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TOTAL AMINO ACID ANALYSIS USING PRE-COLUMN FLUORESCENCE DERIVATIZATION

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SUMMARY

An approach to total amino acid analysis utilizing off-line pre-column fluorescence derivatization is described. The full array of natural primary amino acids was treated with *o*-phthaldialdehyde (OPA) in the presence of mercaptans, and the highly fluorescent reaction products were separated on an ODS 5-μm reversed-phase column with gradient elution development. Due to their instability in solution and the relatively poor response of the OPA derivatives, cysteine and cystine were oxidized with performic acid and separated as the OPA cysteic acid reaction adduct, which is highly fluorescent.

Secondary amino acids, such as proline and hydroxyproline, were reacted with 4-chloro-7-nitrobenzofurazan, and the separation was carried out on the same column. The stability of the reaction adducts was investigated and the derivatization reactions were optimized with respect to reaction time and temperature.

It is shown that the detection limits for most amino acids are in the 0.1–1.0-pmole range. The reproducibility of the method was limited by the derivatization procedure and the gradient elution employed, but, by using an on-line computer data handling system, the retention time could be measured within $\pm$ 0.1% relative standard deviation and the relative peak areas, based on the internal standard calculation methodology, were within $\pm$ 3% or less. The sensitivity of lysine and hydroxylysine was improved by forming a sodium dodecylsulfate micellar solution around these amino acids to protect the fluorescent adducts from rapid decomposition. The sensitivity and separation appeared to be very much dependent on the pH of the mobile phase.

Experimental details for the determination of total amino acid residues in pharmaceutically important proteins, such as thymosin α_1 and insulin, are given. Advantages and disadvantages of the system described are discussed.

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INTRODUCTION

Amino acid analysis is an important technique which finds many applications in biochemistry and related fields. Determination of the primary structure of proteins[1], peptide sequencing[2], determination of completeness of solid-phase peptide synthesis, and structure elucidation[3] all require an accurate method for amino acid determination. The analysis of protein samples, however, presents a formidable problem for the practising liquid chromatographer. A very small amount of sample is usually available; trace amounts of amino acids possessing no significant fluorophores or chromophores are present in a very complex sample mixture where other substances interfere with the analysis. A substantial number of amino acids with diverse polarities and functional groups has to be quantitated. Thus, in general, the chromatographic system should exhibit high specificity, sensitivity, and chromatographic selectivity; in addition, the system should also be versatile, simple to operate, and highly reproducible.

Classical two-column amino acid analyzers[4] based on ion-exchange copolymers and operated in post-column derivatization mode utilized either ninhydrin[5] or other fluorogenic reagents[6–9]. Although the use of these instruments for amino acid analysis has been widely advocated in the past, the major shortcomings observed were long analysis times, poor chromatographic performance of ion-exchange columns, inadequate detection limits, high cost of instrumentation, and the total dedication of the system to only one type of analysis. Besides these complications, the difficulty in the detection of proline, hydroxyproline, cysteine, and cystine limited the use of these analyzers.

High-performance liquid chromatography (HPLC) as practiced in various modes has now become a common technique in the analysis of protein hydrolysates because of the tremendous advances made in the development of HPLC columns and column technology. Promising results have been obtained on the separation of dimethylaminobenzenesulfonyl[10] and phenylthiohydantoin[11,12] derivatives of the amino acids. Recent studies have demonstrated simpler and more rapid reactions with the use of *o*-phthaldialdehyde (OPA) in the presence of either ethanethiol[13] or mercaptoethanol[14–16].

The major aim of this paper was to develop an analytical method for the determination of primary and secondary amino groups containing amino acids. It was also attempted to improve the analytical technique from the point of view of separation, sensitivity, analysis time, and flexibility. Applications of total amino acid analysis to proteins of pharmaceutical importance are also demonstrated.

