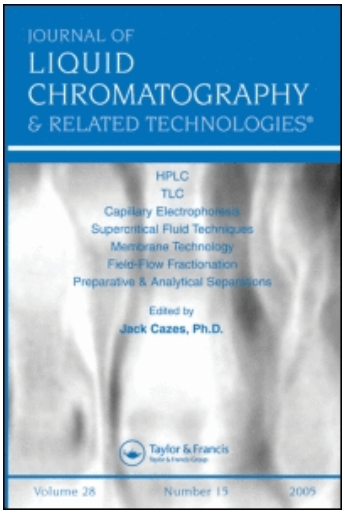

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Investigation of the Influence of Hydrophobic Ions as Mobile Phase Additives on the Liquid Chromatographic Separation of Amino Acids and Peptides

Ziad Iskandarani[a]; Ronald L. Smith[a]; Donald J. Pietrzyk[a]
[a] Department of Chemistry, The University of Iowa, Iowa City, Iowa

To cite this Article Iskandarani, Ziad , Smith, Ronald L. and Pietrzyk, Donald J.(1984) 'Investigation of the Influence of Hydrophobic Ions as Mobile Phase Additives on the Liquid Chromatographic Separation of Amino Acids and Peptides', Journal of Liquid Chromatography & Related Technologies, 7: 1, 111 — 150
To link to this Article: DOI: 10.1080/01483918408073954
URL: http://dx.doi.org/10.1080/01483918408073954

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(1), 111-150 (1984)

INVESTIGATION OF THE INFLUENCE OF HYDROPHOBIC IONS AS MOBILE PHASE ADDITIVES ON THE LIQUID CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS AND PEPTIDES

Ziad Iskandarani, Ronald L. Smith, and Donald J. Pietrzyk

Department of Chemistry
The University of Iowa
Iowa City, Iowa 52242

ABSTRACT

Tetraalkylammonium, R_4N^+, and alkylsulfonate, RSO_3^-, salts were evaluated as mobile phase additives for the separation of amino acids and peptides. The former were used in a basic mobile phase and the latter in an acidic one, conditions which convert the terminal carboxyl or amine groups in amino acids and peptides and the acidic or basic side chains if present into anionic or cationic forms, respectively. Because of the required strongly basic or acidic mobile phase pH, a polystyrene-divinylbenzene copolymer, PRP-1, was used as the reversed stationary phase. The retention is suggested to follow a dynamic interaction involving two major equilibria, namely retention of the hydrophobic ion and an ion exchange between the co-ion accompanying the hydrophobic ion and the amino acid or peptide ion of opposite charge. The effect of amino acid and peptide structure on retention is discussed. Key mobile phase variables are identified; a major one is the optimization of the hydrophobic ion concentration-mobile phase solvent composition to provide a sufficient number of charge sites on the stationary phase due to hydrophobic ion retention. Several separations are shown which focus on the advantages offered by using hydrophobic ions as mobile phase additives. In general,

 0148-3919/84/0701-0111$3.50/0

RSO_3^- salts appear to be more versatile than R_4N^+ salts in improving selectivity and resolution in amino acid and peptide separations.

INTRODUCTION

Reverse phase high performance liquid chromatography (RPLC) has emerged as a powerful technique for the sensitive, rapid, efficient separation of derivatized and underivatized amino acids (AA), peptides, and proteins (1-3). Even preparative applications are feasible since column loadings and sample recovery are often favorable (1,4,5).

The alkyl-modified silicas, in particular, have gained wide acceptance as the stationary phase for the LC separation of AA and peptides (1-3). However, polystyrene-divinylbenzene (PSDB) type stationary phases have also been shown to be useful for these kind of separations (6). Their major advantages lie in the fact that they are stable throughout the entire pH range, unlike the alkyl-modified silicas which have a useful pH range of about pH 2 to 8, and have favorable loading capacities for preparative applications (4,6). In addition to pH, other key mobile phase variables for both types of stationary phases that are manipulated to improve or change selectivity and resolution include mixed solvent ratio, type of solvents, ionic strength, type of buffer components and their concentration, and the use of hydrophilic or hydrophobic ions as mobile phase additives. The need for such a broad range of eluting conditions is important for several reasons. First, AA

and peptides vary widely in structural and functional features because of side chains and mobile phase conditions must have the potential to distinguish between major differences as well as minor differences. The latter are due to minor sequence changes in the peptide as the result of replacement or reduction of one or more subunits in the peptide. Second, the mobile phase conditions must be compatible with sensitive detection devices if analytical LC is the goal or with procedures that easily permit isolation of the peptide from the mobile phase if preparative LC is the goal.

The application of hydrophilic or hydrophobic ions as mobile phase additives has been particularly useful in AA and peptide separations. Typical additives used are alkylsulfonic (RSO_3^-) acids, alkyl sulfates, fluorinated alkyl carboxylic acids, amine salts, tetraalkylammonium (R_4N^+) salts, ClO_4^-, PO_4^{-3}, and others (1,7-16). Secondary equilibria between the additive and the AA or peptide will often bring about significant changes in retention characteristics. In general, retention is augmented due to the presence of the additive. Depending on the mobile phase and stationary phase modifications the nature of the secondary equilibria can range from ion pair formation to an ion exchange selectivity. In the former the interaction is viewed as first formation of an ion pair between the AA or peptide and the additive which is of opposite charge and then retention of the ion pair by the stationary phase. In the latter the additive (if

hydrophobic) is first retained and then the AA or peptide as a charged species exchanges with the co-ion that accompanies the charged additive. More recently it has been shown that under defined mobile phase conditions a dynamic ion exchange (also called ion interaction) type interaction occurs, the details of this and previous model studies are reviewed in detail elsewhere (7-9,17-20). Of particular interest is the recent studies which indicate that under defined mobile phase conditions the ion interaction type retention is present when using both the PSDB and alkyl-modified silica as the stationary phase and RSO_3^- salts or R_4N^+ salts as mobile phase additives (18,19).

Many studies in the past employed a mobile phase pH in the range of about 2.5 to 7 in part because of the pH limitation of the alkyl-modified silica. Thus, AA and peptides range from being partial cations to zwitterions and the full effect of using hydrophobic ions as mobile phase additives is not always realized. In this study we report the use of R_4N^+ salts as mobile phase additives and employ a mobile phase pH (pH > 10) that ensures that the carboxyl-terminus of an AA or peptide and acidic side chains, if present, are in their anionic form. The effect of RSO_3^- salts as mobile phase additives on the retention of AA and peptides from an acidic mobile phase, where the amine terminus and basic side chains, if present, are in their cation form, was also studied. PRP-1, which is a PSDB reverse stationary phase and is stable from pH 1 to 14, was used throughout this investigation.

EXPERIMENTAL

Chemicals and Instrumentation

Amino acids and peptides were obtained from Sigma Chemical Co., Chemalog, Vega Biochemicals, and Research Plus. Tetrapropyl-(TPABr), tetrabutyl-(TBABr), and tetrapentyl-(TPeABr) ammonium bromide and pentyl-(C_5SO_3H), heptyl-(C_7SO_3H), and octyl-(C_8SO_3H) sulfonic acids were purchased from Eastman Kodak and Aldrich Chemical Co. Different anion forms of the R_4N^+ salts were prepared by an anion exchange procedure (18) while the RSO_3H was converted to the salt form by titration with MOH or by cation exchange (19). Organic solvents and water were LC quality while all inorganic salts were analytical reagent grade.

