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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF Dns-AMINO ACIDS IN THE PURITY CONTROL OF PEPTIDES

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SUMMARY

A technique for the rapid determination of the N-terminal amino acids of peptides as dansyl (Dns) derivatives and its application to peptide purity control is suggested. The determination involves three main stages: dansylation, hydrolysis and high-performance liquid chromatography (HPLC), the first two stages being carried out under the conditions suitable for the HPLC of Dns-amino acids. Determination of the N-terminal amino acids takes 2.5 h. Complete separation of 23 mono- and di-Dns derivatives of 20 common amino acids takes 26 min. The sensitivity of detection is 100 pmol (UV, 254 nm).

INTRODUCTION

The determination of 1-dimethylaminonaphthalene-5-sulphonyl (dansyl, Dns) derivatives of amino acids is a conventional technique for protein structure determination. Thin-layer chromatography (TLC) of Dns-amino acids, the only method employed in practice for the identification of these compounds, is a laborious and time-consuming procedure which is difficult to combine with automatic quantitative analysis. Many attempts have been made to apply high-performance liquid chromatography (HPLC) to the determination of Dns-amino acids[1–5]. Nevertheless, it is still not used routinely because HPLC imposes specific requirements on the chemical treatment of the protein (peptide) sample.

This paper describes procedures for the dansylation of peptides, the hydrolysis of Dns-peptides and the HPLC of Dns-amino acids, which together give a rapid, highly sensitive, reproducible and quantitative method for the determination of N-terminal amino acids.

EXPERIMENTAL

Reagents

Bradikinin, insulin B-chain, Dns chloride (Dns-Cl) and Dns-amino acid standards were purchased from Serva (Heidelberg, F.R.G.), acetonitrile from Merck (Darmstadt, F.R.G.), methanol from the Kharkov plant for chemical reagents (Khar-

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kov, U.S.S.R.), lithium carbonate and ethylammonium chloride from Fluka (Buchs, Switzerland) and trifluoroacetic acid (TFA) from Pierce (Rockford, IL, U.S.A.).

Apparatus

The high-performance liquid chromatograph was an Altex Model 322 MP equipped with a Model 153 UV photometer. A Rheodyne Model 7125 injector was connected to an Altex column (Ultrasphere ODS, 5 μm, 250 × 4.6 mm I.D.). The column was jacketed and held at 39°C by means of a U-1 thermostat (G.D.R.). An ISKRA-226 minicomputer (U.S.S.R.) was applied to treat the results. A mixture of dansylation products was stirred by means of a vibromixer (Ika-Vibro-Fix from Janke and Kunkel, F.R.G.) and centrifuged on a CLN-2 centrifuge (U.S.S.R.).

Purification of reagents and solvents

Dns-Cl was recrystallized from isooctane. Acetonitrile was distilled over recrystallized Dns-Cl. Hydrochloric acid (5.7 *M*) was obtained by azeotropic distillation and stored in a dark glass bottle for no longer than 10–14 days. TFA was distilled over chromium trioxide. The water used for preparing aqueous solutions contained no more than 5 pmol of amino acids per 100 μl. All glass ampoules were carefully washed and heated at 400°C for 4 h to decompose contaminating organic compounds.

Preparation of solutions

A stock Dns-Cl solution in acetonitrile (8.7 m*M*) was diluted so that the final solution contained a 5–10-fold excess of Dns-Cl with respect to the α-amino group of the sample to be dansylated. Standard solutions of Dns-amino acids (20 pmol/μl) were prepared by dissolution in methanol and stored at −20°C in tightly screw-capped 1-ml bottles wrapped in aluminium foil. Standard mixtures were prepared using these solutions. Buffers for reversed-phase liquid chromatography (Figs. 3–7) were as follows: buffer A, 25 m*M* TFA, adjusted to pH 7.6 with 1 *M* sodium hydroxide solution and mixed with acetonitrile (90:10, v/v); buffer B, 25 m*M* TFA, adjusted to pH 7.6 with 1 *M* sodium hydroxide solution and mixed with acetonitrile (30:70, v/v).