EXPERIMENTAL

Solvents and reagents

Methanol, acetonitrile, tetrahydrofuran, and high-purity water were obtained from Burdick & Jackson, Muskegon, MI, U.S.A. Amino acid standard H, OPA, 2-mercaptoethanol, ethanethiol, and sodium dodecylsulfate were purchased from Pierce, Rockford, IL, U.S.A. Additional amino acid standards were obtained from Sigma, Morton Grove, IL, U.S.A. and sodium acetate and boric acid were analytical-grade reagents from Mallinckrodt, St. Louis, MO, U.S.A. The sample of thymosin α_1

was obtained from Hoffmann-La Roche, Nutley, NJ, U.S.A. The insulin sample was obtained from Eli Lilly, Indianapolis, IN, U.S.A.

Chromatographic equipment

The instrument used consisted of a Spectra-Physics Model SP-8000 microprocessor-controlled liquid chromatograph coupled to a Schoeffel SF 970 fluorescence detector equipped with a deuterium lamp. The OPA derivatives were detected with the monochromator set at 330 nm and a 418-nm cut-off filter. The sensitivity was set at 1 μA full scale. The 4-chloro-7-nitrobenzofurazan (NBD) reaction adducts were monitored at an excitation wavelength of 220 nm, with a 370-nm cut-off filter, and a sensitivity setting of 0.1 μA.

The detector was connected to a Hewlett-Packard Model HP-1000 computer system with data collection and handling software provided by Computer Inquiry Systems (CIS), Allendale, NJ, U.S.A. Separations were carried out on a 25 cm × 4.6 mm I.D. Ultrasphere column packed with 5 μm ODS particles (Beckman, Berkeley, CA, U.S.A.) connected to a pre-column (5 cm × 4.6 mm I.D.) packed with the same material. The sample was introduced with a Valco air-actuated valve equipped with either a 10- or 100-μl external loop.

Hydrolysis and derivatization procedures

Acid hydrolysis. To *ca.* 100 μg of the protein sample contained in an ignition tube, 200 μl of 4 *N* methanesulfonic acid containing 0.2% of 3-(2-aminoethyl)indole were added and frozen in a slurry of carbon dioxide and isopropanol. Dissolved gases were removed by evaporating the tube by freeze-thawing twice. The tube was then sealed under vacuum and placed in a 110°C oven for 24 h.

Performic acid oxidation. Protein sample (*ca.* 100 μg) was frozen in dry ice and lyophilized. Performic acid was prepared by mixing 1 ml of 30% hydrogen peroxide with 19 ml of 97% formic acid, and allowing the mixture to stand in a closed container for 2 h at room temperature. The mixture was cooled to 0°C and used immediately: 100 μl were added to the sample; the liquid was transferred to a capped vial and was allowed to stand for 2.5 h at 0°C. The reaction was terminated by adding 0.9 ml of cold water. Then 200 μl of this sample were frozen and lyophilized. The dried sample was hydrolyzed by following the hydrolysis procedure described earlier.

Preparation of OPA derivatizing solution. To 50 mg of OPA dissolved in 1.5 ml of methanol, 50 μl of mercaptoethanol and 11 ml of 0.4 *M* borate buffer (pH adjusted to 9.5 with 4 *N* sodium hydroxide) were added. The solution was mixed and flushed with nitrogen to displace dissolved oxygen. The solution was stored in the dark and allowed to stand for 24 h before use. Every 2 days, 10 μl of 2-mercaptoethanol were added. The solution is stable for *ca.* 2 weeks. The OPA derivatizing reagent containing ethanethiol was prepared using the same procedure.

Preparation of the OPA derivatives. A sample of the protein hydrolyzate obtained from acid hydrolysis using methanesulfonic acid was neutralized with 4 *N* sodium hydroxide. For cysteine and cystine determinations, the sample was oxidized to cysteic acid with performic acid. In this case, the oxidation was carried out prior to the acid hydrolysis. The sample was diluted when necessary with 0.4 *M* borate buffer (pH 9.5) to obtain a final concentration of *ca.* 25 nmole/ml of the amino acid with the highest mole ratio in the molecule. An aliquot was withdrawn and mixed with an

equal volume of 4 μg/ml of 2-aminoethanol solution, as an internal standard. One part of this solution was combined with four parts of 1% (w/v) sodium dodecylsulfate in 0.4 *M* borate buffer and four parts of the OPA solution and mixed vigorously for 1 min using a Vortex mixer. Immediately, 5–10 μl were injected onto the column. The commercially available standard amino acid mixture was diluted appropriately to obtain a final concentration of 25 nmoles/ml for each amino acid. The derivatization procedure employed for the protein hydrolyzates was also used for the amino acid standard.

