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RAPID ANALYSIS OF AMINO ACIDS USING PRE-COLUMN DERIVATIZATION

BRIAN A. BIDLINGMEYER*, STEVEN A. COHEN and THOMAS L. TARVIN

Waters Associates, 34 Maple Street, Milford, MA 01757 (U.S.A.)

SUMMARY

A new approach to the pre-column derivatization and analysis of amino acids is described. The method is based upon formation of a phenylthiocarbamyl derivative of the amino acids. The derivatization method is rapid, efficient, sensitive, and specific for the analysis of primary and secondary amino acids in protein hydrolyzates. The liquid chromatographic system allows for the rapid, bonded-phase separation with ultraviolet detection of the common amino acids with 12-min analysis time and a 1-pmol sensitivity.

INTRODUCTION

Amino acid analysis was pioneered by Moore et al. [1]. The approach for this analysis involved a separation on a sulfonated cation-exchange resin using a series of buffers as the eluent. Detection of the separated amino acids was done by colorimetry via a post-column reaction with ninhydrin [2]. This approach has been the mainstay in the protein laboratory for over the last twenty years.

Recently, conventional liquid chromatographs have been configured specifically for amino acid analysis [3]. These approaches have been variations of the classical analysis of amino acids using separation by high-performance ion-exchange resins [3] followed by derivatization with ninhydrin [4], fluorescamine [5, 6], or *o*-phthalaldehyde/2-mercaptoethanol [7—9]. In all of these liquid chromatographic (LC) approaches the post-column derivatization has required special reaction chambers as part of the chromatographic system. While this is an adequate approach it necessarily dedicates the apparatus to one type of analysis and the ion-exchange separation takes approx. 1 h.

Another LC approach to amino acid analysis has been to derivatize the amino acids before the separation. This has been referred to as pre-column derivatization. After the derivative is formed, the separation occurs on a conventional high-performance reversed-phase column. Dansyl (Dns) [10, 11] or phenylthiohydantoin (PTH) [12] derivatives of amino acids are two examples

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of pre-column derivatization methods. In general, the pre-column derivatization technique, especially with reversed-phase columns, offers greater efficiency, ease of use, and higher speed of analysis than the conventional ion-exchange techniques. The LC analysis of the PTH or Dns derivatives of amino acids was relatively rapid and showed good efficiency and sensitivity; however, these techniques suffer from a lack of derivative stability, less than quantitative yields (PTH) or interference from reagent peaks (Dns).

One reagent, *o*-phthalaldehyde (OPA), has been shown to meet many, but not all, of the requirements for a pre-column derivatization agent for primary amino acids [13, 14]. Most primary amino acids form a single, unique, substituted isoindole product that can be detected by fluorescence. However, OPA does not react with secondary amino acids and this can be a significant drawback.

This paper will report on a new approach to the pre-column derivatization and analysis of amino acids. The method is based upon formation of a phenylthiocarbamyl (PTC) derivative of the amino acids which was first demonstrated by Koop et al. [15] for the analysis of free amino acids liberated by carboxypeptidase Y digestion of peptides from cytochrome P-450. We have modified the technique for the application to free amino acids from acid-hydrolyzed proteins. The new method will be shown to be a rapid, efficient, sensitive, and specific technique for the analysis of primary and secondary amino acids in protein hydrolyzates. The method allows for the rapid, bonded-phase separation with ultraviolet detection of the common amino acids with 12-min analysis time and a 1-pmol sensitivity.

EXPERIMENTAL

Materials

Acetonitrile, triethylamine (TEA), phenylisothiocyanate (PITC) and amino acid standards (Pierce H) were obtained from Waters Assoc. (Milford, MA, U.S.A.). Constant boiling hydrochloric acid (Sequanal™ grade) was obtained from Pierce (Rockville, IL, U.S.A.). Sodium acetate was purchased from Mallinkrodt (St. Louis, MO, U.S.A.). Glacial acetic acid was reagent grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). High-purity water was supplied by a Milli-Q™ purification system (Millipore, Bedford, MA, U.S.A.) that was fed with a supply of reversed-osmosis purified tap water. Hydrolysis and derivatization tubes were Pyrex™ brand (Corning, Corning, NY, U.S.A.), while the vacuum vials and resealable enclosures were from Waters Assoc. Proteins were from a variety of sources.