PRP-1 is a 10 μm, spherical PSDV particle with a large surface area and porosity and was obtained as a prepacked column (4.1 mm x 150 mm) from Hamilton Co. A Waters Model 202 LC equipped with a Tracor Model 970 or Spectra Physics 770 variable wavelength detector was used.

Procedures

Peptide and AA sample solutions of about 1 mg/ml were prepared by dissolving mg quantities in H_2O, EtOH, or their mixture in septa sealed vials and refrigerated when not in use. Sample transfer was by syringe. Operating conditions, in general, involved 5 μl sample aliquots, 1 ml/min flow rate, inlet pressures of 700 to 1400 psi depending on mobile phase, detection

at 215 nm, and controlled temperature at 25°C. Mixed solvents are expressed as per cent by volume. Mobile phase pH was controlled by phosphate buffers, HCl, or NaOH. Column void volume, V_o, for a given mobile phase was determined by using analytes that were not retained. Capacity factor, k', was calculated by $k' = (v-v_o)/v_o$ where v is the retention volume of the analyte of interest. Breakthrough volumes were determined as previously described (18).

RESULTS AND DISCUSSION

Two major equilibria influence the retention of a charged analyte on PRP-1 when using hydrophobic ions as mobile phase additives (18-20). One describes the retention of the hydrophobic ion and its co-ion on the PRP-1 surface as a double layer where the hydrophobic ion makes up the primary layer and the co-ion the secondary layer. The second is an ion exchange selectivity between the co-ion and the analyte ion. For a R_4N^+ salt as the additive, A^- its co-anion, and K^- the analyte anion, the retention can be viewed as

$$\left.\begin{array}{lll} (\text{PRP-1}) + R_4N^+A^- \rightleftharpoons (\text{PRP-1})\cdots R_4N^+A^- & (1a) \\ (\text{PRP-1})\cdots R_4N^+A^- + X^- \rightleftharpoons (\text{PRP-1})\cdots R_4N^+X^- + A^- & (1b) \end{array}\right\} \quad (1)$$

If a RSO_3^- salt is the additive, C^+ its co-cation, and M^+ the analyte cation the process is

$$\left.\begin{array}{ll} (\text{PRP-1}) + RSO_3^-C^+ \rightleftharpoons (\text{PRP-1})\cdots RSO_3^-C^+ & (2a) \\ (\text{PRP-1})\cdots RSO_3^-C^+ + M^+ \rightleftharpoons (\text{PRP-1})\cdots RSO_3^-M^+ + C^+ & (2b) \end{array}\right\} \quad (2)$$

Since the hydrophobic salt is at a constant concentration in the mobile phase and the interaction is dynamic the overall process is given by the combination of equations 1a-1b and 2a-2b, respectively, as the analyte passes through the column.

The charge form of an AA and peptide is very dependent on pH because of their acidic and basic terminal groups. The pK_a values, which are also influenced by side chain groups, determine these pH conditions. In general, at pH < 2 the amine terminus is a cation. At a basic pH, pH > 10, the carboxyl terminus is an anion while at an intermediate pH the AA and peptides are zwitterions. If the side chains also contain acidic or basic groups they can provide additional charge sites depending on their pK_a values and the mobile phase pH.

It follows from equations 1 and 2 that in order to separate AA and peptides as anions, R_4N^+ salts and a basic mobile phase would be best while as cations, RSO_3^- salts and an acidic mobile phase would be best. Previous results (18) have already indicated that the zwitterion pH provides the lowest retention and often the poorest selectivity. For these reasons this mobile phase pH or one close to this, which is used most often (7-16) is the least useful of the three particularly when attempting to separate closely related AA and peptides.

Several mobile phase variables have been identified (18-20) which can be manipulated to alter analyte retention and subsequently improve resolution and selectivity while still maintaining favorable analysis times. The data reported in this paper for AA and peptide retention, as well as previous data for the retention of analyte cations and anions (18-20), on PRP-1 from mobile phases containing hydrophobic ions are consistent with these generalizations. Briefly, these are summarized in the following. 1) The structure and concentration of the R_4N^+ or RSO_3^- salt can be altered. As the alkyl chain length, R, increases, retention increases, due to the shift in the equilibrium shown in equations 1a and 2a, leading to an increase in the number of charge sites, which in turn increases analyte retention. Increasing the concentration of the additive in the mobile phase also increases its retention, number of charge sites, and analyte retention. 2) The ion exchange selectivity, indicated by the equilibrium in equation 1b and 2b is responsive to the concentration and type of co-ion in the mobile phase. Thus, the elution order, which is a measure of the selectivity of one ion over another and closely resembles the more traditional ion exchange selectivity (21), follows, in general, the order

$$NO_3^- > Br^- > NO_2^- > Cl^- > \text{formate} > F^- > OH^- \qquad (3)$$

$$M^{+2} > Na^+ > Li^+ > H^+ \qquad (4)$$

where a R_4N^+ salt or a RSO_3^- salt is used, respectively. 3) Mobile phase pH determines the charge on the peptide and AA; in general, as the charge increases retention increases in the presence of a hydrophobic ion of opposite charge. 4) Altering the organic solvent:water ratio sharply changes the retention of the additive on the stationary phase (equations 1a and 2a) which then influences the number of charge sites, and analyte retention; decreasing the organic modifier in the solvent mixture leads to increased additive retention, large number of charged sites, and increased analyte retention. 5) The type of organic modifier used influences the previous variable. The eluting power follows the order CH_3CN > EtOH > MeOH. 6) Ionic strength control can be used to influence analyte ion retention (equations 1b and 2b) since it can provide additional amounts of the same or an indifferent co-ion that accompanies the additive; increasing ionic strength will decrease analyte retention.

Table I demonstrates that AA and peptide retention increases as the hydrophobic ion alkyl chain length increases and that the additive significantly augments analyte retention. When using the R_4N^+ salts the mobile phase is basic (pH = 11) ensuring the analyte is an anion while with the RSO_3^- salts the mobile phase pH is acidic (pH = 2) so that the analytes are cations. If analyte retention is plotted versus C-number, where C-number is equal to the sum of all carbons in the alkyl chain of the R_4N^+ salts, retention increases rapidly and provides or approaches a

TABLE I

Effect of Hydrophobic Ion Alkyl Chain Length on the Retention of Amino Acids and Dipeptides on PRP-1

		Capacity Factor, k'					
		Tetraalkylammonium Salt[a]					
Analyte	No Salt[b]	TMABr	TEACl	TPABr	TBABr	TPeABr	THxABr
D,L-Phe	0.89	0.93	0.95	1.33	3.35	5.81	5.09
D,L-Trp	1.59	1.68	1.75	2.56	7.23	14.4	15.2
L-Leu-L-Tyr	0.63	0.66	0.66	1.19	4.17	12.1	14.3
D-Leu-L-Tyr	0.63	0.67	0.67	1.14	3.49	9.01	10.1

		Alkylsulfonic Acid[c]		
	No Salt[d]	C_5SO_3H	C_7SO_3H	C_8SO_3H
L-Ser	0	0.51	2.18	3.60
L-Thr	0.10	0.80	3.85	6.44
L-Gln	0.10	0.66	3.12	4.91
L-Ser-L-Ser	0.12	0.75	3.43	5.69
L-Ala-L-Ser	0.16	0.98	4.62	7.75
L-Ser-L-Ala	0.24	1.80	9.44	15.9

(a) A 1:9 CH_3CN, 1.00×10^{-3}M R_4N^+ salt, 1.00×10^{-2}M pH=11.0 (phosphate) buffer, and NaCl so that μ=0.10M mobile phase at 1.0 ml/min.

