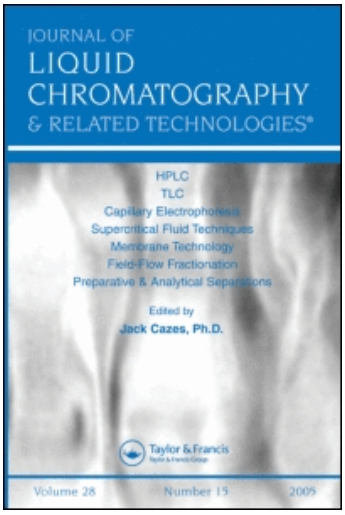

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Ion-Interaction Chromatographic Separation of Free Amino Acids

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ION-INTERACTION CHROMATOGRAPHIC SEPARATION OF FREE AMINO ACIDS

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ABSTRACT

An extensive study of the HPLC separation of 20 free amino acids by the addition of alkanesulfonate salts to the mobile phase was previously reported (1). This paper describes modifications in the procedure that improves the separation and resolution of the 20 free amino acids. Mobile phase variables (type and concentration of alkanesulfonate salt, organic modifier concentration, mobile phase pH, and mobile phase ionic strength), and stationary phase variables (particle size, type of packing) which can affect amino acid separation, resolution and selectivity were studied. Two stationary phases were compared, the 5 μm Hamilton PRP-1 and Phase Separations 3 μm, ODS-2. Longer chain alkanesulfonate salts (octane and decanesulfonate salts) were evaluated as mobile phase additives. A mobile phase gradient of increasing per cent organic modifier was necessary for separating complex mixtures of polar and nonpolar-basic amino acids. It is now possible to separate 19 of 20 free amino acids with this ion-interaction chromatographic procedure.

INTRODUCTION

The separation and identification of amino acids is an important analytical tool in the biological sciences. The

separation and quantitation of amino acids in real samples and hydrolyzed peptides has been studied for many years dating back to Stein and Moore (2-4). Several methods have been used for amino acid analysis, e.g. gas chromatography (5) and ion exchange chromatography (5,6),and reversed phase chromatography with pre-column derivatization (5,6). However, each method is limited.

Amino acids are difficult to separate by gas chromatography unless a volatile derivative is formed prior to injection. The major problem associated with this technique is the irreproducibility in the separation and quantitation of the amino acids due to difficulty in derivatization. Ion exchange chromatography had been limited, until recently, by long analysis times and poor column efficiencies due to difficulty in obtaining small, spherical packings. Pre-column derivatization followed by reversed phase chromatography is by far the most popular method used in amino acid analysis. The amino acid derivatives are not separated according to their structures, but according to the chemical properties of the derivative formed. Quantitation and reproducibility are inherent problems associated with pre-column derivatization. Stability of the amino acid derivative can also affect the chromatography, especially if ortho-phthalaldehyde (OPA) is used for amino acid derivatization. The half-life of the OPA derivative is approximately 15 minutes (7).

One way to eliminate the derivatization step for the separation of amino acids is by the addition of an alkanesulfonate salt (RSO_3^-) to the mobile phase (1). The amino acids are separated according to their own chemical properties rather than

the chemical properties of their derivatives. Therefore, the problems associated with reproducibilty are eliminated.

Chromatographic separations have also been improved by the introduction of small, spherical particles which provide columns of high efficiencies (8). These particles lead to higher loadings of the RSO_3^- salt onto the stationary phase, hence, more ion exchange sites where the cationic amino acids may interact. The increased ion exchange capacity should lead to higher amino acid retention, better selectivity, and improved resolution.

This report focuses on improvements in ion-interaction chromatographic separations of amino acid. These improvements were accomplished by increased column efficiency and the use of longer chain RSO_3^- salt mobile phase additives.

