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Separation and indirect detection of small-chain peptides using chromophoric mobile phase additives

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ABSTRACT

Ruthenium(II) 1,10-phenanthroline, $Ru(phen)_3^{2+}$, and ruthenium(II) 2,2′-bipyridyl, $Ru(bipy)_3^{2+}$, salts were evaluated as mobile phase additives for the liquid chromatographic separation of small-chain peptides on a polystyrene–divinylbenzene copolymeric (Hamilton PRP-1) stationary phase. In a basic mobile phase peptides are anions, and retention, resolution and detection occur because of the interactions between the stationary phase, the Ru^{II} complex and the peptide anion. Since the Ru^{II} complex concentration changes in the analyte band relative to the background eluent Ru^{II} complex concentration, the peptide can be detected by indirect photometric detection using the wavelength where the Ru^{II} complex absorbs. Peptide analyte peaks may be positive or negative depending on the counter-anion and its concentration. Small-chain peptides that do not contain chromophoric side-chains are detected without derivatization at about 0.1 nmol injected at a 3:1 signal-to-noise ratio. Factors that affect retention, resolution and indirect photometric detection are the Ru^{II} complex, its mobile phase concentration, mobile phase pH and solvent composition, and the type and concentration of the mobile phase counter-anion and/or buffer anion.

INTRODUCTION

Three liquid chromatographic (LC) strategies are routinely used for the separation of small-chain peptides. Anion and cation exchangers can be used to separate peptides as anions or cations, respectively[1–3]. A second approach relies on a reversed stationary phase for the separation[4–9]. Mobile phase pH and solvent composition are the major parameters adjusted to affect peptide retention, resolution and elution. The third general strategy is to use a charged hydrophobic ion as a mobile phase additive in combination with a reversed stationary phase[10–13]. Charged peptides are retained because of peptide–hydrophobic ion–stationary phase interactions where quaternary ammonium salts are used for anionic peptide separations and alkyl sulfonate or sulfate salts are used for cationic peptides. Mobile phase parameters, such as pH, buffer ion and counter-ion concentration, hydrophobic ion

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structure and concentration, and solvent composition, are manipulated to enhance peptide retention and improve resolution. In all three strategies the amino acid side-chain structure in the peptide chain strongly influences the peptide elution order. These three approaches have been used in a variety of applications and are reviewed elsewhere[1–13].

If the small-chain peptide contains a subunit with a UV chromophoric side-chain, detection is possible at 250–290 nm. In the absence of the chromophore, detection is possible at a low UV wavelength due to the amide and carboxyl groups. However, the low wavelength also limits the kind and concentration of mobile phase components. Recently, it was shown that analyte anions can be separated on reversed stationary phases and indirectly detected by using 1,10-phenanthroline or 2,2′-bipyridyl metal complexes as mobile phase additives[14–16]. The complex is an ion interaction (pairing) reagent that enhances the analyte anion retention, and by manipulation of the mobile phase parameters complex mixtures of analyte anions can be resolved. Since the complexes are chromophoric and the amount of complex in the analyte band changes in proportion to the amount of analyte relative to the background complex concentration in the mobile phase, indirect photometric detection (IPD) is possible[15]. If the fluorescent Ru^{II} complexes are used indirect fluorometric detection (IFD) is possible[17].

This report focuses on using ruthenium(II) 1,10-phenanthroline ($Ru(phen)_3^{2+}$) and ruthenium(II) 2,2′-bipyridyl ($Ru(bipy)_3^{2+}$) salts as mobile phase additives for the separation and indirect detection of short-chain peptides. A polystyrene–divinylbenzene copolymeric stationary phase is used because a basic mobile phase is required to convert the peptides into their anionic forms.

EXPERIMENTAL

Reagents

$Ru(bipy)_3Cl_2(H_2O)_6$ was purchased from G. F. Smith Chemicals and $Ru(phen)_3^{2+}$ salts were prepared as described[14,16,18]; other counter-anion forms were obtained by anion exchange[14]. Peptides (Sigma and Chemalog Chemical Dynamics) were used as received. Disodium 1,5-naphthalenedisulfonate (1,5-NDS) was purchased from Eastman Kodak. Buffer and/or ionic-strength salts were analytical-reagent grade when possible. Organic solvents were of LC quality and water was treated with a Sybron–Barnstead purification unit. Polystyrene–divinylbenzene columns (PRP-1, 10 μm, 150 mm × 4.1 mm I.D.) were obtained from Hamilton. The LC instrumentation consisted of a Waters 6000A or Spectra Physics 8800 pump, a Rheodyne 7125 injector, a Kratos 773 or Spectra Physics 8450 detector, a Hewlett Packard 3390A integrator and a Bioanalytical Instrument temperature controller.