(b) Same as (a) except R_4N^+ salt is omitted.

(c) A 1.00×10^{-3}M RSO_3H, 0.01M HCl, and 100% H_2O mobile phase at 1.0 ml/min.

(d) Same as (c) except RSO_3H is omitted.

maximum. The maximum, which has been observed before (18), and its location relative to C-number is a function of the retention of the R_4N^+ salt. This retention increases as the CH_3CN concentration in the mobile phase decreases and therefore increases the number of charged sites (according to the equilibrium in equation 1a) on the PRP-1 (18) due to the retained R_4N^+ salt. As the number of sites increase, the maxima shifts to a lower C-number. Since only a few RSO_3^- salts were examined under comparable mobile phase conditions, it was not possible to ascertain whether a maximum was present when using RSO_3^- salts as mobile phase additives. These and other data not shown here, however, clearly indicate that increasing the alkyl chain in the RSO_3^- salt increases the number of charged sites (see equilibrium in equation 1b) due to RSO_3^- salt retention and enhances AA and peptide retention. Also, as the organic solvent concentration decreases in the mobile phase the number of RSO_3^- sites on the PRP-1 surface increases (19).

Tetraalkylammonium Salts

Table I and other preliminary experiments suggested that the optimum R_4N^+ salts were ones where R = propyl, butyl, or pentyl. Retention data for several AA at pH = 11 using these three R_4N^+ salts as additives are shown in Table II. The F^- salts were used because F^- is one of the weakest eluent co-anions (18,20) (see equation 1b; also compare data for Phe in Tables I and II). The presence of the R_4N^+ salt significantly enhances

TABLE II

Effect of R_4N^+ Salts on the Retention of Amino Acids on PRP-1

	Capacity Factor, k'			
	TPAF		TBAF	TPeAF
Amino Acid/% CH_3CN	0%	5%	10%	15%
Nonpolar				
L-Ala	2.15		2.60	5.05
L-Pro	10.8	1.10	2.59	6.11
L-Met		2.67	2.65	5.44
L-Phe		14.0	22.0	
Polar				
Gly	2.15		2.60	5.05
L-Ser	2.04		2.24	4.98
L-Thr	2.73		2.79	5.34
L-Cys		2.02	4.18	
L-Asn	2.18		2.58	4.88
L-Gln	2.63		2.34	4.80
Basic				
L-His	3.37		2.65	5.44
Acidic				
L-Asp		1.60	18.2	
L-Glu		1.64	17.7	
L-Tyr		2.11	16.6	

A 10 μm, 4.1 mm x 150 mm PRP-1 column using a CH_3CN:H_2O, 1.00 x 10^{-3}M R_4NF, 1.00 x 10^{-3}M NaOH, mobile phase at 1.0 ml/min with detection at 215 nm.

AA retention, however, the differences in retention for AA with polar and basic side chains tend to be small. For nonpolar AA differences are large only for those AA with very hydrophobic side chains such as with Phe and Leu.

If the AA side chain is acidic and the mobile phase pH is basic enough to convert this group into an anionic site then the AA is a divalent anion. As seen in Table II a marked increase in retention is found when a R_4N^+ salt is present as a mobile phase additive. In the absence of the R_4N^+ salt there is no retention on PRP-1 as the di-anion. If the alkyl chain length of the R_4N^+ salt is increased the retention of the acidic AA markedly increases in comparison to AA without acidic side chains. In Table II the number of sites (retained R_4N^+ salt) is approximately the same (10 to 13 μmoles of R_4N^+ salt retained/column as determined from breakthrough volumes) for TPAF, TBAF, and TPeAF when the $CH_3CN:H_2O$ ratio is 5:95, 10:90, and 15:85, respectively. Thus, the observed retention trends are not due to differences in loading capacity.

The data in Table II indicate that acidic and very hydrophobic AA can be readily separated from other AA. Of particular interest are the separations involving the acidic AA. In the presence of a R_4N^+ salt these are highly retained and would elute after the polar, basic, or weakly hydrophobic AA; in the absence of the R_4N^+ salt and a mobile phase pH of 11 the acidic AA would elute from PRP-1 before or with these weakly retained AA.

Although not shown AA elution peaks are well defined and exhibit column efficiencies similar to peptide chromatographic peaks shown later.

Similar retention studies were carried out using dipeptides that contained polar subunits and tripeptides that contained weakly hydrophobic side chains as analytes. Often, these are difficult to separate in the absence of hydrophobic ions in the mobile phase. Table III lists retention data for several of the dipeptides. In general, even though retention is enhanced in the presence of the R_4N^+ salt relative to its absence, where in most cases retention on PRP-1 is zero or barely detectable, differences among the dipeptides are small except for those containing very hydrophobic side chains or acidic side chains (see Table IV).

If the mobile phase is at the zwitterion pH retention in the presence of the R_4N^+ salt is still enhanced relative to the absence of the R_4N^+ salt in the mobile phase. However, the level of enhancement is not comparable to that obtained when the mobile phase pH is basic and the carboxyl terminus and acidic side chains, if present, are ionized. The presence of the cationic site in the zwitterion is responsible for this difference.

Figure 1 shows the separation of several tripeptides that are poorly retained in the absence of the R_4N^+ salt and the improvement in resolution that can be obtained by optimizing the mobile phase variables. In Figure 1A poor resolution is obtained in the absence of a R_4N^+ salt. Marked improvement is

TABLE III

Effect of R_4N^+ Salts on the Retention of Polar Dipeptides on PRP-1

	Capacity Factor, k'			
	TPAF		TBAF	TPeAF
Dipeptide/% CH_3CN	0%	5%	10%	15%
Gly-Gly	2.85		2.47	5.87
Gly-L-Ser	2.57		2.39	5.61
Gly-L-Thr	4.33	0.84	2.64	6.06
Gly-L-Lys		1.10	2.72	5.43
Gly-L-Asn	2.65		2.29	5.28
Gly-L-Met		3.33	7.77	14.4
L-Ala-L-Ser	4.24		2.62	6.39
L-Ala-L-Thr			2.98	6.71
L-Ala-L-Lys		1.15	2.29	5.77
L-Ala-L-Asn	4.08		2.44	5.62
L-Ala-L-Met		6.07	11.3	17.9
L-Ala-L-Ala		1.05	3.14	7.03
L-Ser-L-Ser	2.39		2.29	5.25
L-Ser-L-Gly	2.51		2.31	5.34
L-Ser-L-Ala	3.23		2.46	5.57
L-Ser-L-Met		2.57	6.14	11.4
L-Ser-L-Leu		3.69	8.11	14.3
L-Met-L-Gly		4.34	9.00	15.0
L-Met-L-Ser		3.51	7.67	13.3

Column conditions are the same as Table II.

TABLE IV

Retention of Acidic Dipeptides on PRP-1 in the Presence of TPAF

	Capacity Factor, k'	
	pH	
Dipeptide	11.00	8.85
L-Tyr	3.43	1.31
Gly-L-Tyr	4.10	2.70
L-Tyr-Gly	5.01	4.58
L-Ala-L-Tyr	4.67	3.90
L-Tyr-L-Ala	6.79	6.00
L-Arg-L-Tyr	1.97	1.12
L-Tyr-L-Arg	2.98	1.87
L-Lys-L-Tyr	4.36	0.91
L-Tyr-L-Lys	5.04	1.45
L-Glu-L-Tyr	9.83	3.93
L-Tyr-L-Glu	11.8	6.17

Column conditions are the same as Table II except $CH_3CN:H_2O$ is 5:95 and pH=8.85 is by 1.0×10^{-2}M NH_3/NH_4^+ buffer with detection at 215 nm.

