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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XXXIX*. REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF Dns-AMINO ACIDS: COMPARISON OF SEVERAL DIFFERENT ELUTION SYSTEMS

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SUMMARY

Reversed-phase high-performance liquid chromatographic analytical systems are described for the separation and identification of the Dns derivatives of the protein amino acids. The effect of eluents of different pH and composition on solute selectivity on several different types of alkylsilica stationary phases was investigated. Applications of these chromatographic systems to N-terminal analysis of polypeptides are presented.

INTRODUCTION

The characterisation and structural elucidation of peptides and proteins has been revolutionised over the past few years with the advent of reliable reversed-phase high-performance liquid chromatographic (RP-HPLC) procedures. With the increasing use of RP-HPLC as a micro-preparative technique for the isolation of polypeptides and proteins, improved procedures for the separation and analysis of the protein amino acids have become essential. The usual procedure for amino acid analysis has until recently been classical ion-exchange chromatography with detection based on post-column derivatisation with ninhydrin[1,2] or a fluorogenic reagent[3,4]. Adaption of this technique to microbore ion-exchange columns allows the resolution of most amino acids with good limits of detection (low picomole level)[5] particularly when either *o*-phthaldialdehyde (OPA)–2-mercaptoethanol, OPA–ethanethiol or fluorescamine are used as post-column derivatisation reagents. Following on from the recent advances in HPLC instrumentation and column packing materials, alternative approaches for amino acid analysis have been developed which employ pre-column derivatisation. Among these new procedures, pre-column formation and chromatographic separation of the OPA, 1-N,N′-dimethylaminonaphthalene-5-

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sulphonyl (Dns), and N,N-dimethylaminoazobenzenesulphonyl (dabsyl) derivatives of amino acid mixtures satisfy several of the criteria of suitable analytical methods, namely technical simplicity, reproducibility and high detection sensitivity. The Dns derivatives, in particular, have previously been widely used for qualitative amino acid analyses, the determination of the amino acid terminus of peptides, and for manual microsequence determination. The preparation of the Dns-amino acid derivatives is rapid, and under carefully controlled reaction conditions[6] is essentially quantitative. In addition, adequate UV detection of the Dns-amino acid can routinely be achieved near the 100 picomole level and this can be extended with fluorescence detection into the femtomolar range[26] and with chemiluminescence methods ever lower[7]. Recent studies have demonstrated that Dns-amino acid derivatives including D- and L-enantiomers can be analysed with good selectivity and detection sensitivity by both normal- and reversed-phase HPLC[8–13]. In this report, the RP-HPLC separation of the Dns-amino acids with a variety of different elution conditions is further evaluated, and the application of the new chromatographic systems in the determination of N-terminal residues of polypeptides was investigated.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile and methanol were both HPLC grade and obtained from Waters Assoc. (Milford, MA, U.S.A.) or Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was quartz-distilled and deionised using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Orthophosphoric acid and sodium acetate were AnalaR grade from BDH (Poole, Great Britain). Dodecylamine, bovine insulin and sperm whale myoglobin were purchased from Sigma (St. Louis, MO, U.S.A.). Dns-Amino acids were purchased as a kit from BDH or Sigma. Ammonium hydrogen carbonate was from Ajax Chemicals (Sydney, Australia).

Apparatus

All separations were carried out on either a Waters gradient HPLC system, consisting of two M6000A solvent delivery systems, a M660 solvent programmer, a U6K injector and a M450 variable-wavelength UV monitor, or a DuPont Model 850 gradient system with a Model 860 fixed-wavelength (254 nm) detector and a Rheodyne Model 7120 sample injector fitted with a 50-μl sample loop. Omniscribe chart recorders were used with both HPLC systems. Sample injections were made with either Hamilton Model 802 RN syringes (Reno, NV, U.S.A.) or SGE Model 50A syringes (Melbourne, Australia). The pH measurements were performed with a Radiometer pH M64 meter equipped with a combination glass electrode. The reversed-phase packing materials used in this study were: μBondapak-alkylphenyl, -C_{18}, and -C_{30} (all from Waters Assoc. and packed into stainless steel columns, 30 × 0.4 cm I.D., d_p 10 μm); Hypersil 5 μm ODS (Shandon Southern, Great Britain) packed into stainless-steel columns (15 × 0.4 cm I.D.); and Radial-Pak A cartridges (10 × 0.8 cm) were obtained from Waters Assoc. and used in conjunction with a RCM module.