Sample preparation hardware

A new sample preparation module, the Pico-Tag™ Workstation (Waters Assoc.), was developed [16]. The unit incorporates two separate functions in a compact design for the batchwise preparation of protein and peptide hydrolyzates and for derivatization of the free amino acids. A high-efficiency vacuum pump, equipped with cold and acid traps, is connected to a manifold that allows virtually leak-free attachment of a specially designed vacuum vial

(Waters Assoc.) containing twelve 50 × 6 mm sample tubes. The vacuum vials are equipped with a resealable PTFE closure that is drilled through to allow a vacuum to be applied. The use of hydrochloric acid/water vapor for hydrolysis, rather than placing 6 *M* hydrochloric acid in the sample tubes, allows for a cleaner hydrolysis and faster sample drying after hydrolysis. Condensed hydrochloric acid is kept out of the sample tubes by the bulged sides of the vacuum vial. Removal of trace levels of oxygen from hydrolysis vials is easily accomplished by alternate evacuating and nitrogen flushing steps using the manifold controls on the Workstation. In this manner, twelve samples can be sealed under vacuum in less than 5 min.

The Workstation is also equipped with a four-chamber oven with variable-temperature control. Up to four of the vacuum vials, or 48 samples, can be processed simultaneously.

Hydrolysis of proteins and peptides

Standard proteins and peptides were dissolved in water or 0.1 *M* hydrochloric acid. Stock solutions contained 1.0 mg/ml, and for high-sensitivity work the stock solutions were diluted to 20 μg/ml. A volume corresponding to 0.1 to 5.0 μg was pipetted into a 50 × 6 mm tube, and up to twelve tubes were placed in the vacuum vial. The vial was then attached to the Workstation manifold, and the solvent removed under vacuum. After drying, the vacuum was released and 200 μl of constant boiling hydrochloric acid containing 1% (v/v) phenol was pipetted into the bottom of the vacuum vial (not the hydrolysis tubes). The vacuum vial was then reattached to the manifold, evacuated and sealed under vacuum (130—260 Pa). Samples were hydrolyzed in the Workstation either at 150°C for 1 h or at 108°C for 24 h. After hydrolysis, the outside of the 50 × 6 mm tubes were wiped clean and the residual hydrochloric acid inside the vacuum vial removed under vacuum.

Derivatization of amino acids with PITC

Standards of free amino acids, in a mix (Pierce H) or individual samples, containing up to 25 nmol of each amino acid were placed in the 50 × 6 mm tubes and dried under vacuum. Free amino acids and hydrolyzed samples were dried down again after adding 10—20 μl of ethanol—water—TEA (2:2:1) to each tube. When the vacuum reached 8—10 Pa (about 2—3 min), the samples were ready for derivatization.

The derivatization reagent was made fresh daily and consisted of ethanol—TEA—water—PITC (7:1:1:1). The PITC was stored at −20°C under nitrogen to prevent breakdown products from forming. To make up 300 μl of reagent, enough for twelve samples, 210 μl of ethanol were mixed thoroughly with 30 μl each of PITC, TEA, and water. PTC amino acids were formed by adding 20 μl of reagent to the dried samples and sealing them in the vacuum vials for 20 min at room temperature. The reagents were then removed under vacuum using the Workstation.

Using the conditions and Workstation previously described, the reaction of free amino acids with PITC is essentially complete after 20 min at ambient temperature. Longer reaction times and higher temperature do not result in increased yield of any amino acids; however, decreased yields of the acidic amino acids, glutamic and aspartic acids, were observed at elevated tempera-

ture. Furthermore, neither higher reagent concentration or addition of more reagent after 20 min gave increased response.

Reaction times as short as 10 min could be used on a standard amino acid mixture with little change in yield, but an occasional protein sample exhibited anomalous low yields for certain polar residues (e.g. aspartic acid and histidine) with a shorter period of reaction. These slower kinetics might be caused by relatively poor solubility in the largely organic reaction medium.

Substitution of TEA with other tertiary amines was studied. Both trimethylamine and pyridine were used either as the sole amine or in concert with TEA. However, the replacement of part or all of the TEA yielded significant amounts of interference peaks in the chromatographic analysis, especially in high-sensitivity studies. Even the use of redistilled pyridine posed problems in low-level analyses.