Preparation of the NBD derivatives. An aliquot of the neutralized sample used for the preparation of the OPA derivative was diluted, when necessary, to obtain a final concentration of *ca.* 20 nmoles/ml of proline. Equal volumes of the sample, 0.4 *M* borate buffer, and the NBD solution (concentration 2 mg/ml in methanol) were combined and the mixture was heated for 5 min at 60°C in a closed screw-capped vial. The reaction was stopped by cooling the mixture to 0°C. Aliquots of 100 μl were injected onto the column.

Kinetics of the derivatization reactions

The dependence of the NBD–proline reaction on time and temperature was studied. Standard proline solution (20 nmoles/ml) was treated at 50°C for 0, 1, 5, 20, and 30 min, and the reaction time necessary to reach an equilibrium state was determined from the fluorescent response. The effect of temperature on the NBD–proline reaction was also investigated at 30, 40, 50, 60, and 80°C for 5 min. Similar experiments were carried out with the OPA reaction. However, decomposition of the reaction adducts was observed at elevated temperatures. The OPA reagent is known to react within seconds to attain maximum fluorescent intensity[17], and, therefore, subsequent work with the OPA reagent was carried out using a 1-min reaction time and room temperature. Quantitative reactions with both reagents were possible only with a large excess of either reagent, which indicated that chemical equilibrium processes rather than the kinetics of the reactions were the limiting factors.

RESULTS AND DISCUSSION

Although the liquid chromatography of OPA-amino acids has been recently reported[14], the separation of complex protein hydrolyzates necessitated the development of a new HPLC method which would exhibit an improvement in selectivity, efficiency, reproducibility, speed of the analysis, resolution, and detection limits. The recent work of Jones *et al.*[16] indicated that a feasible chromatographic system consists of a 5-μm reversed-phase column operated in a gradient elution mode with a ternary tetrahydrofuran–methanol–buffer mobile phase. Throughout this work, an Ultrasphere ODS column was used.

No simple gradient could resolve all amino acids completely, and a series of isocratic elution steps coupled with gradient elution displacement effects had to be employed. Furthermore, detailed investigation of various solvent mixtures showed that the need for ternary solvent mixtures was well substantiated. Very good resolution of all primary amino acids was obtained with methanol–tetrahydrofuran–0.05 *M* sodium acetate buffer pH 6.6 (15:1:84) as a starting solvent and 80% (v/v) methanol as the final solvent. The separation obtained is illustrated in Fig. 1. Each peak cor-

responds to 20 pmoles of amino acid. For most amino acids, as shown later, the detection limit was in femtomoles. Thus, the analytical method is *ca.* 1000 times more sensitive than amino acid analyzers that employ ninhydrin for the detection. The average peak width at the base was *ca.* 400 μl. Assuming an analysis time of 45 min and a constant peak width, the maximum peak capacity calculated is *ca.* 150. This means that only *ca.* 20% of the available chromatographic peak capacity has been utilized and certainly additional amino acid-type compounds can be separated with this system. This was demonstrated for the homoserine adduct which elutes close to methionine sulfoxide; increasing the second isocratic step by 2 min resulted in total resolution between the two amino acid adducts.

The separation of the NBD derivatives was carried out on the same column, using a similar solvent system. The kinetics of the NBD–proline reaction was examined by derivatizing a standard proline solution at 50°C using different reaction times. The results can be seen in the top part of Fig. 2, where a graph relating fluorescent NBD–proline peak height to reaction time is shown. At 50°C, the reaction reaches an equilibrium state only after a relatively long time of 30 min.