Methods

All chromatograms were carried out at ambient temperatures. Bulk eluents were degassed as reported previously[14], filtered through either Millipore type HA (0.45 μm) filters for aqueous eluents and type FH (0.50 μm) filters for aquo-organic solvent combinations. Both the Waters and DuPont HPLC systems were equipped with pulse dampers for all chromatographic runs at flow-rates at or below 1.0 ml/min. All columns were equilibrated to new mobile phase conditions for at least 30 min. Sample volumes varied between 5 and 25 μl, with all stock solutions of Dns-amino acids made up freshly in the appropriate eluent (or the initial mobile phase for gradient elution). The Dns derivatization procedures were based on the methods of Gray[15] and Tapuhi *et al.*[6]. The retention times for the Dns-amino acids separated on the various alkylsilica columns were measured in the usual way, the relative capacity factors for isocratic and gradient experiments were calculated as reported previously[14,16].

RESULTS AND DISCUSSION

In common with all ionogenic solutes, the Dns-amino acids show pronounced retention time and selectivity changes on reversed phases as the pH of the eluent is varied. From preliminary experiments with several Dns-amino acids including Dns-Asp, Dns-Thr, Dns-Arg and Dns-Phe, it was evident that two compensating pH-dependencies were influencing their retention behaviour over the range pH 2.0–8.0, with the observed variations in retention to alkylsilicas reflecting changes in the ionisation state of the dimethylamino-group and the α- and side chain carboxyl groups. At pH values above the pK of the dimethylamino group (pK = 4.07; ref. 17), the fully Dns derivatized amino acids behave as weak acids with their capacity factors decreasing as the pH is increased up to *ca.* pH 8.0. At values below approximately pH 3.5, where the α- and side chain carboxyl groups (pK *ca.* 3.5) are essentially unionised, retention similarly decreases due to the increased polarity of the Dns-amino acid following protonated dimethylamino-group. Consequently, these opposing pH dependencies result in retention maxima near pH 3.5–4.0. These observations are in accord with the trends previously noted by Wilkinson[9] with selected Dns-amino acids separated on a μBondapak C_{18} column. In order to investigate further the effect of low, *e.g.*, pH 2.3, and neutral, *e.g.*, pH 7.8, pH conditions and buffer composition on retention behaviour, the separation of the common Dns-amino acids on μBondapak-alkylphenyl, -C_{18}, and -C_{30} stationary phases was investigated. Representative data from these experiments carried out using a pH 2.3, 0.1% orthophosphoric acid–acetonitrile–water system are shown in Fig. 1 and comparative data for other buffer and column systems are found in Table I. Under the above low pH elution conditions, the Dns derivatives of the amino acids elute from μBondapak-alkylphenyl, -C_{18} and -C_{30} columns of equivalent dimensions in order of their amino acid side chain hydrophobicities[18] essentially in three groups: (1) the acidic amino acid derivatives which show the least retention; (2) the neutral aliphatic amino acid derivatives, and finally (3) the di-Dns derivatives. At the low pH 2.3 value Dns-Asn is retained less than Dns-Asp and similarly Dns-Gln elutes before Dns-Glu. Due to protonation of the α-amino group of ε-Dns-Lys and the guanidino-group of Dns-Arg at these low pH values, both these Dns derivatives exhibit relatively short retention times. How-

