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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF Dns-AMINO ACID DERIVATIVES AND APPLICATIONS TO PEPTIDE AND PROTEIN STRUCTURAL STUDIES

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SUMMARY

Dns derivatives of amino acids were separated completely by reversed-phase high-performance liquid chromatography employing complex gradient elutions. Three different solvent systems were developed and compared with respect to resolution. Two different detection methods were evaluated. Fluorescence (excitation 320 ± 75 nm, emission 430 nm cutoff) could be monitored for the detection in the low picomole range. The system could be applied to the determination of amino acid compositions of peptides and proteins. The amino acid compositions of peptides and proteins could be determined, including the amino acids proline, cystine and tryptophan. In addition, the system has been satisfactorily applied to N-terminal analyses and Dns–Edman sequence analyses of peptides and proteins.

INTRODUCTION

Dns chloride (Dns-Cl, 1-dimethylaminonaphthalene-5-sulfonyl chloride) is a well characterized fluorogenic agent for the detection of amino compounds such as amino acids[1,2]. It has been used as a highly sensitive reagent in protein chemistry laboratories for the identification on amino acids, peptides and proteins. With the pioneering work of Gray and Hartley[3–6], this technique became one of the powerful methods for the end-group and sequence analysis of peptides and proteins. One of the most commonly used separation methods for the Dns-amino acid derivatives is the two-dimensional polyamide thin-layer chromatography (TLC) of Woods and Wang[7] which provides a sensitivity of the separated Dns-amino acids as low as 10–50 pmoles. However, with a routine analysis, usually the separation of the acidic amino acid pairs, namely, Dns Glu/Asp, the polar Dns Thr/Ser and the Dns His/Arg has been difficult. Hence, alternate methods of separation such as electrophoresis have been applied[8]. All of the thin-layer methods suffer from the relative inability to quantitate the amounts of the Dns derivatives.

Precolumn-labeled amino acid derivatives such as phenylthiohydantoins (PTH) and dimethylaminoazobenzeneisothiohydantoins (DABTH) have been

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successfully separated by high-performance liquid chromatography (HPLC)[9]. It would be rather surprising if derivatized Dns-amino acids were not subjected to this type of separation. As reviewed by Deyl[10], however, the early attempts of separation of Dns-amino acids by HPLC were not compatible to the separation employing simple TLC. More recently, Dns derivatives of amino acids have been separated using more efficient columns such as silica gel normal-phase or chemically bonded phase packings with either ultraviolet of fluorescence detection[11–17]. However, some workers still faced difficulties in resolving all the derivatives in a single chromatographic analysis, while others did not obtain satisfactory response in detecting minute amounts of the sample. A recent communication reported the use of a Hypersil ODS reversed-phase column and a quaternary solvent system to separate all Dns-amino acids[18]. Even though this column material is commercially available, prepacked columns are not. De Jong *et al.*[19] used a Brownlee RP-300 resin and a gradient of methyl ethyl ketone–2-propanol at 55°C for the separation of dansylated amino acids. In spite of the poor resolution for several pairs of Dns-amino acids, these authors pointed out the potential usefulness of the method of precolumn Dns derivatization and fluorescence detection for both amino acid and amino-terminal analyses of peptides. Wiedmeier *et al.*[20] were also able to quantitate Dns-amino acids from body tissues and fluids with the use of an Ultrasphere ODS column.

We report here a complete separation of Dns-amino acids on a reversed-phase packing employing complex gradient elutions with a detection level in the low picomole range. Three different solvent systems are compared with respect to resolution, and two different detection methods are evaluated. Also, examples of determinations of amino acid compositions of peptides and proteins, N-terminal determinations of peptides and proteins and Dns–Edman sequence analyses are reported.

MATERIALS AND METHODS

Materials

Dns-amino acids were purchased from Sigma. N-α-Dns-L-histidine was obtained from Fluka. Standard amino acid mixture, Dns-Cl, trifluoroacetic acid (sequanal grade) and 4 *M* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole were from Pierce. Performic acid oxidized insulin A chain, lysozyme and ribonuclease A were purchased from Millipore. Carboxymethylated lysozyme (CM-lysozyme) was prepared by reduction of the protein with dithiothreitol followed by alkylation with recrystallized iodoacetic acid according to Crestifield *et al.*[21]. Synthetic L-Met–L-Phe–Gly monohydrate and Met-enkephalin were from Travinol. Acetonitrile (UV cutoff 188 nm) and tetrahydrofuran were purchased from MCB Manufacturing Chemists. Other chemicals and solvents were at least analytical grade and were purified by recrystallation or redistillation.

