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COMPARISON OF REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR PRECOLUMN-DERIVATIZED AMINO ACIDS

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ABSTRACT

A comparison was made among five precolumn derivatization techniques for amino acid analysis using reverse-phase high-performance liquid chromatography (HPLC). All chromatographic analyses were conducted using the same instrumentation and a C₁₈ Ultrasphere ODS column (5 μm, 250 x 4.6 mm). The precolumn derivatization methodologies studied included the formation of OPA (o-phthaldialdehyde), DANSYL (dimethylaminonaphthalenesulphonyl), DABSYL (dimethylaminoazobenzenesulphonyl), PTH (phenylthiohydantoin), and PTC (phenylthiocarbonyl) derivatives. The derivatization procedures were evaluated for simplicity, time required, and derivative stability. HPLC analyses of the amino acid derivatives were compared in terms of resolution, sensitivity, reproducibility, and time of analysis.

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INTRODUCTION

In the past, amino acid composition of peptides and proteins was determined by the classical ion-exchange amino acid analyzer introduced by Moore, Spackman and Stein (1). This separation was based on cation exchange using a gradient elution and postcolumn derivatization with ninhydrin. In the 1970s, the development of HPLC and advancement in reverse-phase (RP) columns consisting of a silica stationary phase modified by octadecyl-, octyl-, and other organic adsorbants greatly increased the potential for separation and quantitation of amino acids. Numerous procedures have been developed involving the production of amino acid derivatives capable of fluorescent, visible, or ultraviolet detection. Postcolumn LC derivatization techniques make use of fluorogenic agents such as o-phthalaldehyde (OPA) and fluorescamine (fluram). However, precolumn systems are becoming more popular because of their instrument simplicity, high sensitivity, selectivity and lower cost. HPLC has been used in protein and clinical chemistry for structural elucidation and identification of amino acids in enzymatic or acid hydrolysates of amidated peptides for protein sequencing (2,3).

Primary amines react readily with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (MCE) or ethanethiol to form 1-thiosubstituted-2-alkyl-isoindoles which are highly fluorescent (4,6). These isoindoles have been shown to be well-suited for HPLC separation (2,7-11). Although the OPA derivatization proce-

dure involves a rapid reaction and high sensitivity, the derivatives formed are highly unstable (11), and consequently, the use of OPA may be more suited to postcolumn derivatization which requires additional equipment. Also, OPA fails to react with secondary amino acids, proline and hydroxyproline (Hyp), and yields low fluorescence with lysine and cystine (12).

Other reagents that have been employed which are capable of reacting with both primary and secondary amino acids are dimethylaminonaphthalenesulfonyl chloride (DANSYL-Cl) and dimethylaminoazobenzenesulphonyl chloride (DABSYL-Cl). DANSYL derivatives are fluorescent (13), but more often, they are quantified with lesser sensitivity using ultraviolet detection to avoid problems with quenching (11, 14-16). The derivatization procedure is relatively lengthy, sometimes shown to be dependent on the ratio of derivatizing reagent to amino acids with the formation of both mono- and didansyl derivatives (13). DANSYL derivatives are also photosensitive (17). DABSYL derivatives have been shown to be suitable for HPLC separation (18-20). However, like the DANSYL procedure, DABSYL derivative formation is also concentration dependent and results in the formation of multiple derivatives with several amino acids (19).

For several decades, phenylthiohydantoin (PTH) amino acid derivatives formed by the Edman degradation procedure has been widely used for protein sequencing (21). Recently, PTH derivatives have been shown to be easily separated by HPLC employing both

isocratic and gradient elution (22-26). While the determination of amino acids using PTH derivatives has several advantages such as derivative stability and high HPLC resolution, the laborious task of forming PTH derivatives makes this procedure undesirable. More recently, HPLC separation of phenylthiocarbonyl (PTC) derivatives has been reported (8-27). The use of PTC derivatives has many of the same advantages as PTH derivatives. Since the formation of PTC derivatives is the first step in the Edman degradation procedure used for PTH derivative formation (21), the time of derivatization is reduced from days to minutes (8).

The ideal LC method for amino acid separation and detection should be quantitative, simple, rapid, sensitive, reproducible, and exhibit high resolution (8). Until now, it has been difficult to compare LC techniques for amino acids where single systems were optimized in independent studies. Postcolumn systems using ninhydrin vs. OPA (6) and fluorescamine vs. OPA (12) have been compared in relation to detection limits and reproducibility. In this investigation, five precolumn LC systems were assessed in regards to their ease and time of derivatization, derivative stability, chromatographic resolution, sensitivity, precision, and time of analysis. Such a study is the first to compare separation and detection in fluoro- and chromogenic labeling of amino acids conducted with standardized HPLC equipment.

Numerous papers exist on these various precolumn HPLC techniques for amino acids, and therefore, reproducing all of the

published procedures would have been an insurmountable task. The following procedures were selected because of their applicability with the available instrumentation and C₁₈ RP column. The methods described in these papers were followed as closely as possible; however, this study contains a great deal of new elements since many of the reported methods were modified, in terms of derivatization procedure, stationary phase or slightly different gradients (mobile phase) to optimize separation.

MATERIALS AND METHODS

Equipment

The chromatographic instrumentation utilized was a Beckman Gradient Liquid Chromatograph Model 334 consisting of two Model 110A pumps for solvent delivery and a Model 421 Controller for generation of gradient elutions. The system was equipped with an Altex 210 Injector fitted with a 20 μ l stainless steel injector loop. For all methods, an Altex Ultrasphere-ODS (5 μ m particle size, 250 x 4.6 mm I.D.) C₁₈, reversephase column was employed which was protected with an Altex ODS-5 guard column. When HPLC analyses were conducted at elevated temperatures, the column was enclosed in a column heater (Eldex Model III).