Procedures

Aqueous peptide standards (1.5 mg/ml) were injected as 1–5 μl aliquots by syringe. Mobile phase solvent mixtures are percent by volume. Buffer, ionic strength salts, counter-anion salts and $Ru(bipy)_3Cl_2$ were added by weight while Na_2CO_3, NH_3 or 1,5-NDS solutions were added by volume. Mobile phase pH was adjusted with a dilute NaOH solution and diluted to volume.

Columns were conditioned with at least 50 ml (1 ml/min) of the mobile phase

beyond its breakthrough point[14]. An acetonitrile–water mixture (1:1, *ca.* 200 ml) can be used to remove the retained $Ru(bipy)^{2+}$ salt from the PRP-1 column. The flow-rate as 1.0 ml/min, the column temperature 30°C, the inlet pressure 600–1000 p.s.i. and detection was performed at 448 nm for $Ru(phen)_3^{2+}$ and at 445 nm for $Ru(bipy)_3^{2+}$. Typical column void volumes were 1.1–1.3 ml.

RESULTS AND DISCUSSION

When a mobile phase containing a $Ru(bipy)_3^{2+}$ or a $Ru(phen)_3^{2+}$ salt passes through a PRP-1 column an equilibrium amount of the complex is maintained as a double layer on the PRP-1 surface according to the mobile phase conditions. The $Ru(bipy)_3^{2+}$ occupies the primary layer and a counter-anion, C^-, is present as a diffuse secondary layer. An analyte anion competes with C^- and it is the selectivity difference between the analyte anion and C^- which accounts for analyte anion retention. Peptides ionize under basic conditions, and as an anion, the peptide will also compete with C^- in the diffuse layer. The parameters, which affect these equilibria[14–17], are: (1) Fe^{II} or Ru^{II} complex concentration; (2) type and concentration of counter-anion; (3) mobile phase solvent composition; (4) pH. Optimization of each of these parameters as described elsewhere 14–17 can be used to bring about the retention, selectivity and resolution of small-chain peptide analytes.

The Ru^{II} complexes were used rather then $Fe(phen)_3^{2+}$ salts for the following reasons. (1) The Ru^{II} complexes are stable at basic pH. (2) Absorptivity is high and remains constant at the basic pH; this is required for indirect detection and a favorable detection limit. (3) Retention on PRP-1 follows the order $Ru(phen)_3C_2 > Fe(phen)_3C_2 > Ru(bipy)_3C_2$. Since peptides can be highly retained their retention can be reduced by using a $Ru(bipy)_3^{2+}$ salt. (4) $Ru(phen)_3^{2+}$ and $Ru(bipy)_3^{2+}$ salts are divalent, reversibly retained on PRP-1 and undergo rapid, reversible interaction with analyte anions. (5) Ru^{II} complexes are chromophoric and will fluoresce, thus, both IPD[14,15] and IFD[17] are possible; the origin and parameters affecting IPD are discussed elsewhere[15].

The Ru^{II} complex mobile phase concentration should provide an absorbance of < 0.7. When the absorbance is larger, the detector offset capabilities can be exceeded depending on the detector. Also, IPD sensitivity decreases rapidly as the background absorbance increases.

PRP-1 columns were used because the mobile phases were basic. A given column was used continuously providing efficiency and k' values were reproducible for a standard benzene–phenol sample and acetonitrile–water (9:1) as the mobile phase. In general, column life often exceeded several months.

The IPD strategy produces system peaks (SP), as well as analyte peaks, due to the competing equilibria between the analyte anion and the counter-anion(s) and the effects of these processes on the equilibrium amount of the Ru^{II} complex maintained on the PRP-1 column[15,16]. Each kind of counter-anion present may produce an SP. In general, when other factors are equal, the higher the counter-anion exchange-like selectivity the higher the retention time for its SP. A basic mobile phase contributes to a special SP problem. As the pH is increased to ensure peptides are anions, the OH^- SP moves to a higher retention time and increases in peak area because of the increased OH^- concentration. A compromise is reached by using a pH 9.5 mobile phase. At this

condition the OH^- SP area is small, the SP occurs at a low retention time or prior to the peptide peak, and the fraction of peptide in an anionic form is high. The OH^- SP is minimized further by adding a second counter-anion of high anion-exchange selectivity of suitable concentration to the mobile phase. This also shifts the OH^- SP to a lower retention time because the exchange selectivity for OH^- is small. Adding the second counter-anion also aids the chromatography because peptide anions are highly retained and strong eluent counter-anions are required for their elution. In these studies, except where noted, the OH^- SP appears at a low retention time in the chromatogram.

