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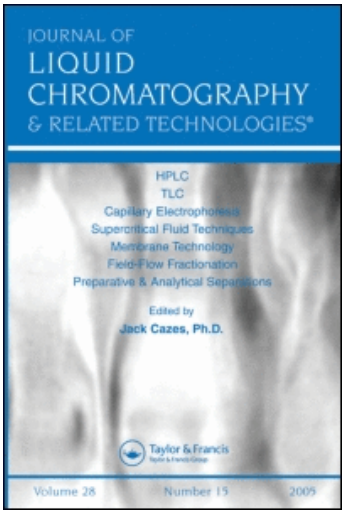

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AMINO ACID PROFILING OF PROTEIN HYDROLYSATES USING LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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ABSTRACT

A liquid chromatography procedure is described for separating the amino acids in protein hydrolysates. The proteins are hydrolyzed with hydrochloric acid and an aliquot of the hydrolysate is derivatized with dansyl chloride reagent. The derivatization procedure takes only 2 minutes using a reaction temperature of 100 °C. The dansylated amino acids are chromatographed using a reversed-phase C_8 column and a multi-step, non-linear gradient elution solvent program which is readily achieved using a microprocessor-controlled liquid chromatograph. Chromatography is complete in approximately 40 min. The procedure is useful for characterizing proteins and may also be used to analyse intact dansylated polypeptides. Chromatograms showing the amino acid profile of chymotrypsin, albumin and histone are given.

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INTRODUCTION

The use of dansyl chloride as a reagent for preparing strongly fluorescent derivatives of a wide range of organic compounds has been well documented (1-5). Dansyl chloride will react readily with primary and secondary amines at a pH of 9 to 9.5, and with phenols at a pH of about 10.5.

We have been developing liquid chromatography procedures in our laboratory using pre-column derivatization with dansyl chloride for determining compounds of biochemical importance (6-10). Two of these reports (9,10) describe the determination of amino acids in biological fluids.

The determination of the amino acid composition of proteins has often been performed using classical ion-exchange liquid

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chromatography. An excellent review of this subject has been published (11). These procedures generally require the use of specialized equipment and a long analysis time. A single sample analysis often takes several hours to complete. The technique does permit resolution of most amino acids and provides good detection limits for them after post-column derivatization with ninhydrin or a fluorescing derivatizing reagent.

More recently, gas chromatography procedures have been developed for determining amino acids. A common requirement of these procedures is the formation of a double derivative of the amino acid to make them sufficiently volatile for GC analysis. Numerous derivatives have been suggested for this purpose. An example is the n-propyl, N-acetyl derivatives (12). These derivatives are formed in a two-step procedure which requires approximately 30 minutes to complete. The relatively large amount of sample handling involved appears to have curtailed the use of this technique on a more routine basis.

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The significant developments in the use of liquid chromatography over recent years has made it the method of choice for many analyses. Very recently a number of publications have appeared describing the use of this technique for separating the amino acids in protein hydrolysates, after dansylating the hydrolyzed amino acids (13,14). Both these procedures make use of reversed-phase chromatography with C_{18} bonded phase columns. An earlier procedure by Bayer, et al. (15), described the use of normal phase chromatography for separating dansylated amino acid standards.

We have developed a liquid chromatography procedure using a 10 μm, C_8, reversed-phase column with a multi-step gradient elution program. The use of the microprocessor controlled Series 3 liquid chromatograph permits very wide flexibility in the choice of a gradient elution profile. The procedure allows the separation of most of the common protein amino acids in approximately 40 minutes.

Norleucine is completely separated from the other amino acids and may be used for internal standardization. The derivatization procedure takes only 2 minutes, thereby providing minimal sample handling.

A problem associated with maintaining retention time reproducibility of the amino acids has in large part been resolved by careful control of the mobile phase pH. We have found that minimal operator attention is required to maintain this reproducibility once the separation has been optimized for a particular chromatographic column. Changing columns will however require an optimization of the chromatography conditions in order to obtain similar retention time characteristics for the amino acids.