EXPERIMENTAL

Amino acids were obtained from Sigma Chemical Company. Alkanesulfonate salts, 2-mercaptoethanol, and ortho-phthaladehyde (OPA) were obtained from Aldrich Chemical Company. HPLC grade acetonitrile was obtained from Matheson Coleman Bell. LC quality water was obtained by passing distilled water through a Millipore water purification unit. A Spectra Physics Model 8700 solvent delivery system, Rheodyne Model 7125 injector, Kratos Model URS051 post-column system, Wescan Model UVIDEC-100-V variable wavelength detector (340 nm) and Spectra Physics Model 4270 integrator were used. Both the analytical and post-column pumps were operated at flow rates of 1.00 mL/min. The reversed phase columns were obtained from Hamilton Company (PRP-1, 4.1 x 150 mm, 5 μm

spherical polystyrenedivinylbenzene copolymer) and Phase Separations, Inc. (Spherisorb ODS-2, 4.6 x 150 mm, spherical, 3 μm, alkyl modified silica).

Aqueous amino acid samples of approximately 1 mg/mL were used. Typical operating conditions involved a 0.5 to 1 hour equilibration period after column breakthrough of the alkanesulfonate salt (RSO_3^-) mobile phase additive. Sample aliquots of 2 to 10 μL were used, and inlet pressures were 2400-3700 psi. The post-column derivatization solution (1,5,6) used for detection was prepared as follows: 0.50 g OPA was dissolved in 30 mL of 95% ethanol. The ethanolic solution was added to 500 mL of a 1.00 M H_3BO_3 and 0.0286 M 2-mercaptoethanol solution (adjusted to pH=10.0 with KOH). The resulting solution was added to a 1L volumetric flask and diluted to volume with LC quality water.

RESULTS AND DISCUSSION

Enhanced retention of inorganic and organic ions on reversed stationary phases from a mobile phase containing a hydrophobic ion of opposite charge is determined by two major equilibria (1, 9-11). The first accounts for the retention of the hydrophobic ion on the stationary phase, the second describes the ion exchange selectivity between the analyte ion and the counterion associated with the retained hydrophobic ion. These equilibria, for an alkyl sulfonate (RSO_3^-) salt as the mobile phase additive, are shown by equations 1 and 2, respectively.

$$A + RSO_3^-C^+ \rightleftharpoons A\cdots RSO_3^-C^+ \quad (1)$$

$$A\cdots RSO_3^-C^+ + X^+ \rightleftharpoons A\cdots RSO_3^-X^+ + C^+ \quad (2)$$

In these equations, A is the stationary phase, C^+ is the counterion provided by the RSO_3^- salt, the buffer, and/or ionic strength salt, and X^+ is the analyte ion. Additional equilibria may also become significant as the hydrophobic center within the mobile phase additive and/or the concentration increases (9-11).

Amino acids are protonated under acidic conditions. Therefore, according to eqs. 1 and 2, enhanced retention should take place in an acidic mobile phase containing an RSO_3^- salt additive. Major mobile and stationary phase parameters that may influence amino acid retention and selectivity include (1, 9-11): the type of stationary phase used, the hydrophobic character of the added RSO_3^- salt, mobile phase ionic strength, the concentration of organic modifier in the mobile phase, the concentration of RSO_3^- salt, and the kind of counterion provided by the buffer and/or ionic strength salts added to the mobile phase.

The optimized mobile phase conditions from previous studies for the separation of amino acids (1) were 0.001 M $RSO_3^-C^+$, 0.010 M HCl (pH=2.0), and 100% H_2O for the polar-acidic amino acids. A two step gradient of 7.5% and 15.0% acetonitrile was required for the nonpolar-basic amino acids, or a mixture of the two groups of amino acids. The nonpolar-basic amino acids were well resolved,

but the polar-acidic amino acids were difficult to resolve because of poor selectivity and low retention. By focusing studies on the latter group, a separation for 19 of 20 amino acids is now possible.

Figure 1 shows the separation of the polar-acidic amino acids where the mobile phase additive is $C_8SO_3^-Na^+$. Two different columns were used. Figure 1A shows a chromatographic separation on a 3 μm, Spherisorb ODS-2 column, while Figure 1B shows the separation on a 5 μm, PRP-1 column. The amino acid separations on both stationary phases are similiar, although the ODS-2 column provided better resolution than the PRP-1 column. The increased resolution can be attributed to the higher efficiency of the ODS-2 column relative to the PRP-1 column. On the ODS-2 stationary phase Gly and L-Ser co-eluted, while L-Ala appeared as a shoulder on the L-Glu peak. With the PRP-1 column, Gly and L-Ser co-eluted as did L-Glu and L-Ala.