Fluorescence was measured with a Gilson Spectra-glow Filter Fluorometer Model 901 with filters for excitation at 360 nm and emission at 455 nm. Both ultraviolet and visible light absorbance were measured with a Hitachi Variable Wavelength Spectrophotometer

Model 100-20, with an Altex Spectrophotometer Flow Cell. Peak areas were recorded and integrated by a Hewlett-Packard Integrator Model 3390A.

Reagents

Standard amino acids, an amino acid standard solution for protein hydrolysates, and PTH amino acid derivatives were obtained from Sigma Chemical Company (St. Louis, Mo). DANSYL amino acid standards were obtained from Pierce Chemical Company (Rockford, Ill). All reagents used were HPLC grade when available. HPLC grade acetonitrile, methanol, ammonium acetate, sodium acetate, pyridine, triethylamine, and phosphoric acid were obtained from Fisher Scientific (Rockville, Md). HPLC grade water was prepared by filtering deionized water through the following sequence: HN organic removal resin (Barnstead, Boston, Mass), Ultrapure DI exchange column (Barnsted, Boston, Mass), and a 0.22- μ m membrane GS filter (Millipore, Bedford, Mass).

OPA Derivatization Procedure

Samples were derivatized according to the method of Jones and Gilligan (2), but rather than using the commercially available fluoraldehyde OPA reagent, the derivatizing reagent consisted of *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (MCE) in a sodium borate buffer (pH 9.5) as previously described (9). Brij 35 (polyoxyethylene lauryl ether) (0.8 mg/mL) was added to enhance the fluorescence of lysine (12). The derivatization procedure as

described by Jones and Gilligan (2) consisted of the following: a 5 μL aliquot of a standard (5 nmol/mL) was mixed with 5 μL of the OPA/MCE derivatizing reagent for exactly 1 min. Immediately 100 μL of 0.1 M sodium acetate buffer (pH 7.0) was added. The sample was rapidly mixed and injected.

OPA Chromatographic Conditions

The method described by Jones and Gilligan (2) was used for separation of the OPA-derivatized standard mixture. Solvents A and B consisted of tetrahydrofuran:methanol:0.1 M sodium acetate, pH 7.2, (5:95:900), and methanol, respectively. The elution gradient used is given below.

<u>Time (min)</u>	<u>% B</u>	<u>Duration of step (min)</u>
0.0	0	
0.1	20	0.9
12.0	45	5.0
20.0	60	2.0
27.0	100	2.0
32.0	0	8.0
40.0	END	

This program is similar to that used by Jones and Gilligan (2) except that solvent B was decreased to 0% more gradually and maintained at 0% for two additional minutes to allow the column to re-equilibrate. This elution gradient was also run at a flow rate of 1.2 ml/min rather than 1.7 to avoid limiting high pressures. The HPLC analyses were run at room temperature.

DANSYL Derivatization Procedure

Preparation of DANSYL derivatives of standard amino acid mixtures was performed according to the procedure of Tapuhi et al. (17). The derivatizing reagent consisted of 5.56 mM DANSYL-Cl in HPLC grade aceto-nitrile. The acetonitrile had previously been distilled over DANSYL-Cl to eliminate possible contaminants. Standard amino acid mixtures were prepared by dissolving 1 mM of individual amino acids in 40 mM lithium carbonate buffer (pH 9.5). Dansylation was performed by adding 1.0 mL of the DANSYL-Cl derivatizing reagent to 2.0 mL of the amino acid standard solution, shaking gently for 2 min, and allowing the sample to react at room temperature until exactly 35 min had elapsed. At this time the dansylation reaction was terminated by the addition of 100 μ L of 2% ethylamine. Standard DANSYL amino acids were dissolved in methanol and analyzed by HPLC. All samples were wrapped in aluminum foil to exclude light since DANSYL derivatives are sensitive to ultraviolet irradiation (17).

DANSYL Chromatographic Conditions

DANSYL amino acid standard mixtures were resolved using the reverse-phase HPLC procedure described by Wilkinson (29). Solvents A and B consisted of 30 mM sodium phosphate buffer (adjusted to pH 7.6 with phosphoric acid), and acetonitrile, respectively. Amino acids were separated with a simple linear gradient as follows:

<u>Time (min)</u>	<u>% B</u>	<u>Duration of step (min)</u>
0.0	10.0	
0.1	45.0	22.9
23.0	10.0	7.0
30.0	END	

Analyses of 20 μL of samples containing 1 nmol/mL were run at a flow rate of 2.0 mL/min at room temperature. Ultraviolet absorption (250 nm) rather than fluorescent detection was used, as is often the case since quenching occurs with the use of an aqueous buffer which is needed for elution of the derivatives (29).

DABSYL Derivatization Procedure

DABSYL derivatives were formed according to the procedure of Chang et al. (19) which involved dissolving mono amino acids in 0.1 M sodium bicarbonate buffer (pH 9.0). Although the procedure called for 1 mg of amino acid in 100 μL buffer, a molar concentration of 200 nmol/ μL was used instead in order to obtain the desired final concentration of 0.1 nmol/ μL . Since dabsylation is highly dependent on pH, the pH of the amino acid standards was checked and adjusted to 9.0 when necessary. One hundred μL of derivatizing reagent, which consisted of recrystallized DABSYL-Cl (2 $\mu\text{mol/mL}$ acetone), was added to 100 μL of a standard amino acid solution. This corresponded to a DABSYL-Cl:amino acid ratio of 1:1. The sample was mixed and then heated in a waterbath for 10 min at 70°C with occasional

mixing. The sample was dried in vacuo and redissolved in 2 mL of 70% (v/v) ethanol, resulting in a final concentration of 0.1 nmol/ μ L.

