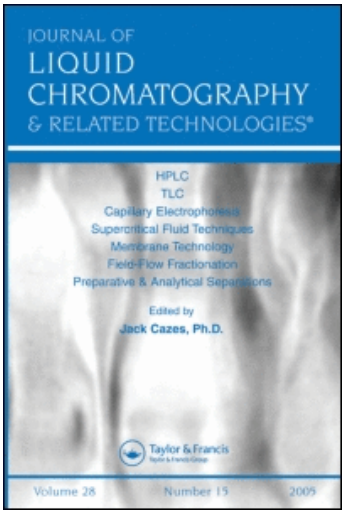

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Ruthenium (II) 1,10-Phenanthroline Salts as Mobile Phase Additives for the Separation and Indirect Detection of Free Amino Acids

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RUTHENIUM (II) 1,10-PHENANTHROLINE SALTS AS MOBILE PHASE ADDITIVES FOR THE SEPARATION AND INDIRECT DETECTION OF FREE AMINO ACIDS

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ABSTRACT

Ruthenium(II) 1,10-phenanthroline, $Ru(phen)_3^{2+}$, salts are used as ion interaction reagents in a basic mobile phase for the retention, resolution, and indirect photometric detection (IPD) of free amino acids on a polystyrene divinylbenzene (Hamilton PRP-1) column. Mobile phase $Ru(phen)_3^{2+}$ concentration and pH and type and concentration of organic modifier and counteranion affect retention and IPD. Underivatized amino acid elution order is influenced by side chain structure typical of ion exchange processes. Detection limits for the separation and detection of free amino acids using an isocratic elution condition are about 0.1 nmole for lower retained amino acids and 0.25 nmole for higher retained amino acids for a 3:1 signal:noise ratio. Gradient elution is possible but at higher detection limits.

INTRODUCTION

The determination of amino acids (AA) has been an important and difficult analytical problem for a long time. This is still

the case because of the increasing need to determine AA at lower detection limits. Since AA usually occur in mixtures, a separation step is required prior to their determination. AA separations and their subsequent detection are often complicated and difficult because AA are similar in structure. Moore and Stein (1,2) dealt with these problems and developed the first useful, automated amino acid analyzer based on a liquid column chromatographic strategy. In the procedure the AA are separated as cations on a cation exchanger, using an acidic increasing pH mobile phase gradient. Following the separation each AA was derivatized postcolumn by a reaction between the AA and ninhydrin. The derivative was then detected photometrically. This approach, which has been used for over 30 years, has undergone several major changes. The major ones are: 1) marvelous improvement in instrumentation, 2) conditions for the separation and detection (including the chemistry of the AA-ninhydrin reaction) have been optimized, and 3) spherical, well characterized cation exchanger stationary phase microparticles of a small, uniform size have been developed and are readily available (2-4).

The emergence of high performance liquid chromatography (HPLC), associated instrumentation, and new column and stationary phase strategies has resulted in many new separation procedures for the determination of AA many of which are applicable to trace quantities (3,5). However, virtually all require a post- or pre-column derivatization step. Thus, in the former the AA are first separated and this is followed by derivatization and detection.

In the latter the AA are first converted into derivatives on line which is followed by separation and detection of the AA-derivatives. In the postcolumn strategies AA can be separated on cation or anion exchangers, on reversed stationary phases, or on reversed phases in combination with ion interaction (ion pairing) reagents. The optimum mobile phase conditions are determined by the AA structure and the specific properties of the selected stationary phase, while conditions favoring the derivatization are established postcolumn. While many derivatization reagents (5) can be used postcolumn for the detection of AA, o-phthaldehyde (OPA) is probably the most versatile and is used the most. The AA-OPA derivatives can be detected photometrically, however, since these derivatives are also highly fluorescent a lower detection limit is obtained for most AA by using a fluorescence detector. When precolumn AA-derivatization is used, the separation of the AA derivatives is generally done by reversed phase and the optimum mobile phase conditions for the separation are determined by the properties of the AA-derivative rather than by the AA properties.

This report focuses on a column/mobile phase procedure for the separation and detection of AA that eliminates both the post- or pre-column derivatization step. The underivatized AA are separated on a reversed stationary phase as anions by using a basic mobile phase containing a ruthenium(II) 1,10-phenanthroline, $Ru(phen)_3^{2+}$, salt as an ion interaction mobile phase additive (6-9). The $Ru(phen)_3^{2+}$ salt not only enhances the retention and selectivity of the AA but it also provides a way for their

indirect detection. Since $Ru(phen)_3^{2+}$ salts are chromophoric and fluorescent, indirect photometric (IPD) and fluorometric (IFD) detection is possible (8,9), thus, permitting separation and detection of underivatized AA at low detection limits. Parameters influencing AA retention and resolution on a polymeric reversed phase and the indirect detection of the AA are established.