Apparatus

The HPLC system used was a Micromeritics 7500 series liquid chromatograph consisting of Model 730 universal injection valve, Model 731 column compartment (with temperature control), Model 740 control module with integration and complex gradient capabilities, Model 750 solvent delivery system, Model 753 ternary solvent mixer, and Model 786 variable-wavelength detector. Fluorescence was monitored by

using either a Waters fluorescence detector Model 420-AC or a Varian Fluorochrom fluorocence detector. Excitation was through a 320 ± 75 nm bandpass filter and the emission filter was a 430 nm cutoff. All separations were conducted at 40°C on a 250 × 4 mm I.D. BioRad Bio-Sil ODS-5S column which was protected with a cartridge-type guard column.

Chromatographic procedures

For the separation of Dns-amino acids three solvent systems were developed, each with a flow-rate of 1.0 ml/min. The chromatographic conditions for the three solvent systems are summarized in Table I.

System 1. Solvent A was 10 m*M* sodium acetate, pH 5.2, and solvent B was acetonitrile. The method developed using these solvents consists of two linear gradients in tandem. Initial conditions were 100% solvent A. Before the first gradient, there was a 15-min isocratic step at the initial conditions (100% A). The first gradient was from 0% to 24% B over 22 min, followed by a 10-min hold at this concentration. The second gradient lasted 12 min, and the concentration of solvent B was increased to 40% during this period. An 8-min hold followed the second gradient.

System 2. Solvent A used in this system was 10 m*M* sodium acetate, pH 5.2, 0.075% trifluoroacetic acid (TFA) and 5% tetrahydrofuran (THF), and solvent B was 10% THF in acetonitrile. The chromatography was initiated with the mobile phase consisting of 92% solvent A and 8% solvent B. A gradient was initiated 13 min after the injection of the sample; solvent B concentration was increased linearly from 8 to 30% over a period of 8 min, which was followed by a 3-min isocratic period at 30% B. After this isocratic step a second linear gradient was applied —from 30 to 32% over 12 min, followed by another isocratic hold at 32% B (for 6 min). The third linear gradient (from 32 to 80% B) lasted for 10 min, followed by an 8-min hold at the final conditions (80%).

System 3. This system consisted of solvent A, 30 m*M* sodium phosphate, pH 6.5, and solvent B, acetonitrile. Initial conditions for the chromatography were 90% solvent A and 10% solvent B. The concentration of B was linearly increased to 22% over a period of 45 min, and then maintained at 22% for 13 min. This step was followed by another linear gradient (from 22 to 40% B) which lasted 20 min. The level of B was maintained at 40% for an additional 12 min.

Hydrolysis of peptides and proteins

Peptides and proteins were hydrolyzed in constant-boiling 6 *M* hydrochloric acid containing 0.2% redistilled phenol for 24 h at 110°C *in vacuo*. The samples containing tryptophan were hydrolyzed in 6 *M* hydrochloric acid containing 3% mercaptoacetic acid and 0.2% phenol or in 4 *M* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[22] for 24 h at 110°C *in vacuo*. After hydrolysis, samples containing 6 *M* hydrochloric acid were dried under vacuum. The samples containing 4 *M* methanesulfonic acid were neutralized with equal volume of 4 *M* sodium hydroxide.

Dansylation of amino acids

The conditions for the dansylation of amino acids were similar to those used by Tapuhi *et al.*[23] with some minor modifications. A Dns-Cl solution (usually 2.5–

TABLE I

CHROMATOGRAPHIC CONDITIONS*

System 1	*Solvent A* 10 m*M* sodium acetate, pH 5.2	*Solvent B* Acetonitrile	*Initial conditions* 0% B (15 min)	*First gradient* 0 24% B (22 min)	*First isocratic hold* 10 min at 24% B
	Second gradient 24 40% B (12 min)	*Second isocratic hold* 8 min at 40% B	*Total time* 67 min		
System 2	*Solvent A* 10 m*M* sodium acetate, pH 5.2, 0.075% TFA, and 5% THF	*Solvent B* 10% THF in Acetonitrile	*Initial conditions* 8% B (13 min)	*First gradient* 8 30% B (8 min)	*First isocratic hold* 30% B (3 min)
	Second gradient 30 32% B (12 min)	*Second isocratic hold* 6 min at 32% B	*Third gradient* 32–80% B (10 min)	*Third isocratic hold* 8 min at 80% B	*Total time* 60 min
System 3	*Solvent A* 30 m*M* sodium phosphate, pH 6.5	*Solvent B* acetonitrile	*Initial conditions* 10% B (0 min)	*First gradient* 10–22% B (45 min)	*First isocratic hold* 13 min at 22% B
	Second gradient 22 40% B (20 min)	*Second isocratic hold* 12 min at 40% B	*Total time* 90 min		

* Numbers in parentheses are the length of time to each step.