Chromatography

The system was a Model ALC 204 liquid chromatograph (Waters Assoc.) which consisted of two Waters M6000A solvent delivery systems and an M440 fixed-wavelength detector (254 nm) controlled with an M720 controller. The temperature was controlled within ± 1°C with a column heater (Waters Assoc.). Samples were injected in volumes ranging from 1 to 40 μl using an M710B WISP^{TM} auto injector (Waters Assoc.). The columns were application-specified Pico-Tag columns, packed in 15 cm × 3.9 mm hardware, and quality-controlled for rapid, high-efficiency, bonded-phase separations. Eluents were kept under a blanket of helium with an Eluent Stabilization System (Waters Assoc.). The solvent system consisted of two eluents: (A) an aqueous buffer and (B) 60% acetonitrile in water. The typical buffer was 0.14 *M* sodium acetate containing 0.5 ml/l TEA and titrated to pH 6.35 with glacial acetic acid. A gradient which was run for the separation consisted of 10% B traversing to 51% B in 10 min using a convex curve (number 5). After this, a washing step was programmed to 100% B so that any residual sample components would be cleaned from the column.

RESULTS AND DISCUSSION

Resolution and analysis time

The amino acid standards were derivatized using the procedure previously described. The separation of the amino acids at the 250-pmol level is shown in Fig. 1. All of the amino acids are well resolved. The gradient shape was chosen to optimize the spacing of the separated peaks in the minimum analysis time. A more complete discussion of the development of the separation is given elsewhere [17].

Other common amino acids can be analyzed using the same conditions. PTC-carboxymethyl cysteine elutes at 2.7 min and PTC-hydroxyproline at 3.1 min, both well resolved from other derivatives. PTC-tryptophan elutes after PTC-phenylalanine and is also well resolved. Yet to be studied are the derivatives of asparagine, glutamine and the amino sugars that are often found in protein hydrolyzates. Reagent purity is important and if dirty or old TEA reagents are used there can be spurious peaks occurring between peak number

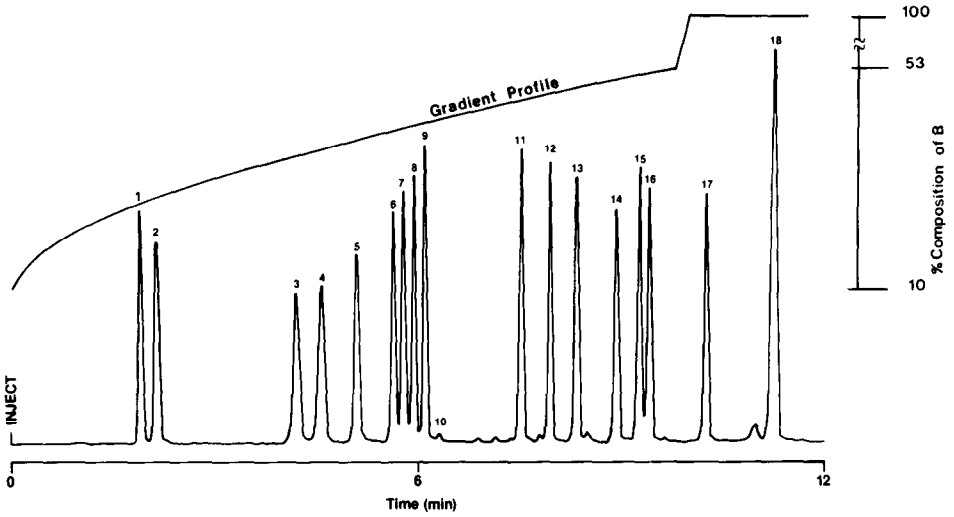

Fig. 1. Separation of amino acid standards (Pierce H). Eluent A: 0.14 *M* sodium acetate, 0.5 ml TEA, pH 6.4; eluent B: 60% acetonitrile in water; gradient: 10% B to 53% B in 10 min on curve 5; flow-rate: 1.0 ml/min; column: Pico-Tag analysis column; detector: ultraviolet (254 nm) at 0.1 a.u.f.s. For peak identification see Table I; 10 = ammonia.

13 and 14. The peak between peaks 17 and 18 is an unknown peak and is present in the analysis of a blank derivative.