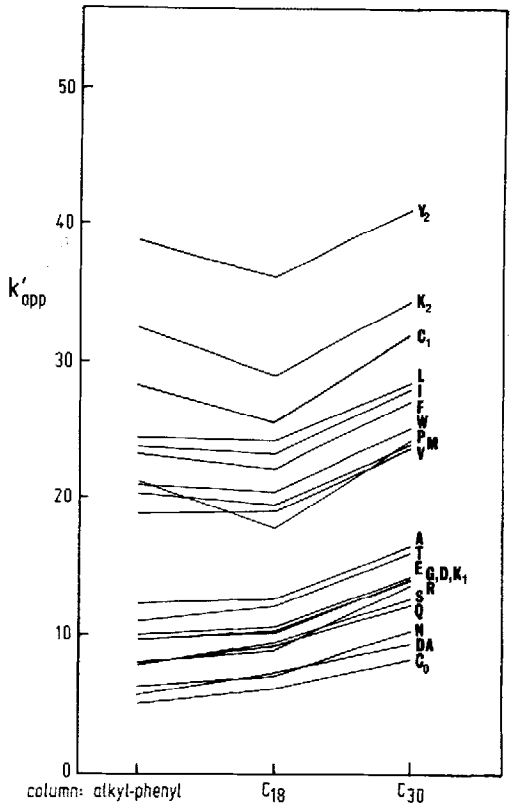

Fig. 1. Comparative retention behaviour of the common Dns-amino acids separated on different μBondapak reversed phases. Chromatographic conditions: flow-rate, 2 ml/min; temperature, 20°C, mobile phase, 60-min linear gradient generated from A (water–0.1% orthophosphoric acid) and B (80% methanol–20% water–0.1% orthophosphoric acid) starting at 10% B. The key to the various Dns-amino acids is given in Table I.

ever, on all the stationary phases examined in this study under these low pH elution conditions, these two derivatives co-eluted with Dns-Asp or Dns-Glu, respectively. Despite the overall similarities in retention behaviour, the resolution of the Dns derivatives of the amino acids is in several respects superior on the μBondapak alkylphenyl support to that attainable with the μBondapak C_{18} support, notably in terms of peak shape which permitted lower detection limits and better reproducibility from column to column. In accord with earlier observations[9], the only significant variations in elution order obtained on these three alkylsilicas by changing the mobile phase from a low pH phosphate system to a pH 6.5, 50 m*M* sodium acetate based eluent (condition 2, Table I) were reversals for Dns-Asn/Dns-Asp and Dns-Gln/Dns-Glu, *i.e.*, both Dns-Asp and Dns-Glu show larger relative decreases in retention, due to ionisation of the side chain carboxyl groups at pH 6.5, than Dns-Asn or Dns-Gln respectively.

Fig. 2 shows the separation of a standard mixture containing approximately 0.5 nmol of each Dns-amino acid derivatives separated on a Hyperil ODS column using a 200 m*M* ammonium hydrogen carbonate aquo-acetonitrile gradient mobile

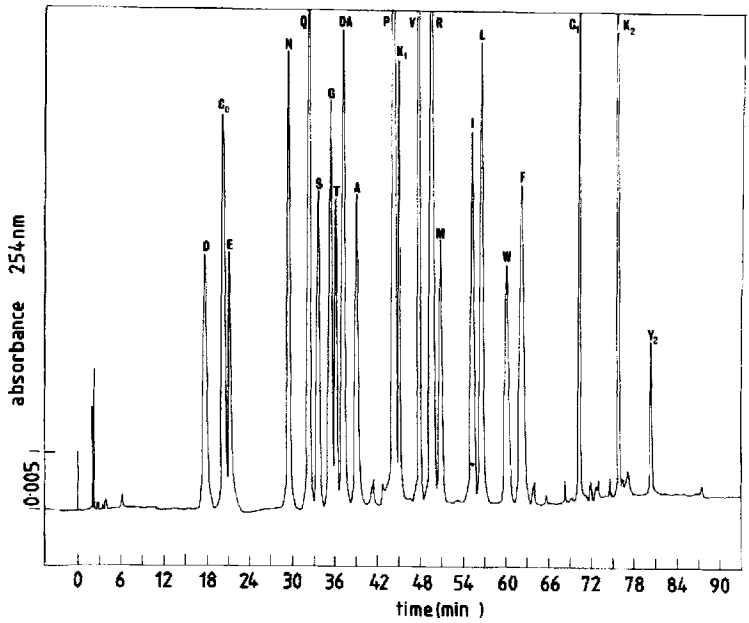

Fig. 2. Gradient elution of Dns-amino acids from a column (150 × 4.6 mm) of Hypersil-ODS (5 μm). Conditions: flow-rate, 1.0 ml/min; temperature, 18°C; solvent A, 10% acetonitrile–200 m*M* ammonium hydrogen carbonate, pH 7.8; solvent B, 45% acetonitrile–200 m*M* ammonium hydrogen carbonate, linear gradients from 0–30% B over 25 min, 30–45% B over 20 min, 45–100% B over 15 min followed by 100% B for 15 min. Sample size: 0.5 nmol of each derivative injected in a total volume of 25 μl. The key to the Dns-amino acids is given in Table I.