5.0 mg/ml, 7.26–14.53 m*M*) was freshly prepared weekly in HPLC grade acetonitrile; 50 m*M* potassium borate buffer was prepared by diluting Dionex Fluoropa Diluent (1 *M*, pH 10.5) with distilled water and adjusting the pH to 9.5 with 1 *M* hydrochloric acid. The ratio of Dns-Cl to buffer was 1:2 and the volume used varied according to the total concentration of Dns-susceptible amino groups present in the sample. The optimal conditions for the dansylation reaction were found to be at a ratio of Dns-Cl to amino acids of 5:1 to 10:1. A similar ratio range was previously reported by Tapuhi *et al.*[23] and De Jong *et al.*[19] using lithium carbonate as the reaction buffer. The standard amino acid mixture (1–10 nmoles) or peptide and protein hydrolysates (1–5 nmoles) were treated with the Dns-Cl solution at 37°C for 60 min or at elevated temperatures (65°C for 10 min). The derivatized samples were then subjected to HPLC analysis or stored at −40°C until analyzed.

N-Terminal analysis

Peptide or protein samples (1–5 nmoles) were dansylated using similar conditions as described for dansylation of amino acids. The derivatized sample was then dried under vacuum and hydrolyzed with 6 *M* hydrochloric acid containing 3% mercaptoacetic acid and 0.2% phenol or with 4 *M* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. The sample was then subjected to HPLC analysis or stored at −40°C until analyzed.

Dns-Edman sequence analysis

Sequence analysis of peptides using the Dns–Edman method was modified from that reported by Hartley[4]. The sample (10–20 nmoles) was placed in a stoppered Pierce Reacti-Vial (1 ml) and evaporated and then 150 μl of water were added. At this point, an aliquot was taken and N-terminal analysis was conducted as described above. After the addition of 150 μl of 5% phenyl isothiocyanate (PITC) in pyridine, the vial was flushed with nitrogen and incubated for 45 min at 55°C. To effect partial removal of the side products, the reaction mixture was extracted with 200 μl of *n*-heptane–ethyl acetate (2:1) and then the residual sample was evaporated to dryness over phosphorus pentoxide. To effect the cleavage, 100 μl of trifluoroacetic acid were added and incubated for 30 min at 45°C under nitrogen. After the acid was evaporated over sodium hydroxide, 100 μl of water were added to the sample. The sample was extracted twice with 200 μl of butyl acetate, and a portion of the sample was taken for N-terminal analysis.

RESULTS AND DISCUSSION

Although the HPLC separations of Dns-amino acid derivatives have been recently reported with potential applicability[19,20], the separation of complex samples necessitates improvement of new HPLC method(s) which exhibit satisfactory selectivity, sensitivity, reproducibility and resolution. Recent work[14,18,20] indicated the use of reversed-phase HPLC in a gradient elution mode is a feasible chromatographic system for the separation.

Resolution

Three different solvent systems were compared with respect to the separation

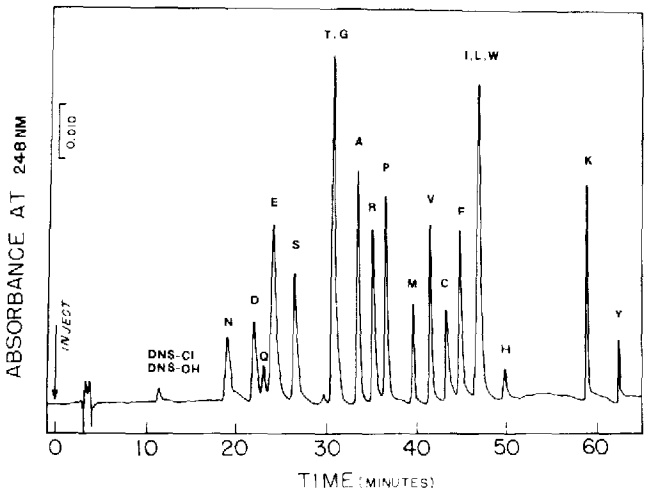

Fig. 1. HPLC separation of Dns-amino acids with solvent System 1. For experimental details see Materials and methods. Single-letter designation for amino acids is used in the identification of the peaks. The effluent is monitored with a UV detector at 248 nm.