A second problem is CO_2 absorption by the basic mobile phase which introduces a CO_3^{2-}/HCO_3^- SP. The location (higher retention time than OH^- because of its greater anion selectivity) and peak area are concentration-dependent and depend also on mobile phase preparation and storage. Alternatively, CO_3^{2-}/HCO_3^- can be added to the mobile phase as a counter-anion to fix the CO_3^{2-}/HCO_3^- SP retention time and peak area since the CO_2 absorbed is insignificant compared to the added CO_3^{2-}/HCO_3^-.

Table I lists retention data for a series of dipeptides for three mobile phase conditions. All the mobile phases were basic (pH 9.5), contained the strong eluent 1.5-NDS counter-anion and differed in other parameters. If the pH was increased, the retention increased and appeared to correspond to the fraction of the peptide present in the anionic form. However, the OH^- SP and its affect also became more significant. When the pH was lowered the fraction of peptide as an anion decreased and retention and peak area (detection limit is less favored) decreased rapidly. When organic modifier was increased peptide retention decreased. In Table I the retention difference between mobile phases 1 and 2 is partly due to the solvent composition and partly due to the differences in counter-anion. When the concentration of the 1,5-NDS was increased the retention decreased. Switching to a different counter-anion changed the dipeptide retention according to counter-anion selectivity. Two other trends in Table I are: (1) dipeptide retention is significantly lower in a $Ru(bipy)_3^{2+}$ salt mobile phase compared to one containing a $Ru(phen)_3^{2+}$ salt, (2) dipeptide retention is structure-dependent and correlates to hydrophobicity effects.

In Table I, mobile phase 3, the equilibrium amount of a $Ru(bipy)_3^{2+}$ salt on the PRP-1 surface is 6 μmol per column compared to 34 μmol per column when using a $Ru(phen)_3^{2+}$ salt, mobile phase 2. This difference is responsible for the sharp drop in dipeptide retention when using the $Ru(bipy)_3^{2+}$ salt. A well defined peak shape as well as the favorable feature of IPD and detection limit are still retained. Thus, peptide retention can be reduced by using the $Ru(bipy)_3^{2+}$ complex and by increasing organic modifier and/or counter-anion concentration.

In general, retention increases as hydrophobic amino acid (AA) units are introduced into the dipeptide. When the dipeptides in Table I are grouped into families of similar structure (Gly-AA and AA-Gly) two trends are apparent. First, retention increases as the AA side-chain hydrophobicity increases. This trend, even though it involves anion-exchange-like interactions, is observed in dipeptide retention on reversed stationary phases[6]. Second, the dipeptide with the side-chain AA in position 1, or near the terminal amine group, has a higher retention when compared to the corresponding dipeptide with the AA in position 2. This trend is also observed for reversed-phase dipeptide retention[6]. Acidic side-chains enhance retention of the

TABLE I

RETENTION OF DIPEPTIDES

Analyte	*Capacity factor (k')*		
	Mobile phase[a]		
	1	*2*	*3*
Gly-Gly	2.26[b]		
Gly-L-Thr	2.24		
Gly-L-Ser	2.11[b]		
L-Ser-Gly	2.07[b]		
Gly-L-Ala	2.50		
L-Ala-Gly	2.57		
Gly-L-Pro	3.97		
Gly-L-Val	6.14		
L-Val-Gly	8.46(−)[c]		
Gly-L-Asp	12.4(−)	6.43	1.8[b]
L-Asp-Gly	13.4(−)	6.47	1.7[b]
Gly-L-Glu	13.4(−)	6.43	1.8[b]
L-Glu-Gly	14.0(−)	6.30	1.7[b]
Gly-L-Met	13.1(−)	5.16	2.26
L-Met-Gly	15.1(−)	6.36	2.77
Gly-L-Leu	17.0(−)	7.36	3.37
L-Leu-Gly	27.4(−)	10.1	4.77
Gly-L-Phe		20.7	9.04
L-Phe-Gly		33	14.6(−)[c]
L-Ala-L-Phe		35	15.1(−)
OH^-, Cl^- SP	2.0	2	0.7(−)
CO_3^{-2}/HCO_3^- SP	21	3	1.4(−)

[a] Mobile phase 1: 0.050 m*M* $Ru(phen)_3(HCO_3)_2$, 0.10 m*M* $Na_2CO_3/NaHCO_3$, 0.050 m*M* Na_2-1,5-naphthalenedisulfonate, acetonitrile–water (2:98), pH 9.5; mobile phase 2: 0.050 m*M* $Ru(phen)_3Cl_2$, 0.20 m*M* NH_3, 0.050 m*M* Na_2-1,5-naphthalenedisulfonate, acetonitrile–water (5:95), pH 9.5; mobile phase 3: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.20 m*M* Na_2-1,5-naphthalenedisulfonate, acetonitrile–water, (5:95) pH 9.5.