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MATERIALS AND METHODS

Apparatus

We used a Perkin-Elmer Model Series 3 liquid chromatograph equipped with a Model LC-65T variable wavelength uv detector and oven module, a Model LC-420 automatic sample injector with a 10 µL injection loop and a Model 650-10LC fluorescence detector. An RP-8 reversed-phase column (Perkin-Elmer part no. 258-1484, 0.46 x 25 cm; particle size 10 µm) was used. All chromatograms were recorded using a Perkin-Elmer Model 56 recorder.

Special glassware included: 16 x 100 mm PTFE-lined screw-capped tubes, 2 mL PTFE-lined capped sample vials, 10 µL, 20 µL and 50 µL disposable glass pipets and a 1 mL Hamilton gas-tight syringe (Perkin-Elmer Part No. 023-0118). A test tube heating block and an evaporation manifold with a source of dry air were also used. A Model W-185 sonifier, from Branson Sonic Power Co., Danbury, CT, was used for degassing the mobile phase solutions.

Reagents and Standards

Acetonitrile, distilled in glass, uv grade, and acetone, distilled in glass, were obtained from Burdick and Jackson Laboratories, Inc., Muskegan, MI 49442. Dansyl chloride, 100 g/L in

acetone, was obtained from Pierce Chemical Co., Rockford, IL 61105. Sodium bicarbonate, "analyzed reagent", was obtained from Fisher Scientific Co., Pittsburgh, PA 15219. Sodium hydroxide, hydrochloric acid and phosphoric acid, reagent grade, were obtained from J.T. Baker Chemical Co., Phillipsburg, NJ 08865.

The individual amino acid standards were obtained from Calbiochem, San Diego, CA 92112. Polypeptides and protein materials were obtained from Sigma Chemical Co., St. Louis, MO 63178.

Individual standard stock solutions of each amino acid were prepared by dissolving the amino acid in 0.01N HCl to provide a concentration of 20 mmol/L. A working solution of norleucine was prepared at a concentration of 2 mmol/L. Solutions of the polypeptides and proteins were prepared in 0.01N HCl to provide a concentration of 1 mg/mL. The solutions were stored at 4 °C.

The dansyl chloride working solution was prepared by diluting 0.1 mL of the dansyl chloride stock solution to a volume of 8 mL with acetone. The reagent was prepared freshly each day. The stock solution of dansyl chloride was stored at 4 °C. The buffer was prepared by adjusting the pH of a 0.1 mol/liter sodium bicarbonate solution to 10.5 with 0.5N NaOH. The buffer solution was passed through a 0.45 µm filter (Millipore Corp., Bedford, MA 01730) before use.

The mobile phase solutions were prepared as follows. Pump A contained acetonitrile to which acetic acid was added to provide a concentration of 0.1 mL/L and phosphoric acid was added to provide 0.77 mL/L. Pump B contained 10 mmol/L sodium acetate buffer to which was added acetic acid to provide a concentration of 0.1 mL/L. The pH of the mobile phase solution in pump B was adjusted to 3.00 by addition of phosphoric acid. The deionized water was passed through a 0.45 µm filter before use. The mobile phase solvents were degassed using sonification at 90 w for 5 minutes.

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Protein Hydrolysis

One mg of the protein was placed into a 16 x 100 mm PTFE-lined screwcapped tube and 1 mL of 6N HCl was added. The tube was capped tightly and heated for 16 hours at 110 °C in a block heater. The tube was cooled and 20 µL of the hydrolysate was transferred to a 2 mL sample vial. Ten µL of the norleucine internal standard solution was added to the vial and the contents evaporated to dryness.

Derivatization Procedure

To the evaporated amino acid sample contained in a 2 mL sample vial was added 40 µL of pH 10.5 buffer followed by 100 µL of dansyl chloride reagent working solution. The vial was capped and mixed vigorously for 15 sec. The vial was heated at 100 °C for 2 min in a block heater. The vial was removed and allowed to cool. One mL of a 50:50 (v/v) mixture of the two mobile phase solutions was added to the vial using a 1 mL gas-tight syringe and mixed for 15 sec. Ten µL was injected into the liquid chromatograph.