of the Dns-amino acids with reversed-phase HPLC. Figs. 1–3 show the chromatograms obtained for the three solvent systems. System 1 consists of 10 m*M* sodium acetate, pH 5.2 (solvent A), and acetonitrile (solvent B). Utilizing this solvent system and a complex gradient, most of the Dns-amino acids were resolved except the Thr/Gly pair and Trp/Ile/Leu triplet (Fig. 1). In System 2 all the Dns-amino acids were resolved except the Glu/Ser and Ile/Leu pairs. The resolution of these pairs was satisfactory to allow quantitation. The arginine and histidine peaks were relatively

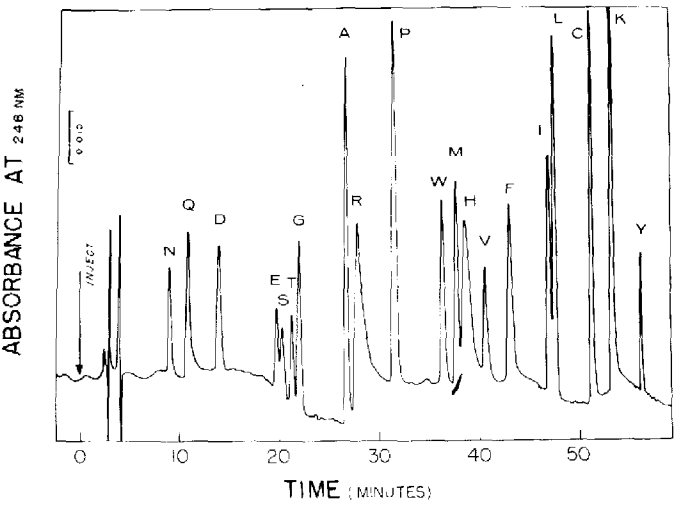

Fig. 2. HPLC separation of Dns-amino acis with solvent System 2. For experimental details see Materials and methods. Single-letter designation for amino acids is used in the identification of the peaks. The effluent is monitored with a UV detector at 248 nm.

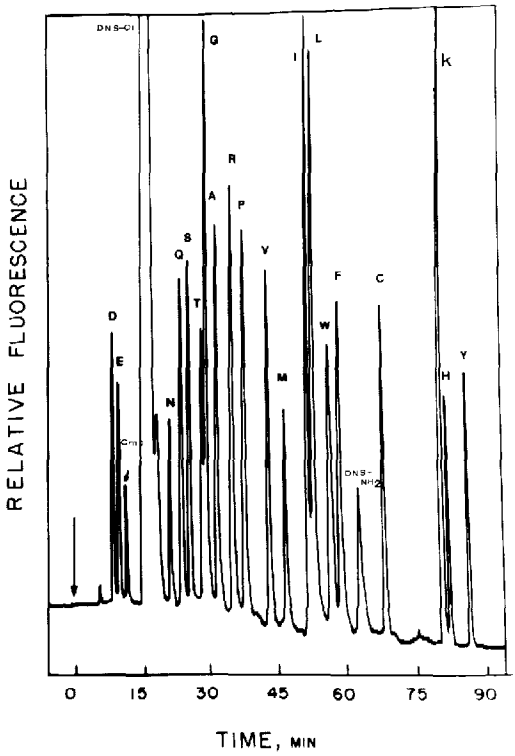

Fig. 3. HPLC separation of Dns-amino acids with solvent System 3. For experimental details see Materials and methods. Arrow marks the injection. Single-letter designation for amino acids is used in the identification of the peaks. The effluent is monitored with a fluorescence detector (excitation 320 + 75 nm, emission 430 nm cutoff).

broader (Fig. 2). The retention time of these two basic amino acid derivatives were very sensitive to slight changes in solvent configuration. Therefore in utilizing this system, it is important to pay special attention to the preparation of solvents, to minimize changes from one batch of solvent to the next. The separation of Dns derivatives of amino acids utilizing System 3 with the aid of a complex gradient, is shown in Fig. 3. The separation of the Ile/Leu pair was much better than in the other two systems. The only pair of Dns-amino acids which failed to result in baseline resolution was the Thr/Gly pair. The reproducibility of the chromatogram was excellent; there was almost no change from one batch of solvent to the next.

Quantitation

If a chromatographic system is to be used for quantitative analysis, the technique must be reproducible, possess high sensitivity, and give a linear response in the concentration range required. All amino acid derivatives tested gave linear responses in the concentration range of 5–100 pmole. At least in this concentration range, the system could be used to obtain quantitative results.

Reproducibility

The reproducibility of the system was evaluated by injecting the standard mixture of Dns-amino acids utilizing System 3. Five chromatographic runs were conducted on different days; retention times and peak areas of the individual amino acids were measured. The average deviation in the retention times was ±3.0%. The average relative deviation in the measured peak areas was ±6.5%. If an internal standard was used, the average deviation could be lowered much further.

