in both the reversed phase and size exclusion HPLC of polypeptides. The efficacy of alkylamines to mask coloumbic solute-silanol interactions as well as to participate in pairing ion phenomena with polar solutes has been well documented[1,2]. In this context it is interesting to note the similarity of the plots for the iodoamino acids in the absence and presence of 15mM triethylamine under the various elution conditions studied. The most noticable differences in elution behaviour in the presence and absence of triethylamine were not associated with abolition of the enhanced retention at higher solvent content (a finding which suggests that the polar phase selectivity seen at high ψ_s values is not due to direct electrostatic interaction between ionised silanol and solute groups) but rather were associated with more subtle changes in selectivity such as those associated with reversal of the elution order of thyronine (T_o) and diiodotyrosine (DIT) or the percentage organic modifier at which overall selectivities become changed.

The data obtained with these solutes provide further support for the proposition that the mechanism of retention of ionised solutes, such as amino acid derivatives or peptides, on alkylsilicas involves composite reversed phase and polar phase adsorption components. The importance of each class of adsorption phenomenon will reflect the relative accessibility of appropriate 'solvophobic' or 'silanophilic' binding sites on the stationary phase surface as the water content is varied. As demonstrated elsewhere[1,4] the interplay of such composite retention processes can prove advantageous as far as the control over selectivity. For example, with hydrocarbonaceous stationary phases, such as the μBondapak C_{18} supports where enhanced retention is observed with polar solutes at high organic solvent content, then retrogradients can be employed from high to intermediate solvent content with very non-polar amino acid or peptide derivatives[2]. Such elution

TABLE

Structures and Calculated Retention Parameters of Iodoamino Acids Used in Present Study

No.	Structure	Abbreviation	Elution System 1		Elution System 2	
			s_i	$\log k'_w$	s_i	$\log k'_w$
1.	3,3',5,5'-Tetraiodothyronine	T_4	7.07	2.77	6.95	2.80
2.	3,3',5-Triiodothyronine	T_3	6.42	2.27	5.28	1.90
3.	3,5-Diiodothyronine	T_2	5.72	1.81	2.75	0.70
4.	Thyronine	T_0	3.77	0.90	1.50	0.0
5.	3,5-Diiodotyrosine	DIT	3.07	0.60	2.63	0.45
6.	3-Iodotyrosine	MIT	1.88	0.02	1.25	-0.10
7.	Tyrosine	Y	0.48	-0.58	0.18	-0.59

procedures are usually nor required with the iodoamino acids since adequate selectivity can be maintained under regular reversed phase conditions to resolve all the known iodothyronines and thyroacetic acids[10] with a single gradient of increasing organic solvent composition. However, retrogradients may be usefully employed to permit resolution of biological conjugates such as the N-acetyl derivatives or alternatively the phenolic O-glucuronide or O-sulphate derivatives since polar phase selectivity is maintained in this elution mode.

As is evident from the Table, the s-values for the iodoaminoacids ranged between 1.2 and 7.1. Introduction of iodo-groups into the thyronine nucleus results in increased relative hydrophobicity of the solutes and is also associated with progressively larger s-values (compare data for compounds 1-4). The incremental effect of the iodo-group on the retention of the iodoamino acids to alkylsilicas has been recognised in several previous studies. For example, plots of logk' versus molecular hydrophobic surface area[11], and logk' versus iodine atom per aromatic nucleus[12] have been found to follow linear relationships for these compounds. The above retention data confirm that with homologous solutes the overall hydrophobicity of a solute is a key parameter in determining the magnitude of the solute's s-value. Furthermore, the calculated s-values of compounds 1-7 eluted with either elution system from octadecylsilica are similar to those observed for small polar organic molecules[6] but considerably smaller than s-values of polypeptides and proteins where s-values in the range of 20-30 have been described[3,4,7,8]. The practical consequences of low s-values (and also low k'_w-values) with these iodoamino acids are regular reversed phase chromatographic selectivity with good peak shape and symmetry over a wide range of eluent compositions. In addition, low s-values for a solute imply linear elution development over a considerable range of eluent compositions and a relative insensitivity of the capacity factor to small changes in organic solvent content with water-rich mobile

phases. Such solvent composition-capacity factor relationships are important requirements where reproducible analytical precision is essential, e.g. in the assay of thyronine derivatives in biological samples.

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