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CHROMATOGRAPHY

The mobile phase flow rate was 1.5 mL/min and the column temperature was maintained at 50 °C. The Model 650-10LC fluorescence detector was set to an excitation wavelength of 298 nm and an emission wavelength of 546 nm. The bandwidths of both monochromators were set at 10 nm.

Fig. 1 shows a profile of the multi-step mobile phase gradient we used. The gradient consisted of a five step solvent program which allowed us to increase significantly the separation of the amino acids over that which had been obtainable when single step linear gradients were evaluated. After purging the system at 100% acetonitrile for 4 min the column was equilibrated with the initial starting mobile phase composition for 15 min. The use of the Model LC-420 sample injector provided automatic injection of the sample at pre-set times and allowed identical equilibration periods

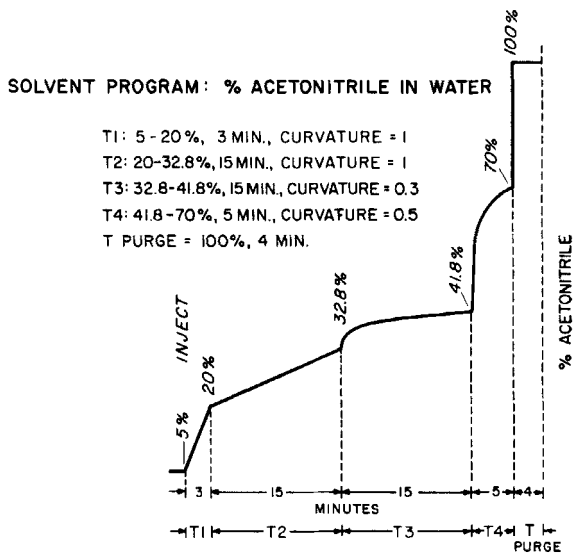

Figure 1 Schematic showing the mobile phase gradient elution program used.

between sample injections. It should be noted that Fig. 1 shows the profile established by the pumps. The actual acetonitrile percentage at the head of the column at any time during the gradient run is slightly less due to the void volume of the chromatographic system.

Fig. 2 shows a chromatogram obtained from the injection of a 20 µL aliquot of a reaction mixture containing approximately 17.4 nanomoles of 24 different amino acid standards. This corresponds to approximately 13 to 44 ng of each amino acid injected on column, depending on the molecular weight of the amino acid.

RESULTS

Table I lists the retention time and the relative retention, r_{is}, of each compound studied using norleucine as the reference. The last amino acid eluting under these conditions is tyrosine which has a retention time of about 40 min. Therefore, the chroma-

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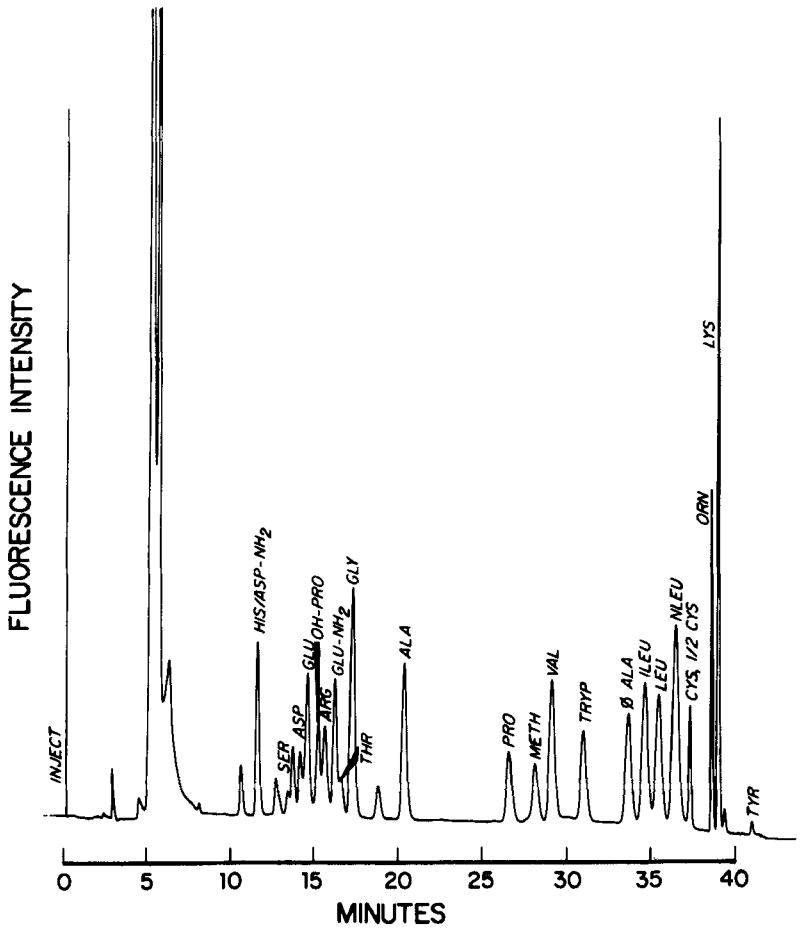

