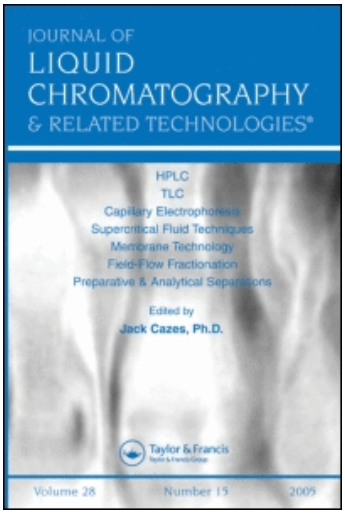

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IONISATION EFFECTS IN THE REVERSED PHASE LIQUID CHROMATOGRAPHIC SEPARATION OF THYROMIMETIC IODOAMINO ACIDS.*

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ABSTRACT

The influence of ionisation equilibria on the retention behaviour of iodoamino acids and related compounds on microparticulate octadecylsilica supports has been examined. The chromatographic data for these ionogenic solutes have been discussed in terms of current concepts for reversible solvophobic interactions with the hydrocarbonaceous stationary phase. This treatment permits the conditional effects of the mobile phase composition and pH on solute retention to be assessed and the relationship between the molecular surface area of a solute and its retention to a non polar stationary phase evaluated.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity over the past few years

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for the separation of ionic substances, particularly those of biological origin. The large majority of these substances contain ionogenic functional moieties such as carboxyl, amino or phosphate groups. Ion exchange chromatography has traditionally been the method of choice for the separation of such components. Recent developments in RP-HPLC have demonstrated that this technique is eminently suitable for the separation of amino acids, peptides and proteins provided due regard is placed on the role which secondary chemical equilibria can play in the chromatographic distribution process. Attention has been focused on the use of RP-HPLC with eluents containing a variety of anionic or cationic reagents in hydro-organic solvent mixtures. Excellent control over selectivity for amino acids and peptides on chemically bonded microparticulate alkylsilicas can be achieved with these reagents via specific pairing ion interactions. The more lipophilic of these pairing ion reagents are known to act as surfactants and modify the non-polar surface of the sorbent to the equivalent of a dynamic liquid-liquid ion-exchanger. It has also been demonstrated in a variety of studies that the level of solute ionisation plays a very significant role in the retention processes when ionogenic substances are chromatographed on alkylsilicas. Detailed experimental studies with the protein amino acids and small peptides have shown [1-5] that the physico-chemical basis of their chromatographic sorption-desorption processes can be interpreted in terms of solvophobic theory [6,7]. This exacting theoretical treatment allows the parameters affecting solute retention to be individually evaluated. In earlier studies we have described [8-10] methods for the separation of the thyromimetic iodoamino acids by RP-HPLC. Subsequently, these methods have been extended [9,11-13] to the analysis of the iodoamino acids in biological samples. In this paper we examine the effect of protic ionisation on the chromatographic retention of these

solutes and evaluate the chromatographic behaviour in terms of the solvophobic model for solute retention.

MATERIALS

Chromatographic Apparatus.

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which consisted of a M6000A solvent delivery unit, a U6K universal liquid chromatograph injector and a M440 UV absorbance detector coupled to a Rikadenki double channel recorder. The μBondapak C_{18} columns (10μm, 30 x 0.4cm I.D.) were purchased from Waters Assoc. (Aus.) Pty. Ltd. Sample injections were made with Pressure-Lok liquid syringes (0-25μl) Series B110 from Precision Sampling (Baton Rouge, La., U.S.A.). Solvents and dissolved iodoamino acid samples were filtered using AP2500 filters from Millipore Corp. (Bedford, Mass., U.S.A.)

Reagents.

Methanol was supplied by Waters Assoc. (Aus.) Pty. Ltd. Orthophosphoric acid and potassium dihydrogen phosphate were from May and Baker (Dagenham, Great Britain). The iodoamino and thyroacetic acids were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and from Henning (Berlin, G.F.R.). Stock solutions of the iodoamino and thyroacetic acids were prepared by dissolving the compounds in 1% methanolic NH_4OH at a concentration of ca 10mg/ml.