EXPERIMENTAL

Materials. The $Ru(phen)_3^{2+}$ salts were synthesized (6,10,11) and converted into different counteranion forms by anion exchange. Amino acids were purchased from Sigma Chemical Co. Disodium 1,5-naphthalene-disulfonate, 1,5-NDS, was obtained from Eastman Kodak Chemical Co. Buffer and/or ionic strength salts were analytical reagent grade while organic solvents were LC quality. A Sybron/Barnstead unit was used to prepare LC quality water. Prepacked PRP-1 columns, 10 μm, spherical, polystyrenedivinyl-benzene particles in a 4.1 X 150 mm column were obtained from Hamilton Co. A Spectra-Physics 8800 pump, a Rheodyne 4125 injector, a Kratos 773 or Spectra Physics 8450 detector, a Hewlett Packard 339A integrator and a Bioanalytical Instrument Temperature Controller were used.

Procedures. Aqueous AA standards, 1 mg/mL, and mixtures of standards were injected by syringe (<15 μL). Mobile phase solvent mixtures were prepared as percent by volume, while $Ru(phen)_3^{2+}$ salt was added by weight when possible. Mobile phase buffer, ionic strength, and counteranion salts were prepared as standard

solutions and added by volume. The pH was adjusted with dilute NaOH when necessary prior to dilution to volume.

Columns were conditioned with the mobile phase beyond the breakthrough point until the detector signal was constant. Breakthrough data (6,8) were used to establish the amount of $Ru(phen)_3^{2+}$ salt (about 25 μmole/column depending on mobile phase conditions) maintained on the PRP-1. A 1:1 $CH_3CN:H_2O$ mixture was used to remove the retained $Ru(phen)_3^{2+}$ salt. Column performance and stability were monitored continuously by determining column efficiency by reversed phase chromatography (9:1 $CH_3CN:H_2O$ and a benzene, toluene mixture as a standard sample). Flow rate was 1.0 mL/min, column was 30°C, inlet pressure was 600 to 1000 psi, column void volume was 1.1 to 1.3 mL, and IPD was at 448 nm.

RESULTS AND DISCUSSION

When a $Ru(phen)_3^{2+}$ salt mobile phase passes through the reversed phase PRP-1 column an equilibrium amount of the $Ru(phen)_3^{2+}$ salt is maintained as a double layer on the stationary phase surface according to the mobile phase conditions. The $Ru(phen)_3^{2+}$, which makes up the primary layer, is held by reversed phase interactions while counteranions, C^-, occupy a diffuse secondary layer. As an analyte anion, such as AA^-, passes through the column, it competes with the counteranion in the diffuse layer according to an anion ion exchange like selectivity leading to retention and subsequently resolution. These equilibria can be described by eqs. 1 and 2, respectively.

$$\text{PRP-1} + \text{Ru(phen)}_3^{2+} + 2\text{C}^- \rightleftharpoons \text{PRP-1}\cdots\text{Ru(phen)}_3\text{C}_2 \quad (1)$$

$$\text{PRP-1}\cdots\text{Ru(phen)}_3\text{C}_2 + 2\text{AA}^- \rightleftharpoons \text{PRP-1}\cdots\text{Ru(phen)}_3\text{X}_2 + 2\text{C}^- \quad (2)$$

The parameters, which influence the direction of eqs. 1 and 2 are therefore the ones that must be optimized to enhance AA retention and indirect detection (6-9). These are: mobile phase pH, buffer, ionic strength, organic modifier, and $Ru(phen)_3^{2+}$ salt concentration. A high pH favors the formation of the anionic form of the AA and will increase AA retention. Increasing the organic modifier decreases AA retention. Choosing a mobile phase counteranion of low anion exchange selectivity (see eq. 2) increases AA retention. Increasing ionic strength will increase AA retention initially because eq. 1 is shifted to the right, however, the mass action effect of the counteranion in eq. 2 rapidly becomes the dominate factor causing retention to decrease. Increasing the $Ru(phen)_3^{2+}$ concentration like the ionic strength initially increases AA retention (shifts eq. 1 to the right), however, the mass action effect of its counteranion eventually reverses the trend. A discussion of the significance of each parameter on anion retention and IPD is described elsewhere (6-9).