Fluorescence detection

An excitation wavelength at 330 nm can be used to produce a fluorescence emission above 500 nm for the Dns-amino acids[4,25]. However, the UV absorbance of these Dns derivatives at 248 nm is at least 3 times greater than the absorption at 330 nm. Hence the detection of Dns derivatives of amino acids can be accomplished with a UV detector at 248 nm. UV detection is satisfactory for analysis at the 100–200 pmole level. For studies with smaller amounts of protein and/or peptides, it is necessary to increase the sensitivity further. In these cases, System 3 was used as the separation procedure, and fluorescence detection utilized. With fluorescence detection, the limit can be lowered to below the 10 pmole level. Since the ionization of N,N-dimethylamino group (pK_a = 4) quenches the fluorescence[4], detection of Dns derivatives of amino acids reaches maximum between pH 6 and 9. Therefore, when

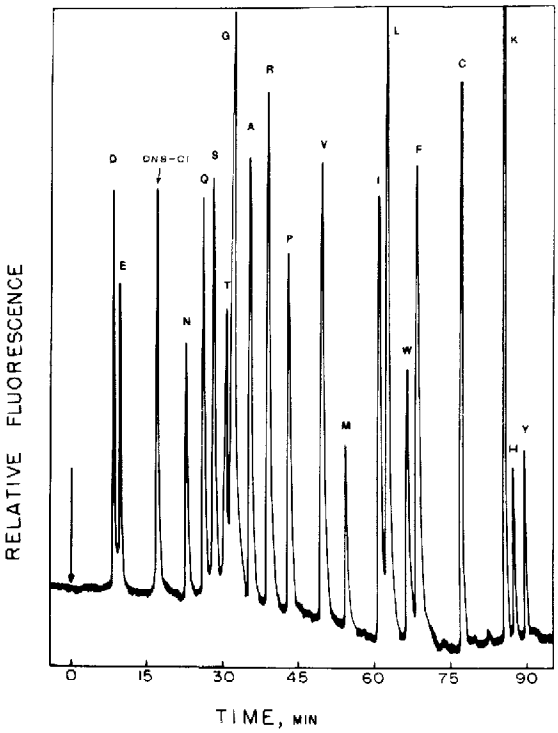

Fig. 4. HPLC separation of Dns-amino acids and detection at 10 pmole level. Arrow marks the injection. Single-letters designation for amino acids is used in the identification of the peaks. Fluorescence detection is utilized under the same conditions as explained in the legend to Fig. 3, except the sensitivity of the detector is increased.