METHODS

A flow rate of 2ml/min was used throughout this study. The solvent reservoirs, precolumn delivery systems and columns were maintained at 18^{o}. All bulk and eluting solvents were prepared and degassed as reported previously[8]. All columns were equilibrated to new elution conditions for at least 30 min. The capacity factors were calculated in the usual way. The retention time of an unretained solute can be readily calibrated

with $NaNO_3$ or D_2O. The analysis of the data by the least squares method was performed on a Burroughs 6700 computer with a modified Biomedical Computing programme (BMD-07R, Univ. of California, Ca. U.S.A.) written in Fortran language. The ionic strengths of the eluents were chosen on the basis of previous experimental studies [3,8,14-16] with simple acids, amines and peptides.

RESULTS AND DISCUSSION

Theoretical Considerations.

In the basic ligand adsorption model for the separation of iodoamino acids and related ionogenic solutes, the chromatographic process is viewed as a reversible association of the solute, S_i, with the hydrocarbonaceous octadecyl ligand L, bound to the surface of the microparticulate, porous silica. The equilibrium association constant K_i, of the solute S_i, is given by

$$K_i = \frac{[S_iL]}{[S_i][L]} \qquad ....(1)$$

If it is assumed that the sorption process does not involve ionic or hydrogen bonding interactions between the solute and the stationary phase, then the capacity factor of the solute S_i can be expressed by

$$k_i' = \psi \cdot K_i \qquad ....(2)$$

where ψ is the phase ratio of the column.

The capacity factor for a diprotic acid, such as diiodothyroacetic acid, as a function of pH can be evaluated[14] from

$$k' = \frac{k_o + k_1 \frac{K_{a_{i,m}}}{[H^+]_m} + k_2 \frac{K_{a_{i,m}} K_{a_{2,m}}}{[H^+]_m^2}}{1 + \frac{K_{a_{1,m}}}{[H^+]_m} + \frac{K_{a_{i,m}} K_{a_{2,m}}}{[H^+]_m^2}} \qquad ....(3)$$

where k_o, k_1 and k_2 are the capacity factors of the undissociated, mono-dissociated and fully dissociated diprotic acid and $K_{a_{1,m}}$ and $K_{a_{2,m}}$ are the corresponding acid dissociation constants.

Similarly, the capacity factor for tyrosine and related ampholyte analogues as a function of pH is given [3,10,14] by

$$k' = \frac{k_o + k_1 \frac{[H^+]_m}{a_{1,m}} + k_2 \frac{K_{a_{1,m}}}{[H^+]_m} + k_3 \frac{K_{a_{1,m}} K_{a_{2,m}}}{[H^+]_m^2}}{1 + \frac{[H^+]_m}{K_{a_{1,m}}} + \frac{K_{a_{2,m}}}{[H^+]_m} + \frac{K_{a_{2,m}} K_{a_{3,m}}}{[H^+]_m^2}} \quad \ldots.(4)$$

where k_o, k_1, k_2 and k_3 are the capacity factors of the zwitterionic, cationic, anionic and doubly anionic charged species, respectively, and $K_{a_{1,m}}$, $K_{a_{2,m}}$ and $K_{a_{3,m}}$ are the three dissociation constants. A given polyprotic ampholyte will in general be characterised by a set of limiting capacity factor values corresponding to the undissociated (zwitterionic, isoelectric) form, k_o, the monodissociated form k_1, the di-dissociated form, k_2, ... fully dissociated from, k_n, with a corresponding set of pK_{a_1}, pK_{a_2} ..... pK_{a_n} values. By choosing appropriate values of the k_o, k_1 ..... k_n and pK_{a_1}, pK_{a_2} .....pK_{a_n} parameters, limiting conditions of the k′ versus pH_m dependency for ionisable solutes can be assessed. Figure 1 illustrates the computed curves for the pH dependence of the capacity factor for some typical cases of diprotic acids and ampholytes using this approach.