Derivative stability

After the reagent is removed under vacuum the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. Dissolved in solution prior to injection into the LC system, degradation will occur if the samples are not kept cold. At ambient temperature a 5% drop in response will occur for cystine, valine, and isoleucine after 6 h. After 10 h under the same conditions, most of the other derivatives have lost 5% in response. If kept cold, no noticeable loss in chromatographic response occurred after three days.

Response linearity and reproducibility

For any chromatographic analysis, it is important to demonstrate reaction linearity in the range of analytical interest. Amounts varying from 200 to 5000 pmol were derivatized, and 10% of the total sample was analyzed. Samples were run in triplicate, and the average areas were calculated. The data are plotted in Fig. 2, and show that linear response is indeed obtained in the range 20—500 pmol. Correlation coefficients for these data exceeded 0.999. The poorest linearity was for cystine. The reason for this is unknown at present.

In order to determine the reproducibility of the entire chromatographic procedure several analyses were performed. Individual samples of the amino acid standard mixture were derivatized and analyzed on the liquid chromatograph. The results are shown in Table I. The data have been normalized to the internal standard norleucine. As can be seen the retention time had an average

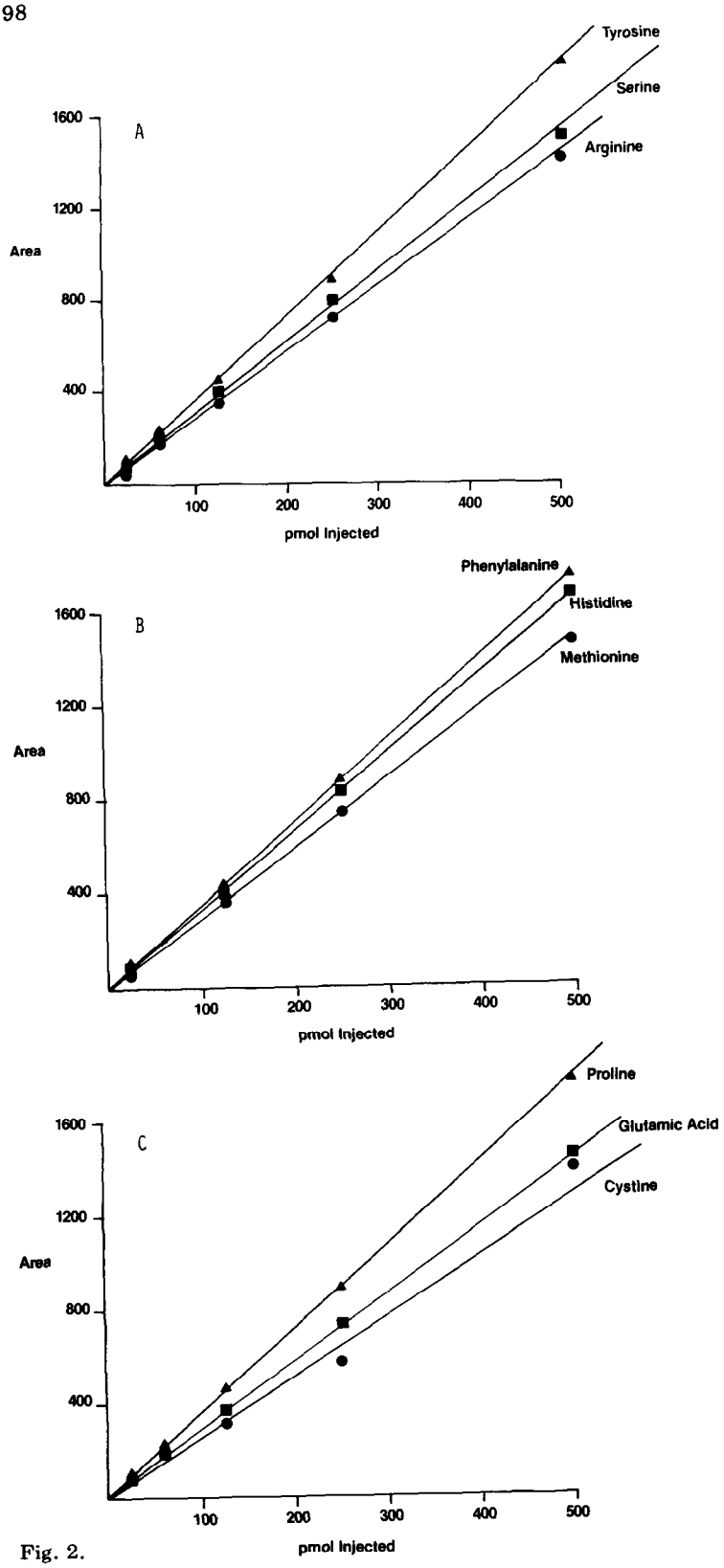
Tyrosine
Serine
Arginine
A
1600
1200
Area
800
400
100
200
300
400
500
pmol Injected
Phenylalanine
Histidine
Methionine
B
1600
1200
Area
800
400
100
200
300
400
500
pmol Injected
Proline
Glutamic Acid
Cystine
C
1600
1200
Area
800
400
100
200
300
400
500
pmol Injected