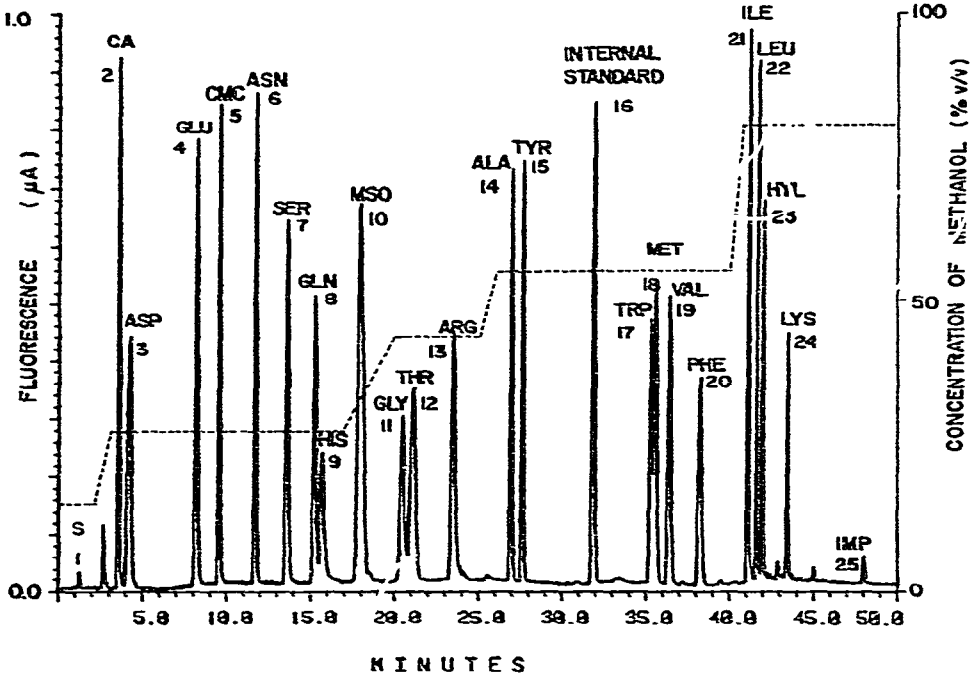

Fig. 1. Separation of OPA-amino acid standards. Operating conditions: column, Ultrasphere ODS 25 cm × 4.6 mm I.D., 5 μm particle size; flow-rate, 1.5 ml/min; solvent A, tetrahydrofuran–0.05 *M* sodium acetate pH 6.6 (1:99); solvent B, methanol; gradient program, 15% B for 2 min from the beginning of the program, linear step to 28% B for 1 min, isocratic elution step at 28% B for 14 min, linear step to 44% B in 3 min, isocratic elution step at 44% B for 5 min, linear step to 56% B in 1 min, isocratic elution step at 56% B for 9 min, linear step to 80% B in 1 min, and isocratic development at 80% B for 19 min; sample volume, 10 μl; excitation at 330 nm; emission filter 418 nm. Peaks: 1 = solvent; 2 = cysteic acid; 3 = aspartic acid; 4 = glutamic acid; 5 = carboxymethylcysteine; 6 = asparagine; 7 = serine; 8 = glutamine; 9 = histidine; 10 = methionine sulfoxide; 11 = glycine; 12 = threonine; 13 = arginine; 14 = alanine; 15 = tyrosine; 16 = 2-aminoethanol; 17 = tryptophan; 18 = methionine; 19 = valine; 20 = phenylalanine; 21 = isoleucine; 22 = leucine; 23 = hydroxylysine; 24 = lysine; 25 = unknown impurity.