The bis amino acids, histidine, lysine, and tyrosine were derivatized by treating 100 μ L of an amino acid standard (50 nmol) in 0.1 M sodium bicarbonate (pH 9.0) with 200 μ L of DABSYL-Cl derivatizing reagent (4 nmol/mL acetone) which resulted in a DABSYL-Cl:amino acid ratio of 16:1. Samples were treated identically to the mono amino acids except that the dried residue was taken up in 500 μ L 70% (v/v) ethanol resulting in a final concentration of 0.1 nmol/ μ L.

DABSYL Chromatographic Conditions

The separation of a dabsylated amino acid standard mixture was conducted according to the method described by Chang et al. (19). Solvents A and B consisted of 0.04 M sodium acetate buffer (5.44 g sodium acetate trihydrate, 7.7 mL acetic acid, diluted to 90 mL, pH 4.13) and acetonitrile, respectively. The elution program, conducted at room temperature and at a flow rate of 1.2 mL/min, is given below:

<u>Time (min)</u>	<u>% B</u>	<u>Duration of step (min)</u>
0.0	20	
0.1	70	25
32.0	25	5
40.0	END	

PTH Derivatization Procedure

Since the formation of PTH amino acid derivatives by the Edman degradation process is so lengthy, details of the procedure will not be given here but are reported by Edman and Henschen (21). The initial step, the formation of phenylthiocarbonyl (PTC) derivatives, was accomplished by dissolving amino acids in a pyridine:water (1:1) mixture, adjusting the pH of the samples, heating, and adding phenylisothiocyanate (PITC), the derivatizing reagent. Excess reagents were removed by benzene extraction, followed by cooling, and acidification of the samples. For conversion of PTC to PTH derivatives, the samples were dissolved in HCl, heated, cooled, and repeatedly extracted with ethyl acetate. The extracts were dried in vacuo, and the PTH derivatives were then recrystallized.

PTH Chromatographic Conditions

HPLC analysis of PTH amino acids was conducted using the procedure described by Zimmerman et al. (26). A standard mixture of PTH amino acids (5 nmol dissolved in methanol) was separated using 0.01 N sodium acetate (pH 4.5) and acetonitrile as solvents A and B, respectively. The gradient elution program used is given below.

<u>Time (min)</u>	<u>% B</u>	<u>Duration of step (min)</u>
0.0	24	
0.1	44	5.9
14.0	24	11
25.0	END	

HPLC analyses were conducted using a flow rate of 1.0 mL/min and a column temperature of 62°C.

PTC Derivatization Procedure

PTC amino acid derivatives were formed following the procedure of Henrikson and Meredith (8). Standard amino acids were dissolved in 0.1 N HCl (2.5 μ mol/mL) to avoid problems with the low solubility of certain amino acids in water. A Sigma standard amino acid mixture was also derivatized for comparison. A 10 μ L aliquot of a standard amino acid mixture (in 0.1 N HCl) was pipetted into a 25 mL boiling flask. The sample was dried in vacuo and the residue was dissolved in 100 μ L of coupling buffer (acetonitrile:pyridine:triethylamine:water, 10:5:2:3). The sample was evaporated to dryness again to remove the HCl. The residue was redissolved in 100 μ L of coupling buffer. Five μ L of phenylisothiocyanate (PITC), which was kept tightly sealed in a Pierce Reactival, was added. The sample was mixed and allowed to react for five min at room temperature. The sample was again evaporated to dryness and the PTC derivatives were taken up in 250 μ L of acetonitrile:water (2:7) for HPLC analysis.

PTC Chromatographic Conditions

The method described by Henrikson and Meredith in System III (8) was followed for HPLC analyses. Solvent A consisted of 0.115 M ammonium acetate, pH 6.0, and solvent B was 0.23 M

ammonium acetate, pH 6.0, in acetonitrile:methanol:water (44:10:46). The ionic strength of both eluents was increased over that originally described which was 0.05 and 0.1 M ammonium acetate in solvents A and B, respectively. The solvent pH was adjusted with phosphoric acid to pH 6.0 rather than 6.8 as recommended by the senior author (personal communication). The HPLC analyses were conducted at room temperature rather than at 52°C as recommended. The elution gradient, conducted at a flow rate of 1.0 mL/min, is given below:

<u>Time (min)</u>	<u>% B</u>	<u>Duration of step (min)</u>
0.0	0	
0.1	15	14.9
15.0	50	15
30.0	100	4
37.0	0	13
50.0	END	

This program is slightly longer than the original program of Heinrikson and Meredith (8). It was necessary to decrease % B to 0% more gradually to elute the PTC amino acids off the column and to allow for column re-equilibrium.

RESULTS AND DISCUSSION

OPA Derivatives

The OPA derivatization procedure was very simple and rapid, involving only the mixing of a standard solution of amino acids with the OPA/MCE derivatizing reagent. Consistency in timing proved to be critical because of the instability of the OPA

derivatives (26). The sodium acetate buffer (pH 7.0) was added, not for termination of the reaction, but for the purpose of preserving the life of the column (9). It was confirmed that Brij 35 enhanced the fluorescence of Lys-OPA. Because Lys- and Gly-OPA are extremely unstable (9,31), a precise reaction time (60 sec) was selected to maximize reproducibility.

The separation of an OPA derivatized standard mixture of 18 amino acids (500 pmol) is shown in Fig. 1. Two of the protein amino acids were excluded from the mixture; proline because OPA does not react with α -imino groups (12) and cystine because of low fluorescence yield without the addition of iodoacetic acid (11). Resolution of 16 out of 18 OPA derivatives was achieved including separation of asparagine and glutamine which were not investigated by Jones and Gilligan (12). Glycine and threonine co-eluted even though Jones et al. (9) found that with the addition of an organic modifier, tetrahydrofuran, resolution between Gly and Thr could be substantially improved on an Altex Ultrasphere ODS column (the same column used in this study).