Dansylation

Dansylation was carried out according to the modified procedure of Tapuhi *et al.*[6] using an acetonitrile solution of Dns-Cl and lithium carbonate. The ratio of organic solvents to the aqueous buffer was 1:2 and the volume of the reaction mixture was 6 μl (4 μl of lithium carbonate solution of buffer plus 2 μl of Dns-Cl in acetonitrile).

Peptide (solution of 100–200 pmol per 5 μl of an appropriate solvent) was placed on the bottom of an ampoule (2 × 80 mm) with a calibrated capillary, dried *in vacuo* at 60°C and evaporated to dryness twice from 5 μl of water. Lithium carbonate buffer (4 μl, 40 m*M*, pH 9.5) and Dns-Cl in acetonitrile (2 μl) were placed in the same ampoule, which was then wrapped in aluminium foil; the mixture was stirred on a vibromixer, centrifuged at 1000 *g* for 1 min, kept in darkness for 35 min at 20°C and then 2 μl of 0.1% ethylammonium chloride solution were added. The solution obtained was agitated, centrifuged at 1000 *g* for 1 min and dried *in vacuo* at 60°C. A mixture of 5.7 *M* hydrochloric acid and TFA (2:1, v/v; 5 μl) was added to the

dried precipitate and the resulting solution was frozen. The ampoule was evacuated, filled with argon, evacuated, sealed, thermostated at 166°C (50 min) or 190°C (15 min), centrifuged at 1000 *g* for 2 min, opened and dried *in vacuo* at 60°C. Dry methanol (4 μl) was added to the ampoule and the mixture obtained was evaporated *in vacuo*, then the addition and evaporation of methanol were repeated once more. The sample was dissolved in methanol (6–10 μl) and injected into the column.

HPLC of Dns-amino acids

A 5-μl volume of the solution containing 50–200 pmol of Dns-amino acids was injected into the column. For quantitative injection of the sample into the injector loop, the sample was followed by 3 μl of methanol. Dns-amino acids were separated on an Ultrasphere ODS column at 39°C in a gradient of acetonitrile. The effluent was monitored at 254 nm. Retention times and peak areas were determined using a minicomputer.

RESULTS AND DISCUSSION

HPLC of Dns-amino acids

Reversed-phase (RP) HPLC seems to be the most promising approach. The main problem here is the choice of optimal parameters (composition, molarity and

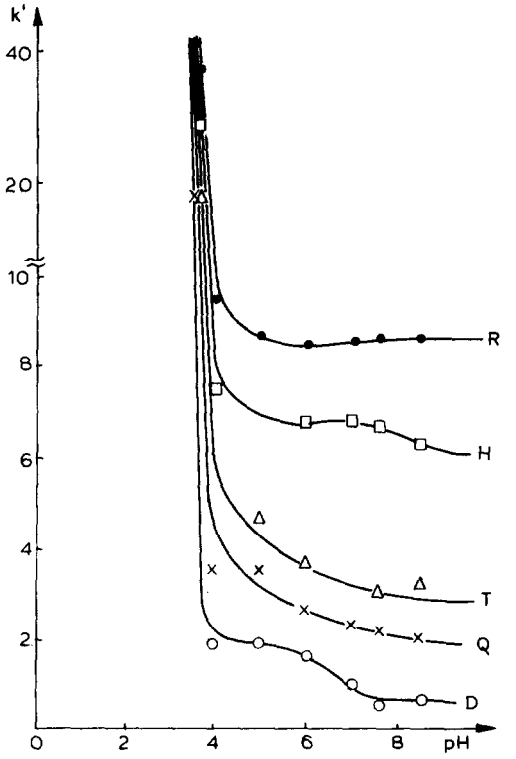

Fig. 1. Influence of pH on the *k'* values of Dns-amino acids (Ultrasphere ODS, 5 μm, column 250 × 4.6 mm I.D.). Isocratic elution system, acetonitrile–sodium TFA buffer (25 m*M*, pH 3.7–8.5) (20:80). Flow-rate, 1.5 ml/min; temperature, 39°C. One-letter code used for amino acids: R = Arg; H = His; T = Thr; Q = Gln; D = Asp.

pH of the buffer, slope of the gradient) for the rapid and complete separation of Dns derivatives of all 20 amino acids without overlapping with side-products (Dns-Cl, Dns-OH, Dns-NH_2). A TFA-containing buffer, highly selective for the separation of peptides[7–9] and phenylthiohydantoin (PTH) derivatives of amino acids[10], was used; as far as we know, it has not previously been applied to the determination of Dns-amino acids.