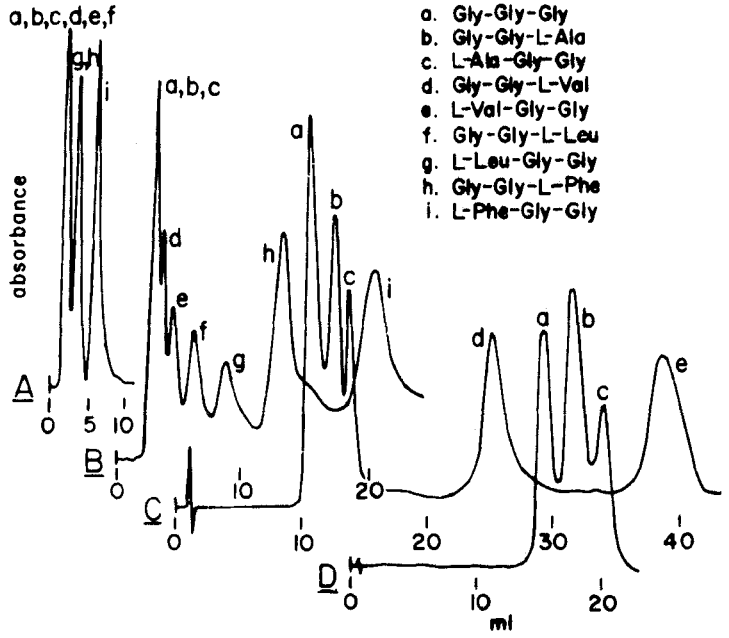

FIGURE 1

Effect of Mobile Phase Variables on Peptide Separations on PRP-1 Using a R_4N^+ Salt as a Mobile Phase Additive

A 4.1 mm x 150 mm, 10 µm, PRP-1 column and a 5:95 $CH_3CN:H_2O$ mobile phase solvent containing (A) pH=11.0 PO_4^{-3} buffer with no R_4N^+ salt, (B) $1.00x10^{-3}$M TPeABr, pH=11.0 (PO_4^{-3}), (C) $1.00x10^{-3}$M TPeABr, pH=11.0 (NaOH), and (D) $1.00x10^{-3}$M TPeAF, pH=11.0 (NaOH) at a flow rate of 1.0 ml/min.

obtained, Figure 1B, when using TPeABr in the mobile phase. However, this mobile phase contains several strong eluent co-anions (18,20) (Br^-, PO_4^{-3}, and Cl^- provided by the R_4N^+ salt, the buffer which yields pH = 11, and NaCl which provides ionic strength control, respectively) which will reduce retention

and affect resolution. By switching to NaOH which provides the necessary pH, PO_4^{-3} is replaced by the very weak eluent co-anion, OH^- (18,20). Thus, resolution in Figure 1C, where only the least retained tripeptides are considered, is significantly improved as a result of increased retention and selectivity. An additional improvement is made by using TPeAF instead of the Br^- salt since F^- is also a very weak eluent co-anion (18,20); this is shown in Figure 1D where the three tripeptides that appear in the first peak in Figure 1B are now separated. Although not shown here a very modest change in CH_3CN:H_2O ratio will also strongly affect retention and resolution, particularly for those analytes that are highly retained.

The observation that the greatest effect on AA and peptide retention is due to ionization of additional acidic sites is consistent with the equilibria indicated in equation 1, particularly the exchange equilibrium shown in equation 1b. It is this exchange selectivity between the analyte anion and the co-anion provided by the R_4N^+ salt or any other salt in the mobile phase which appears to account for the major difference in retention. Although the presence of nonpolar, polar, and basic side chains can influence retention, this effect is small, except for very hydrophobic groups, and the major influence is the result of additional anionic charge sites.

The effect of one or two acidic side chains on dipeptide retention is illustrated in Table IV. When only one acidic

side chain is present the other AA subunit provides a very polar side chain and its effect would be one to also sharply decrease retention in the absence of the R_4N^+ salt. In all cases the mobile phase pH is basic enough so that both the acidic side chain group(s) and the carboxyl terminus are ionized. Thus, all analytes in Table IV are di-or trivalent anions and retention is significantly enhanced due to these additional anionic charges. At the $CH_3CN:H_2O$ ratio used and pH = 11 retention of all the dipeptides is negligible in the absence of a R_4N^+ salt; even at 100% H_2O, pH = 11 retention in most cases is small or barely detectable.

If the mobile phase pH in Table IV is reduced to a pH where the Tyr side chain is not ionized retention is reduced. Retention also correlates to the hydrophobicity provided by the second AA subunit (without the acidic side chain) in the dipeptide. The more hydrophobic the side chain the greater the retention is. Thus, retention for corresponding dipeptides is in the order Ala > Lys > Gly > Arg. For the Glu-Tyr dipeptides three anionic sites are present at pH = 11 and retention of these dipeptides is higher.

It should be noted that retention is always the largest when the acidic side chain is at subunit 1 in the dipeptide or when the side chain anionic site is far removed from the terminal $-CO_2^-$ site. Although not shown in Table IV, this trend was observed when comparing Asp-Gly to Gly-Asp and

Glu-Gly to Gly-Glu. These observations demonstrate that the behavior is not specific to Tyr.

Selectivity in Table IV is very favorable and many separations are possible via the use of a R_4N^+ salt as a mobile phase additive. The separation of a 8-component mixture of tyrosyl dipeptides is shown in Figure 2A where TPAF is used as the mobile phase additive. As in previous separations

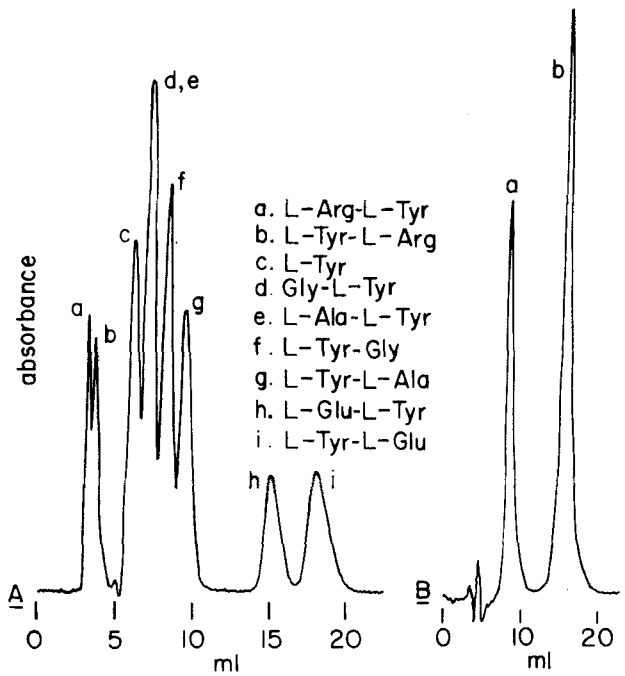

FIGURE 2

Separation of Tyr-Containing Acidic Dipeptides

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a (A) 5:95 $CH_3CN:H_2O$, 1.00×10^{-3}M TPAF, pH=11.0 (NaOH) mobile phase at a flow rate of 1.0 ml/min; (B) same as (A) except 100% H_2O.

excellent peak symmetry and favorable column efficiency is obtained. Resolution can be improved for given pairs of dipeptides by altering the CH_3CN:H_2O. For example, a baseline separation, Figure 2B, is obtained for the first two dipeptides in the separation of Figure 2A by using 100% H_2O. If the TPAF is omitted from the mobile phase in Figure 2 all of these very polar dipeptides would coelute with the dead volume peak on the PRP-1 when using a 5:95 CH_3CN:H_2O mobile phase at pH = 11.0.