Indirect detection of the AA is possible because the amount of $Ru(phen)_3^{2+}$ within the analyte band differs (eq. 1 shifts to the left to give a positive peak or to the right to give a negative peak) from the background amount of $Ru(phen)_3^{2+}$ in the mobile phase (7). Thus, it is the change in the $Ru(phen)_3^{2+}$ concentration in the analyte band that is detected not the AA itself. Two key factors, the change in ionic strength in the analyte band relative

to the background and the difference in the anion exchange like selectivity between the analyte anion and the counteranion, are responsible for the change. When these and the other parameters are optimized the detector signal is proportional to the amount of AA present and the detection limit is favorable. Also, if a mobile phase counteranion that has a much higher anion exchange like selectivity than the analyte anion is used, the analyte peak will be positive. The optimum $Ru(phen)_3^{2+}$ mobile phase concentration is also determined by the background absorbance, if IPD is used (or fluorescence if IFD is used), and the detector's offset capabilities. In general, background absorbance should not exceed about 0.7 absorbance units. If the background absorbance is too high the electronic offset capability of the detector will be exceeded and the ability of the detector to respond to the absorbance change over the background is negated (7).

All AA can participate in anion exchange when the terminal carboxyl group is dissociated. Side chain properties affect dissociation and can also contribute to stationary phase matrix-AA interactions. Side chain features, such as dissociation, polarity, hydrophobicity, and size are therefore responsible for differences in AA anion exchange retention. In general, in anion exchange of AA the polar side chain AA are least retained while the more hydrophobic and/or acidic side chain AA will have higher retention (3,5). The retention order of AA on PRP-1, a reversed stationary phase, from a basic mobile phase containing a $Ru(phen)_3^{2+}$ salt as a mobile phase additive is consistent with the

retention order of AA anions on anion exchangers. This is illustrated in Table I where AA retention data are listed for several mobile phases that differ in the counteranion present in the mobile phase. A 10 μm, PRP-1 column was used rather than a silica bonded reversed phase column because of the requirement of a basic mobile phase. A 5 μm, PRP-1 column when used, provided only minor improvement in peak efficiency indicating that equilibria in the retention process (see eqs. 1 and 2) are probably the limiting factor in determining peak efficiency. All AA were detected by IPD at 448 nm; although not shown detection by IFD at λ_{ex} 465 nm, λ_{em} 600 nm is also feasible (8). However, IFD is less sensitive than IPD for AA detection because typical fluorescent detectors are not designed to detect a fluorescence change on a highly fluorescent background.

The AA analytes in Table I can be divided into three groups according to their elution order (effect of side chain). The first or least retained group include the monovalent polar and basic AA: Arg, Lys, Pro, Gly, Ala, Ser, Asn, Gln, Thr, His, and Val. In Table I Arg and Lys are not retained under any of the conditions studied. The intermediate group are the nonpolar and divalent AA: Ile, Leu, Met, Asp, Glu, and Tyr. The OH^- system peak (SP) (due to the basic pH) and the CO_3^{2-}/HCO_3^- SP (added as a counteranion or due to CO_2 absorption by the basic mobile phase) are in the group 1 and group 2 AA, respectively. The OH^- SP appears early because of its low anion exchange selectivity while the CO_3^{2-}/HCO_3^- SP is intermediate in anion exchange selectivity and its SP occurs at an intermediate region. Other counteranions

used in Table I, Trp^-, Phe^-, phthalate, and ClO_4^-, have large anion exchange selectivities and their SP appear well beyond the region of chromatographic interest. The third and highest retained group of AA include Cys, Phe, and Trp (in Table I their k' values are > 35 for all mobile phases).

The strong eluent power of the four counteranions is demonstrated in Table I. If the mobile phase counteranion is only CO_3^{2-}/HCO_3^- AA retention is sharply increased, particularly the more highly retained AA. Both anion exchange-like selectivity and other types of interactions are evident. For example, phthlate, which is a divalent anion at the pH used, is a stronger eluent counteranion than Phe^-, Trp^-, or the $ClO_4^-/CO_3^{2-}/HCO_3^-$ mixture for the elution of the weakly retained AA but intermediate for the more highly retained AA. If a weak eluent counteranion, such as Cl^-, is used AA retention is increased, a Cl^- SP will appear close to the OH^- SP, and AA peak direction will be affected as discussed elsewhere (7). Although not shown in Table I increasing CH_3CN concentration and/or counteranion concentration will decrease AA retention. The $Ru(phen)_3^{2+}$ concentration and pH will also influence AA retention, however, these two parameters were optimized according to their combined affects on retention, resolution, and IPD. If the pH is too high, which favors anionic AA formation, the OH^- SP retention time and area increase. This will then become a major interference since this SP occurs in a key chromatographic region of interest. Too high of a $Ru(phen)_3^{2+}$ concentration, which increases the AA retention because the number