The dependence of the capacity ratio (k') for Dns-amino acids on pH and the molarity of the sodium TFA buffer was studied. Fig. 1 shows the influence of buffer pH on k' for Dns derivatives of Asp, Gln, His, Arg, Thr; the k' values for the derivatives of these amino acids depend strongly on pH. A sharp decline in the k' curves between pH 3.5 and 4.5 made the analysis irreproducible. The k' values do not depend strongly on pH over the range 7–8, and hence the reproducibility is improved. A poor separation of Dns-Asp and Dns-Glu was observed at low pH values; on the other hand, dissolution on the silica gel matrix of the column support starts at pH > 8. For these reasons, we used a buffer of pH $\leqslant 7.6$.

Fig. 2 shows the dependence of k' on buffer molarity for the same derivatives. Low k' values and, consequently, rapid analysis, were observed at low molarity of TFA-containing buffers (*ca.* 5 m*M*). However, low k' values resulted in incomplete separation of some derivatives. The steep slope of the k' curves over the low molarity region indicated that even minute changes in buffer molarity (from batch to batch) shifted the peak positions on the chromatograms, which could result in misinterpretation of the separation results. Good and reproducible separations could be achieved at higher buffer concentrations where the k' values of different Dns-amino acids

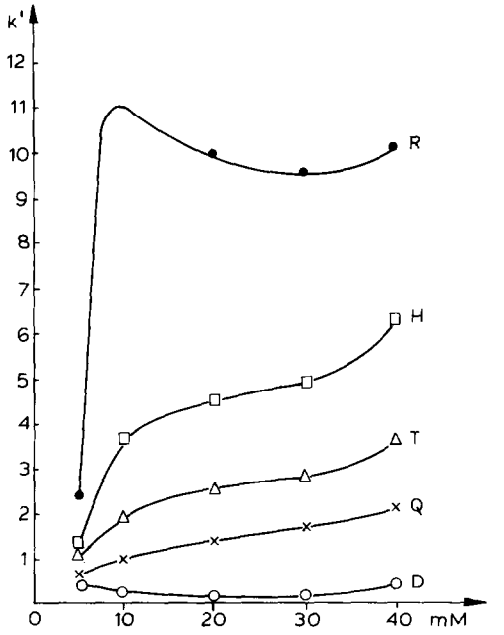

Fig. 2. Influence of molarity of TFA buffer on the k' values of Dns-amino acids (Ultrasphere ODS, 5 μm, column 250 × 4.6 mm I.D.). Isochratic elution system, acetonitrile–sodium TFA buffer (pH 7.6, 5–40 m*M*) (20:80). Flow-rate, 1.5 ml/min; temperature, 39°C. Abbreviations as in Fig. 1.

differed considerably and to a smaller extent depended on buffer molarity. It was found that 25 m*M* was the optimal buffer concentration. A mixture of 23 Dns-amino acids was separated completely on an Ultrasphere ODS column using 25 m*M* sodium TFA buffer (pH 7.6) (Fig. 3). It is noteworthy that O-Dns-Tyr and ε-Dns-Lys produced diffuse peaks; the Dns-Asp and Dns-Glu peak areas were half those of equimolar amounts of other derivatives.