The above discussion on the dependence of k′ on pH does not take into account the effect of an organic solvent on the pH of an aquo-organic solvent mixture or on the pK of an ionogenic solute. It is now well recognised that the pK values of a particular ionogenic solute varies from eluent to eluent by virtue of specific solvation effects, as well as, changes in dielectric properties of the mobile phases [17]. With simple

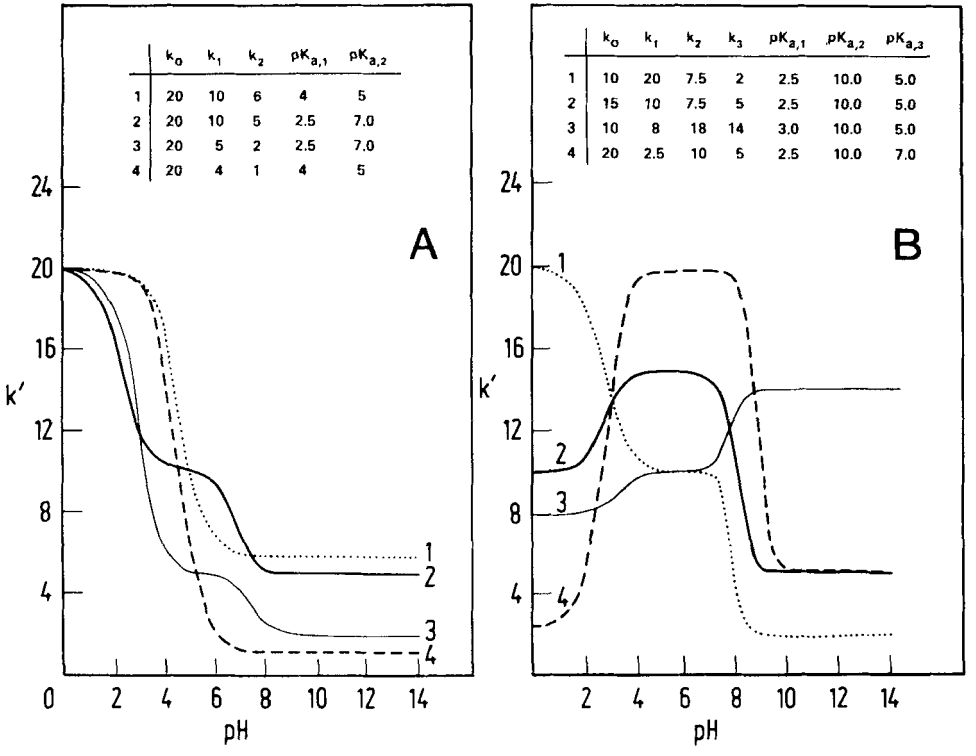

Figure 1.

Plots of the capacity factor of diprotic weak acids (A) and triprotic ampholytes (B) versus the pH of the mobile phase. The curves were calculated from eqns. 3 and 4 using the limiting values of the parameters shown on each graph.

monoprotic acids, theory predicts an increase in pK_{a_1} with increasing organic solvent content. This has been observed experimentally although ΔpK_a variations with solvent percentage in general are non-linear. For example, a one unit increase in pK_a for benzoic acid occurs[18] with water-methanol mixtures when the methanol content is increased from 20% to 60%. When the pHs of hydro-organic mobile phases are near a pK value of a solute significant differences in selectivity and retention

behaviour are thus expected for eluents of different organic solvent content.