A separation was also attempted on all 20 amino acids. A acetonitrile step gradient was used in order to elute the amino acids in a reasonable amount of time. Post-column derivatization with ortho-phthalaldehyde (OPA) was used with detection at 340 nm, because direct detection of the amino acids at 210 nm resulted in a drifting baseline and extraneous peaks. L-Cys and L-Pro can not be detected with OPA because the OPA adduct of L-Cys has a low molar absorptivity, and L-Pro will not react with OPA in the absence of sodium hypochlorite.

Figure 2 shows the step gradient separation, using the ODS-2 column, in which mobile phase A was composed of 0.001 M $C_8SO_3^-Na^+$,

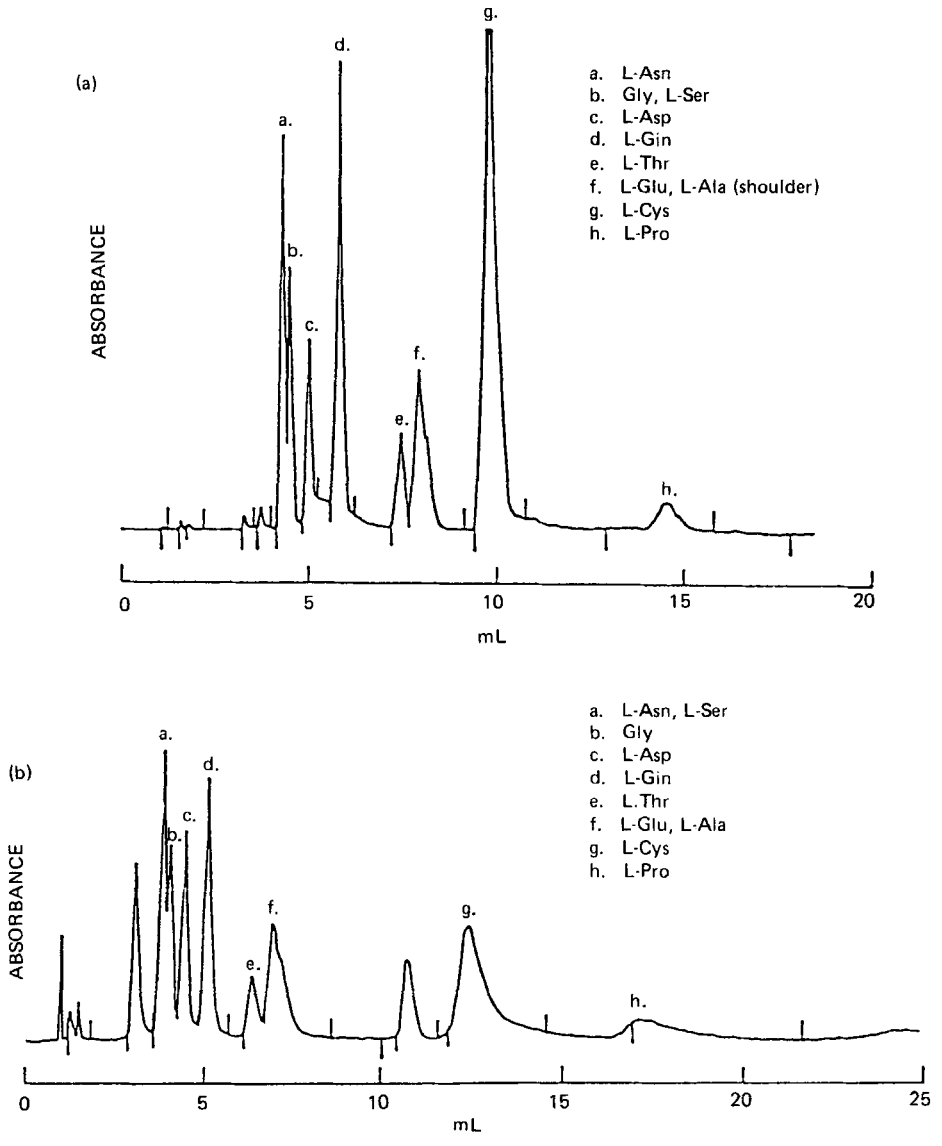

FIGURE 1

Separation of Polar Amino Acids on C-18 and PRP-1 Stationary Phases using $C_8SO_3^-Na^+$ as the Mobile Phase Additive

An aqueous mobile phase of 0.0010 M $C_8SO_3^-Na^+$, 0.010 M HCl, μ=0.011 M with a flowrate of 1.0 mL/min: (A) 4.6 x 150 mm C-18 column and (B) 4.1 x 150 mm PRP-1 column.