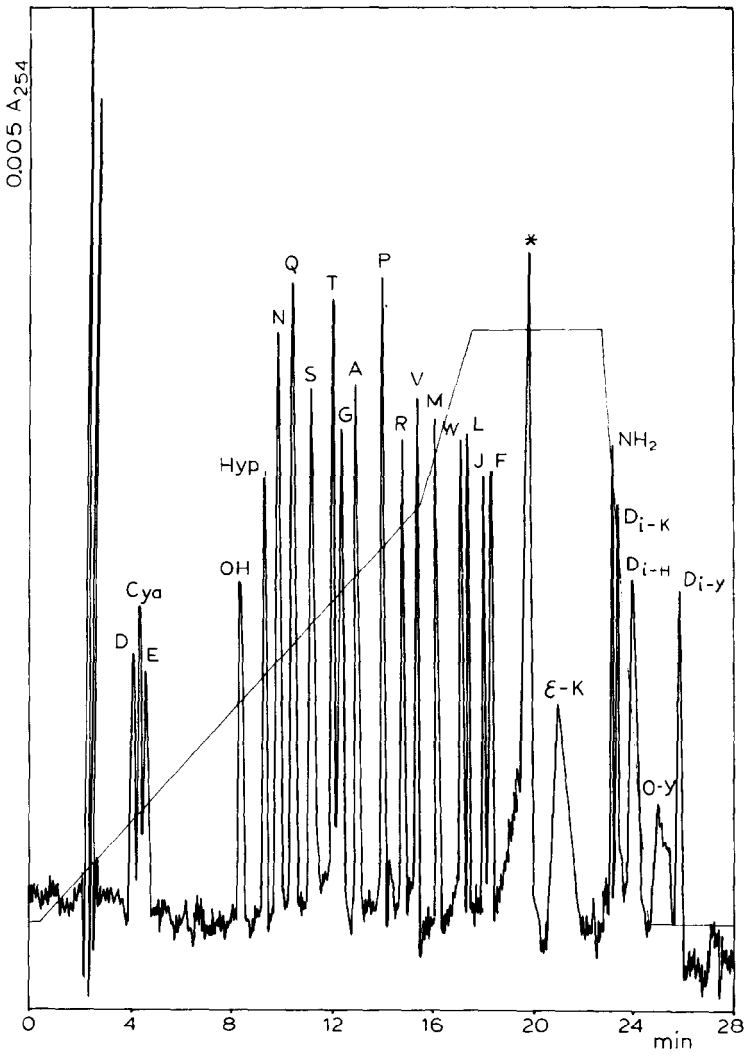

Fig. 3. Reversed-phase HPLC of Dns-amino acids on an Ultrasphere ODS (5 μm) column (250 × 4.6 mm I.D.), 100 pmol of each derivative, except the di-derivatives (60 pmol of each). Solvent A, acetonitrile–sodium TFA buffer (25 m*M*, pH 7.6) (10:90); solvent B, acetonitrile–sodium TFA buffer (25 m*M*, pH 7.6) (70:30); gradient from 7 to 65% B. Solvent program: 0 min after injection, 7% B; 1 min, 7%; 16 min, 48%; 18 min, 65%; 23 min, 65%; and 25 min, 7%. Detector range, 0.005 a.u.f.s. at 254 nm. Flow-rate, 1.2 ml/min; temperature, 39°C. One-letter code for amino acids (D = Asp; E = Glu; N = Asn; Q = Gln; S = Ser; T = Thr; G = Gly; A = Ala; P = Pro; R = Arg; V = Val; M = Met; L = Leu; I = Ile; F = Phe) is used except for the following abbreviations: Cya = cysteic acid; Hyp = hydroxyproline; Di-K = Di-Dns-Lys; Di-H = Di-Dns-His; Di-Y = Di-Dns-Tyr; ε-K = ε-Dns-Lys; O-Y = O-Dns-Tyr; OH = Dns-sulphonic acid; NH_2 = Dns-amide. The peak marked with an asterisk corresponds to an unknown compound.

Dansylation of peptides

Under classical conditions (sodium hydrogen carbonate, pH 9; acetone–water, 2:1) a large molar excess of Dns-Cl is required for complete dansylation. These conditions are not compatible with the subsequent HPLC of Dns-amino acids because of the overlap of broad peaks of Dns-OH and Dns-NH_2, masking the peaks of some Dns-amino acids. Tapuhi *et al.*[6] and others[10,11] found that in lithium carbonate buffer (pH 9.5) and at an acetonitrile–water ratio of 1:2 (opposite to classical conditions) the decomposition of Dns-amino acids and Dns-NH_2 formation were suppressed and a large excess of Dns-Cl was not necessary for complete dansylation.