Fig. 2.

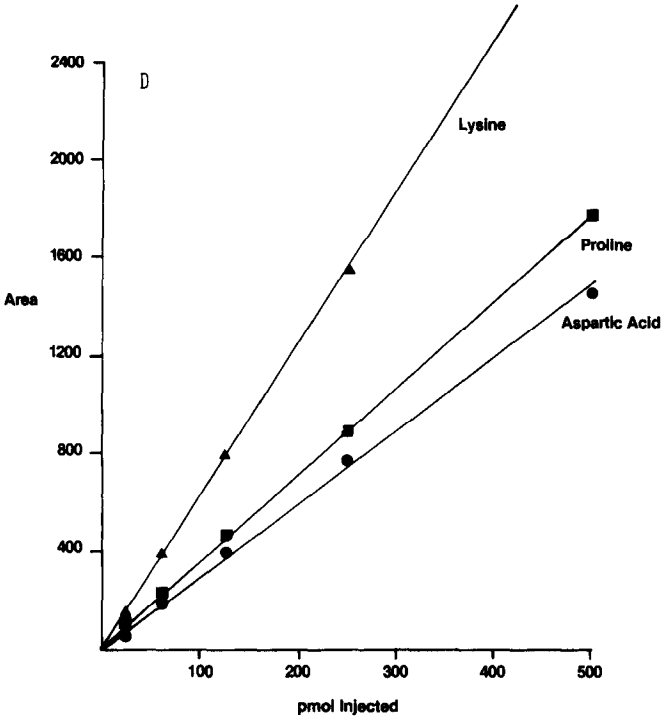

Fig. 2. Linearity of response. See text for details.

TABLE I

REPRODUCIBILITY OF METHODOLOGY

Amino acid	Percent relative standard deviation (n = 8)		
	Retention time	Peak area	
		Uncorrected	Normalized
1. Aspartic acid	0.50	1.86	0.97
2. Glutamic acid	0.45	1.90	1.05
3. Serine	0.41	1.89	0.85
4. Glycine	0.37	1.44	1.48
5. Histidine	0.19	1.82	1.15
6. Arginine	0.23	1.44	1.22
7. Threonine	0.13	2.55	1.56
8. Alanine	0.21	2.07	0.96
9. Proline	0.16	2.39	1.46
11. Tyrosine	0.37	2.15	1.78
12. Valine	0.22	2.03	0.95
13. Methionine	0.22	1.84	0.87
14. Cystine	0.14	4.25	3.85
15. Isoleucine	0.07	2.38	1.23
16. Leucine	0.08	2.04	1.07
17. Phenylalanine	0.11	2.31	1.55
18. Lysine	0.06	4.76	4.60
Average	0.23	2.30	1.48

percentage relative standard deviation of ± 0.23. The largest percentage relative standard deviation occurred for the early eluting peak aspartic acid; however, This was a reflection of a retention time of 1.9 with a variation of less than ± 0.01 min. The average percentage relative standard deviation in peak area was ± 1.48.