The sensitivity of the OPA derivatives was evaluated with serial dilutions of an amino acid standard mixture. The sensitivity in this study ranged between 10 and 200 pmol for various amino acids of the standard mixture. Other studies have reported similar detection limits (7,12) with linearity of response in the range of 5-100 pmol (9). Hill et al. (31)

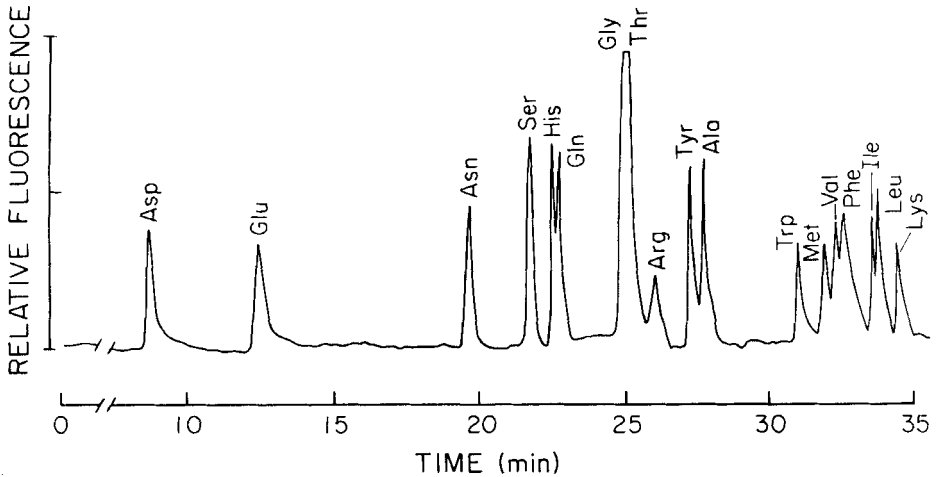


FIGURE 1. HPLC analysis of a standard mixture of OPA-derivatized amino acids (500 pmol).

reported that the use of ethanethiol in place of mercaptoethanol increased sensitivity. However, Lindroth and Mopper (10) were unable to confirm such claims. The detection limit, is, of course, dependent on the intensity of the fluorescence in relation to baseline stability. The background noise encountered with these samples was often quite high. It is unknown whether this was due to contaminants or quenching problems.

The precision using OPA derivatives was determined by repeated derivatization and HPLC analysis of the same amino acid standard mixture. The coefficients of variation (C.V.) for eight replications of a standard mixture are reported in Table I on an individual amino acid basis. Precision values were not calculated for amino acids which co-eluted. C.V.

TABLE 1

Coefficients of Variation (C.V.) on Repeated Determinations of
of Individual Amino Acid Components of a Standard Mixture
Subjected to Five Derivatization Procedures.

Amino acid*	Derivative				
	OPA	DANSYL	DABSYL	PTH	PTC
	-----C.V. (%)-----				
Alanine	6.0	2.2	3.9	3.7	7.7
Arginine	6.4	n.d.	3.3	6.5	9.9
Asparagine	4.0	n.d.	15.1	6.4	5.6
Aspartamate	3.4	1.6	7.7 [#]	7.8	1.4
Cysteine	n.d. [†]	n.d.	3.7	n.d.	4.4
Glutamate	3.3	1.5	23.6	n.d.	5.1
Glutamine	17.0	n.d.	5.1	n.d.	8.4
Glycine	n.d.	1.5	3.2	3.2	3.4
Histidine	3.7	n.d.	n.d.	6.9	7.3
Hydroxyproline	n.d.	n.d.	n.d.	n.d.	8.4
Isoleucine	18.0	n.d.	n.d.	5.7	8.0
Leucine	10.1	n.d.	3.8	5.6	7.0
Lysine	12.1	n.d.	n.d.	n.d.	6.1
Methionine	7.0	14.9	2.7	6.0	5.4
Phenylalanine	5.4	10.0	7.9 [#]	5.5	4.9
Proline	n.d.	1.5	3.2	5.7	4.2
Serine	2.5	1.8	6.1 [#]	n.d.	7.2
Threonine	n.d.	1.6	11.0	9.1	5.6
Tryptophan	3.7	11.8	3.0 [#]	5.6	7.1
Tyrosine	5.6	n.d.	n.d.	6.9	4.7
Valine	17.3	1.5	3.0	4.3	6.7
Average	7.8	4.5	6.6	5.9	6.1

*50.0 nmole sample of each amino acid derivative

[†]n.d. = not determined

[#]determined on standards containing an individual amino acid
only (50.0 nmole sample of the amino acid derivative)

values for the individual OPA derivatives ranged from 2.5 - 18.0% with an average of 7.8%. High C.V. values, and thus poor reproducibility, were obtained with glutamine, valine, isoleucine, leucine, and lysine. Low precision might be expected because of the instability of the OPA derivatives. However, of these five amino acids with high C.V. values, only the lysine derivative has an extremely short half-life, which was 3.7 min in the OPA/MCE derivatizing reagent reported by Turnell and Cooper (11). Glutamine, valine, isoleucine, and leucine had comparatively long half-lives ranging from 20.3 to 43.5 min. The most likely explanation for the poor reproducibility of these individual amino acids is the lack of complete separation between these and other amino acids. Glutamine occurred as a shoulder on the histidine peak, and valine as a shoulder on the phenylalanine peak. The poor precision observed with isoleucine and leucine can also be due to a lack of resolution. Chromatographic resolution, R_s between two peaks can be expressed as the ratio of the difference of retention times (t) to the average baseline peak width (w) (15):

$$R_s = \frac{2(t_2 - t_1)}{w_2 + w_1}$$

Satisfactory resolution was obtained for all OPA derivatives except His/Gln ($R_s = 0.30$), Tyr/Gly ($R_s = 0$), Met/Val ($R_s = 0.54$), Val/Phe ($R_s = 0.31$) and Ile/Leu ($R_s = 0.60$). Co-elution

of Tyr/His has also been reported with a Varian Micropack MCH 5 and Gly/Thr and Val/Met on a Waters μ Bondapak (7). Still, the average C.V. value for all the OPA/MCE amino acids of 7.8% is acceptable as C.V. values of 9 to 13% are not uncommon with the classical ion-exchange amino acid analyzer (28).