Table V lists retention data for several Tyr containing peptides in the presence and absence of TPAF. In basic solution retention of the peptides in the presence of TPAF is greatly

TABLE V

Retention of Tyrosine Peptides on PRP-1 in the Presence of TPAF

	k'			k'	
Peptide	No Salt[a]	TPAF[b]	Peptide	No Salt[a]	TPAF[b]
L-Tyr	0.29	4.46	Gly-Gly-Gly	0.29	1.15
Gly-Gly	0.28	1.07	Gly-Gly-L-Tyr	0.37	6.54
Gly-L-Tyr	0.35	5.86	Gly-L-Tyr-Gly	0.69	8.75
L-Tyr-Gly	0.35	8.00	L-Tyr-Gly-Gly	0.66	10.1

A 10 μm, 4.1 mm x 150 mm PRP-1 column using (a) 100% H_2O, 1.00×10^{-3}M NaOH, 1.0×10^{-1}M NaCl or (b) a 2.5:97.5 CH_3CN:H_2O, 1.00×10^{-3}M TPAF, 1.00×10^{-3}M NaOH mobile phase at 1 ml/min with detection at 215 nm.

enhanced due to the additional anionic charge of the Tyr side chain. Furthermore, the effect increases as this charged Tyr side chain is moved further from the charged $-CO_2^-$ terminus. Figure 3 demonstrates that TPAF in the mobile phase improves the resolution of peptide mixtures where the peptides have acidic side chains and that a modest change in solvent composition significantly changes retention and alters resolution (compare Figures 3A and 3B where the % CH_3CN is decreased by 2.5%, respectively). Very little retention is observed when attempting to separate the peptides in Figure 3 when omitting the TPAF from the mobile

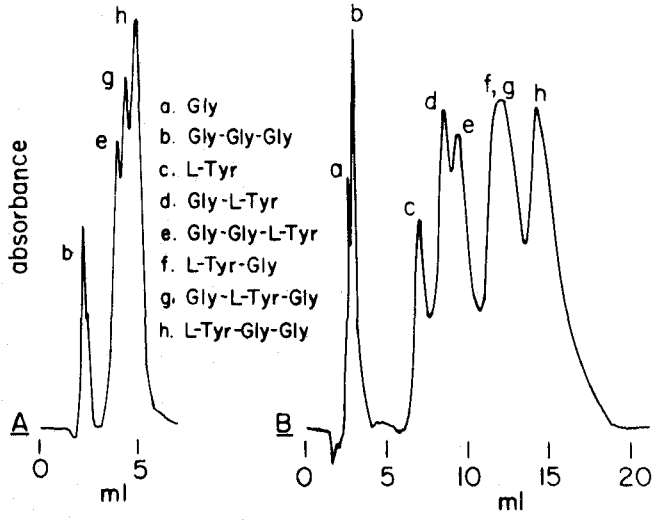

FIGURE 3

Effect of Mobile Phase Solvent Composition on Resolution of Tyr-Containing Acidic Peptides

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 1.00×10^{-3}M TPAF, pH = 11.0 (NaOH), (A) 5:95 or (B) 2.5:97.5 $CH_3CN:H_2O$ mobile phase at 1 ml/min.

phase. Although not shown in Table V, determination of peptide retention at the zwitterion pH and in the presence of TPAF indicated an enhanced retention over the absence of TPAF. However, the increase does not compare with that obtained at the basic pH.

Retention was determined for a series of Tyr peptides that also contain a Trp subunit as a function of solvent composition in the absence and presence of TPABr at pH = 11. At this pH all the Tyr-peptides are dianions. Since retention is high due to the very hydrophobic side chain from Trp, the Br^- salt was used to provide a stronger eluent co-anion. These data are summarized in Table VI. In the absence of the R_4N^+ salt retention is high and drops off as the CH_3CN concentration increases in the mobile phase. With the R_4N^+ salt present in the mobile phase retention is sharply enhanced and also drops rapidly with increasing CH_3CN in the mobile phase. These trends correlate with the decrease in retention of TPABr, these data are provided elsewhere (6), on the PRP-1 and are consistent with the equilibrium shown in equation 1a. That is, as the CH_3CN concentration increases the number of charged sites on the PRP-1 surface provided by the retained R_4N^+ salt decreases which then also decreases analyte retention. Thus, these data as well as others indicate that optimum retention and resolution require very careful control of the mobile phase solvent mixture and R_4N^+ concentration so that there are at least a minimum number of retained charge sites; our experiments

TABLE VI

The Effect of Solvent Composition on the Retention of Tyrosine Peptides on PRP-1

	Capacity Factor, k'					
	No TPABr[a]		1.00 x 10^{-3}M TPABr[b]			
Peptide 1% CH_3CN	2.5%	10%	7.5%	10%	13%	15%
L-Tyr		0	0.72	0.40	0.25	0.20
L-Trp	8.12	1.61	9.32	4.21	2.07	1.46
L-Trp-L-Tyr	11.5	1.31	27	8.02	2.85	1.85
L-Trp$(Gly)_1$L-Tyr	16.4	1.38	26	7.78	2.76	1.80
L-Trp$(Gly)_2$L-Tyr	18	1.41	26	7.46	2.62	1.79
L-Trp$(Gly)_3$L-Tyr	21	1.56	28	8.05	2.80	1.80
L-Trp$(Gly)_4$L-Tyr	25	1.77	30	8.38	2.87	1.75
	Encaphelins					
L-Tyr$(Gly)_2$L-Phe-L-Leu		3.95[a]			16.1[c]	
$(Gly)_2$L-Phe-L-Leu		6.46[a]			6.25[c]	
L-Tyr$(Gly)_2$L-Phe-L-Met		2.92[a]			12.2[c]	
$(Gly)_2$L-Phe-L-Met		4.77[a]			6.68[c]	

A 10 µm, 4.1 mm x 150 mm PRP-1 column using (a) a CH_3CN:H_2O, 1.00 x 10^{-3}M NaOH, 1.00 x 10^{-3}M NaF mobile phase, (b) a CH_3CN:H_2O, 1.00 x 10^{-3}M TPABr, 1.00 x 10^{-3}M NaOH mobile phase, or (c) same as (b) except TPAF and 12:88 CH_3CN:H_2O at a flow rate of 1 ml/min with detection at 215 nm.

indicate that for a 150 mm PRP-1 column the range is about 10 to 25 μmole/column. The retention minimum in Table VI that occurs in the presence of the TPABr when Gly units are inserted is probably the result of peptide coiling and the subsequent location of the terminal hydrophobic Trp subunit relative to the terminal divalent anionic charge due to the Tyr subunit.

Data for the 4 encaphelin peptides in Table VI illustrate how an R_4N^+ salt can be used to reverse elution order. In the absence of the TPAF the Tyr containing encaphelins are less retained on PRP-1 than the des-Tyr encaphelins at pH = 11. This is due to the anionic charge site from the Tyr subunit and its reducing effect (6) on the hydrophobic type retention that occurs in the absence of the TPAF. In the presence of the TPAF this same anionic charge site causes such a sharp increase in retention for the Tyr encaphelins that they are now more retained than the des-Tyr encaphelins whose retentions undergo little change. This is consistent with an increased exchange effect between the analyte di-anion and the retained TPAF as shown in equation 1b. Chromatograms illustrating this elution reversal, which is so favorable that baseline separations are easily obtained, are shown in Figure 4.