Figure 2 Chromatogram showing the separation of a 24 amino acid mix. Each peak corresponds to approximately 0.3 nanomoles of each amino acid on column. (The symbols used are listed in Table I)

tography time together with the derivatization and mobile phase equilibration times permits the analysis of one sample approximately every hour.

Figs. 3,4 and 5 illustrate the chromatograms we obtained when we processed three hydrolyzed protein samples: albumin,

TABLE I

Retention Time and Relative Retention of Derivatized Amino Acid Standards

AMINO ACID	SYMBOL	RETENTION TIME (MIN)	r_{is}*
Histidine	HIS	11.32	.277
Asparagine	ASP-NH_2	11.47	.281
Serine	SER	13.43	.338
Aspartic Acid	ASP	13.92	.352
Glutamic Acid	GLU	14.31	.363
Hydroxyproline	OH-PRO	15.03	.384
Arginine	ARG	15.42	.396
Glutamine	GLU-NH_2	16.02	.413
Threonine	THR	16.38	.424
Glycine	GLY	17.00	.442
Alanine	ALA	20.11	.532
Proline	PRO	26.34	.713
Methionine	METH	27.86	.757
Valine	VAL	28.89	.787
Tryptophan	TRYP	30.69	.839
Phenylalanine	ϕALA	33.44	.919
Isoleucine	ILEU	34.42	.948
Leucine	LEU	35.29	.973
Norleucine	NLEU	36.22	1.000
Cystine	CYS	37.09	1.025
Cysteine	1/2 CYS	37.09	1.025
Ornithine	ORN	38.35	1.062
Lysine	LYS	38.79	1.075
Tyrosine	TYR	40.73	1.131

*Relative to Norleucine

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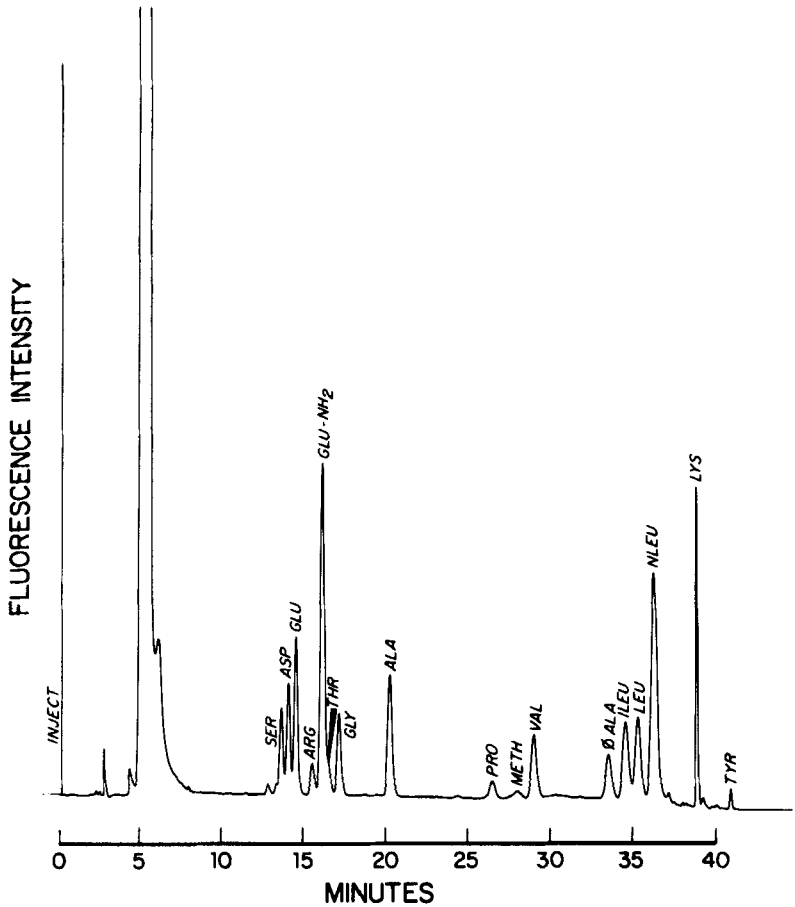