All of the 18 OPA derivatives eluted from the column within 35 min and the entire program was completed by 40 min using a flow rate of 1.2 ml/min. This is comparable to most other precolumn analyses of OPA derivatized amino acids using gradient elution ranging from 30 to 45 min (7,9,31,32). Pressure buildup exceeding 5,000 p.s.i. necessitated the use of a slower flow rate. Consequently, HPLC analysis time was several minutes longer than with the original program described by Jones and Gilligan (2). If shorter analysis time is desired, a 3 or 5 μ m particle size 150 mm column could be used (2). Nonetheless, an analysis time of 40 min for the separation of OPA derivatives is a substantial time savings over classical ion-exchange methods.

The advantages in using OPA derivatives for amino acid detection are: (1) the derivatives exhibit high fluorescent yield, (2) they are soluble and stable in aqueous buffer, (3) the reaction occurs quickly, and (4) OPA is not fluorescent itself, interfering with the reaction. The major drawback with OPA is that it does not form fluorophors with secondary amino acids. However, secondary amino acids can be detected in the

presence of sodium hypochlorite (NaOCl) in postcolumn systems, but NaOCl diminishes the response of many primary amino acids (7,9,28,30). Also, double peaks have been reported for the OPA derivatives of His, Lys and ornithine (7,10).

DANSYL Derivatives

The dansylation procedure of Tapuhi et al. (16) is a relatively simple, straightforward method involving the addition of DANSYL-Cl to amino acids dissolved in lithium bicarbonate buffer adjusted to pH 9.5. Although it previously had been reported that derivative yield was dependent on the ratio of the concentrations of DANSYL-Cl to amino acids (32), Tapuhi et al. (17) found that with the use of lithium carbonate rather than sodium carbonate, and acetonitrile rather than acetone and water, the degree of derivatization did not differ even with a 1000-fold increase in the DANSYL-Cl concentration. The authors, however, recommended using a ratio of 5-10:1.

DANSYL-Cl reacts with the primary amino acids, as well as proline and hydroxyproline. With this dansylation procedure, amino acids reacted with DANSYL-Cl to form monodansyl derivatives except histidine, lysine, tyrosine, and cystine which formed didansyl derivatives as expected. The entire derivatization procedure was relatively lengthy compared to other methods. The DANSYL-Cl reaction required 35 min to obtain maximum DANSYL derivative yields (17). The timing for termination of the reaction by addition of ethylamine was critical.

The DANSYL derivatives formed were stable for 12 to 24 hr which confirms the results of Tapuhi et al. (17). All samples were covered with Al foil to exclude light since DANSYL derivatives are known to be sensitive to ultraviolet light irradiation (17).

HPLC analysis of a standard mixture of commercially prepared DANSYL amino acids with sample concentrations of 1.0 and 0.5 nmol for the mono- and didansyl derivatives, respectively, is illustrated in Fig. 2. Of the 18 DANSYL amino acids within the mixture, asparagine/hydroxyproline and leucine/isoleucine were not resolved. Poor resolution was also noted for Thr/Gly ($R_S = 0.04$), Phe/Trp ($R_S = 0.04$) and Trp/Cys ($R_S = 0.08$).

Since DANSYL derivatives are fluorescent the detection limit of the derivatives could be quite low, in the sub-pmol range. However, because of fluorescence quenching with the use of an aqueous buffer for elution, ultraviolet detection is more commonly used although it greatly decreases the sensitivity of the HPLC analysis. The detection limit measured by UV absorbance at 250 nm, ranged from 50 to 500 pmol for the various DANSYL derivatives which is similar to that reported by Wilkinson (29) of 100 pmole. The precision values obtained for the individual DANSYL derivatives ranged from 1.2 - 14.9% with an average C.V. of 4.5% (Table I). The time of analysis for separation of the DANSYL amino acids was fairly short, requiring about 30 min for elution and column re-equilibration.

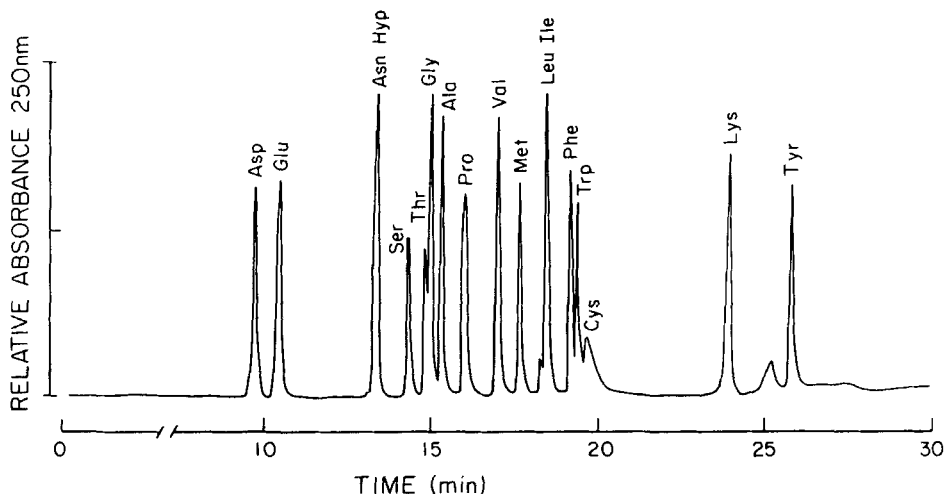


FIGURE 2. HPLC analysis of a standard mixture of DANSYL amino acids (1.0 and 0.5 nmol of mono- and didansyl derivatives, respectively).