The effect of R_4N^+ salts on the retention of $(L\text{-Ala})_n$, where n = 1 to 6, was studied. In the presence of TPAF retention was enhanced relative to its absence and increased as n, the number of Ala subunits in the peptide, increased. However, selectiv-

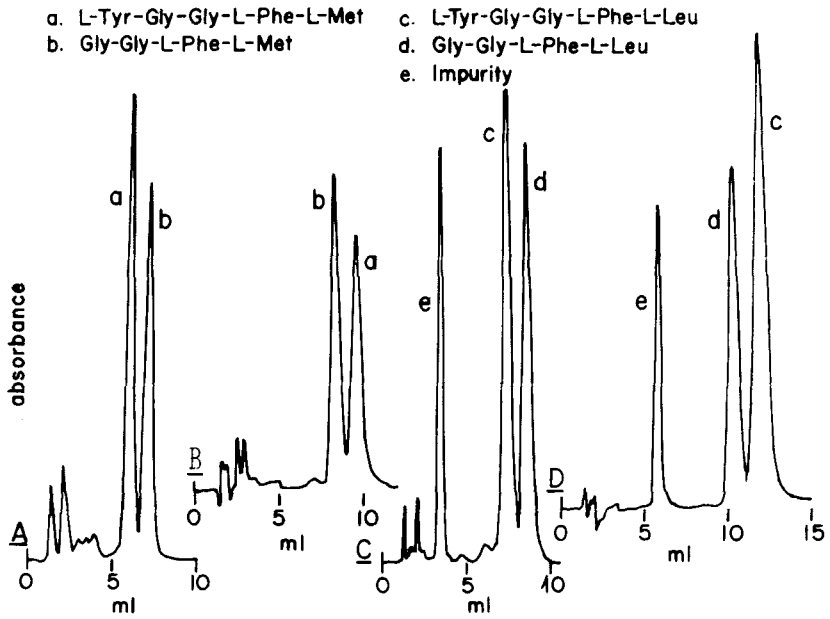

FIGURE 4

Separation of Encaphelin Peptides on PRP-1

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a (A,B) 13.5:86:5 $CH_3CN:H_2O$ or (C,D) 1:9 $CH_3CN:H_2O$, pH = 11.0 $(NaOH)_3$mobile phase in the presence (B,D) and absence (A,C) of 1.00×10^{-3}M TPAF at a flow rate of 1 ml/min.

ity, when compared to mobile phase conditions where the TPAF is omitted (6), does not improve.

When retention of dipeptide diastereomers were studied the presence of a R_4N^+ salt did not improve the separation of L,L and D,D enantiomers from L,D and D,L enantiomers even though retention was enhanced. In most cases selectivity was actually poorer than that obtained in the absence of a R_4N^+ salt (6).

Although the number of examples were limited it appeared that this was also true for dipeptide diastereomers that also contained an AA subunit with an acidic side chain.

Alkylsulfonate Salts

In the presence of a RSO_3^- salt and a mobile phase pH of pH < 2, AA retention is enhanced in comparison to the absence of the RSO_3^- salt with the largest increase occurring for AA with very hydrophobic or basic side chains. At this acidic pH both the amine terminus and basic side chains are in the cation form. The co-cation accompanying the RSO_3^- salt also influences retention. These two trends are illustrated in Figure 5 where retention of several polar AA can be compared in the presence of H^+, Li^+, and Na^+ salts of $C_8SO_3^-$ at pH = 2. Retention for the AA is significantly enhanced since in the absence of the $C_8SO_3^-$ salt retention is zero or barely detectable. Furthermore, unlike AA retention in the presence of R_4N^+ salts, retention in Figure 5 differs for the polar AA studied. This is a useful property since these AA are the most difficult to separate by RPLC in the absence of mobile phase additives. It should be noted that in Figure 5 the mobile phase solvent of 100% water is the weakest eluent solvent possible.

The effect of the added Na^+, Li^+, and H^+, which is provided by the $C_8SO_3^-$ salt, is compared at a 0.001M concentration in Figure 5. The full co-cation effect can not be realized since all three solutions also contain 0.01M HCl (pH = 2) so that the

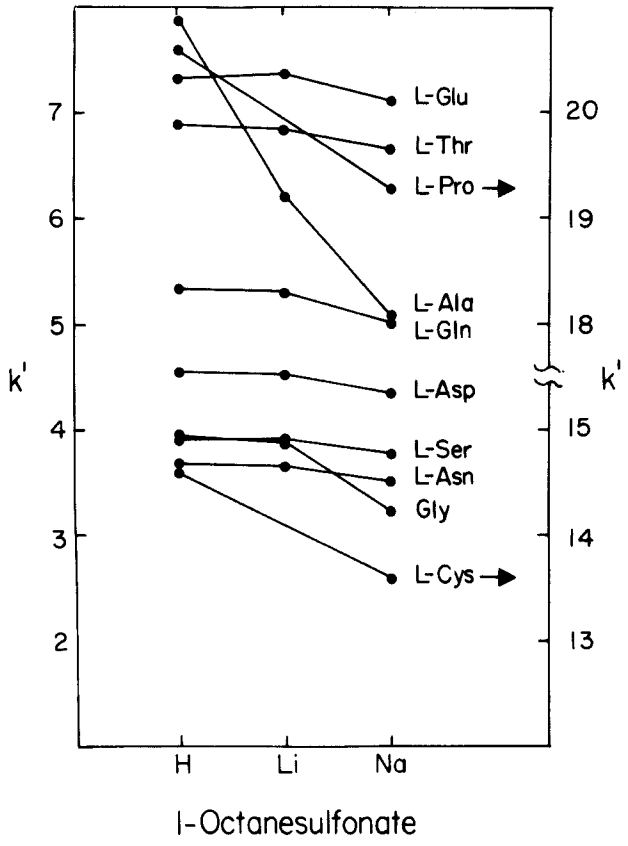

FIGURE 5

Effect of Co-Cation on Amino Acid Retention on PRP-1

A 4.1 mm x 150 mm, 10 µm, PRP-1 column and a 1.0×10^{-3} M $C_8SO_3^-M^+$ where $M^+ = H^+$, Li^+, or Na^+, 1.0×10^{-2} M HCl, 100% H_2O, mobile phase at a flow rate of 1 ml/min.

AA are in their cationic charged form. Even at this low level the co-cation effect, where the co-cation competes in the secondary layer with the AA cation for the $C_8SO_3^-$ anion site in the primary layer (see the exchange equilibria in equation 2b) is evident. Thus, the selectivity follows the order $Na^+ > Li^+ > H^+$, which is typical of the classical ion exchanger selectivity observed for these cations (21). As indicated by Table I, decreasing the alkyl chain length in the RSO_3^- salt decreases retention; the co-cation effect is also still observed.

Figure 6 illustrates the separation of several very polar AA as cations by using a C_8SO_3H, pH = 2, aqueous mobile phase. In the absence of the C_8SO_3H little or no retention is found for these AA, and in general, these are very difficult to separate by RPLC. As indicated in Figure 5, many other separations are also possible. Retention times and resolution are readily altered by using different RSO_3^- salts, modest addition of organic solvent to the mobile phase, and altering the RSO_3^- salt concentration.