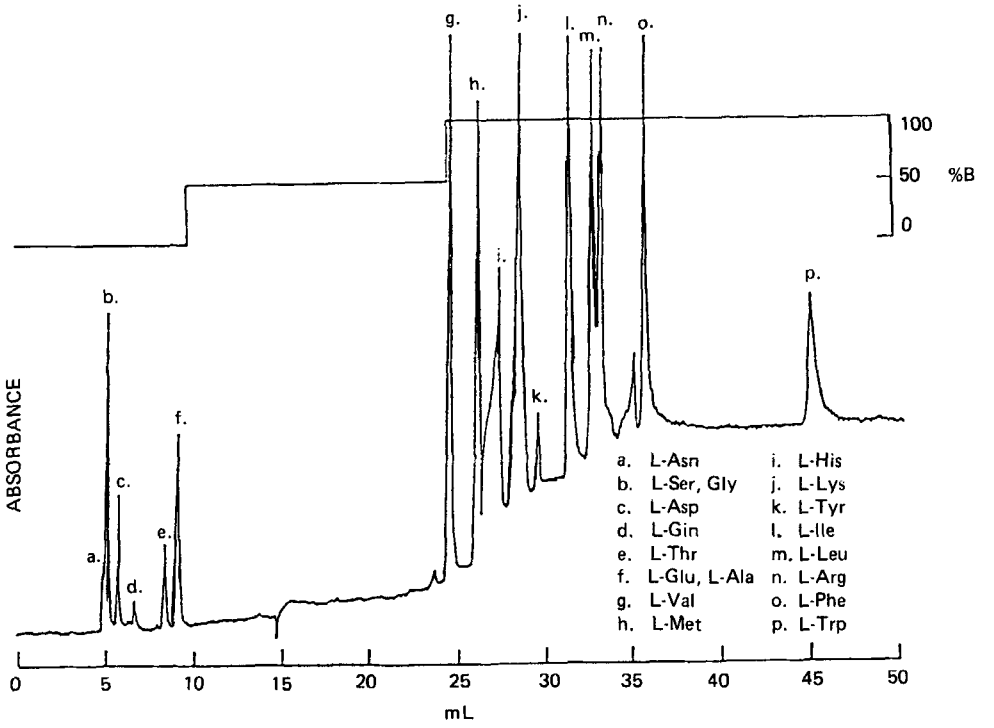

FIGURE 2
Separation of Amino Acids on C-18 Stationary Phase using a Step Gradient

A step gradient with an A solvent of 0.0010 M $C_8SO_3^-Na^+$, 0.010 M HCl, 100% H_2O and a B solvent of the same except 15:85 CH_3CN:H_2O at 1.0 mL/min.

0.010 M HCl, 100% H_2O. Mobile phase B differed from mobile phase A only in that the former contained 15% acetonitrile. The column was re-equilibrated for 30 minutes with 100% mobile phase A prior to injection. The ODS-2 stationary phase provided an excellent separation of the amino acids. A typical separation was observed

for the polar-acidic amino acids with L-Ser, Gly and L-Glu, L-Ala co-eluting. L-Cys and L-Pro, if detected by OPA post-column detection, would have retention times of 10 and 14.5 minutes, respectively. The nonpolar-basic amino acids were well resolved and no co-elution was observed. The elution order of the nonpolar-basic amino acids differed from that observed on a strong cation exchange column (12) and is typical of reversed phase amino acid chromatography (13-15). This difference is due to an increase in mobile phase CH_3CN concentration after the step gradient, which removes $C_8SO_3^-$ from the stationary phase. During this stage of the chromatography, the cation exchange sites are being removed from the stationary phase. The faster CH_3CN is added to the mobile phase, the faster the $C_8SO_3^-$ is removed from the column and the greater the affect on amino acid elution order. Retention of the nonpolar-basic amino acids are solvent dependent and are influenced by amino acid side chain structure, amount of CH_3CN in the mobile phase and the amount of $C_8SO_3^-$ sorbed onto the stationary phase (13-16).