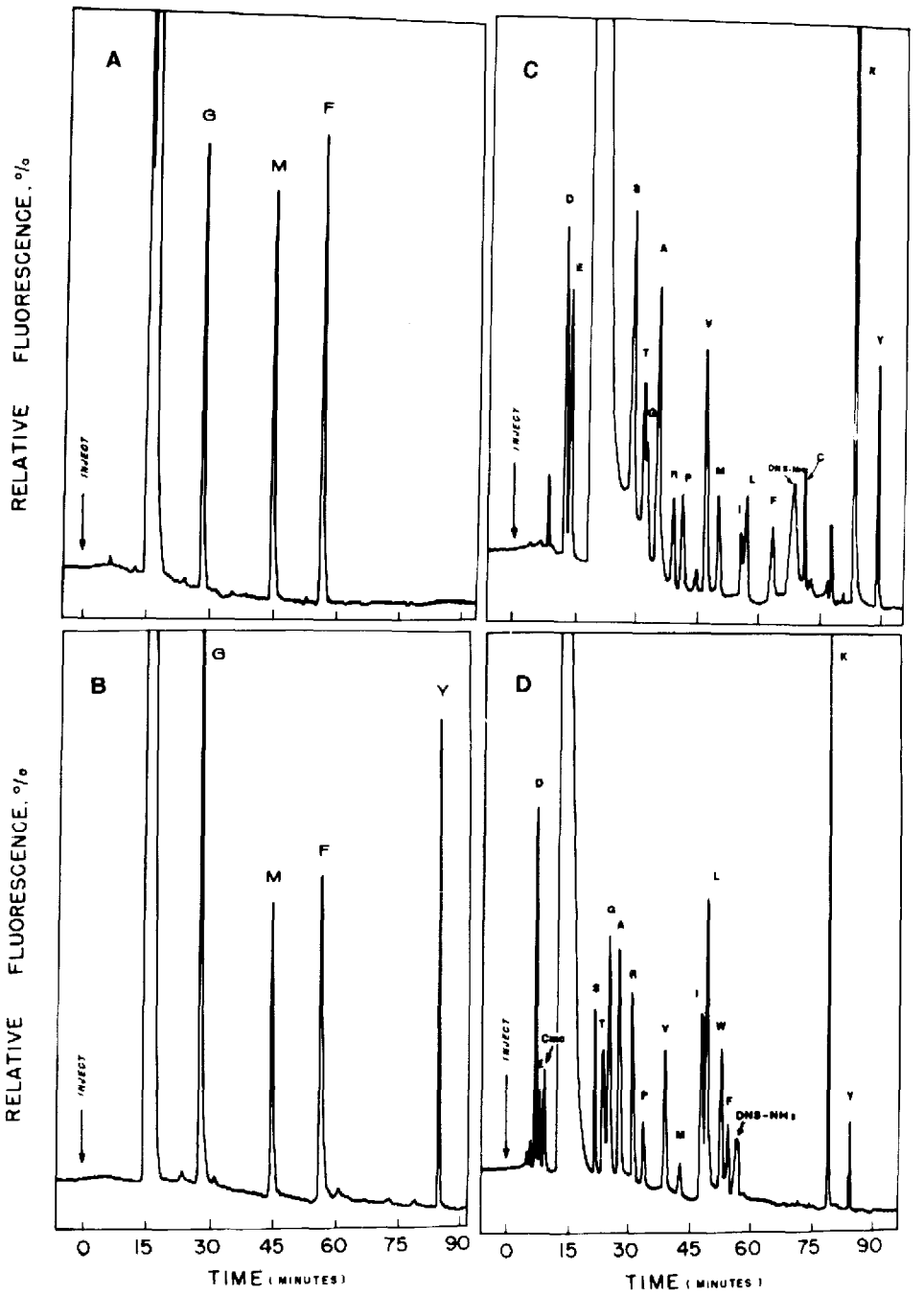

Fig. 5. HPLC analysis of the amino acid compositions of peptides and proteins. The preparation of the samples are explained in Materials and methods. Panels A and B are for the peptides Met–Phe–Gly and enkephalin, respectively; panels C and D are the chromatograms obtained for the proteins RNase A and CM-lysozyme, respectively.

fluorescence detection is to be used, System 2 is not feasible since TFA in solvent A lowers the pH below the fluorescence detection range. System 3 containing phosphate buffer at pH 6.5, on the other hand, meets the requirements to obtain maximal quantum yield and can be applied successfully. The detection limit for the System 3 was determined by serial dilutions of the standard mixture. Fig. 4 shows the standards chromatographed at 10 pmole level. It is obvious from this figure that the detection limit of the system is well below 10 pmoles.

Other considerations

If appropriate precautions are taken, over 500 runs can be conducted with one column before it "wears out". The precautions are to change the cartridge in the guard column after each 200 runs, and to filter the samples before injection, to avoid injecting solid particles.

Application to amino acid analyses

The utility of an analytical procedure depends on the applicability of the procedure to actual samples rather than standards. But whenever the results are to be evaluated quantitatively, the response factor for each amino acid needs to be determined. Therefore, a known amount of standard amino acid mixture was dansylated according to the procedure explained in Materials and methods. This Dns-amino acid mixture was then subjected to analysis. All the amino acid derivatives, including proline, cystine and tryptophan, gave similar responses except α,ε-di-Dns-lysine,

TABLE II

AMINO ACID COMPOSITIONS OF SOME PEPTIDES AND PROTEINS DETERMINED BY DANSYLATION PROCEDURE

	Enkephalin		*Met-Phe-Gly*		*RNase A*	
	Expected	*Exper.*	*Expected*	*Exper.*	*Expected*	*Exper.*
D	–	–	–	–	15	16.02
E	–	–	–	–	12	14.13
CM-C	–	–	–	–	–	–
S	–	–	–	–	15	15.66
T	–	–	–	–	10	10.80
G	2	1.97	1	1.09	3	3.78
A	–	–	–	–	12	12.96
R	–	–	–	–	4	4.14
P	–	–	–	–	4	4.32
V	–	–	–	–	9	9.81
M	1	0.95	1	0.93	4	3.87
I	–	–	–	–	3	2.79
L	–	–	–	–	2	2.34
W	–	–	–	–	–	–
F	1	0.99	1	0.98	3	3.06
C-C	–	–	–	–	4	4.40
K	–	–	–	–	10	9.99
H	–	–	–	–	4	3.40
Y	1	1.09	–	–	6	6.30