TABLE I. Retention of Amino Acids as a Function of Eluent Counteranion

	Capacity Factor, k'			
	Mobile Phase[a]			
Amino Acid	1	2	3	4
Lys	0	0	0	
Arg	0	0	0	
Pro	1.27	2.51	1.6(s)[b]	
Gly	2.17	2.94	1.66	2.87
Ala	2.03	2.97	1.74	2.80
Ser	3.40	3.84	2.26	3.99
Asn	3.87	4.63	2.40	4.43
Gln	3.84	4.64	2.39	4.23
Thr	3.96	4.87	2.74	4.31
His.HCl[c]	4.49/4.67	5.59/6.56	3.86	6.06/6.67
Val	7.17	11.0	7.97	8.23
Ile	17.6(-)	30(-)	26	>100
Leu	20.2(-)	34(-)	31	
Met	20.3(-)	30(-)	27	
Asp	22.8(-)	38(-)	17.6	
Glu	25.2(-)		19.8	
Tyr	28.1(-)		30	
OH^- SP	5.4(s)[b]	4.1(s)[b]	2.9(s)[b]	5.0(s)[b]
CO_3^{2-}/HCO_3^- SP	29	44	15(-)	34(-)

a. Mobile phase 1: aqueous 0.050 mM $Ru(phen)_3(HCO_3)_2$, 0.10 mM Trp, pH = 9.5; Mobile phase 2: same as 1 except 0.10 mM Phe; Mobile phase 3: same as 1 except 0.10 mM phthlate; Mobile phase 4: 0.075 mM $Ru(phen)_3(ClO_4)_2$, 0.50 mM $NaClO_4$, 0.10 mM Na_2CO_3, 1:99 $CH_3CN:H_2O$, pH = 9.5. b. A split peak. c. Cl^-, His^- peak.

of ion interaction sites increases, causes background absorbance to increase beyond the offset capabilities of the detector and eliminates the indirect detection. Resolution of chiral AA was not obtained with the $Ru(phen)_3^{2+}$ mobile phase when individual D- and L-AA were used as standards.

The elution time interval between group 1 and group 2 AA is about 10 to 20 minutes depending on the mobile phase. The elution interval between the second and third group is excessive (see Table I) and the successful elution of group 3 AA requires either a very strong mobile phase or a reduction in the number of ion interaction sites maintained on the stationary phase. Each AA group can be retained and eluted with a single mobile phase. A single mobile phase can also be used to separate a group 1-2 mixture but a gradient elution is required to elute all three groups in a convenient elution time.

Gradient elution using detector active ion interaction reagents, such as $Ru(phen)_3^{2+}$ salts, with indirect detection is more complicated than detection in ordinary LC-gradient strategies. Since the baseline of the detection is based on the absorbance of the mobile phase, any change in the concentration of the absorbing ion interaction reagent will cause the baseline to shift. As indicated the equilibrium amount of $Ru(phen)_3^{2+}$ maintained on the stationary phase surface is mobile phase dependent. Any mobile phase parameter that is altered to generate an increase in mobile phase eluting power will also alter the equilibrium amount of $Ru(phen)_3^{2+}$ on the stationary phase. The

column, therefore, is like a reservoir for the $Ru(phen)_3^{2+}$ in that it either comes off (produces a positive baseline change) or is retained (produces a negative baseline change) by the column as the mobile phase variable changes. As discussed elsewhere (10,12) the two most useful types of gradients from an AA retention/resolution point of view is to either increase the mobile phase organic modifier concentration or the mobile phase counteranion concentration with time while all other factors are held constant. Neither are satisfactory because the first produces a sharp rise in background absorbance while the latter produces a sharp decrease in background absorbance with time leading to chromatograms with significant distortion in the baseline. However, computer concellation of the baseline, since it is reproducible, or a dual column strategy employing baseline cancellation within the detector is possible (10,12).

Inorganic counteranions, such as CO_3^{2-}/HCO_3^-, PO_4^{3-}/HPO_4^{2-}, and ClO_4^-, are suitable for elution of group 1 AA but do not have anion exchange selectivities that are large enough to conveniently elute group 2 AA. One of the AA from group 2, such as Trp, or divalent phthlate are suitable counteranions for group 2 AA elution while a strong eluent counteranion, such as 1,5-NDS (9), is useful for group 3 AA elution. A reduction in ion interaction sites, which reduces group 3 AA retention, can be achieved by using a $Ru(bipy)_3^{2+}$ salt as the mobile phase additive (8-10). When NH_3 is the buffer in combination with the phthlate counteranion and the mixture is used for group 2 AA elution, the CO_3^{2-}/HCO_3^- SP

due to CO_2 absorption appears before the group 2 AA peaks and all group 2 AA peaks are positive; group 1 AA peaks, Thr and lower, give negative peaks. A Trp/CO_3^{2-}/HCO_3^- mobile phase resolves group 1 and 2 AA and shifts the CO_3^{2-}/HCO_3^- SP conveniently beyond the group 2 AA, however, this also causes all group 2 AA peaks to be negative. The effect of counteranion on peak direction is discussed elsewhere (7). A phthlate/CO_3^{2-}/HCO_3^- mixture, while producing all positive AA peaks, yields a CO_3^{2-}/HCO_3^- SP that can interfere in the middle range of the group 2 AA elution. When a strong eluent counteranion, such as 1,5-NDS, is used both the OH^- and CO_3^{2-}/HCO_3^- SP appear early in the chromatogram and neither SP interferes in elution of the highly retained AA.