Since co-elution of the polar-acidic amino acids was still observed with the more efficient ODS-2 column, a longer chain RSO_3^- salt was studied to determine whether the longer chain salt would increase the number of cation exchange sites on the packing; thereby increasing amino acid retention, and improving selectivity and resolution. Figure 3 shows the separation of the polar-acidic amino acids on a PRP-1 and an ODS-2 column, where $C_{10}SO_3^-Na^+$ was added to the mobile phase. The mobile phase conditions in both

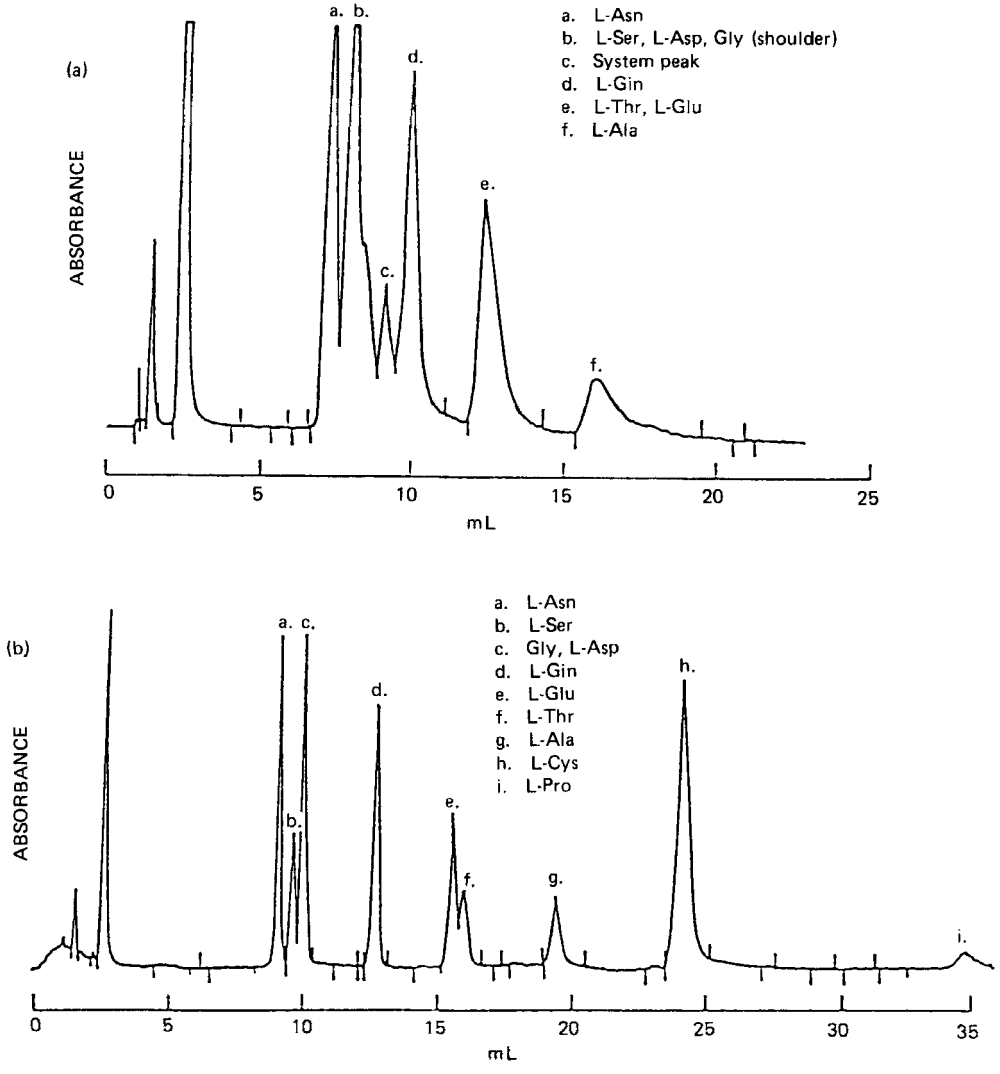

FIGURE 3

Separation of Polar Amino Acids on C-18 and PRP-1 Stationary Phases using $C_{10}SO_3^-Na^+$ as the Mobile Phase Additive