According to solvophobic theory, solute retention can be expressed in terms of the overall standard unitary free energy changes associated with the transfer of the solute from the mobile to the stationary phase such that

$$\ell n\ k_i' = \ell n\ \psi - \frac{\Delta G_{assoc,i}}{RT} \qquad ....(5)$$

Horvath and his coworkers [6,7] have evaluated $\Delta G_{assoc,i}$ in terms of the association of the solute and the ligand in the gas phase and the transfer of the solute, the ligand and the complex individually into the eluent. The capacity factor of an ionised solute can then be expressed as

$$\ell n\ k_i' = \text{const} - \frac{\Delta G_{es,i}}{RT} - \frac{\Delta G_{cav,i}}{RT} \qquad ....(6)$$

where the electrostatic component $\Delta G_{es,i}$, is the energy associated with placing an ionic species into the solvent system and $\Delta G_{cav,i}$ is the free energy required to make a cavity with a surface area identical to that of the solute. The free energy term associated with cavity formation can be evaluated from

$$-\Delta G_{cav,i} = \gamma[N\Delta A_i + 4.836N^{1/3}(\kappa^e - 1)V^{2/3}] \qquad ....(7)$$

where γ is the surface tension of the eluent, N is Avogadro's number, V is the molar volume of the eluent, κ^e the microscopic cavity factor and ΔA_i is the hydrophobic contact area. With ligands of relatively large molecular dimensions and eluents of similar molecular structure, i.e. isocratic phases, ΔA_i is likely to be proportional to the hydrophobic surface area, A, of the solute. It follows that plots of $\ell n\ k'$, corrected for electrostatic effects, versus the hydrophobic surface areas of the various solute molecules should yield straight lines under elution conditions where the mobile phase composition is fixed. Further-

more, the slope of the plots of ℓn k′ versus the total molecular surface area, A_W, of the solutes will remain constant for eluents of the same composition but different pH only in the absence of pronounced polar interactions in the sorption process or solvation, molecular aggregation and stacking effects specific to the solutes. The slope values will thus respond to changes in the surface tension and dielectric constant of the mobile phase.

Effect of pH on k′.

Because of the chemical instability of octadecylsilica stationary phases to exposure to aqueous solutions at pH values higher than pH 7.5 for extended periods of time, we restricted our investigations to mobile phases have the operational upper limit of pH 7.0. As a consequence, it was not possible to

TABLE 1

Structures of Iodoamino Acids and Related Compounds.

No.	Abbreviation	Structure
1.	Diac	3,5-Diiodothyroacetic acid
2.	Triac	3,3',5-Triiodothyroacetic acid
3.	Tetrac	3,3'5,5'-Tetraiodothyroacetic acid
4.	Tyr	Tyrosine
5.	MIT	3-Iodotyrosine
6.	DIT	3,5-Diiodotyrosine
7.	T_0	Thyronine
8.	T_2	3,5-Diiodothyronine
9.	T_3	3,3',5-Triiodothyronine
10.	T_4	3,3',5,5'-Tetraiodothyronine

examine the retention behaviour of the compounds listed in Table 1 over the pH range where the phenolic groups were completely ionised, i.e. greater than 99.9% in the phenoxide form, or the amino groups were fully deprotonated. Previous studies [14,16] with hydroxylphenylacetic acids and aromatic amino acids have demonstrated, however, that sufficient data can be obtained to allow the retention behaviour of simple ionogenic solutes over this restricted pH range to be analysed in terms of the above theoretical considerations.

The pH dependence of the capacity factors for the thyroacetic acids, (1) - (3), is shown in Fig.2. In common with related studies on mono- and di-protic weak organic acids, sigmoidal shapes for the dependence of the capacity factors of compounds (1) - (3) on the apparent pH of the mobile phase were observed. Least squares fit of the data confirmed that the the experimental data was essentially in agreement with the relationship explicit to eqn. 3. At values below pH 3.0, some divergence from expected behaviour was evident. This effect may be due to the influence that silanophilic interactions have on the retention process under these low pH conditions [5,19]. With diprotic solutes which exhibit widely separated pK_a values, as is the case with the thyroacetic acids, the shape of the k' versus pH plot can be considered a composite of the respective dissociation curves. However, over the pH range examined, the ionisation of the 4'-hydroxy- group of compounds (1) - (3) does not appear to significantly perturb the basic sigmoidal behaviour characteristic of mono-protic acids. Similar observations have been noted [14] with hydroxyphenylacetic acid analogues. As the pH is increased both the carboxylic and phenolic groups will progressively become more ionised. Since solute retention is dependent on the extent of ionisation, a progressive decrease in the k' values will ensue until limiting values are reached.