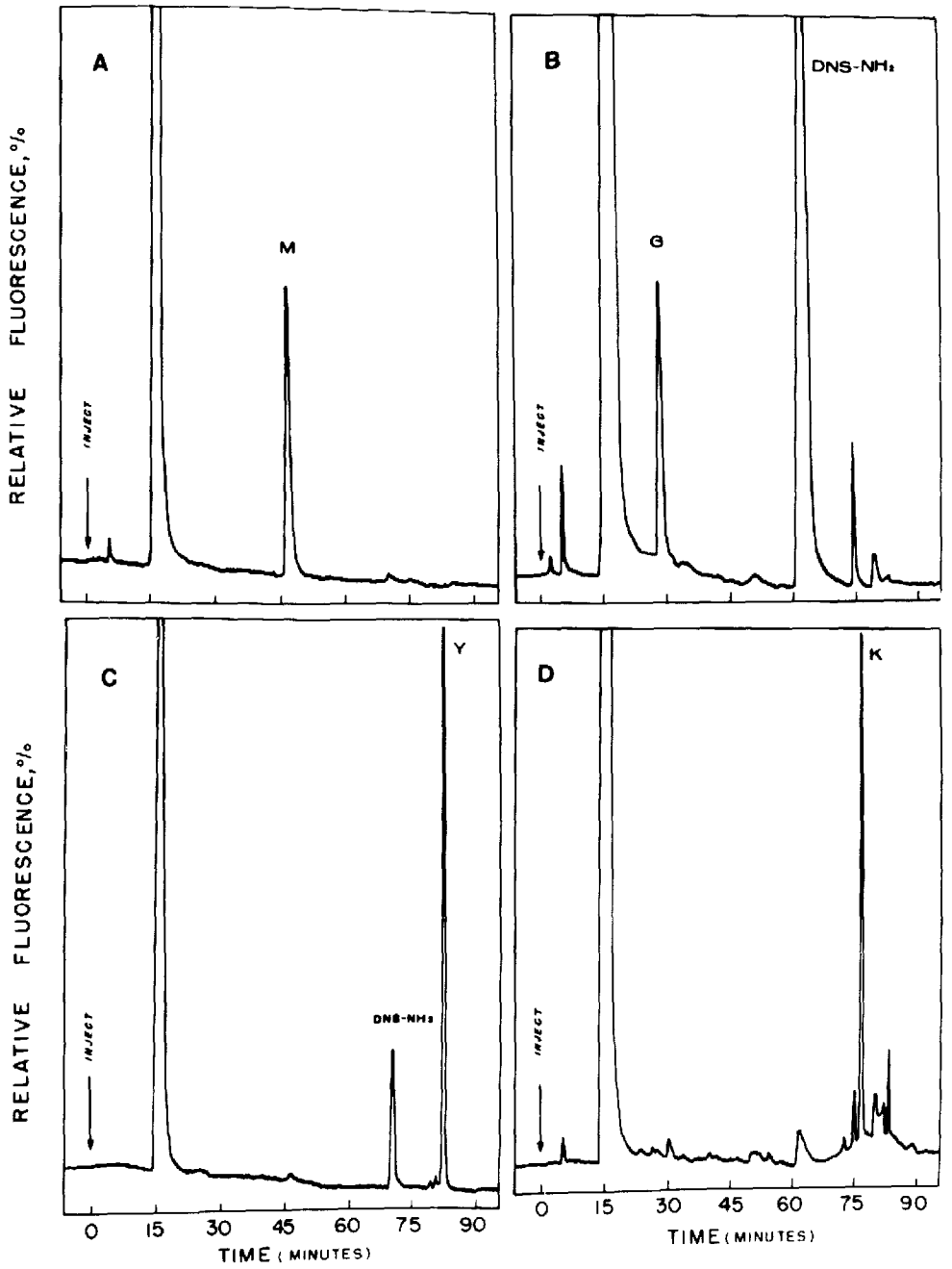

Fig. 6. N-Terminal analysis of peptides and proteins. The preparation of samples are explained in Materials and methods. The chromatograms are for Met–Phe–Gly (A), insulin chain A (B), enkephalin (C) and RNase A (D).

which exhibited a much higher fluorescence emission compared to the rest of the amino acids.

Fig. 5 shows the chromatograms of the amino acid compositions of two peptides and two proteins. Panels A and B are for peptides Met–Phe–Gly and enkephalin, respectively; panels C and D are the chromatograms obtained for RNase A and CM-lysozyme, respectively. In the case of the peptides, it is obvious that the responses obtained are very satisfactory, since the ratio Met:Phe:Gly is very close to 1:1:1. Table II shows the expected amino acid compositions and the experimental results (response factors for each amino acid already included) for the two peptides and RNase A. For Met–Phe–Gly, enkephalin and RNase A, the agreement between the expected and experimental values are very good. It should be noted that, in the case of CM-lysozyme (panel D), a carboxymethylcysteine peak (CM-C) is observed eluting after glutamic acid; and also a peak for tryptophan is observed since this sample was hydrolyzed with the precautions explained in Materials and methods.