An aqueous mobile phase of 0.0010 M $C_{10}SO_3^-Na^+$, 0.010 M HCl, μ=0.011 M with a flowrate of 1.0 mL/min: (A) PRP-1 column and (B) C-18 column.

chromatograms consisted of 0.001 M $C_{10}SO_3^-Na^+$, 0.010 M HCl, 100% H_2O. Figure 3A shows the amino acids separation using the ODS-2 column. It is observed that resolution and selectivity are improved, and that 9 of 10 amino acids are resolved. These mobile phase conditions, combined with the Sherisorb 3 μm ODS-2 column provided the best overall separation of the polar-acidic amino acids. Figure 3B shows the separation of the amino acids on the PRP-1 column, where the resolution and selectivity of the amino acids was much poorer with $C_{10}SO_3^-Na^+$ as compared to the $C_8SO_3^-$ salt. In both cases 210 nm was used instead of post-column detection in order to detect L-Cys and L-Pro.

A two step gradient elution (using the ODS-2 columns) was also performed to determine how the $C_{10}SO_3^-$ salt would affect the elution order of the nonpolar-basic amino acids relative to the $C_8SO_3^-$ salt. The two mobile phases were identical to those used in Figure 2 with detection at 340 nm (OPA). The step gradient provided favorable elution of all the amino acids with the exception of Gly and L-Asp. A chromatogram of the separation is shown in Figure 4. L-Trp, although not included in this separation, was injected as a single analyte and found to have a retention time of 95 minutes. L-Cys and L-Pro, which were studied at 210 nm, had retention times of 21 and 26.5 minutes, respectively. A change in the baseline was observed just before the L-Arg peak, and was observed only when L-Arg was injected. We currently have no explanation for this phenomenon,but it may be due to a mixed retention mechanism for L-Arg. Re-equilibration of 45 minutes was required in order to obtain reproducible results.

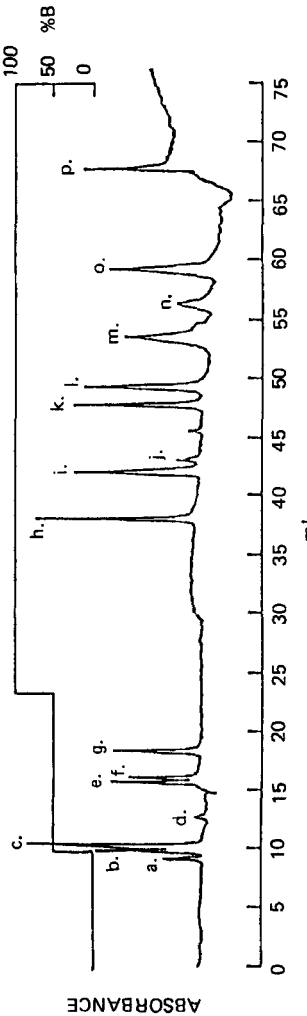

FIGURE 4

Separation of Amino Acids on C-18 Stationary Phase using a Step Gradient

A step gradient with an A solvent of 0.0010 M $C_{10}SO_3^-Na^+$, 0.010 M HCl, 100% H_2O and a B solvent of the same except 15:85 $CH_3CN:H_2O$ at 1.0 mL/min.

Overall, the separation obtained with the $C_{10}SO_3^-$ salt provided excellent resolution and good selectivity, with co-elution of only one amino acid pair.

CONCLUSION

The separation of 19 of 20 free amino acids by the addition of an RSO_3^- salt to the mobile phase can be accomplished in a reasonable amount of time, with good resolution and selectivity, and without the need for pre-column derivatization. The higher percent of carbon bonded to the 3 μm ODS-2 column relative to other bonded phase columns led to an increase in amino acid resolution. This is most likely due to an increase in the amount of RSO_3^- salt sorbed to the stationary phase, which increases the number of cation exchange sites. The ODS-2 column provided better amino acid separations than the PRP-1 column did due to increased efficiency. The method is reproducible from run to run, provided adequate re-equilibration of the weaker eluent is allowed.

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