Polar dipeptide retention is significantly enhanced on PRP-1 from an acidic, RSO_3^- salt containing mobile phase where the amine terminus is converted into the cationic form. Data illustrating this are shown in Table VII. In the absence of the C_8SO_3Na retention on PRP-1 is barely detected even when using 100% H_2O. The enhanced retention provides a sufficient selectivity so that many polar dipeptides are more easily separated. Two examples are shown in Figure 7. It should be noted that the

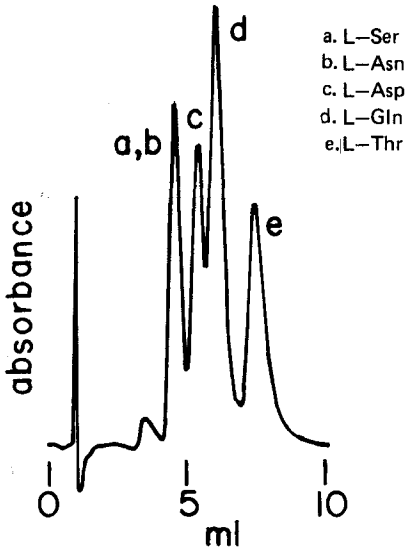

FIGURE 6

Separation of Polar Amino Acids on PRP-1

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 1.0 x 10^{-2}M HCl, 1.00 x 10^{-3}M $C_8SO_3^-H^+$, 100% H_2O mobile phase at a flow rate of 1.0 ml/min.

polar diastereomers of DL-Ala-DL-Ser (Figure 7B) are readily separated. In both separations no retention is observed in the absence of the RSO_3^- salt. Although the number of examples are limited it appears that an acidic, RSO_3^- salt, mobile phase is superior to a basic, R_4N^+ salt, mobile phase for separating dipeptide diastereomers. The former mobile phase also appears to be superior to RPLC (6) for dipeptide diastereomer separation particularly when the dipeptides contain polar side chains. Also, there is an advantage over RPLC (6) for these kinds of

TABLE VII

Retention of Polar Dipeptides on PRP-1 in the Presence of C_8SO_3H

Peptide/C_8SO_3Na	Capacity Factor, k'	
	0[a]	0.001M[b]
L-Ser-L-Ser	0.12	2.38
Gly-L-Ser	0.13	2.39
L-Ser-Gly	0.12	2.68
L-Ala-L-Ser	0.16	2.77
L-Ser-L-Ala	0.24	4.27
Gly-L-Thr	0.23	3.40
L-Ala-L-Thr	0.24	3.80
Gly-L-Asn	0.12	2.28

A 10 μm, 4.1 mm x 150 mm PRP-1 column using (a) a $1.0x10^{-2}$M HCl, $1.0x10^{-2}$M NaCl, 100% H_2O mobile phase and (b) a $1.0x10^{-2}$M HCl, $1.0x10^{-3}$M C_8SO_3Na, 5:95 CH_3CN:H_2O mobile phase at a flow rate of 1 ml/min with detection at 215 nm.

separations, particularly when separating dipeptide diastereomers that contain polar AA subunits since these are the most difficult to separate. The limited number of examples tested indicate that the L,L and D,D enantiomers always coelute first.

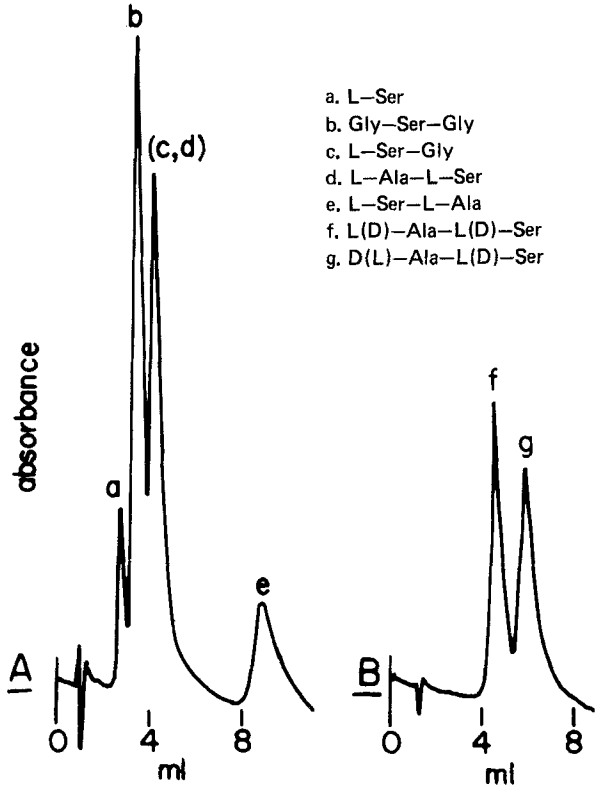

FIGURE 7

Separation of Polar and Diastereomeric Dipeptides on PRP-1

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 2.5×10^{-2}M PO_4^{-3} pH = 2.2 buffer, 5.00×10^{-3}M $C_8SO_3^-Na^+$, 100% H_2O mobile phase at a 1.0 ml/min flow rate.

A major enhancement in retention occurs if the AA, or peptide contains a basic side chain and the mobile phase pH is acidic enough to convert this group, in addition to the terminal amine group, into its cationic form. This additional charge

site (see the exchange equilibrium in equation 2b) accounts for the enhancement. This is illustrated in Table VIII where retention data in the presence of C_5SO_3Na are shown; when the $C_5SO_3^-$ salt is omitted from the mobile phase ittle or no retention is found. The enhanced retention follows the order Arg > His > Lys and is followed (Table VIII) even when considering comparable Gly peptides of the three. Table VIII also reveals that the largest retention is favored when the charged basic side chain is in position 1 in the peptide or is in the same unit as that which provides the terminal $-NH_3^+$ charge site. This is opposite to the results found when using R_4N^+ salts, a basic mobile phase pH, and peptide analytes containing acidic side chain subunits. If the peptide contains several basic side chains, the increased number of charge sites sharply increases retention. This is particularly noticeable when comparing the retention of $(L\text{-}Lys)_n$, where n = 1 to 5, peptides. Data are shown in Table VIII only for n = 2. For n = 3 retention times appear to be at least 90 minutes; if the RSO_3^- salt is omitted no retention is observed.

Several chromatograms, which focus on the separation of peptides containing basic side chains, are shown in Figure 8. In addition to high retention and favorable selectivity (in the absence of the RSO_3^- salt no retention is observed), chromatographic peaks, column efficiency, and analysis times are generally favorable.

TABLE VIII

Retention of Basic Peptides on PRP-1 in the Presence of C_5SO_3Na

Peptide	k'[a]	Peptide	k'[a]
L-Lys	2.15	Gly-Gly-L-His	11.3
L-His	2.70	Gly-L-His-Gly	18
L-Arg	7.70	L-His-Gly-Gly	19
$(L\text{-}Lys)_2$	15.2[b]	L-Ala-L-His	12.9
Gly-L-Lys	6.62	L-His-L-Ala	22
L-Lys-Gly	6.92	L-His-L-His	35
Gly-Gly-L-Lys	8.30	Gly-L-Arg	15.5
Gly-L-Lys-Gly	9.10	L-Arg-Gly	19
L-Ala-L-Lys	8.60	L-Arg-L-Ala	42
L-Lys-L-Ala	16.1	L-Arg-Gly-Gly	32
Gly-L-His	9.50	Gly-L-Arg-Gly	24
L-His-Gly	10.4	Gly-Gly-L-Arg	19

A 10 μm, 4.1 mm x 150 mm PRP-1 column using (a) a 2.5×10^{-3}M C_5SO_3Na, 1.0×10^{-2}M HCl, 100% H_2O mobile phase at a flow rate of 2 ml/min and detection at 215 nm; for (b) the C_5SO_3Na was 1.0×10^{-3}M; no retention in the absence of C_5SO_3Na.