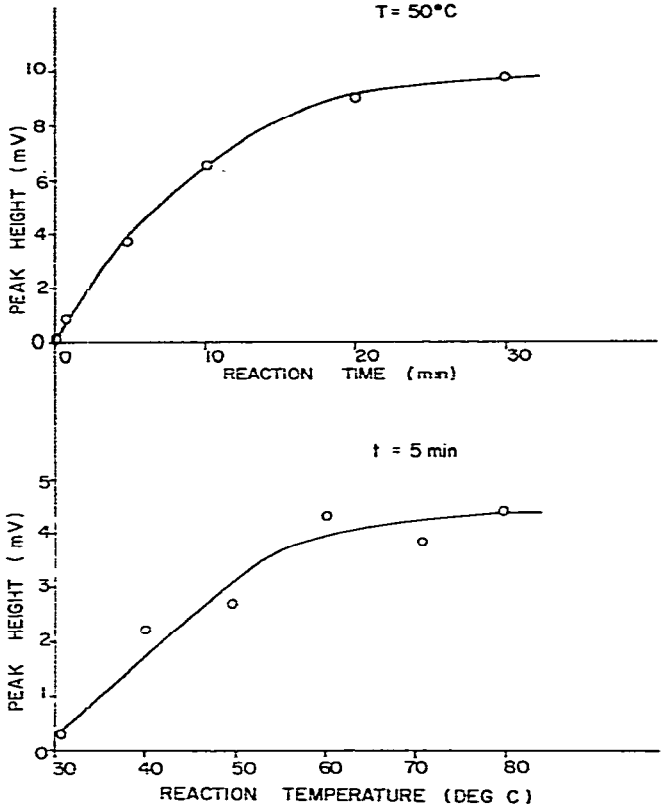

Fig. 2. Graphs relating reaction time and temperature of NBD–proline derivatization to fluorescent yield.

The effect of reaction temperature on the peak height at a constant reaction time of 5 min is shown on the bottom part of Fig. 2. The maximum fluorescent response is attained using temperatures of 60–80°C. Since the reagent blank peak increases with increasing temperature, all NBD–proline reactions were carried out at 60°C, where minimum interference from the reagent was observed. A typical separation of proline from cysteine and hydroxyproline can be seen in Fig. 3. Each peak represents 2 nmoles of amino acid, and the separation was achieved using a simple linear gradient.

Detection limits of OPA and NBD derivatized amino acids are shown in Table I. It can be noted that most amino acids exhibit detection limits below 1 pmole, except in the case of the proline derivative where the detection limit is *ca.* 6 pmoles. Since the NBD reagent peak can be separated from the NBD–proline, the reaction may be carried out at 80°C, where the detection limit for proline would also fall below 1 pmole. The much higher fluorescence response observed for secondary amino acids as compared to primary amino acids agrees well with the work of Ahnoff *et al.*[18], Krol *et al.*[19], and Roth[20], who demonstrated slower reaction kinetics for primary amino acids. The linearity of the proline derivative response was found to be excellent in the 5–25 nmoles/ml range.

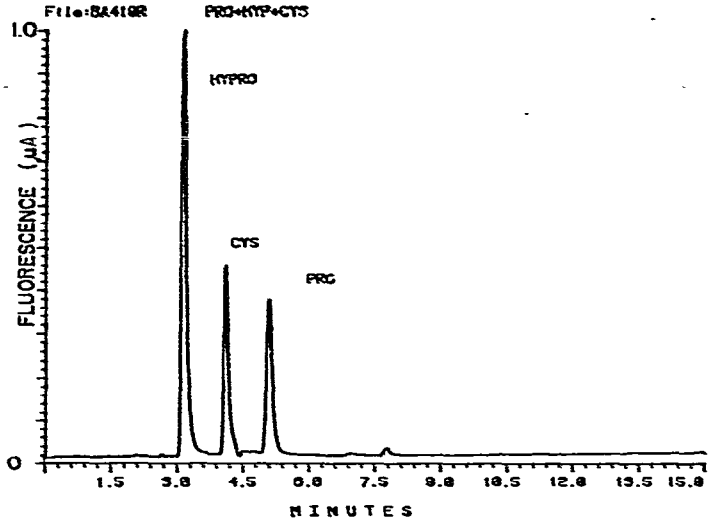

Fig. 3. Separation of proline, cysteine, and hydroxyproline using NBD derivatization. Conditions same as in Fig. 1, except for the gradient program which is linear gradient from 30% B to 45% B, rate 1.5% B per minute; flow-rate, 1.0 ml/min; sample volume, 100 μl; excitation at 220 nm; emission at 370 nm.

TABLE I

DETECTION LIMITS OF AMINO ACIDS

Sample volume, 10 μl.