[b] Overlapped with system peak (SP).

[c] A negative peak direction.

dipeptides through additional ionization at these sites. However, at pH 9.5, ionization is not high and large side-chain hydrophobicity, such as provided by Phe and Leu, is greater than anionic charge contribution due to acidic side-chains. If the pH is increased the acidic side-chain dissociation increases and the difference between the two effects is less pronounced.

Increasing the number of hydrophobic side-chain units increases the retention (see Table II where the retention of (L-Ala)$_n$ peptides for three mobile phases is listed). The effects of counter-anion, concentration of counter-anion and solvent composition on retention are consistent with dipeptide retention. The retention times for the longer-chain peptides can be excessive for the $Ru(phen)_3^{2+}$ salt. These are reduced by using a $Ru(bipy)_3^{2+}$ salt (see mobile phase 3 in Table II).

Table III lists tripeptide retention for two mobile phases containing $Ru(bipy)_3Cl_2$

TABLE II

RETENTION OF (L-ALA)$_n$ PEPTIDES

Analyte	*Capacity factor (k')*		
	Mobile phase[a]		
	1	*2*	*3*
L-Ala	2.60	3.09(−)[b]	2.13
(L-Ala)$_2$	7.09	17.3	8.40
(L-Ala)$_3$	9.00	25.2	14.3
(L-Ala)$_4$	12.2	38.8	24
(L-Ala)$_5$	29.9(−)[c]	67.5	50
OH^- SP	4.5	5.5(−)	3.5
CO_3^{-2}/HCO_3^- SP	34(−)	44(−)	16(−)

[a] Mobile phase 1: 0.050 m*M* Ru(phen)$_3$(HCO$_3$)$_2$, 0.10 m*M* L-Trp, acetonitrile–water (1:99), pH 9.5; mobile phase 2: 0.050 m*M* Ru(phen)$_3$(ClO$_4$)$_2$, 0.40 m*M* NaClO$_4$, 0.20 m*M* NH$_3$, aqueous, pH 9.5; mobile phase 3: 0.050 m*M* Ru(bipy)$_3$(ClO$_4$)$_2$, 0.40 m*M* NaClO$_4$, 0.20 m*M* NH$_3$, aqueous, pH 9.5.

[b] A negative peak direction.

[c] Overlapped with CO_3^{-2}/HCO_3^- system peak (SP).

which was used to reduce retention. If acetonitrile or counter-anion concentration is increased, the retention time decreases. The effect of side-chain hydrophobicity is consistent with the trends for dipeptide retention. For the tripeptide series, Gly-Gly-AA and AA-Gly-Gly, retention increases as the AA side-chain hydrophobicity increases. Second, retention is greater when the AA subunit is in position 1 near the terminal amine group rather than position 3 close to the $-CO_2^-$ site. Furthermore, the retention difference between tripeptide pairs, such as AA-Gly-Gly and Gly-Gly-AA, is greater than the difference for AA-Gly and Gly-AA dipeptides. For tripeptides where the subunit is in position 2, or Gly-AA-Gly, retention is intermediate to examples where the AA unit is in position 1 or 3. When two or more side-chains are present (see Table III) retention increases depending on hydrophobicity of the side-chains.

Retention of hydrophobic peptides will still be high even when using a $Ru(bipy)_3^{2+}$ salt. Increasing the organic modifier and/or using a counter-anion of higher anion selectivity reduces peptide retention. However, the equilibrium amount of the Ru^{II} complex retained on the PRP-1 also increases as counter-anion selectivity increases which increases peptide retention. The effect of counter-anion selectivity on exchange is greater resulting in a net decrease in analyte anion retention. Counter-anion selectivity at pH 9.5 was shown to follow the order:

p-OH-benzoate < L-Tyr < carbonate < succinate < phosphate < benzoate < L-Phe < phthalate < ClO_4^- < *p*-CH_3-benzoate < *p*-CH_3-benzenesulfonate < *p*-NO_2-benzoate < L-Trp < *p*-Cl-benzoate < *p*-Cl-benzenesulfonate < 1,5-naphthalenedisulfonate

In Tables I–III L-Trp and 1,5-NDS mobile phases are the stronger eluents when considering only counter-anion effects.