Our experiments confirmed that the completeness of dansylation did not depend on the excess of Dns-Cl (5–100-fold), and subsequently we used 5–10-fold excesses of Dns-Cl relative to the peptide α-amino group. We did not change the volume of the reaction mixture where dansylation occurred; this increased the reproducibility of the analysis. To remove excess of Dns-Cl and to stop dansylation, 0.1% ethylammonium chloride was added. The Dns-ethylamine (Dns-NHEt) formed was eluted at the end of the separation profile and did not hinder the interpretation of the results. To evaluate the completeness of dansylation, we used a hydroxyproline-containing buffer, hydroxyproline being the internal standard for both the dansylation reaction and the chromatography of Dns-amino acids.

Hydrolysis of Dns-peptides

Classical conditions for the acidic hydrolysis of Dns-peptides are 5.7 *M* hydrochloric acid and heating at 105°C for 12 h. Such prolonged hydrolysis results in partial decomposition of Dns-Pro, Dns-Met and Dns-Gly and, moreover, does not meet the requirements of rapid routine analysis. We decided to accelerate the hydrolysis of Dns-peptides by using the hydrochloric acid–TFA mixture. This mixture was proposed for peptide bond hydrolysis but was not used for the hydrolysis of Dns-peptides[12]. We found the conditions that were optimal for the hydrolysis of peptides (166°C, 25 min) to be unsuitable for the hydrolysis of Dns-peptides. The optimal hydrolysis time appeared to be 50 min (at 166°C). More prolonged heating at 166°C led to a decrease in the yield of the N-terminal Dns-amino acid (Fig. 4).

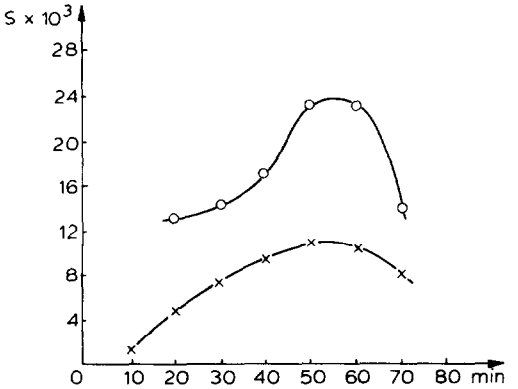

Fig. 4. Dependence of the yield (peak area, arbitrary units) of Dns-F (×) (the N-terminal amino acid of Dns-insulin B-chain) and of Dns-I (O) (the N-terminal amino acid of Dns-I-P-G-E) on the time of hydrolysis with hydrochloric acid–TFA = (2:1) at 166°C. Abbreviations as in Fig. 3.

Optimization of the hydrolysis conditions was carried out not only with respect to time, but also temperature. Heating at 190°C allowed the time of hydrolysis to be reduced to 15 min without a substantial decrease in the N-terminal amino acid yield (Fig. 5). On comparing the hydrolysis conditions [hydrochloric acid–TFA (2:1) (Fig. 6C) and 5.7 *M* hydrochloric acid (Fig. 6D)], it was found that the results of hydrolysis were the same. A disadvantage of the high-temperature hydrolysis (hydrochloric acid–TFA, 166°C) was the increased decomposition of Dns-Hyp compared with the yield of Dns-amino acids in 5.7 *M* hydrochloric acid at 105°C.

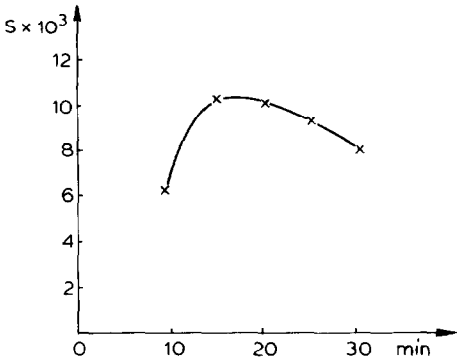

Fig. 5. Dependence of the yield (peak area, arbitrary units) of Dns-F (the N-terminal amino acid of Dns-insulin B-chain) on the time of hydrolysis with hydrochloric acid–TFA (2:1) at 190°C.