phase combination. In contrast to the low pH elution conditions described above, with this separation system all the common Dns derivatives are well resolved (>90%) with analysis time under 90 min. Although absolute retention times vary by up to *ca.* 15 sec from run to run with this and the other gradient systems, we have not experienced difficulties in making peak assignment or identification. As anticipated on the basis of ionisation considerations, the Dns derivatives of the acidic amino acids (aspartic, glutamic and cysteic) show minimal retention at pH 7.8 with this system. Similar elution orders were observed (Fig. 3) with the same ammonium bicarbonate based mobile phase combination for the Dns-amino acids on the Radial-Pak A support, a spherical octadecylsilica stationary phase packed into flexible walled polyethylene cartridge. Considerably higher flow-rates can be used with these cartridges (Fig. 3 shows a separation at a flow-rate of 7.0 ml/min) with consequently shorter analysis times. However, as is evident from Table I, this column is more retentive for the Dns-amino acids due to the combination of lower linear elution velocities which can be achieved and the involvement of silanol effects[19]. The latter effects probably account for the significant increase in retention seen with Dns-Arg on this alkylsilica support (cf. Figs. 2 and 3).

Manipulation of selectivity on reversed phases for polar, amphoteric solutes via pairing ion interactions is a well established procedure. The addition of hydrophobic co- or counter-ions to the mobile phase is known to influence significantly the extent and order of retention of amino acids and peptides to alkylsilicas[16,20,21]. When

TABLE I

COMPARATIVE RETENTION BEHAVIOUR OF SELECTED Dns-AMINO ACIDS UNDER SEVERAL DIFFERENT CHROMATOGRAPHIC CONDITIONS

Condition 1: column, μBondapak-alkylphenyl; flow-rate, 2.0 ml/min; solvent A, water–15 m*M* orthophosphoric acid, pH 2.3; solvent B, 15 m*M* orthophosphoric acid, methanol–water (80:20); linear 60-min gradient from 10 to 100% B.
Condition 2: column, μBondapak-alkylphenyl; flow-rate, 2.0 ml/min; solvent A, water–50 m*M* sodium acetate, pH 6.5; solvent B, 50 m*M* sodium acetate, methanol–water (80:20); linear 60-m gradient from 10 to 100% B.
Condition 3: column, μBondapak-alkylphenyl; flow-rate, 1.0 ml/min; solvent A, 2 m*M* dodecylamine, 15 m*M* orthophosphoric acid, acetonitrile–water (25:75); solvent B, 2 m*M* dodecylamine, 15 m*M* orthophosphoric acid, acetonitrile–water (65:35); gradient, solvent A isocratic for 25 min followed by concave gradient (curve No. 8, M660 programmer) to 100% B over 35 min.
Condition 4: column, Hypersil-ODS; flow-rate, 1.0 ml/min; solvent A, 200 m*M* ammonium hydrogen carbonate, acetonitrile–water (10:90); solvent B, 200 m*M* ammonium hydrogen carbonate, acetonitrile–water (45:55); linear gradients from 0 to 30% B over 25 min, 30–45% B over 20 min, 45–100% B over 15 min, followed by 100% B for 15 min.
Condition 5: column, Radial-Pak A; flow-rate, 7.0 ml/min; solvents A and B as in *Condition 4*; linear gradients from 10 to 20% B over 8 min, 20–50% B over 12 min, 50–100% B over 3 min, followed by 100% B for 5 min.
Condition 6: column, μBondapak-C_{18}, flow-rate 2 ml/min; solvent A, 30 m*M* sodium phosphate, pH 7.7; solvent B, acetonitrile; linear gradient from 10 to 40% B over 23 min, data calculated from ref. 9.
nd = Not determined.