Figure 3 Chromatogram showing the amino acid profile of albumin hydrolysate. (The symbols used are listed in Table I)

chymotrypsin and histone. Each chromatogram is the result of injecting approximately 200 ng of hydrolyzed protein onto the column. In each case, norleucine was added prior to derivatization to provide relative retention data.

To further demonstrate the potential of this technique in protein work, we dansylated some intact polypeptide materials and

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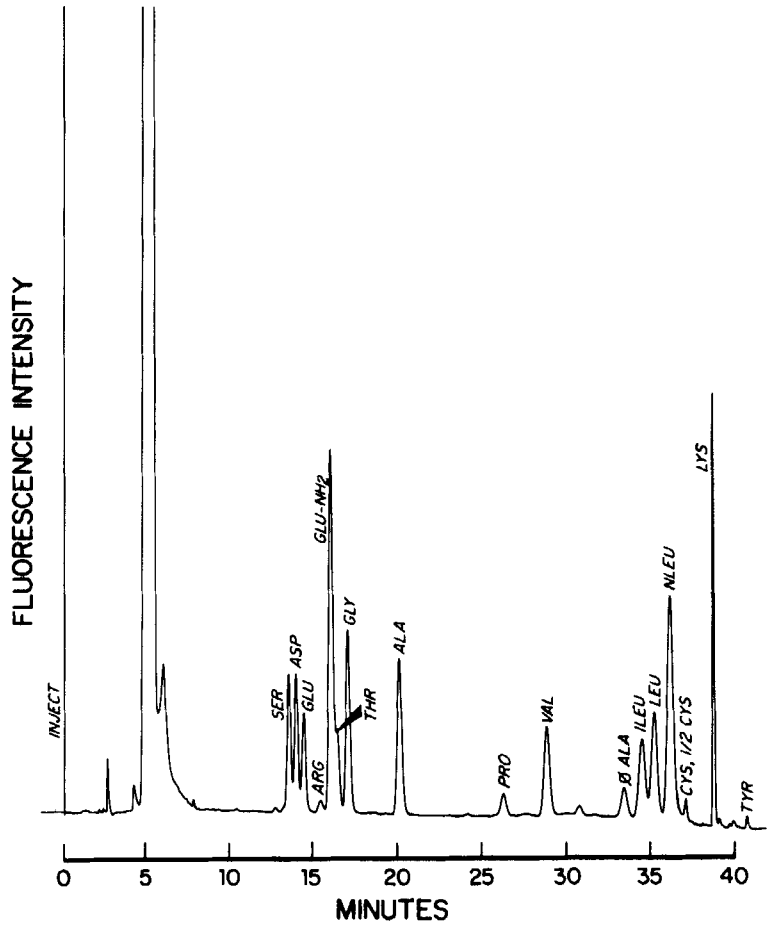

Figure 4 Chromatogram showing the amino acid profile of chymotrypsin hydrolysate. (The symbols used are listed in Table I)

chromatographed them without prior hydrolysis. Figure 6 shows a chromatogram illustrating the separation of the dansyl derivatives of some dipeptides. Figure 7 shows the separation of a mixture of polypeptides containing varying numbers of glycine residues. The