To evaluate the yield of the N-terminal residue (Phe) of insulin B-chain, the amino acid Phe (100 pmol) was dansylated; the reaction products were subjected to chromatography (Fig. 6A); Dns-Phe (100 pmol) was hydrolysed and chromatographed (Fig. 6B); insulin B-chain (100 pmol) was dansylated and hydrolysed under the suggested conditions [hydrochloric acid–TFA (2:1), 166°C, 50 min] (Fig. 6C) and under classical conditions (5.7 *M* hydrochloric acid, 105°C, 4 h) (Fig. 6D). Analysis of the N-terminal amino acid of insulin B-chain (Phe) resulted in a yield of Dns-Phe of 80% (compared with the results of the chromatographic analysis of 100 pmol of Dns-Phe).

Under the conditions described here for peptide dansylation, Dns-peptide hydrolysis and Dns-amino acid separation, we determined the N-terminal amino acids and the purity of several standard polypeptides of different size: bradikinin (9 amino acids), 18-membered peptide II-7 from oligomycin sensitivity-conferring protein (OSCP) (beef heart mitochondria), melittin (26 amino acids, bee venom) and 65-membered toxin (*Buthus eupeus* scorpion venom). The results are shown in Fig. 7.

The use of lithium carbonate buffer, a 5–10 molar excess of Dns-Cl in acetonitrile, a ratio of organic solvent to water of 1:2 and a constant volume of the reaction mixture allowed the dansylation reaction to be carried out quantitatively with minimal formation of Dns-OH and Dns-NH_2, the small amounts of which greatly facilitated subsequent HPLC separation. Ethylammonium chloride as a Dns-Cl quencher decreased the formation of Dns-OH and increased the reproducibility of the dansylation reaction. Application of the hydrochloric acid–TFA mixture to the hydrolysis of Dns-peptides accelerated the analysis (50 min hydrolysis at 166°C or