The HPLC analysis of DANSYL amino acids was judged to be moderate in terms of separation, sensitivity, precision, and time of analysis. However, when individual standards of amino acids were derivatized, various unidentified peaks were encountered which often coeluted with other derivatives, thereby making quantitation difficult. Interfering peaks were particularly abundant with lysine, histidine, and tyrosine, the amino acids which typically form didansyl derivatives. It is unknown whether both mono- and didansyl derivatives were being formed or whether other reaction products were causing the extraneous peaks. Difficulties were also encountered by Needle

and Pollitt (32) who reported that quantitative dansylation of free amino acids was not possible.

Although the chromatographic analysis of DANSYL amino acids appears to be a good separation method if modifications to optimize resolution are made, the DANSYL derivatives formed by the method of Tapuhi et al. (17) may not be suitable for this particular chromatographic separation. Wilkinson (29), using a different derivatization procedure, also reported the presence of extraneous peaks with a dansylated peptide sample and suggested that the use of DANSYL derivatives may not be appropriate in various biological applications.

DABSYL Derivatives

The DABSYL derivatization procedure was relatively simple and fairly rapid, requiring approximately 20-25 min to mix the standard and derivatizing reagent, heat the sample, evaporate it to dryness, and redissolve the derivative residue. One major drawback of this derivatization method was the formation of multiple derivatives with the bis amino acids, histidine, lysine, tyrosine, and cystine. Lin and Chang (33) also reported the formation of multiple derivatives with low DABSYL-Cl: amino acid ratios and high pH values. With a ratio of 5:1, they observed only single derivatives of the bis amino acids. Chang et al. (19) suggested using DABSYL-Cl:amino acid ratios of 4:1 - 80:1. However, their mono amino acid dabsyla-

tion procedure, which was used in this study, specified reagent and amino acid concentrations that produced a ratio of 1:1.

Unlike the DANSYL, the DABSYL derivatives are photostable when dissolved in sodium bicarbonate buffer (33). The derivatives were stable for 24 to 48 hr at room temperature as measured by peak areas of a standard mixture that was repeatedly injected over time. The DABSYL-Cl derivatizing agent, if stored under N_2 and refrigerated, was reported to be stable for long periods of time (20).

DABSYL amino acid derivatives were detected using visible light (436 nm). Resolution of 12 out of 16 mono DABSYL amino acid derivatives of a standard mixture (2 nmol) was achieved within 25 min (Fig. 3). Only mono amino acids were included in this standard mixture because dabsylation of the bis amino acids histidine, lysine, tryrosine, and cystine resulted in the production of 3, 3, 4, and 2 derivatives, respectively. Although the major peaks for histidine, lysine, and tyrosine had retention times of 27.8, 28.2, and 30.8 min, respectively, and therefore were resolvable, the secondary peaks produced coeluted with several of the mono amino acid peaks and interfered with quantitation. Both isoleucine and hydroxyproline were also omitted from the standard mixture because of coelution with leucine and glycine, respectively. Aspartate and serine were not resolved ($R_S = 0$) as was also reported by Chang et al. (19). This particular program also did not separate

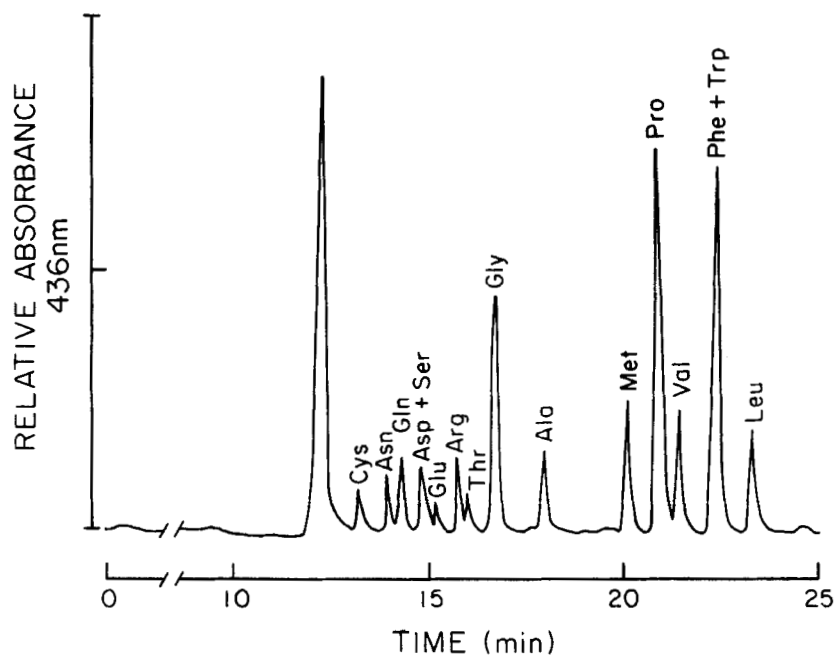


FIGURE 3. HPLC analysis of a standard mixture of DABSYL-derivatized amino acids (2nmol).

phenylalanine and tryptophan ($R_s = 0$). Poor resolution was noted for the following DABSYL derivatives: Asn/Gln ($R_s = 0.12$), Asp/Glu ($R_s = 0.30$), Ser/Glu ($R_s = 0.30$) and Arg/Thr ($R_s = 0.24$). Both glutamine and asparagine, which were not included in the mixture used by Chang et al. (19), were separated from the other mono derivatives. The large peak which eluted first before the amino acid peaks is excess DABSYL-C1 (Fig. 3).