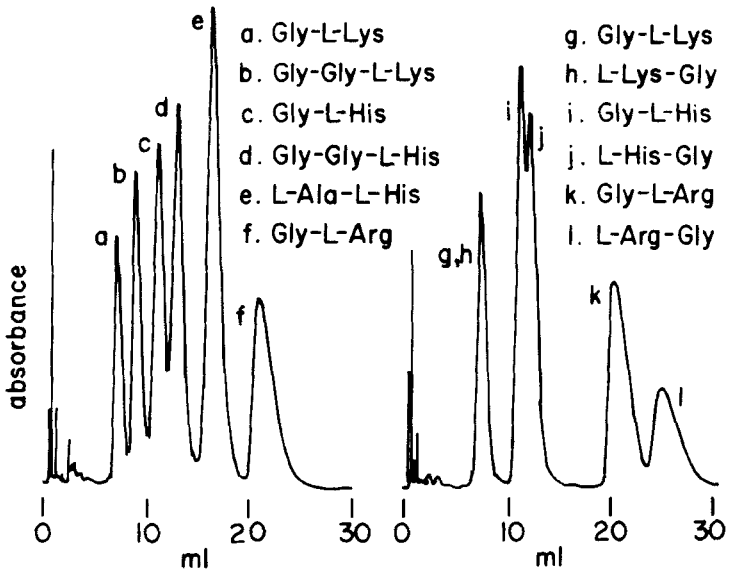

FIGURE 8

Separation of Basic Peptides on PRP-1

A 4.1 mm x 150 mm, 10μm, PRP-1 column and a 5.0×10^{-3}M $C_5SO_3^-Na^+$, 1.0×10^{-2}M HCl, 100% H_2O mobile phase at a flow rate of 2.0 ml/min.

Conclusions

The data reported here indicate that the major interactions influencing retention of AA and small chain peptides on PRP-1 from a mobile phase containing a hydrophobic ion are retention of the hydrophobic ion by the stationary phase and an exchange between its co-ion and the charged form of the AA or peptide. Evidence suggesting ion pair formation as a major contributing factor was not found. There is, however, the possibility that a hydrophobic interaction between the stationary phase and the AA

or peptide can also occur even though the analytes are charged as the result of mobile phase pH control. Since the amount of retained hydrophobic ion is small (usually less than 25 μmole/column), surface coverage on the PRP-1 (surface area is about 420 m^2/g and there is approximately 1 g PRP-1/column) is very small. Experiments with longer chain peptides particularly with those that also contain very hydrophobic side chains, suggest this possibility. Also, recent studies (22), where superficially charged ion exchangers were used as the stationary phase, strongly suggest this added interaction.

These data indicate several other generalizations, in addition to those cited earlier in this report, concerning AA and peptide separations. These are summarized in the following. It is best to start at a hydrophobic ion (R_4N^+ salt in a basic solution or a RSO_3^- salt in an acidic solution) concentration, organic solvent:water ratio, and an ionic strength that gives a loading of about 15 to 25 μmole of hydrophobic ion per column (15 cm). This is controlled by the hydrophobicity of the R groups, the concentration of the hydrophobic ion, the type of organic solvent, and the ratio of organic solvent to water in the mobile phase. From preliminary separations the concentrations of each of these variables can be adjusted to provide the best column performance in terms of resolution and analysis time. The largest effect of the hydrophobic ion in the mobile phase is realized when separating either acidic AA or basic AA or

peptides containing either of these side chains. In general, the RSO_3^- salts in an acidic mobile phase are more versatile than the basic, R_4N^+ salt mobile phases since the former has a more favorable effect on the selectivity of polar AA and peptides. Even diastereomers are more readily separated. Although F^-, OH^-, and H^+ are the weakest eluent co-ions when using R_4N^+ salts or RSO_3^- salts, respectively, and yield the most enhanced retention, stronger eluent co-ions can be used to reduce separation times, particularly when dealing with very highly retained AA or peptides. Finally, elution of AA and peptides in the presence of hydrophobic ions does not necessarily follow the same retention order as in the absence of the mobile phase additive; this is particularly true when the AA or peptides contain acidic or basic side chains and are ionized due to the mobile phase pH.

ACKNOWLEDGEMENTS

This investigation was supported by Grant CHE 79-13203 awarded by The National Science Foundation. Initial results were reported at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, March, 1983, as paper number 465.

REFERENCES

1. Majors, R. E., Barth, H.G., and Lochmüller, C. H., Anal. Chem., 54, 323R (1982).

2. Hearn, M. T. W., Regnier, F. E., and Wehr, C. T., Am. Lab., 14(10), 18 (1982).

3. Regnier, F. E., and Gooding, K. M., Anal. Biochem., 103, 1 (1980); 126, 1 (1982).

4. Pietrzyk, D. J., Cahill, Jr., W. R., and Stodola, J. D., J. Liq. Chromatogr., 5, 443 (1982).

5. Kullman, W., J. Liq. Chromatogr., 2, 1017 (1979).

6. Iskandarani, Z., and Pietrzyk, D. J., Anal. Chem., 53, 489 (1981).

7. Tomlinson, E., Jefferies, T. M., and Riley, C. M., J. Chromatogr., 159, 315 (1978).

8. Hearn, M. T. W., "Advances in Chromatography," J. C. Giddings, E. Grushka, J. Cazes, P. Brown, Eds., Dekker, New York, Vol. 18, 1980,pg 59; Vol. 20, 1982, pg 1.

9. Hearn, M. T. W. and Hancock, W. S., Biological/Biochemical Applications of Liquid Chromatography, Vol II, G. L. Hawk, Ed., M. Dekker, New York, 1979, pg. 243.

10. Kraak, J. C., Jonker, K. M., and Huber, J. F. K., J. Chromatogr., 142, 671 (1977).

11. Hancock, W. S., Bishop, C. A., Prestidge, R. L., Harding, D. R. K., and Hearn, M. T. W., J. Chromatogr., 153, 391 (1978).

12. Rivier, J. E., J. Liq. Chromatogr., 1, 343 (1978).

13. Hearn, M. T. W., Grego, B., and Hancock, W. S., J. Chromatogr., 185, 429 (1979).

14. Hancock, W. S., Bishop, C. A., Battersby, J. E., Harding, D. R. K., and Hearn, M. T. W., J. Chromatogr., 168, 377 (1979).

15. Bennett, H. P. J., Browne, C. A., Solomon, S., J. Liq. Chromatogr., 3, 1353 (1980).

16. Hearn, M. T. W., and Grego, B., J. Chromatogr., 203, 349 (1981).

17. Bidlingmeyer, B. A., J. Chromatogr.Sci., 18, 525 (1980).

18. Iskandarani, Z., and Pietrzyk, D. J., Anal. Chem., 54, 1065 (1982).

19. Smith, R. L., Iskandarani, Z., and Pietrzyk, D. J., Submitted Anal. Chem.

20. Iskandarani, Z., and Pietrzyk, D. J., Anal. Chem., 54, 2427 (1982).

21. Helfferich, F., Ion Exchange, McGraw-Hill, New York, 1962.

22. Iskandarani, Z., and Pietrzyk, D. J., Unpublished results.