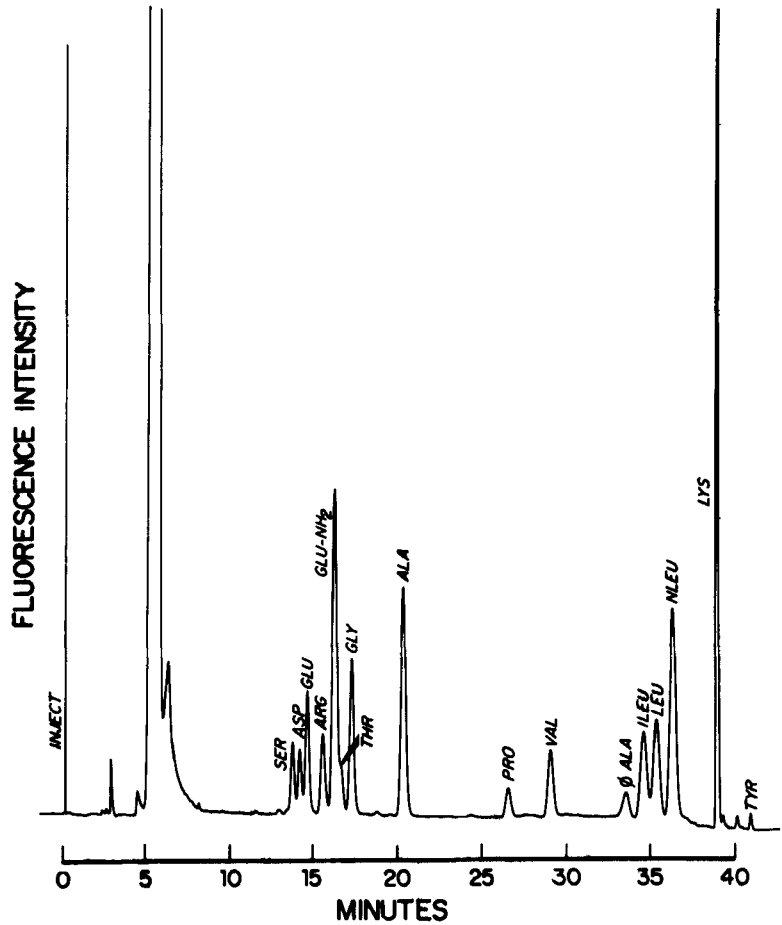

Figure 5 Chromatogram showing the amino acid profile of histone hydrolysate. (The symbols used are listed in Table I)

actual chromatography conditions used for separating the glycine polypeptide mix is listed in the figure legend.

DISCUSSION

The use of modern liquid chromatography for separating the protein amino acids offers a useful alternative to the more fre-

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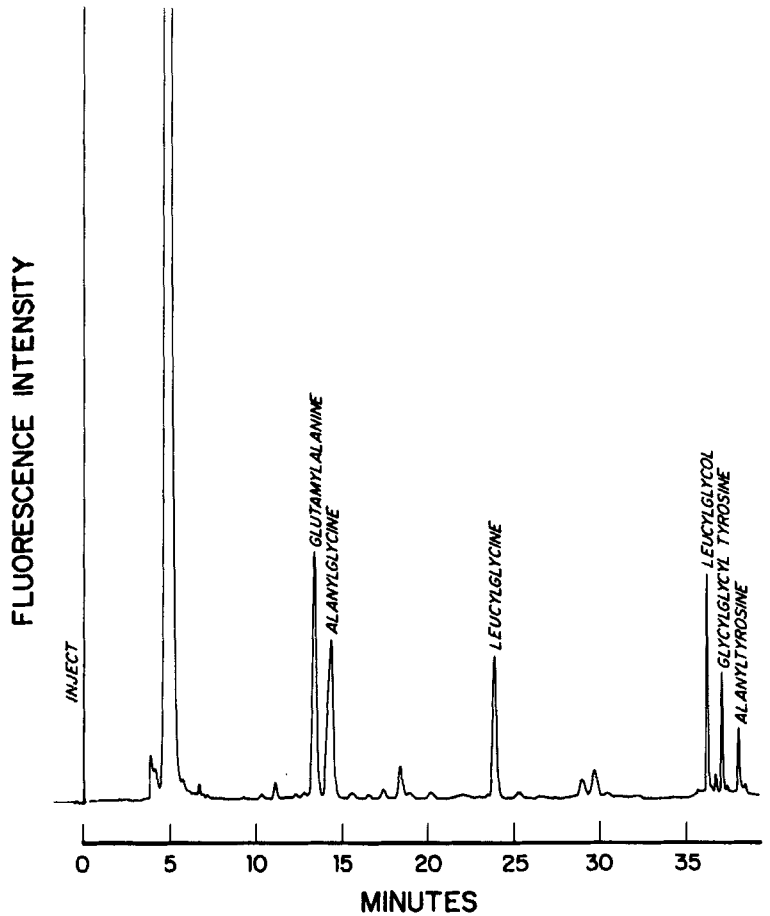