Dns-amino acid	*Key*	*Chromatographic system*										
		Condition 1		*Condition 2*		*Condition 3*		*Condition 4*		*Condition 5*		*Condition 6*
		Elution order	k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*
Cysteic acid	C_0	1	15	1	8.0	11	4.7	2	8.8	2	6.0	nd
Dns-NH2	DA	2	5.8	2	9.4	5	2.8	9	16.8	9	18.3	6
Asparagine	N	3	6.3	7	14.6	3	2.2	4	13.1	4	11.2	3
Serine	S	4	8.0	4	12.8	7	3.1	6	15.1	6	14.7	5
Arginine	R	5	8.1	5	12.9	1	0.6	14	22.7	17	37.3	11
Glutamine	Q	5	8.1	9	16.2	4	2.5	5	14.5	5	13.0	4
Aspartic acid	D	7	9.8	3	10.0	10	3.9	1	7.6	1	5.0	1
ε-Lysine	K_1	7	9.8	8	14.7	2	1.3	12	20.5	12	27.0	17
Glutamic acid	E	9	10.0	5	12.9	8	3.3	3	9.1	2	6.0	2
Glycine	G	9	10.0	10	6.4	12	4.8	7	16.0	7	15.8	7
Threonine	T	11	11.0	11	17.0	9	3.7	8	16.3	8	16.9	6
Alanine	A	12	12.3	12	19.1	13	5.3	10	17.7	10	20.0	8
Valine	V	13	18.9	13	19.7	14	10.7	13	21.9	13	28.8	10
Methionine	M	14	20.4	14	20.6	14	10.7	15	23.3	14	31.7	12
Tryptophan	W	15	21.1	15	21.1	18	14.3	18	27.7	18	38.7	nd
Proline	P	16	21.2	16	21.4	6	2.8	11	20.1	11	24.7	9
Phenylalanine	F	17	23.2	17	23.0	19	14.5	19	28.7	19	39.5	15
Isoleucine	I	18	23.7	18	23.7	17	13.8	16	25.4	15	34.7	13
Leucine	L	19	24.4	19	24.1	16	13.7	17	26.1	16	35.7	14
Cysteine	C_1	20	28.3	20	26.8	20	16.6	20	32.6	20	46.3	nd
α,ε-Lysine	K_2	21	32.4	21	29.1	22	17.8	21	35.2	21	51.2	18
α,3-Histidine	H_2	22	33.7	22	30.3	nd	nd	22	35.6	nd	nd	nd
α,0-Tyrosine	Y_2	23	38.7	23	32.5	21	17.5	23	37.4	22	53.2	19

very hydrophobic surfactants are used, such modified reversed-phase systems behave like conventional ion-exchange systems. Of particular recent interest for the resolution of amino acid enantiomers, commonly as the Dns derivatives, has been the use of chiral hydrophobic additives and ligand exchange chromatographic approaches often with Cu(II), Zn(II) or Ni(II) employed as the complexing metal[12,13]. In previous studies, we demonstrated[21,22] that low concentrations of *n*-alkylamines, as their phosphate or acetate salts, could be successfully used for the resolution of peptides on alkylsilicas. At low pH values, these reagents distribute on the surface of alkyl-