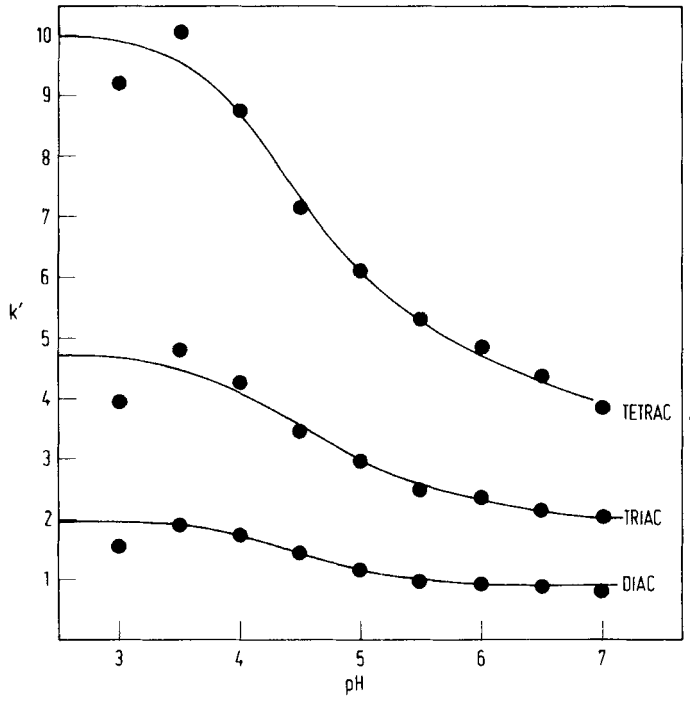

Figure 2.

Capacity factors for several thyroacetic acids as a function of the pH of the eluent. Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; eluent, 50% methanol-50% water-25mM KH_2PO_4. The legend to the acronyms is given in Table 1.

The data for the capacity factors of the iodoamino acids (4) - (10) are shown in Figs. 3 and 4. Over the pH range studied with these iodoamino acids, the level of amino group protonation will remain essentially constant, i.e. the pK_{NH_2}-pH will generally be larger then 3.0 with the percentage in the protonated form ranging from ca 99.9990 to ca 99.90. Following the procedures outlined earlier, the data were evaluated by least squares fit analysis in terms of eqn. 4.

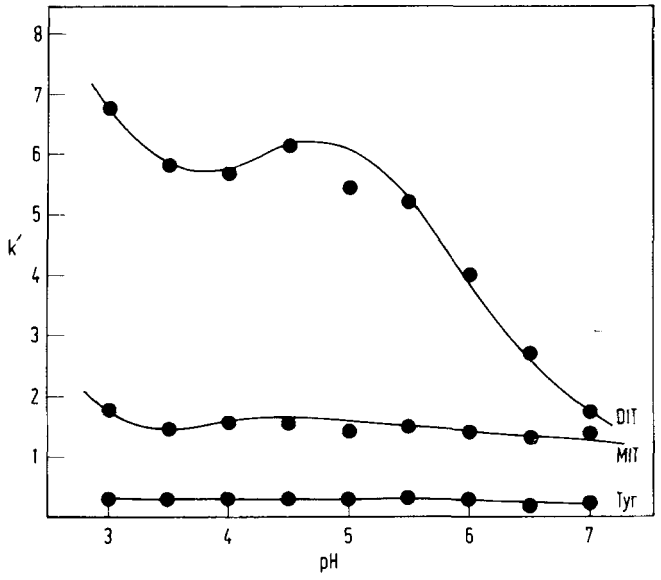

Figure 3.

Plots of the capacity factor versus the pH of the eluent for tyrosine and the iodotyrosines. Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; eluent 5% methanol-95% water-50mM KH_2PO_4. The legend to the acronyms is given in Table 1.