Amino acid	*Peak height (mV)*	*Detection limit* (pmoles)*	*Sensitivity (relative to histidine)*
CA**	7.700	0.17	6.5
ASP	7.379	0.17	6.3
GLU	6.600	0.20	5.5
CMC**	5.408	0.24	4.5
ASN	6.233	0.21	5.2
SER	4.629	0.28	3.9
GLN	2.475	0.51	2.1
HIS	1.192	1.07	1.0
MSO**	6.692	0.19	5.6
GLY	3.025	0.42	2.5
THR	4.492	0.29	3.7
ARG	5.362	0.24	4.5
ALA	5.042	0.25	4.3
TYR	5.133	0.25	4.3
TRP	2.750	0.46	2.3
MET	4.537	0.28	3.8
VAL	5.087	0.25	4.3
PHE	3.758	0.34	3.1
ILE	6.967	0.18	5.9
LEU	5.546	0.23	4.7
HYL	2.796	0.46	2.3
LYS	1.192	1.07	1.0
PRO	1.890	5.95	—

* Detection limit given here is expressed as minimum amino acid mass placed on the column corresponding to two times signal-to-noise ratio.

** Non-standard abbreviations: CA = cysteic acid; CMC = carboxymethylcysteine; MSO = methionine sulfoxide.

The stability of the proline derivative was also found to be excellent, especially when the sample was reacted in the dark. Using the experiments described previously, no significant change in the fluorescent intensity was observed during a 5-h period. Light-exposed solutions of NBD–proline exhibited an increase in degradation, but more than 95% of the derivative remained unchanged after 5 h of light exposure. An increase in methanol concentration of the mobile phase appeared to improve the stability of all the derivatives, while a decrease in the pH of the mobile phase produced greater instability of the OPA-amino acid adducts. At pH values below 6, the detection of lysine and hydroxylysine has been a problem. To protect these derivatives from rapid decomposition, the suggestion of Jones *et al.*[16] to use sodium dodecylsulfate (SDS) in the reaction mixture was investigated. The lipophilic molecules of SDS

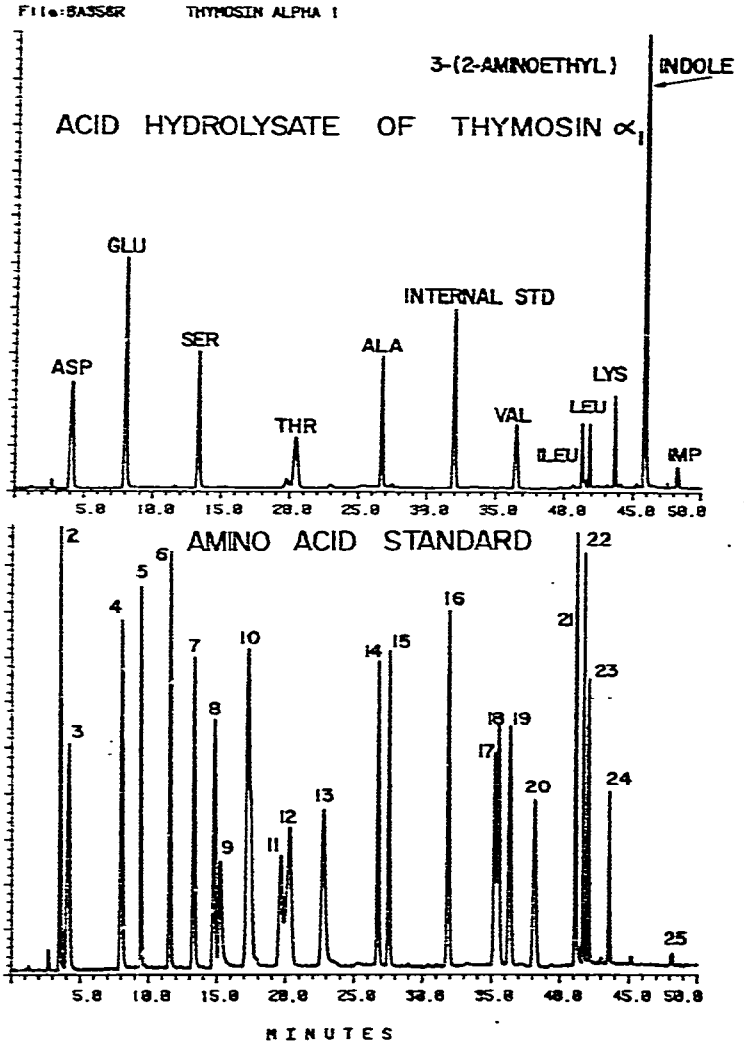

Fig. 4. Amino acid analysis of thymosin α_1. Conditions same as in Fig. 1. Top, acid hydrolysate of thymosin α_1; bottom, amino acid standard.