Figure 1 illustrates the decrease in AA retention that occurs as Trp^-, a counteranion with a large anion exchange selectivity, increases in concentration in a CO_3^{2-}/HCO_3^- buffered mobile phase. Group 1 AA are positive chromatographic peaks while group 2 AA are negative. The OH^- and CO_3^{2-}/HCO_3^- SP are in a convenient location in the chromatogram and cause no interference under these conditions. The OH^- SP occurs between the two groups while the CO_3^{2-}/HCO_3^- SP occurs after the group 2 AA. While a decrease in AA retention is expected as Trp^- concentration increases, the rate of decrease for group 2 AA is more pronounced than for group 1 AA. The CO_3^{2-}/HCO_3^- SP is affected like the group 2 AA and its SP is located at a retention time that is larger than for the most retained group 2 AA. If more CO_3^{2-}/HCO_3^- is added to the mobile phase in Fig. 1, AA retention decreases, the OH^- SP retention is

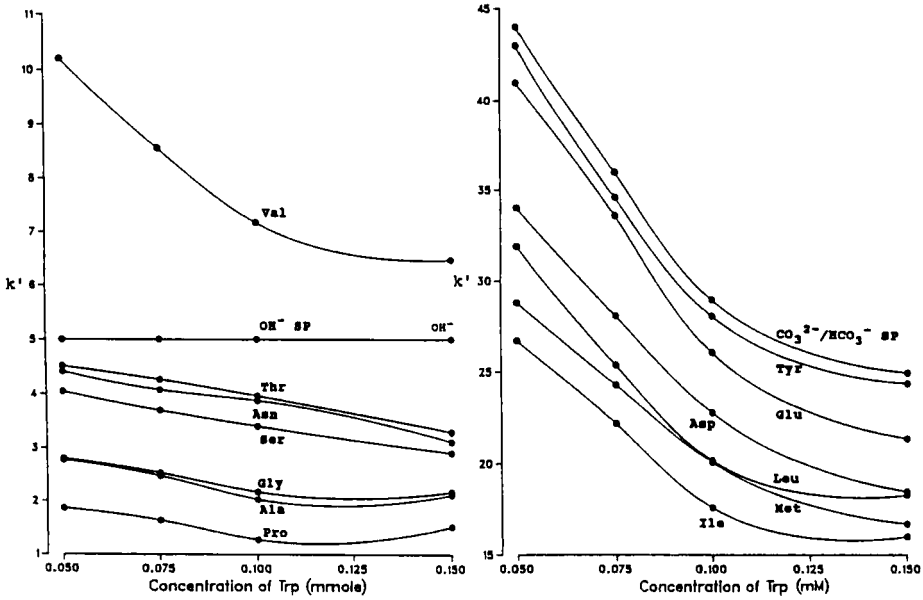

FIGURE 1. Effect of Trp Concentration on the Retention of Group 1 and Group 2 Amino Acids. Mobile phase 1 in Table I with added Trp.

about the same, and the CO_3^{2-}/HCO_3^- SP shifts to a higher retention and increases in peak area.

The effect of increasing pH on elution was studied with aqueous 0.050 mM $Ru(phen)_3(ClO_4)_2$, NaOH mobile phases. As NaOH concentration (pH) is increased AA retention is influenced by two competing affects. First, a high pH favors AA anion formation which will increase AA retention. Second, and a competing effect, is that mass action of the OH^- will decrease AA retention. The OH^-, however, has a low anion exchange selectivity and, hence, is a weak eluent counteranion. A large OH^- concentration is required

to signficantly affect the retention of the group 2-3 AA. For example, at 1.0 mM NaOH (pH = 11), group 1 AA have k' values of less than 4.0 but for valine it is 19 or significantly larger than found for all the mobile phases used in Table I. Increasing the NaOH concentration does not improve the resolution of the group 1 AA (except for Gly, Ala). The large OH^- concentration, however, will cause two chromatographic problems. First, the OH^- SP is shifted to a larger retention time at high pH and all group 1 AA peaks, which appear prior to the OH^- SP are negative. Second, the increase in mobile phase ionic strength as OH^- increases reduces detection sensitivity (7). It is for these two reasons that a mobile phase pH of 9.5 is recommended.