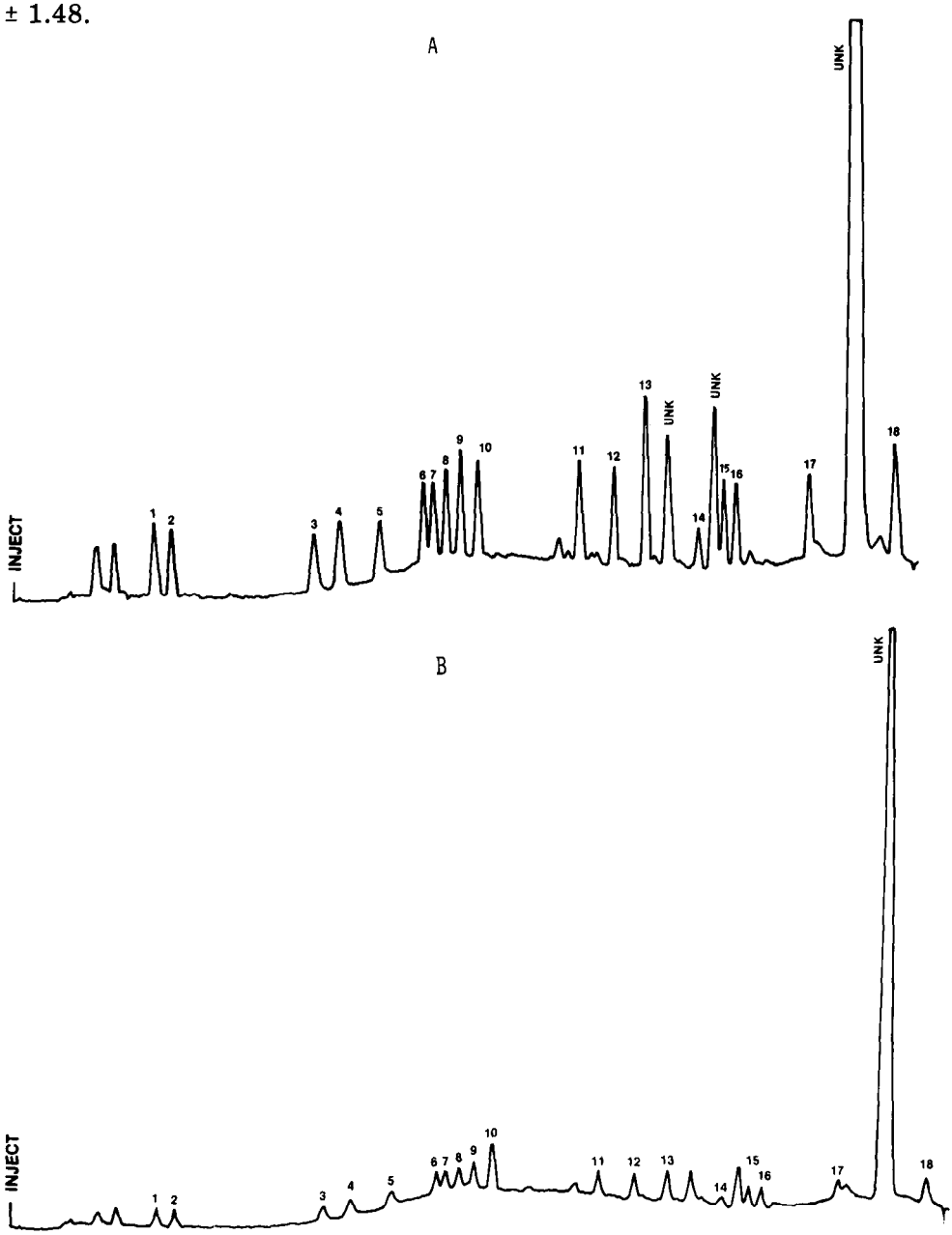

Fig. 3. High-sensitivity amino acid analysis. (A) Amino acid standards at 4 pmol, (B) amino acid standards at 1 pmol. Conditions as in Fig. 1 except gradient is 10% B to 51% B in 10 min on curve 5 and the detector was at 0.005 a.u.f.s. For peak identification see Table I: 10 = ammonia; UNK = unknown.

Detection limits

Fig. 3 shows the analysis of the standard amino acid mixture (Pierce H) at 4- and 1-pmol levels. By defining the detection limit as having a signal-to-noise ratio of 5, the detectable limit for most of the amino acid derivatives can be considered to be 1 pmol. However, because of background amino acids being contributed from solvents, chemicals, glassware, and pipet tips, quantitation of real samples at these levels (hydrolysis and derivatization included) is not practical. It has been the authors' experience that starting with greater than 500 ng of protein before hydrolysis is best suited for the amino acid analysis of proteins. This range is readily handled by the system described earlier using the ultraviolet detector at 0.005 a.u.f.s. With more sophisticated data devices the level of detectability should be much lower.