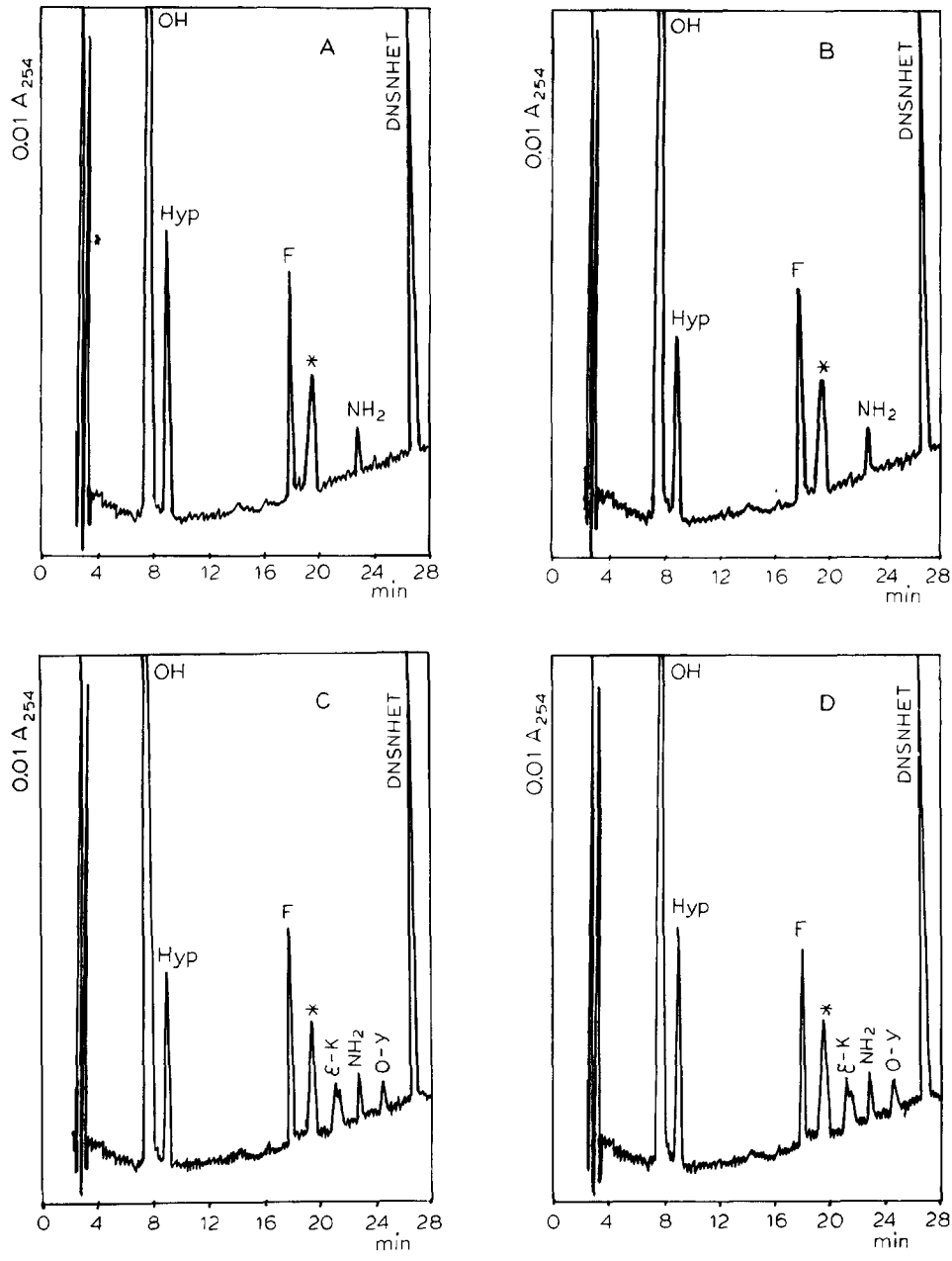

Fig. 6. Influence of hydrolysis conditions on the yield of Dns-F. (A) Dansylation of 100 pmol of F in buffer containing 100 pmol of Hyp; (B) hydrolysis [hydrochloric acid–TFA (2:1), 166°C, 50 min] of 100 pmol of Dns-F and 100 pmol of Dns-Hyp; (C) dansylation of 100 pmol of insulin B-chain and 100 pmol of Hyp followed by hydrolysis [hydrochloric acid–TFA (2:1), 166°C, 50 min]; (D) dansylation of 100 pmol of insulin B-chain and 100 pmol of Hyp followed by hydrolysis (5.7 *M* hydrochloric acid, 105°C, 4 h). 80% of the sample was injected. Abbreviations: DNSNHET = Dns-ethylamide; ε-K = ε-Dns-Lys; O-Y = O-Dns-Tyr. Peaks marked with an asterisk represent unknown compounds.