TABLE III

RETENTION OF TRIPEPTIDES

Analyte	*Capacity factor (k')*	
	Mobile phase[a]	
	1	*2*
Gly-Gly-Gly	1.61	2.51
L-Ala-Gly-Gly	1.92	3.31
L-Pro-Gly-Gly	7.31	14.2(−)[b]
L-Val-Gly-Gly	8.62	18[c]
L-Tyr-Gly-Gly	18.2	35
L-Ile-Gly-Gly	29	
L-Leu-Gly-Gly	33	
Gly-Gly-L-Ala		2.97
Gly-Gly-L-His		3.85
Gly-Gly-L-Val	4.1[c]	7.77(−)
Gly-Gly-L-Glu		12.4(−)
Gly-Gly-L-Tyr	8.31	20
Gly-Gly-L-Ile		24
Gly-Gly-L-Leu	12.9	29
Gly-L-Tyr-Gly	11.9	
Gly-L-Ser-L-Ala		2.62[d]
Gly-L-Pro-L-Ala	4.23[c]	7.69(−)[d]
Gly-L-Tyr-L-Ala	16	33[d]
OH^-, Cl^- SP	1.3 to 1.8(−)	1.7 to 2.2(−)
CO_3^{-2}/HCO_3^- SP	2.7 to 3.2(−)	16 to 18

[a] Mobile phase 1: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.20 m*M* Na_2-1,5-naphthalenedisulfonate, 0.20 m*M* NH_3, acetonitrile–water (1:99), pH 9.5; mobile phase 2: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.05 m*M* L-Trp, 0.10 m*M* Na_2CO_3, acetonitrile–water (1:99), pH 9.5.

[b] A negative peak direction.

[c] Overlapped with CO_3^{-2}/HCO_3^- system peak (SP).

[d] Mobile phase was the same as mobile phase 2 except that 0.075 m*M* L-Trp was used.

Two other types of eluent counter-anions were examined. Table IV lists tripeptide retention data using L-Asp-L-Asp, L-Phe-L-Leu and L-Leu-L-Phe (or D,L-Leu-D,L-Phe) as counter-anions. L-Asp-L-Asp has the potential to be highly charged because of the three carboxyl groups. However, this counter-anion also reduces the equilibrium amount of the Ru^{II} complex on the PRP-1; for mobile phase 1 in Table IV the equilibrium amount was 10 μmol of $Ru(bipy)_3^{2+}$ salt per column. The other two dipeptides are strong counter-anions because of side-chain hydrophobicity. Table IV indicates that the more significant retention decrease is caused by the hydrophobic counter-anion rather than the multiple-charged counter-anion. Other hydrophobic dipeptides or longer-chain peptides can be used providing they are soluble at the chromatographic conditions. In Table IV acetonitrile was required to dissolve L-Phe-L-Leu and this contributed to reduced retention.

Short-chain peptide diastereomers can be separated and detected as shown in Table V. Several trends are indicated. (1) The L-L and D-D dipeptide coelute first followed by L-D and D-L coelution. Only the L-Leu-L-Tyr was found to have a higher

TABLE IV

EFFECT OF DIPEPTIDE COUNTER-ANION ON THE RETENTION OF TRIPEPTIDES

Analyte	*Capacity factor (k')*	
	Mobile phase[a]	
	1	*2*
Gly-Gly-Gly	1.8[b]	
Gly-Gly-L-Ala	2.1[b]	
Gly-Gly-L-His	2.67	
Gly-Gly-L-Val	6.60	
Gly-Gly-L-Glu	7.60	
Gly-Gly-L-Tyr	13.5	
Gly-Gly-L-Ile	20	
Gly-Gly-L-Leu	24	
L-Ile-Gly-Gly		4.13
L-Leu-Gly-Gly		4.44
Gly-Gly-L-Phe		6.56
Gly-L-Phe-Gly		14.0
L-Phe-Gly-Gly		13.3
Gly-L-Phe-L-Ala		15.8
OH^-, Cl^- SP	1.5(−)	
CO_3^{-2}/HCO_3^- SP	4.0(−)	1.8

[a] Mobile phase 1: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.10 m*M* L-Asp-L-Asp, acetonitrile–water (1:99), pH 9.5; mobile phase 2: 0.05 m*M* $Ru(bipy)_3Cl_2$, 0.10 m*M* L-Phe-L-Leu, acetonitrile–water (2:98), pH 9.5.

[b] Overlapped with OH^-, Cl^- system peak (SP).