Figure 2 illustrates the separation and detection of several of the group 1 AA using a ClO_4^- and CO_3^{2-}/HCO_3^- (also the buffer) mixture as the counteranion. Gly and Ala coelute, Asn, Gln, and Thr overlap, and the basic AA, Arg and Lys, are not retained. The Cl^- peak comes from the His·HCl sample. If a HCl hydrolysis step is used, for example in the determination of AA in peptides, the Cl^- area will be large and dominating in this region unless the Cl^- content in the sample is reduced prior to the separation. Using other counteranions allows adjustment of AA retention and resolution and the location and area of the SP. For example, when Trp is used as the counteranion both group 2 AA and group 1 AA are separated (see Fig. 3). If CO_3^{2-}/HCO_3^- is also added to the mobile phase in Fig. 3, the CO_3^{2-}/HCO_3^- SP is shifted to a higher retention volume and causes less interference.

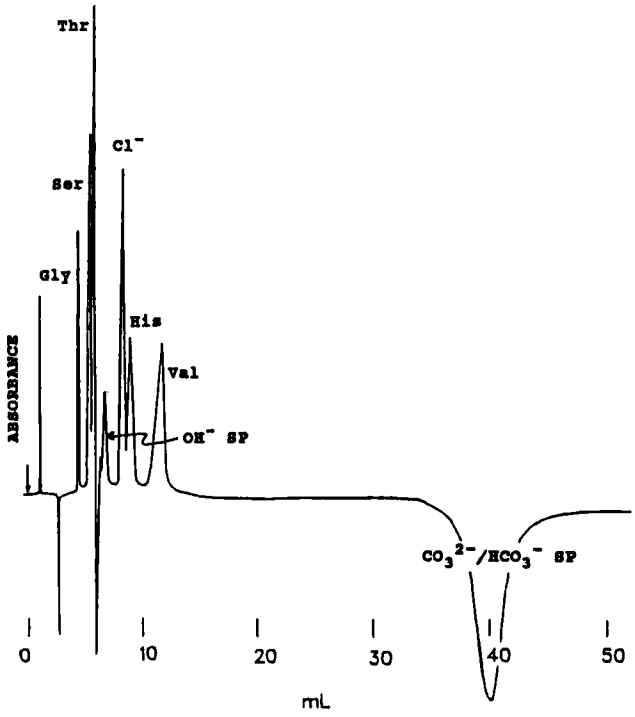

FIGURE 2. Separation of Group 1 Amino Acids. Mobile phase 4 in Table I.

A mobile phase that is more optimum for an enhanced retention and resolution of the group 2 AA is illustrated in Fig. 4. Phthalate is the counteranion and NH_3 is the buffer. Resolution of several of the group 1 AA and the OH^- SP is also possible with this eluent. If 0.1 mM Na_2CO_3 is included in the mobile phase rather than NH_3 at a pH of 9.5 retention of group 2 AA is still obtained, however, retention is lower and resolution of Asp/Glu and Ile/Tyr is not as good. At these conditions all group 1 and group 2 AA appear as positive peaks.

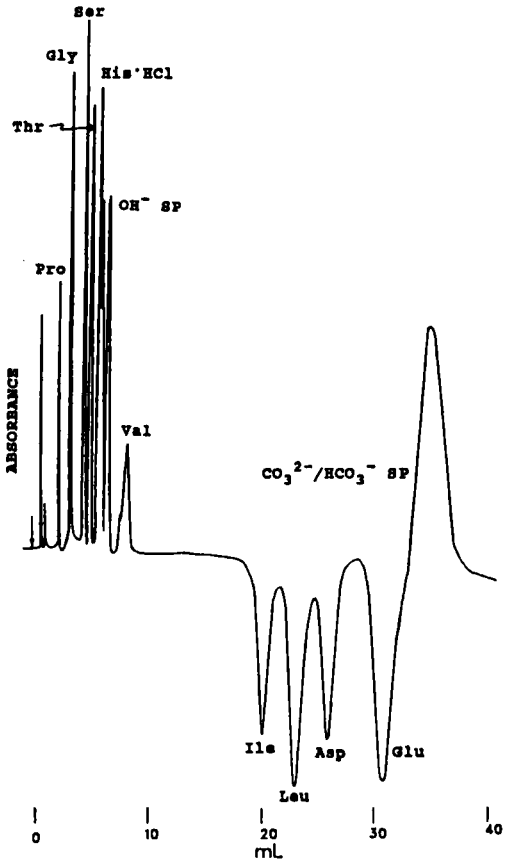

FIGURE 3. Separation of Group 1 and Group 2 Amino Acids. Mobile phase 1 in Table I.

Elution of the group 3 AA requires a strong eluent. This is illustrated in Fig. 5 where a combination of 1,5-NDS, a strong eluent counteranion because of its large anion exchange selectivity, and a high CH_3CN concentration is used for retention and resolution of the AA. Under these conditions most group 1 and group 2 AA have little or no retention.