agglomerate to form micelles, which can entrap the decaying lysine derivative. The critical micelle concentration of SDS, determined from surface tension[21] and conductivity measurements, was found to be 0.2% (w/v). The addition of SDS improved the stability of lysine and hydroxylysine by *ca.* 30%.

The system reproducibility was determined from three replicate injections of an amino acid standard mixture using the conditions described in Figs. 1 and 3. The retention time, as determined by the CIS computer data handling system, could be measured for most amino acids within ±0.2% relative standard deviation (RSD).

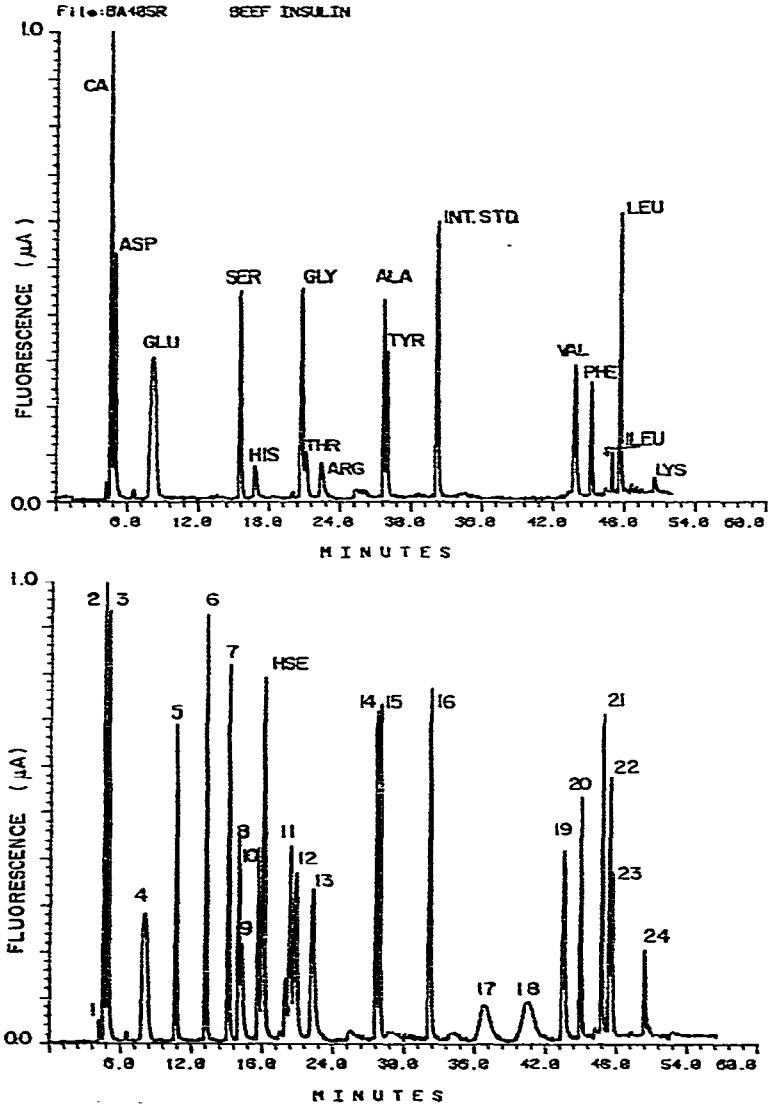

Fig. 5. Amino acid analysis of beef insulin. Conditions same as in Fig. 1. Top, acid hydrolysate of beef insulin; bottom, amino acid standard.

Even with the complex gradient elution system, the relative peak area could be reproduced within ±3% RSD, thus permitting the characterization and quantitation of complex amino acid mixtures.