TABLE V

RETENTION OF DIASTEREOMERIC DI- AND TRIPEPTIDES

Analyte	*Capacity factor k'*	*Mobile phase*[a]	*Amount per column*[b] *(μequiv.)*
D,L-Val-D,L-Val[c]	11.6/14.4	1	25
D,L-Ala-D,L-Leu[c]	4.8/5.9	2	36
D,L-Ala-D,L-Phe[c]	19/21	1	25
D,L-Leu-D,L-Tyr[c]	8.9/7.6	3	11
D,L-Leu-D,L-Leu[c]	32/38	3	11
Gly-D,L-Leu-D,L-Ala[c]	6.4/10.2	1	25
L-Ala-L-Ala-L-Ala	4.3	4	11
L-Ala-L-Ala-D-Ala	8.3	4	11
D-Ala-D-Ala-D-Ala	4.2	4	11
CO_3^{-2}/HCO_3^- SP	2 to 3(−)[d]		

[a] Mobile phase 1: 0.050 m*M* $Ru(bipy)_3Cl_2$, 0.075 m*M* D,L-Leu-D,L-Phe, acetonitrile–water (1:99), pH 9.5; mobile phase 2: same as 1 except that 0.10 m*M* L-Leu-L-Phe was used; mobile phase 3: same as 1 except that 0.10 m*M* D,L-Leu-D,L-Phe and acetonitrile–water (6:94) were used; mobile phase 4: same as 1 except that 0.10 m*M* D,L-Trp was used.

[b] Equilibrium amount of $Ru(bipy)_3^{2+}$ salt maintained on the PRP-1 surface.

[c] First *k'* value is for the L,L- and D,D-diastereomer.

[d] Location of system peak depends on the mobile phase; a negative peak direction.

retention over the D-L diastereomer. (2) Resolution appears to be dependent on side-chain hydrophobicity. (3) Resolution of tripeptide diastereomers containing two chiral centers is better than for dipeptides with two chiral centers. (4) Resolution of tripeptides containing three chiral centers is possible. Peak confirmation in Table V was established by using diastereomeric standards.

Figs. 1–4 illustrate that resolution is favorable, peak shapes are well defined, IPD is applicable to peptide analytes, and that mobile phase parameters can be altered in a predictable way to affect resolution. In general, peptide quantities injected were in the range 1–3 μg.

In Fig. 1 dipeptides were separated using a basic mobile phase and a $Ru(phen)_3^{2+}$ salt (Fig. 1A and B) or a $Ru(bipy)_3^{2+}$ salt (Fig. 1C). The counter-anion and acetonitrile concentration was low in Fig. 1A because these peptides have low retention. When the concentrations are increased higher retained peptides are more easily separated (Fig. 1B). If the mobile phase in Fig. 1B contains the counter-anion CO_3^{2-}/HCO_3^- instead of NH_3, eluent strength is increased and retention of hydrophobic dipeptides is