Figure 6 Chromatography of a dansylated dipeptide mix.

quently used methodologies which use ion-exchange systems or gas chromatography. The fast reaction times which may be utilized for preparing the dansyl derivatives and the use of automated sample injection offers a liquid chromatography procedure which allows unattended operation.

The chromatography conditions described permits separation of many of the amino acid derivatives. The procedures we have used does

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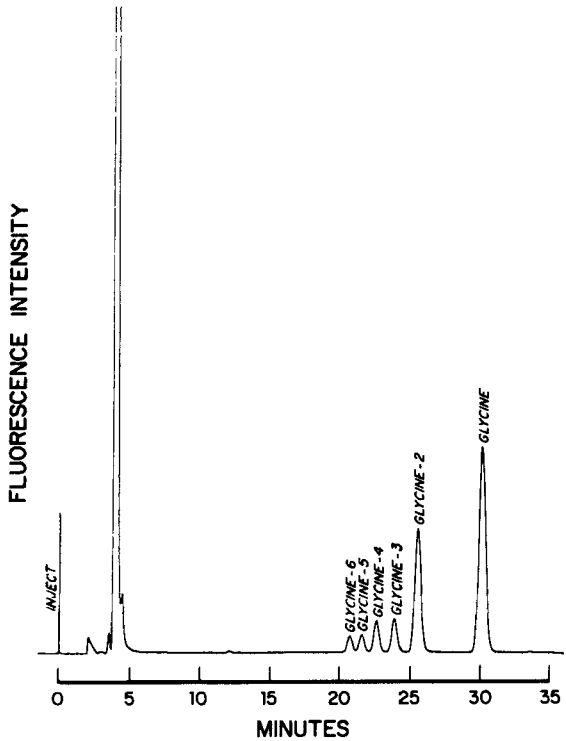

Figure 7 Chromatography of dansylated glycine polypeptides. Mobile phase linear gradient from 5-50% acetonitrile at 1%/minute. Flow rate is 1.5 mL/min and column temperature is 50 °C.

not separate the dansyl derivatives of histidine and asparagine but this does not present a problem for most applications. Cystine and cysteine are not separated, perhaps because with these reaction conditions the cystine is reduced to cysteine during the derivatization procedure. Also, a reagent peak was found to coelute with glutamine.

As with any chromatographic procedure which separates many compounds in a relatively short period of time, careful control of

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the operating conditions must be exercised. Even small changes in mobile phase pH will shift even the relative retention of some of the amino acids. We routinely prepared our mobile phase pH to exactly 3.00 and by doing so have been able to maintain quite acceptable long term reproducibility. The use of other columns of the same packing material may require slight optimization of the chromatography conditions to permit similar separations as we have described.

It should be pointed out that some amino acids are partially or completely destroyed during acid hydrolysis. Tryptophan, for example, cannot be determined after acid hydrolysis. Alternate hydrolysis procedures have been developed when specific amino acids must be determined. For example, alkaline hydrolytic procedures using barium hydroxide will permit tryptophan to be recovered (16). However, acid hydrolysis has been shown to provide adequate recoveries of most amino acids to provide useful characterization of proteins.

Reversed-phased columns display significant variations in selectivity even for nominally similar packing materials. Thus the chromatography conditions must be optimized when a new column is used. The procedure for such optimization has been discussed (17).

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