The chromatographic behaviour of the iodoamino acids under the experimental conditions employed in this study is consistent with the general form of the expression for the k′ of polyprotic ampholytes as a function of the pH of the mobile phase, taking into account solvent effects on ionisation. With ampholytes, theory predicts major differences in the shapes of the k′ versus pH plots even when the relevant limiting parameters of the solutes are similar. By using stationary phases with higher operational upper limits for the mobile phase pH, complete k′ versus pH plots can be achieved [16] and precise pK_a values determined from the chromatographic data.

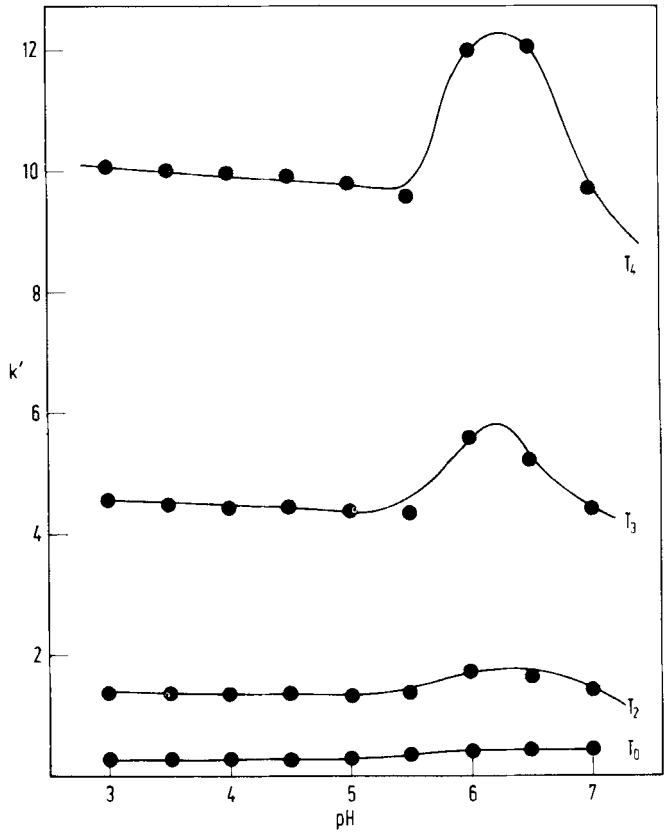

Figure 4.

Capacity factors for several thyronine derivatives as a function of the pH of the eluent. Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; eluent, 40% methanol-60% water-50mM KH_2PO_4. The legend to the acrynoms is given in Table 1.

Effect of Hydrophobic Surface Area on k'.

There is now general consensus that amino acid and peptide retention in RP-HPLC can be interpreted in terms of hydrophobic interactions between these ionogenic solutes and the hydrocarbonaceous ligand attached to the surface of the silica matrix.

With underivatised amino acids, selectivity depends, inter alia, upon the lipophilicity of the side chains, i.e. their chemical functionality and differences in ionisation state. Solvophobic theory predicts that the k´ values for a series of ionogenic solutes eluted under isocratic conditions should follow changes in the relative interfacial surface area, ΔA_i, of the solute in contact with the stationary phase which can be indicated by the molecular hydrophobic surface area, A, of the solute. If eqn. 7 holds, then linear relationships of ℓn k´ for compounds (1) - (10) as a function of A are anticipated. Shown in Fig. 5 are

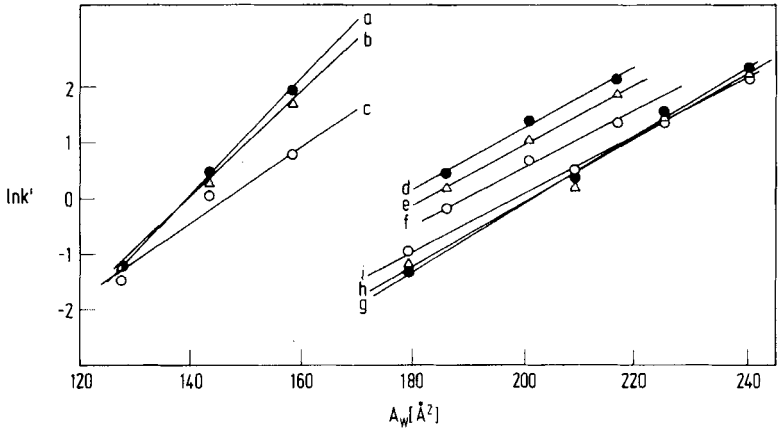

Figure 5.