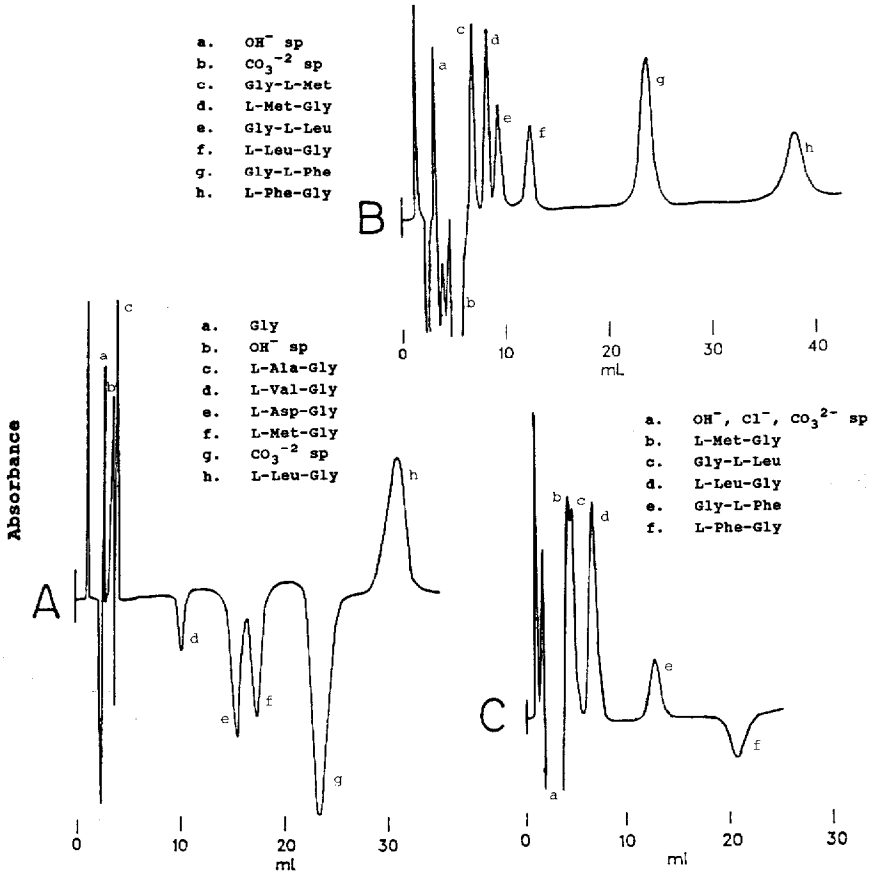

Fig. 1. Separation of dipeptides. (A) Mobile phase 1, Table I; (B) mobile phase 2, Table I except that 0.20 mM Na_2-1,5-naphthalenedisulfonate was used; (C) mobile phase 2, Table I, except that 0.050 mM $Ru(bipy)_3Cl_2$ was used.

decreased. Fig. 1C illustrates a lowering of retention by about 50% by using a $Ru(bipy)^{2+}$ salt which reduces the equilibrium amount of interaction sites on the PRP-1.

Separation of tripeptides and longer-chain peptides using a $Ru(bipy)_3^{2+}$ salt to reduce retention is shown in Fig. 2. Changes in mobile phase solvent composition and counter-anion concentration can also be used to alter retention. The side-chain effect on separation is illustrated in Fig. 2A where the side-chain structure changes at subunit position 1. The effect of the side-chain at subunit 3 is illustrated in Table III; these tripeptides can also be separated (see Fig. 3A). If 1,5-NDS is the counter-anion all peaks are positive. Fig. 2C shows the separation of $(L\text{-Ala})_n$, where $n = 1$–5, using a $Ru(phen)_3^{2+}$ salt. For a $Ru(bipy)_3^{2+}$ salt retention times are reduced by about 25%. L-Trp, a strong eluent counter-anion with a system peak of » 60 min, is used and this lowers retention.

When L-Asp-L-Asp, which is multiple charged, is the counter-anion (Fig. 3A), resolution is favorable but this counter-anion offers little advantage over a hydro-

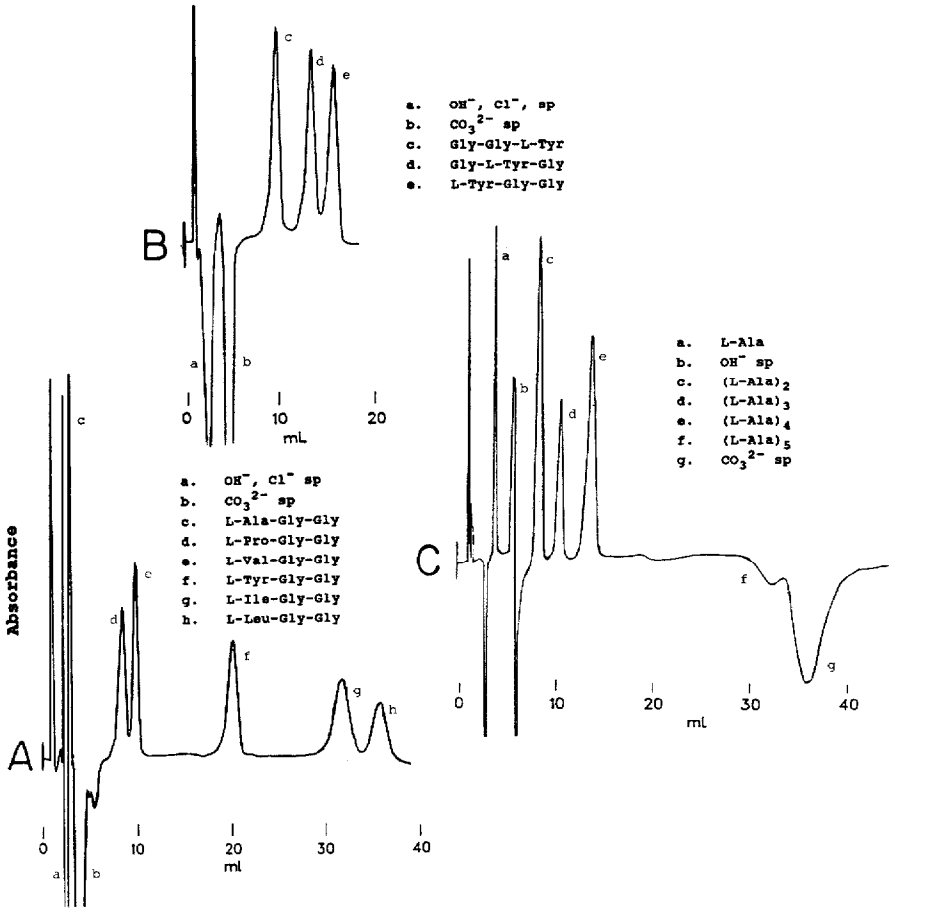

Fig. 2. Separation of small-chain peptides. (A) Mobile phase 1, Table III; (B) mobile phase 1, Table III; (C) mobile phase 1, Table II.