High sensitivity analysis

The feasibility of using PITC derivatization for the analysis of samples in the nanogram range was investigated. The results show a favorable comparison between compositional analyses performed on 100 ng and 5 μg of oxidized bovine insulin B chain. The 100-ng sample amount was equivalent to approx. 33 pmol of sample being hydrolyzed and 3.3 pmol of the sample injected. Chromatography of 10% of the hydrolyzed samples (10 ng and 500 ng, respectively) gave the results calculated in Table II. As is evident, there is slight loss in reliability at this extremely low level, especially for threonine, proline, phenylalanine and leucine. The results for these amino acids may be high owing to contaminating levels that are evident in blank samples. Nonetheless, it should be emphasized that the low-nanogram-level accuracy demonstrated is sufficient for compositional analysis.

TABLE II

HIGH-SENSITIVITY ANALYSIS OF INSULIN B CHAIN

Amino acid	Calculated ratio of amino acids		Theoretical composition
	10 ng injected*	500 ng injected*	
Aspartic acid	1.2	1.1	1
Glutamic acid	2.4	3.2	3
Serine	1.2	1.0	1
Glycine	3.2	3.0	3
Histidine	1.6	1.9	2
Arginine	1.3	1.0	1
Threonine	1.3	0.9	1
Alanine	2.1	2.0	2
Proline	1.3	1.0	1
Tyrosine	1.5	2.0	2
Valine	2.5	2.9	3
Methionine	0	0	0
Isoleucine	0	0	0
Leucine	2.0	4.0	4
Phenylalanine	2.1	2.9	3
Lysine	0.8	1.0	1

*The injected amount was 10% of total hydrolyzed peptide.

Comparison to ion-exchange analysis

Amino acid analysis is a major analytical need in numerous scientific studies. A new procedure for amino acid analysis must be equivalent or better in speed, sensitivity, precision, and ease of operation when compared to present ion-

TABLE III

AMINO ACID ANALYSIS OF TRYPSIN (0.2 mg HYDROLYSATE)

Amino acid	Mol percent	
	Pico-Tag	Ion-exchange
Aspartic acid	9.6	10.2
Glutamic acid	8.1	8.7
Serine	11.9	10.6
Glycine	15.6	15.3
Histidine	1.7	1.7
Arginine	2.1	2.3
Threonine	4.3	4.4
Alanine	7.7	8.4
Proline	5.7	6.1
Tyrosine	3.3	3.3
Valine	5.1	5.4
Methionine	0.9	0.9
Cystine	4.8	4.0
Isoleucine	5.4	5.0
Leucine	7.3	7.4
Phenylalanine	1.8	1.7
Lysine	4.6	4.5

TABLE IV

AMINO ACID ANALYSIS OF HUMAN INSULIN

Amino acid	Known	Calculated residues per mol*	
		Pico-Tag	Ion-exchange
Aspartic acid	3	3.0	2.9
Glutamic acid	7	6.9	6.8
Serine	3	2.8	2.8
Glycine	4	4.0	4.0
Histidine	2	1.8	1.8
Arginine	1	1.1	1.1
Threonine	3	3.0	2.8
Alanine	1	1.2	1.1
Proline	1	1.0	1.0
Tyrosine	4	4.0	3.7
Valine	4	3.3	3.0
Methionine			
Cystine	6	5.0	4.9
Isoleucine	2	1.3	1.2
Leucine	6	6.2	5.9
Phenylalanine	3	3.1	2.8
Lysine	1	1.1	1.0

*Based on 4.0 residues per mol glycine.

TABLE V

AMINO ACID ANALYSIS OF OXYTOCIN

Amino acid	Known	Calculated residues per mol (Pico-Tag)
Aspartic acid	1	0.8
Glutamic acid	1	1.0
Serine		
Glycine	1	1.0
Histidine		
Arginine		
Threonine		
Alanine		
Proline	1	1.0
Tyrosine	1	0.9
Valine		
Methionine		
Cystine	2	1.5
Isoleucine	1	0.9
Leucine	1	1.0
Phenylalanine		
Lysine		

exchange analyzers. Clearly the new procedure described here exceeds these needs. However, it is also essential that equivalent results be obtained with the new method compared to the traditional method. In Tables III—V the results of the new derivative procedure is compared to the results of ion-exchange analysis [18]. In all cases, the results compare favorably suggesting that the PITC amino acid methodology described in this communication provides an alternative to current ion-exchange analyzers.

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