Journal of Chromatography, 166 (1978) 555–561

CHROM. 11,319

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF Dns-AMINO ACIDS AND APPLICATION TO PEPTIDE HYDROLYSATES

KUO-TING HSU and BRUCE L. CURRIE

Department of Medicinal Chemistry, College of Pharmacy, University of Illinois Medical Center, P.O. Box 6998, Chicago, Ill. 60680 (U.S.A.)

(First received March 13th, 1978; revised manuscript received July 4th, 1978)

SUMMARY

The amino acid composition of several sample peptides was analyzed by high-performance liquid chromatography on reversed-phase columns. Peptides were acid hydrolyzed and the resulting amino acids were converted to the corresponding 5-dimethylaminonaphthalenesulfonyl (Dns) derivatives and then separated on Partisil PAC. Dns-histidine was analyzed on a column of Vydac polar phase. Aqueous acetonitrile or methanol solutions containing acetic acid were used isocratically to effect the separations of the Dns-amino acid mixtures obtained from four peptides ranging in size from three to eleven amino acids in length.

INTRODUCTION

The determination of the amino acid composition and sequence of peptides ranging in size from two to thirty residues in length is a common problem when studying the structure of naturally occurring peptides and proteins. Since the isolation and purification of these peptides is often tedious and difficult, it is desirable to obtain as much structural information as possible from the least amount of material. In some cases, the paucity of material inhibits structural studies by established methods[1,2]. The development of analytical procedures that can reduce the peptide sample size needed for analysis yet still yield the necessary structural information is therefore required.

Derivatives of amino acids can be syntheiszed, that are fluorescent and /or have intense ultraviolet absorption, as an aid to detection during chromatographic analysis. The 5-dimethylaminonaphthalenesulfonyl (Dns, dansyl) derivatives have been used extensively for this purpose and are useful for the determination of the amino terminus of peptides during sequencing and for micro amino acid analysis. The formation of Dns derivatives of amino acids by reaction with Dns chloride was originally developed by Gray and Hartley[3–7] in 1963 for the purpose of qualitative amino acid analysis.

Dns-amino acids are both fluorescent and ultraviolet absorbing, and can be utilized effectively at the nanomolar level.

Recent technological advances in high-performance liquid chromatographic (HPLC) instrumentation have provided a useful tool to be applied to heretofore difficult separations. Recent success has been achieved in the separation by HPLC of the phenylthiohydantoin (PTH) derivatives[8–12] of amino acids obtained from peptide sequencing methods.

The HPLC separation of mixtures of Dns derivatives of the naturally occurring amino acids from proteins seemed feasible. We therefore investigated the application of HPLC to the separation and determination of the composition of mixtures of Dns-amino acids. These methods were then applied to the analysis of the composition of hydrolysates of several synthetic peptides. Our investigations of polar reversed-phase type column materials are reported using isocratic aqueous mobile phases.

Previous attempts to separate Dns-amino acids on polyamide, polyvinylacetate and silica columns have been published[13–17].

EXPERIMENTAL

A Model ALC-201 analytical liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) with an M6000 solvent delivery system was combined with an LC-25 variable wavelength ultraviolet detector (Waters-Beckman) at 250 nm and a recorder (Beckman) or a Model 440 detector (Waters Assoc.) at 254 nm and a recorder (Texas Instruments).

Columns used included: (1) Partisil PAC, 25 cm × 4.6 mm I.D. × (10 μm particle size, pre-packed, Whatman, Clifton, N.J., U.S.A.); (2) Poragel PN, 3 ft. × $^1/_8$ in. O.D. (37–75 μm particle size, dry packed, Waters Assoc.); and (3) Vydac polar phase, 3 ft. × $^1/_8$ in. O.D. (37–75 μm particle size, dry packed, Separations Group, Hesperia, Calif., U.S.A.).

Acetonitrile and methanol were UV grade, "distilled in glass", obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.), glacial acetic acid was reagent grade, water was deionized and distilled. Dns-amino acid standards were obtained from NBC Biochemicals (Cleveland, Ohio, U.S.A.) Dns chloride and Dns amide were obtained from Sigma (St. Louis, Mo., U.S.A.), <Glu-His-Pro-OH was obtained from Peninsula Labs. (San Carlos, Calif., U.S.A.), Tyr-D-Ala-Gly-Phe-Met-NH_2 was obtained from Beckman, Bioproducts Department (Palo Alto, Calif., U.S.A.), and <Glu-Leu-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 and <Glu-His-Glu-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 were generously provided by Dr. Karl Folkers of the Institute for Biomedical Research, Austin, Texas, U.S.A.

Hydrolyses were carried out in 6 *N* HCl (200 μl) at 120° for 20–24 h in sealed Pyrex tubes (50 mm × 6 mm O.D.) Hydrolysates were lyophilized, adjusted to pH 8.5 with $NaHCO_3$ buffer (200 μl) and treated with a 10–20 fold molar excess of Dns chloride in acetone, incubated at 40° for 1 h, lyophilized and dissolved in 1.0 ml of 50% acetonitrile in water or 50% methanol in water prior to liquid chromatography.