The application of the analytical system described to the amino acid analysis of two important protein hydrolysates, thymosin α_1 and beef insulin, can be seen in Figs. 4 and 5, where chromatograms of OPA-amino acid adducts shown in comparison to the standard amino acid mixture are illustrated. The hydrolysis of beef insulin and thymosin α_1 was carried out with methanesulfonic acid. In order to quantitate cysteine in beef insulin, the sample was treated with performic acid prior to the hydrolysis which converted cysteine into cysteic acid. The fluorescent derivative of cysteic acid was then easily separated from other amino acid adducts.

A summary of the results obtained from the quantitative analysis of the abovementioned proteins can be seen in Table II. In each case, the data obtained were consistent with the description of the protein in question. It can be observed from Table II that, after the performic acid oxidation of beef insulin, the correct mole ratio for cysteic acid was obtained. However, tyrosine and valine were partially destroyed during the oxidation. When the protein samples were hydrolyzed with 4 *N* methanesulfonic acid containing 0.2% (w/v) of 3-(2-aminoethyl)indole, very good agreement with the theoretical amino acid composition was found, and this is demon-

TABLE II
AMINO ACID COMPOSITON OF BEEF INSULIN AND THYMOSIN α_1

Amino acid	*Beef insulin mole ratio*		*Thymosin α_1 mole ratio*	
	*Found**	*Expected*	*Found***	*Expected*
CA	6.2	6	—	—
ASP	3.3	3	3.6	4
GLU	6.5	7	5.7	6
SER	3.3	2	2.9	3
HIS	1.7	2	—	—
GLY	5.5	5	—	—
THR	1.4	1	2.6	3
ARG	1.0	1	—	—
ALA	3.5	3	3.0	3
TYR	2.5	4	—	—
MET	—	—	—	—
VAL	3.8	5	2.6	3
PHE	2.7	3	—	—
ILE	1.0	1	1.1	1
LEU	7.0	6	1.1	1
LYS	0.8	1	4.2	4
PRO	1.0	1	—	—
TRP	—	—	—	—

* Sample treated with performic acid followed by hydrolysis in 4 *N* methanesulfonic acid containing 0.2% of 3-(2-aminoethyl)indole. The amino acid composition was found to be in good agreement with the predicted values except for tyrosine and valine, which are known to be partially destroyed when pretreated with performic acid.

** Sample hydrolyzed with 4 *N* methanesulfonic acid containing 0.2% (w/v) of 3-(2-aminoethyl)indole.

strated on a thymosin α_1 sample shown in Table II. Thus, the hydrolysis is an extremely important step for the correct determination of total amino acids in any given protein. The amino acid mole ratios of the above-mentioned proteins were independently checked using a post-column derivatization system based on ninhydrin[4]. In all cases, an excellent agreement between the pre-column and the post-column methods was found.

The chromatographic system designed for quantitative analysis of primary and secondary amino acids can be extended to other applications, including tryptic mapping. When hydrolyzed samples of proteins extracted from biological tissues were chromatographed, it was possible to obtain separation of the protein fragments. In the system described, these fragments eluted after the lysine peak. Because of the high selectivity and sensitivity of the system described, the qualitative and quantitative analysis of protein fragments would be quite feasible and would require only a minor change in the gradient elution program.

CONCLUSIONS

The chromatographic system for the quantitative analysis of primary and secondary amino acids described shows all the advantages of the pre-column derivatization method. Elimination of the post-column reactor yields a less expensive, more versatile system where rapid analysis and high sensitivity can be achieved. Application of gradient elution in conjunction with computer data handling gives high reproducibility of the analyses. The use of high-efficiency reversed-phase columns assures high selectivity and minimum band broadening.

The introduction of an aromatic highly fluorescent ring into the amino acid molecule not only lowers the solute detection limit, but also improves the system selectivity by increasing the dispersion forces between the solute and the hydrophobic stationary phase. The minimum solute detectability for amino acids could be improved even further by exciting the OPA-amino acid adducts at 230 nm[13]. Future improvement in the total amino acid analysis may come from the development of a detection system or a fluorogenic reagent which can achieve very high sensitivity for both primary and secondary amines.

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