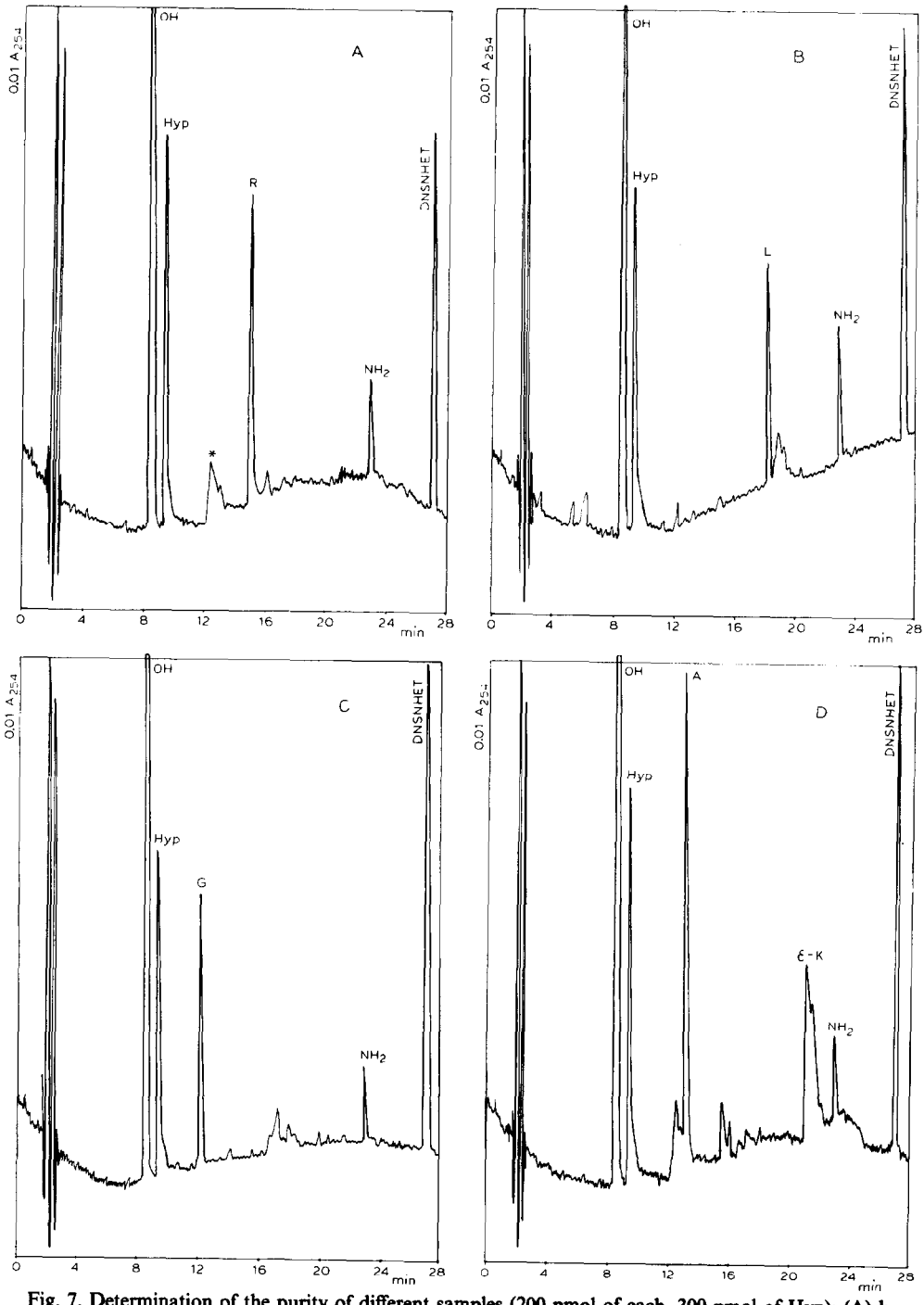

Fig. 7. Determination of the purity of different samples (200 pmol of each, 300 pmol of Hyp): (A) bradikinin; (B) octadecapeptide II-7 of the OSCP protein; (C) melittin; (D) 65-membered toxin CA-2 from *Buthus eupeus* scorpion venom. 80% of the sample was injected. Abbreviations as in Fig. 6.

15 min at 190°C) without decreasing the yield of the N-terminal Dns-amino acid. Optimization of the separation of Dns-amino acids with regard to buffer composition, molarity and pH made it possible to achieve complete and reproducible separation of a mixture of 23 Dns-amino acids in 26 min. The relative standard deviation of the retention time did not exceed 0.6%. It is noteworthy that the peaks of ε-Dns-Lys and O-Dns-Tyr were diffuse even with the standard mixture and the results of their determination in routine analysis often did not correspond to the real content of internal Lys and Tyr residues.

The total time required for the N-terminal amino acid determination is about 2.5 h, including the time for "secondary" operations (evaporation of samples before the analysis and after all the chemical treatments, centrifugation, etc.). Conventional Dns-amino acid analysis takes 12–18 h (TLC) or 3–6 h (HPLC)[3,11].

The use of a TFA-containing buffer allows the detection of components of the reaction mixtures as sharp, high peaks, so that the sensitivity of the routine analysis of N-terminal amino acids is about 100 pmol (UV, 254 nm). The sensitivity of the technique is limited by using a UV photometer and might be increased (to 1.0–0.1 pmol) by using a flow fluorimeter[13].

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