Plots of the logarithm of the capacity factors versus molecular surface area for the compounds (1)-(10). Data obtained for the tyrosine derivatives at pH 3.0, pH 5.0 and pH 7.0 are shown in plots (a)-(c) with the following r^2 and slope co-ordinates from a least squares analysis, (a), 0.9942, 0.1038; (b) 0.9980, 0.0953; (c) 0.8316, 0.0715. The data for the thyroacetic acids and thyronine derivates obtained under similar pH conditions are shown in plots (d)-(f) and (g)-(i) respectively with the following r^2 and slope values: (d) 0.9930, 0.0534; (e) 0.9900, 0.0537, (f) 0.9853, 0.0507; (g) 0.9956, 0.0602; (h) 0.9946, 0.0591 and (i) 0.9868, 0.0529. Chromatographic conditions are given in Figures 1-3.

the plots of ℓn k′ for compounds (1) - (10) determined at pH 3.0, 5.0 and 7.0, versus the total molecular surface areas, A_W, calculated from the group surface increments of Bondi with the appropriate crowding corrections [20,21]. Each family of compounds - tyrosines, thyronines and thyroacetic acids - yielded linear plots for ℓn k′ versus A_W and similar relationships were evident with plots of ℓn k′ versus hydrophobic surface areas calculated by summation of the area increments for carbon, hydrocarbon and iodo-groups only. The displacement of the lines for the three families of compounds can be accounted for by the differences between the dipole moments of the solutes and the composition of the eluent. According to eqn. 7 the slope of the plots will be predominantly controlled by the surface tension γ, of the eluent. The observation that the slopes of the plots for the tyrosine derivatives (determined at 5% methanol) are noticably different to the corresponding values for the thyronines (determined at 40% methanol) and the thyroacetic acids (determined at 50% methanol) is in accord with the anticipated trend. The changes in slope for the plots of ℓn k′ versus A_W, evident for both iodoamino acid families as the pH of the eluent was varied, are suggestive of the participation of silanophilic and pH dependent specific solvation effects in the retention process. In fact, it is likely that solute-silanol interactions and specific solvation of ionized groups in general play important secondary roles in selectivity modulation for amino acids and peptides on reversed phases. For example, such effects are probably involved in the elution order reversals experienced by some isomeric peptides when the pH of the mobile phase is changed as well as the selectivity deviations seen with polypeptides with mobile phases of high organic solvent content[4,19,21].

Although the three families of solutes are not homologous, it can be seen from the data that the iodo-group effectively has a constant incremental effect on retention for all three groups.

The progressive enhancement of retention, arising from the hydrophobic nature of the iodo-group, has been recognised in previous studies on the separation of iodo-compounds by RP-HPLC. For example, we have demonstrated [8,9] that the plots of ℓn k′ versus number of iodine atoms per aromatic nucleus and ℓn k′ versus partition coefficients also follow linear relationships for these compounds. Collectively, these results can all be ascribed to the common physicochemical phenomenon that governs the retention of amino acids, peptides and proteins to chemically bonded reversed phases. Knowledge of the effects of ionisation, and other secondary equilibrium processes which these ionogenic molecules undergo in solution can be usefully applied in the selection of mobile phase conditions which allow optimal chromatographic resolution. The application of such optimisation strategies has recently been discussed [2,4,5] for the separation of amino acids, peptides and other ionogenic solutes by RP-HPLC.

ACKNOWLEDGEMENTS

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