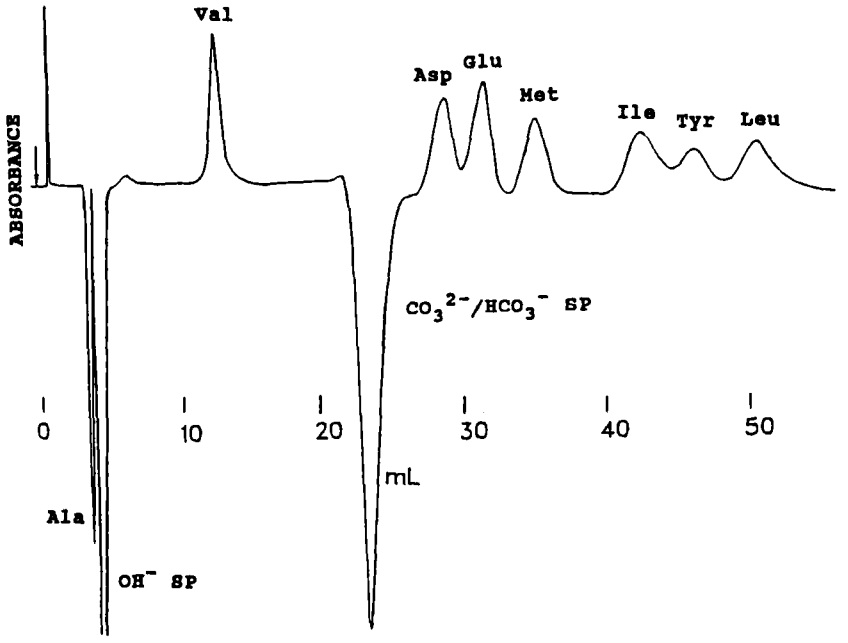

FIGURE 4. Separation of Group 2 Amino Acids. An aqueous 0.050 mM $Ru(phen)_3$phthlate, 0.10 mM NH_3, pH = 9.5 mobile phase.

Figure 6 illustrates that gradient elution and indirect detection of free AA is feasible when using a $Ru(phen)_3^{2+}$ salt as a mobile phase additive. The AA mixture contains AA from all three AA elution groups. The gradient used employs a mobile phase change where both the solvent (CH_3CN) and counteranion (CO_3^{2-}/HCO_3^-) concentration increase with elution time. Both of these mobile phase changes will increase eluent strength. However, each change will also cause a significant distortion in the baseline due to a change in background absorbance. The first causes $Ru(phen)_3^{2+}$ removal from the stationary phase surface which results in an

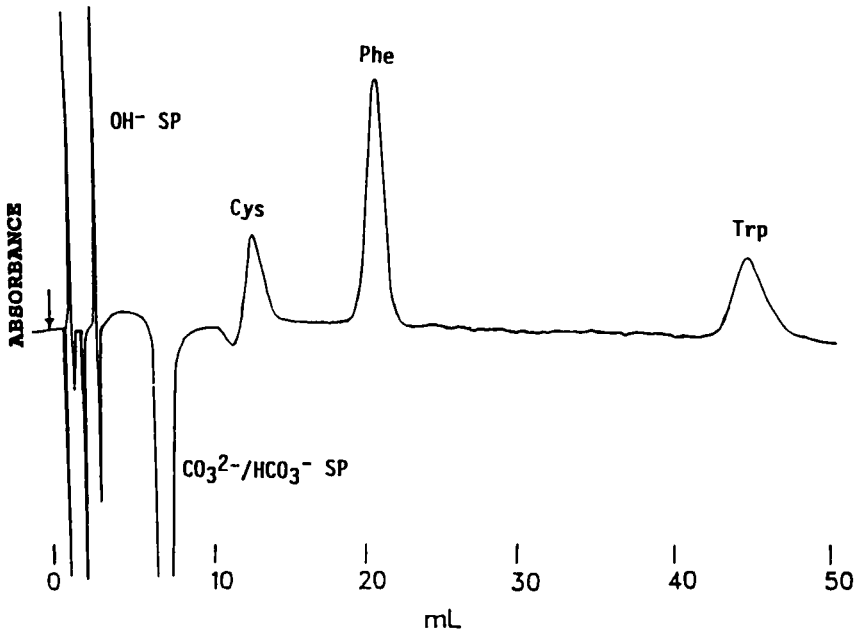

FIGURE 5. Separation of Group 3 Amino Acids. An aqueous 0.050 mM $Ru(phen)_3(HCO_3)_2$, 0.10 mM 1,5-NDS, 5:95 $CH_3CN:H_2O$, pH = 9.5 mobile phase.