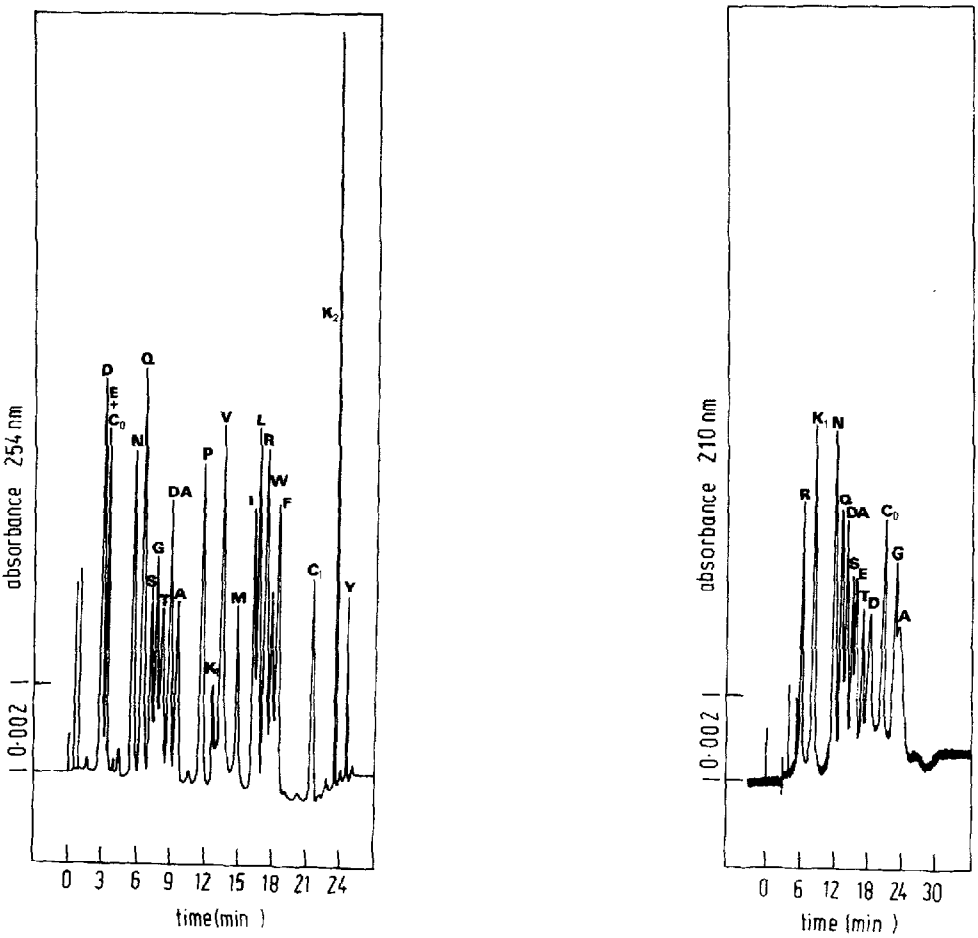

Fig. 3. Separation of Dns-amino acid derivatives on a Radial-Pak A cartridge. Conditions: flow-rate, 7.0 ml/min; temperature, 18°C; solvent A, 10% acetonitrile–200 m*M* ammonium hydrogen carbonate, pH 7.8; solvent B, 45% acetonitrile–200 m*M* ammonium hydrogen carbonate; linear gradients from 10–20% B over 8 min, 20–50% B over 12 min, 50–100% B over 3 min followed by 100% B for 5 min. Sample size was 0.25 nmol of each derivative injected in a total volume of 25 µl. The key to the Dns-amino acids is given in Table I.

Fig. 4. Separation of Dns-amino acid derivatives (0.1 nmol) in the presence of dodecylammonium phosphate. Chromatographic conditions: column, µBondapak-alkylphenyl; flow-rate, 1.0 ml/min; eluent, 2 m*M* dodecylamine, 15 m*M* orthophosphoric acid, pH 4.0, acetonitrile–water (25:75). The key to the various Dns-amino acids is given in Table I.

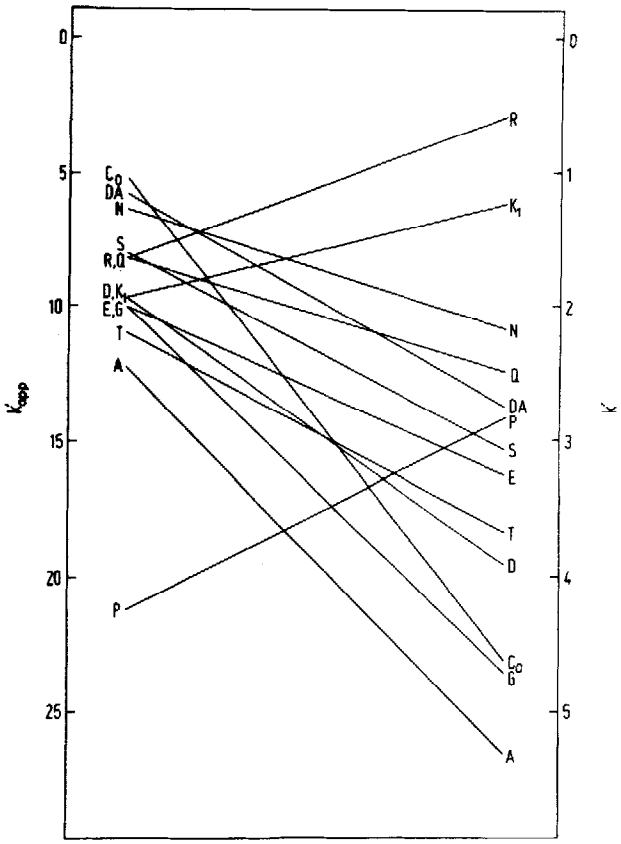

Fig. 5. Comparative retention behaviour of the common mono-Dns-amino acids separated on a μBondapak-alkylphenyl column in the presence and absence of 2 m*M* dodecylamine. Chromatographic conditions: left, elution condition 1 (see legend to Table I, standard 15 m*M* orthophosphoric acid system, pH 2.3) and right, elution condition 3 (see legend to Table I, 2 m*M* dodecylamine system, pH 4.0). The key to the various Dns-amino acids is also given in Table I.