The detection limit of the mono DABSYL amino acid derivatives ranged from 50-500 pmol for the individual amino acids. Chang et al. (19) reported detection as low as 2 to 5 pmol. The lesser sensitivity observed in this study was probably due to poor baseline stability of the detector at its most sensitive settings. The precision data (Table I) shows that the coefficients of variation ranged from 2.7 to 23.6% with an average C.V. of 6.8%. The large C.V. value associated with glutamate is most likely due to the fact that glutamate occurred as a small peak shouldering on the aspartate/serine peak. Lack of resolution is also probably responsible for the high C.V. obtained with threonine. Asparagine was the only other derivative that did not exhibit good reproducibility.

All of the mono DABSYL derivatives were eluted off the column within 25 min, and the bis derivatives by 32 min. The entire program, including re-equilibration of the column took 40 min which is similar to that for OPA analysis. However, only 12 DABSYL amino acids were resolved using the chromatographic conditions described by Chang et al. (19). A more complicated gradient might improve the resolution of the DABSYL amino acid derivatives; however, reproducibility, particularly in reference to retention time, typically suffers with the use of complicated gradient programs.

The major shortcoming of the DABSYL derivatization method is the fact that dabsylation of the bis amino acids resulted in

the formation of multiple derivatives which elute from the column at the same time as other mono-derivatives.

Consequently, quantitation of unknown samples would be nearly impossible because the formation of these multiple derivatives is not consistent, but appears to be dependent upon the ratio of DABSYL-Cl to amino acids.

PTH Derivatives

A major disadvantage with the use of PTH derivatized amino acids is the lengthy derivatization procedure. Whereas some derivatization techniques require only several minutes, this derivatization is a most laborious task which can take up to several days. The fact that certain amino acids such as serine, threonine, arginine, and histidine had to be treated differently from the majority of the amino acids made the derivatization process even more undesirable.

The PTH derivatives are known to be rather stable except when subjected to long periods of ultraviolet light exposure (21). No loss of peak area was observed on samples run repeatedly over the course of several days in this study.

Fifteen of the 18 commercially prepared PTH amino acid standards (5 nmoles) were resolved (Fig. 4) using the procedure of Zimmerman et al. (26). Glutamine, glutamate, and serine coeluted rather than being resolved as in the original paper. Poor resolution was also noted between Arg/Tyr ($R_s =$

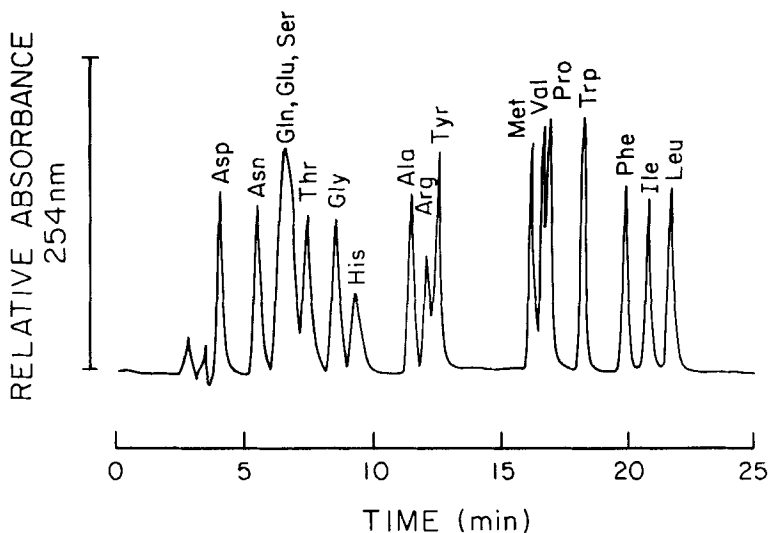


FIGURE 4. HPLC analysis of a standard mixture of PTH amino acids (5 nmol).

0.05) and Val/Pro ($R_s = 0.02$). Because more time was required to elute the PTH amino acids off our column, the program used was slightly longer than that in the original paper (26). The sensitivity of the PTH derivatization procedure ranged between 50 and 500 pmol for the individual PTH amino acid derivatives. The reproducibility of the PTH amino acids ranged from 3.2 to 9.1% with an average C.V. of 6.1% (Table I). The variation associated with the PTH amino acid derivatives was more consistent than with previous derivatization procedures where C.V. values were sometimes quite high for one or several amino acids. In this case, only the PTH threonine derivative showed poor reproducibility which, most likely, is due to the fact

that it eluted as a small peak shouldering a larger peak representing glutamine, glutamate, and serine. The C.V. values for the PTH derivatives are lower than the 9-13% C.V. typically encountered with classical ion exchange methods.

One advantage with PTH derivatives was the short HPLC analysis time of 25 min needed to elute all the derivatives off of the C_{18} column. Zimmerman et al. (26) reported that even a shorter program of only 20 min was necessary to resolve all 20 PTH amino acid derivatives.

PTC Derivatives

Formation of PTC amino acids by the method of Henrikson and Meredith (8) was a relatively simple, rapid derivatization procedure requiring 20-25 min. The evaporation step after the initial addition of coupling buffer was found to be necessary to prevent the appearance of a peak apparently associated with the presence of HCl. Redistillation of the pyridine and triethylamine, even of HPLC grade reagents, was shown to be necessary in diminishing background noise. This derivatization procedure requires a strong vacuum pump for evaporation, particularly with the final drying after derivatization with PITC. The presence of even trace amounts of coupling buffer prevented the resolution of amino acid derivatives during HPLC analysis.