increase in baseline absorbance. The latter change increases the amount of $Ru(phen)_3^{2+}$ retained on the stationary phase surface which produces a decrease in baseline absorbance with time. The solvent change is the dominate one (12) and thus, a baseline absorbance increase is observed for the gradient used in Fig. 6. Cancellation of the baseline distortion is possible, as described elsewhere (10,12), by using either: 1) a two column split stream elution where one column serves as the separator column and the other as a reference column, 2) a two channel sample and reference detection in the detector, or 3) a computer baseline cancel-

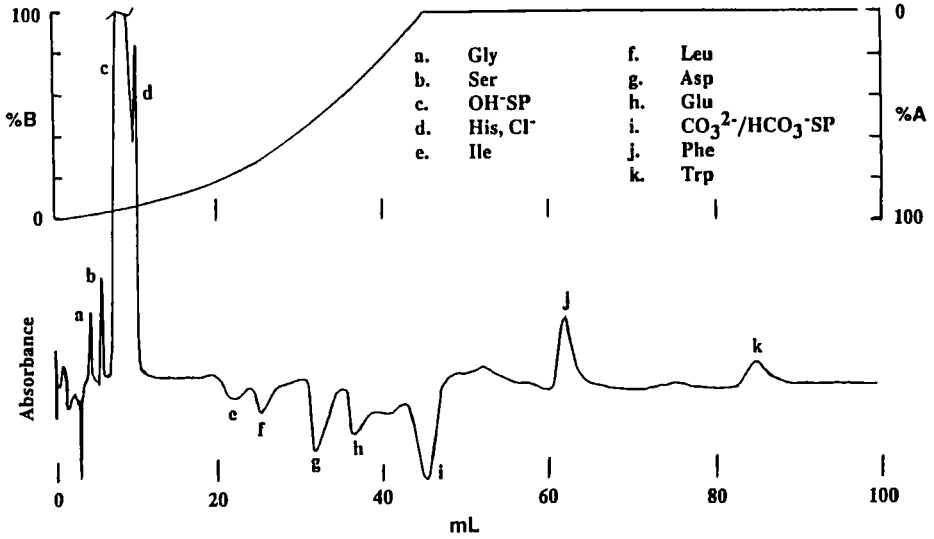

FIGURE 6. Gradient Separation of Several Group 1 to 3 Amino Acids Using a Two PRP-1 Column Baseline Compensated Technique. An (A) mobile phase of 0.050 mM $Ru(phen)_3(HCO_3)_2$, 0.050 mM $Na_2CO_3/NaHCO_3$, 2:98 $CH_3CN:H_2O$, pH = 9.5 and a (B) mobile phase of 0.050 mM $Ru(phen)_3(HCO_3)_2$, 0.50 mM $Na_2CO_3/NaHCO_3$, 6:94 $CH_3CN:H_2O$, pH = 9.5.

lation. Apparently, the presence of the two SP causes two changes in analyte peak direction for the same reasons as in the case where the mobile phase is capable of producing only one SP (7).

Calibration curves were prepared for several AA to establish detection limits and reproducibility for the isocratic separation and indirect detection of mixtures of AA. Depending on mobile phase conditions and the AA, the detection limit was about 8 to 50 ng for a 3:1 signal:noise ratio. Lower retained AA usually had lower detection limits (0.1 nmole for Ala) while higher retained

AA had higher detection limits (0.25 nmole for Ile). For Ala up to 22 μmole injected and a 0.050 μM $Ru(phen)_3(HCO_2)_2$, 0.10 mM $Na_2CO_3/NaHCO_3$, 0.050 mM Trp, 1:99 $CH_3CN:H_2O$, pH = 9.5 mobile phase the linear calibration curve corresponded to the equation Ala peak area X 10^5 = 0.0193 + 13.3 μg Ala with a linear coefficient of 1.00. For Ile up to 76 μmole injected and the same mobile phase the linear curve was defined by the equation Ile peak area X 10^5 = 0.123 + 5.42 μg Ile with a linear coefficient of 1.00. The upper limit of linearity was not determined in either case. Detection limit for a gradient elution is less favorable and is dependent on the gradient and baseline cancellation procedure used.

CONCLUSION

Separation and detection of AA are achieved through the use of a chromophoric ion interaction reagent, a $Ru(phen)_3^{2+}$ salt, as a mobile phase additive. No derivatization is involved either pre- or post-column and detection limits of 8 to 50 ng, depending on AA and eluent conditions, by the IPD strategy are favorable. Altering mobile phase solvent composition and type and concentration of counteranion can be used to change AA retention. While gradient chromatography is feasible, the procedure is complex because of the large effect of the gradient change on the background mobile phase absorbance. The IPD strategy using a $Ru(phen)_3^{2+}$ salt as a mobile phase additive is a universal detection strategy for analyte anions. Interference by other anions in AA determinations will depend on their selectivity and

the mobile phase eluent strength (6-8) and a sample matrix clean up to remove other sample anions may be required.

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