bonded stationary phases which effectively can act as dynamic cationic phases. As a consequence, basic peptides show reduced retentions associated with improved peak shapes. Using 2 m*M* dodecylamine, it is possible to achieve reproducible isocratic or gradient analyses of the mono-Dns-amino acic derivatives. Fig. 4 shows a typical isocratic separation of a number of Dns-amino acids on a μBondapak-alkylphenyl column using dodecylammonium phosphate at pH 4.0. Under these conditions, Dns-Arg and ε-Dns-Lys elute well before either Dns-Asp or Dns-Glu. These changes in selectivity are clearly apparent from an examination of Fig. 5 where the comparative retention behaviour for the mono-Dns-amino acids on a μBondapak-alkylphenyl column using a 15 m*M* orthophosphoric acid gradient eluent, pH 2.3, and a 2 m*M* dodecylamine–15 m*M* orthophosphoric acid isocratic eluent, pH 4.0, is shown.

The applicability of the chromatographic systems developed in this study for N-terminal analysis of peptides has been evaluated using a variety of natural polypeptides. Typical results for the N-terminal analyses of bovine insulin and sperm whale apomyoglobin are shown in Fig. 6. Small samples (0.1 nmole) of these poly-

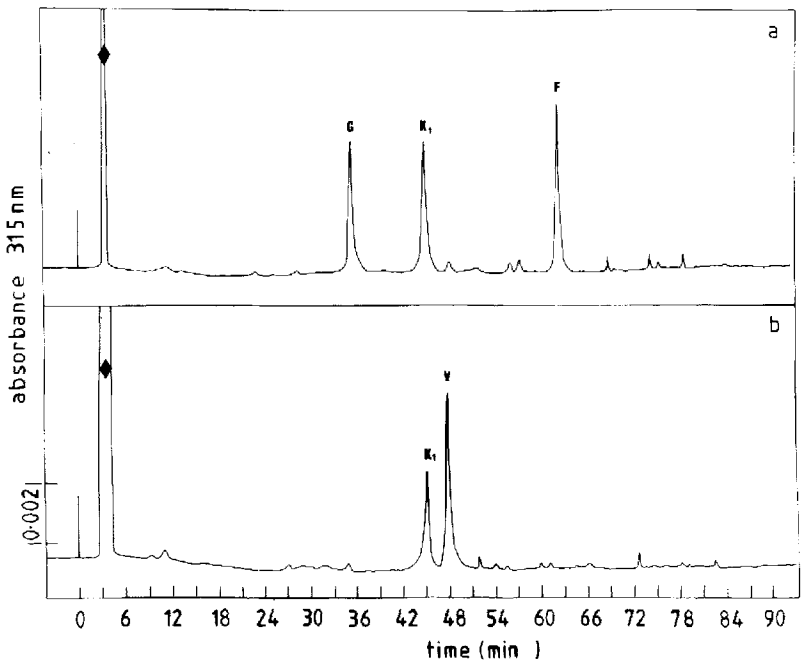

Fig. 6. Chromatographic profiles for the hydrolysates of the Dns-derivatized product from: (a) bovine insulin, and (b) sperm whale myoglobin and the identification of the N-terminal residues. The chromatographic conditions of these analyses are as described in Fig. 3. The major by-products derived from the reagent are marked with an asterisk.

peptides were Dns derivatized, hydrolysed and aliquots analysed. The results obtained for the N-terminal reidues of bovine insulin were in complete agreement with the known N-terminal amino acid residues (A-chain, ^{1}Gly; B-chain, ^{1}Phe)[23] as was also the case with sperm whale apomyoglobin (N-terminal residue, Val)[24]. The ε-Dns-Lys derivative as expected also forms under the Dns-derivatization condition, but can be readily separated from the other Dns derivatives and does not interfere in the chromatographic analysis.

In summary, several new elution and column conditions are described for the RP-HPLC separation of the Dns-amino acids. The procedures also provide versatile methods for N-terminal analysis of peptides. Similar procedures should be applicable to the separation and identification of the Dns derivatives of biologically important amines. Because of the high detection sensitivities which can be achieved with these analytical systems, we have found these procedures are particularly useful in the micropreparative isolation and identification of specific peptides, for example, peptide hormones in biological extracts[25].

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