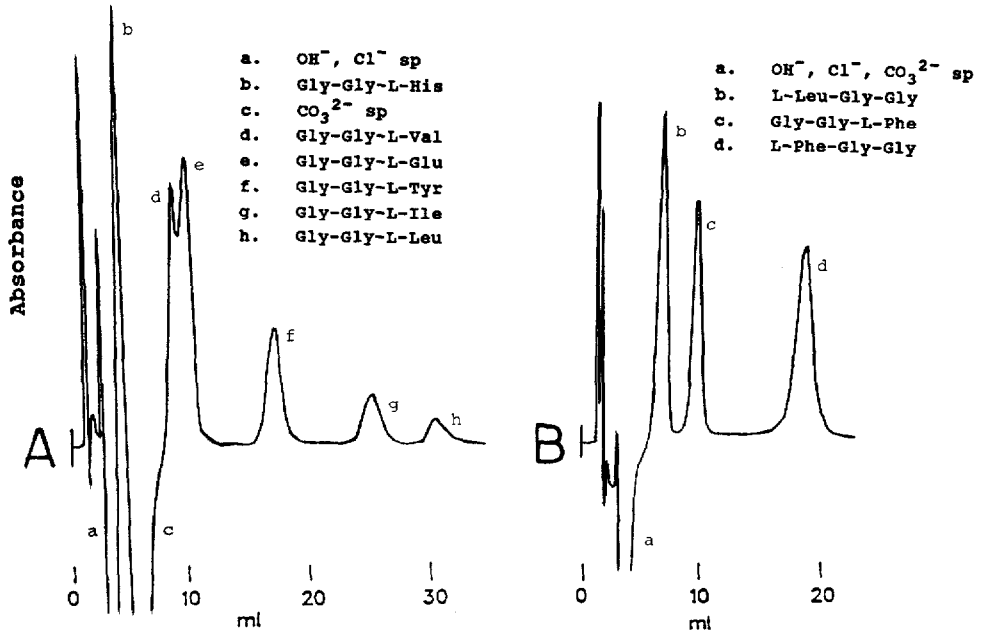

Fig. 3. Effect of counter-anion on the separation of tripeptides. (A) Mobile phase 1, Table IV; (B) mobile phase 2, Table IV.

phobic counter-anion such as L-Trp (see Table III). However, when hydrophobic L-Phe-L-Leu or L-Leu-L-Phe is the counter-anion, the retention is sharply reduced and highly retained tripeptides are resolved in a favorable analysis time (see Fig. 3B). D,L-Leu-D,L-Phe is preferred because it is inexpensive and readily available, and using a dipeptide diastereomer as a counter-anion offers no advantage. In both cases the dipeptide counter-anion system peak is at a high retention time and does not interfere. Fig. 4 illustrates the separation of peptide diastereomers; other examples are listed in Table V.

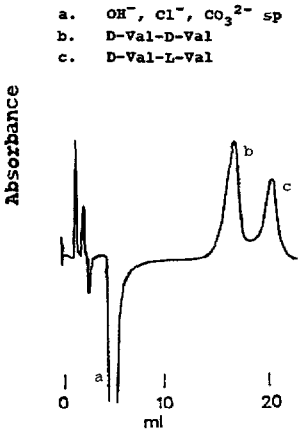

Fig. 4. Separation of peptide diastereomers. Mobile phase 1, Table V.

Calibration curves were prepared using Gly-L-Phe, Gly-Gly-L-Glu, L-Val-Gly-Gly and Gly-Gly-L-Phe standards over the range 0.2–100 nmol per 10 μl. Mobile phases in Fig. 1A (except 0.10 m*M* 1,5-NDS), 1 in Table III, in Fig. 2A and Fig. 3B were used, respectively. Calibration curves were linear and the upper limit of linearity was not determined. Change in peak retention time over this concentration range was modest and corresponded to less than a 10% difference between the low and high sample quantity. The major factor determining the linear calibration curve slope was mobile phase ionic strength; as ionic strength increased, the slope decreased which is consistent with previous studies[15]. In general, the detection limit for the four standards was *ca.* 0.1 nmol at a 3:1 peak height signal-to-noise ratio.

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