The PTC amino acid derivatives formed were quite stable as peak areas of derivatized standards remained constant for two

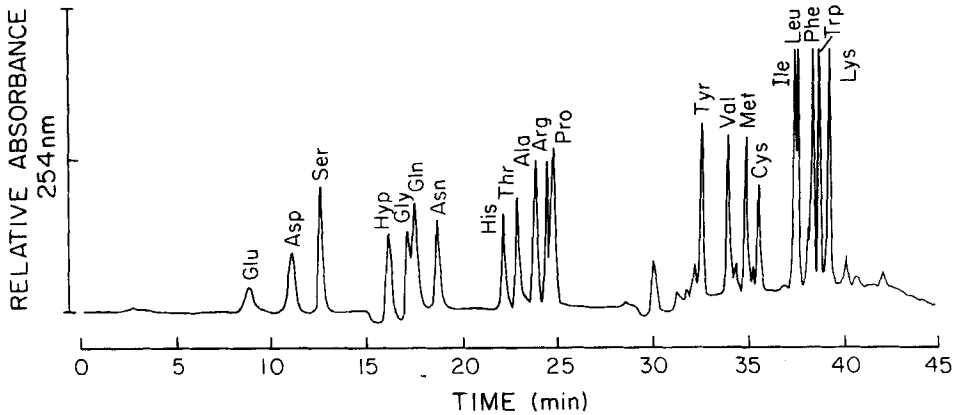


FIGURE 5. HPLC Analysis of a standard mixture of PTC-derivatized amino acids (2 nmol).

or more days. PTC amino acid derivatives were reported to be stable for months if stored dry in a freezer (8). As with the PTH derivatization, PITC, the derivatizing reagent, must be kept tightly sealed to avoid exposure to air. The coupling buffer was usable for several weeks at room temperature, and even longer if stored refrigerated.

All 20 protein amino acids and hydroxyproline (2 nmoles) were resolved within approximately 40 min using the chromatographic conditions for PTC derivatives described by Henrikson and Meredith (8) (Fig. 5). However, resolution was somewhat poor between Gly/Gln ($R_s = 0.03$), Arg/Pro ($R_s = 0.04$), Ile/Leu ($R_s = 0.04$) and Phe/Trp ($R_s = 0.02$). PTC derivatives of asparagine, glutamine, tryptophan, cysteine, and hydroxypro-

line which were not included in mixtures studied by Henrikson and Meredith (8) were also separated as well as the 16 derivatives they investigated. The detection limits of the individual PTC derivatives ranged from 50 to 500 pmol. The precision values obtained with this method ranged from 1.4 to 9.2% with an average C.V. of 6.1% (Table I). The C.V. values for the PTC method, like the PTH method, were fairly consistent among the derivatives with no particularly high values occurring for any specific amino acid. Although the formation of PTC derivatives and the HPLC analysis required 20-25 min and 50 min, respectively, totaling approximately 70-75 min per sample, the PTC methodology was the only method capable of resolving all 20 protein amino acids as well as hydroxyproline.

CONCLUSIONS

Each of the five amino acid derivatization methodologies has certain advantages or a particular facet which renders one method more favorable for derivatization, HPLC analysis, or both, depending on one's priorities (Table II). For sensitivity, the formation of fluorescent derivatives using OPA was most desirable. The derivatization procedure was simple and rapid with a chromatographic analysis of intermediate length (40 min). The main disadvantage of the OPA method lies in the fact that OPA does not react with the secondary amino acids, proline and hydroxyproline. The OPA method may also not

TABLE 2.

Comparison of Precolumn Derivatization HPLC Techniques
in Terms of the Specified Analytical Parameters

SENSITIVITY:	OPA > DABSYL = DANSYL > PTH = PTC
PRECISION:	DANSYL > PTH = PTC > DABSYL > OPA
STABILITY:	PTC = PTH > DABSYL = DANSYL > OPA
RESOLUTION:	PTC > PTH > OPA > DABSYL = DANSYL
TIME OF ANALYSIS:	PTH = DABSYL < DANSYL < OPA < PTC

be best-suited for precolumn derivatization due to the instability of the derivatives formed.

Although the use of DANSYL derivatives is fairly common, this method offers few advantages. DANSYL-Cl is capable of reacting with the secondary amino acids. The total time required, 35 min for derivatization and 30 min for HPLC separation, makes this method favorable in terms of speed of analysis when compared to the PTH method. Fairly good sensitivity and reproducibility was obtained using commercially prepared DANSYL derivatives. However, dansylation of amino acid standards resulted in the formation of both mono- and didansyl derivatives and in the production of extraneous peaks which interfered with quantitation.

Dabsylation of amino acids offers no particular advantage except possibly the relatively short analysis time (60-65 min

total) and reaction of DABSYL-Cl with the secondary amino acids. However, problems encountered with this procedure, particularly in the derivatization which is pH and concentration dependent, detracts from the use of DABSYL derivatives. The multiple derivatives formed during dabsylation of the bis amino acids makes this procedure most unattractive for use in quantifying unknown biological samples.

The use of PTH derivatives is quite common and offers the advantage of a rapid HPLC analysis, fairly good resolution, sensitivity, precision, and derivative stability. However, the lengthy task of forming PTH derivatives makes this amino acid methodology undesirable.

The PTC derivatization and chromatographic analysis is not the most rapid method available, requiring approximately 70-75 min total per sample. This procedure, however, was capable of resolving 21 amino acids, and had fairly good sensitivity, reproducibility, and derivative stability. Therefore, the use of PTC (phenylthiocarbonyl) derivatives for reverse-phase HPLC analysis of precolumn-derivatized amino acids is the preferred method of the derivatization techniques studied.

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