Application to N-terminal analyses

The results of N-terminal analyses conducted on Met–Phe–Gly (A), insulin chain A (B), enkephalin (C) and RNase A (D) are shown in Fig. 6. As expected, Met was the N-terminal amino acid for Met–Phe–Gly, Gly for insulin A, Tyr for enkephalin and Lys for RNase A. It is important to note that when N-terminal analyses are being conducted on proteins, the size of the Dns-amide (Dns-NH_2) peak may be larger than the amino acid peaks (*e.g.*, insulin chain A). There is also the possibility of formation of side products like ε-Dns-lysine, O-Dns-tyrosine and imidazole-Dns-histidine. Since imidazole-Dns-histidine is completely destroyed during the hydrolysis, it does not interfere with the analysis. ε-Dns-lysine elutes between the leucine and tryptophan, and O-Dns-tyrosine between serine and threonine. Therefore, it is possible to determine the N-terminal amino acid of proteins even in the presence of the Dns-Cl, Dns-NH_2, ε-Dns-lysine and O-Dns-tyrosine.

Application to sequencing

The results of the Dns–Edman sequence analysis conducted on the tripeptide Met–Phe–Gly are shown in Fig. 7. The N-terminal amino acid was observed to be Met (panel A). The results of the second cycle of Dns–Edman degradation (panel B), shows that the second amino acid is Phe; and the third cycle (panel C) is Gly. It is obvious that this procedure can satisfactorily be coupled to Dns–Edman degradation in the determination of amino acid sequences of peptides and proteins.

The instability of Dns-amino acids to acid hydrolysis has been previously evaluated[19,24]. The Dns derivatives of proline and tryptophan are completely destroyed under hydrolysis conditions using 6 *M* hydrochloric acid at 110°C for 18 h *in vacuo*. Dns derivatives of serine, glycine and methionine are recovered in less than 40% yield; and the rest of the amino acid derivatives, though partially destroyed, are recovered with higher yields. Therefore, a shorter hydrolysis time, such as 4 h, has been recommended[19]. In this study, we attempted to utilize a hydrolysis procedure which would permit a more satisfactory recovery of most amino acids for the N-terminal analyses and sequencing. The Dns-amino acid mixture was treated with 4 *M* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 24 h *in vacuo*. Most of the Dns-amino acids including methionine and glycine were re-

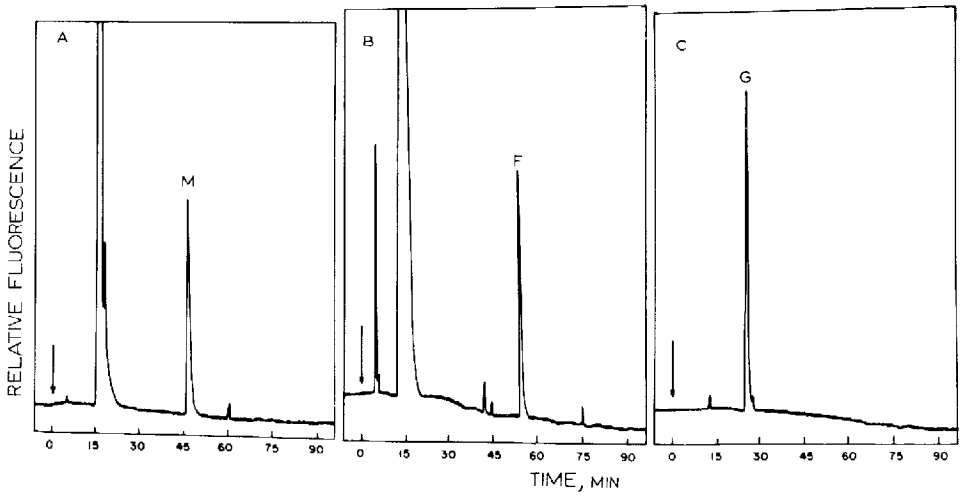

Fig. 7. Dns-Edman sequence analysis of tripeptide Met–Phe–Gly. The preparation of the samples are explained in Materials and methods. Arrows mark the injections. The chromatograms are for the N-terminal analysis (panel A), second cycle of Dns-Edman degradation (panel B) and the third cycle (panel C).

covered in reasonably high yields (greater than 80%), although Dns-tryptophan was completely destroyed. The recoveries of Dns-proline (45%) and -serine (50%) were increased dramatically. Thus, this hydrolytic procedure was used for the N-terminal and Dns–Edman analyses.

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