RESULTS AND DISCUSSION

In order to avoid the problem of reproducibility that arises from gradient elution of columns, the best possible resolution of the Dns-amino acids under isocratic conditions was sought. It was found that the addition of glacial acetic acid to the solvent systems used resulted in faster elution of the dansyl amino acids, but more

importantly reduced tailing of the peaks. This was particularly helpful for the peaks that were retained the longest, *e.g.* Dns-glutamic acid.

The results obtained from the chromatography of standard dansyl amino acids on three different columns and in the indicated solvent systems are shown in Tables I–III.

TABLE I

Dns-AMINO ACID STANDARDS: PARTISIL PAC

Amino acid derivative	*Capacity factor in solvent system (all aqueous, 1% acetic acid)*			
	Acetonitrile (%)			*30% Methanol*
	50	*40*	*30*	
Dns-Arg	0.1			0.1
Dns-NH_2	0.4	0.4	0.4	0.4
Dns-Ileu	1.1	1.4	1.8	
Dns-Trp	1.2	1.3		
Dns-Leu	1.4			3.8
Dns-Tyr	1.7, 2.0	1,7, 2.0	2.0, 2.6	2.6
Dns-Gly	3.1	3.4	3.6	6.4
Dns-Thr	3.2	3.2		
Dns-Hyp	3.3	3.4		
Dns-Pro	3.4	3.4		4.9
Dns-Phe	3.5			
Dns-Met	4.0			
Dns-Ala	4.8			
Dns-Ser	4.9			6.6
Dns-Glu	19.4			16.5
Dns-Asp	23.2			
Dns-OH	28.6			11.2

TABLE II

Dns-AMINO ACIDS: VYDAC POLAR PHASE

Column: 3 ft. × 1/8 in.; solvent system: acetonitrile–water–acetic acid (10:90:1); flow-rate: 1.0 ml/min; chart speed: 0.1 in./min; UV monitor at 250 nm, 0.1 a.u.f.s.

Amino acid derivative	*Capacity factor*
Dns-Gly	3.2
Dns-Ala	3.6
Dns-Met	4.1
Dns-Tyr	4.4
Dns-Glu	4.6
Dns-Pro	6.8
Dns-Phe	7.5
Dns-His	18.0

Chromatography on Poragel PN can result in partial resolution of Dns-amino acid standards. Separation into classes of acidic, neutral and basic amino acid side chain can be achieved in acetonitrile–water–acetic–acid (30:70:1). Use of longer columns or a smaller particle size range may give better results. The separation obtained seems to be primarily due to the weak acid ion exchange effect of Poragel PN. Basic amino acids, such as arginine are strongly retained.

TABLE III

Dns-AMINO ACIDS: PORAGEL PN

Solvent system: acetonitrile–water–acetic acid (30:70:1); flow-rate: 0.5 ml/min; other conditions as in Table II.

Amino acid derivative	*Capacity factor*
Dns-Glu	1.2
Dns-Gln	1.4
Dns-Gly	1.4
Dns-Thr	2.0
Dns-Asp	2.1
Dns-Ser	2.4
Dns-Ala	2.4
Dns-Pro	2.4
Dns-Lys	3.0, 15.2
Dns-Val	3.2
Dns-Asn	3.6
Dns-Leu	3.6
Dns-Ileu	3.8
Dns-Tyr	3.8, 7.2
Dns-Trp	5.0
Dns-Phe	5.2
Dns-Met	6.0
Dns-Arg	7.0

Partisil PAC proved to be the most satisfactory column for generally good separations of the Dns-amino acid standards. Again a class separation into basic, neutral and acidic side chain groups being eluted in that order. The individual amino acid derivatives within the classes can also be resolved in most cases.

Aqueous methanol and aqueous acetonitrile systems containing acetic acid proved to be quite practical with retention times varying slightly when changing the organic component from 30% to 50%. In some cases a few amino acids will change their relative order of elution, *e.g.* Dns-proline.

Sharp peaks were obtained with little tailing in the system of acetonitrile–water–acetic acid (50:50:1). As little as 0.5 ng (1.4 pmole) of Dns-proline can be detected easily in this system on Partisil PAC.

Peak height is proportional to concentration for the amino acid derivatives. A linear relationship of peak height to sample size is obtained using standard samples of each Dns-amino acid sample to be determined. These graphs can then be used to determine the concentration of the Dns derivatives formed from a peptide hydrolysate. The slope of the line is usually different for each amino acid derivative.

Several synthetic peptides of known structure were chosen to illustrate the potential utility of the separation methods that have been developed.

Small samples, 5 to 15 μg, of the peptides were hydrolysed and derivatized and then aliquots of the mixture were chromatographed. Two analogs of the luteinizing hormone-releasing hormone, LH-RH, were used; <Glu-Leu-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, an antagonist, and <Gly-His-Gly-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, an agonist-antagonist[18,19]. A long acting analog[20], Tyr-D-Ala-Gly-Phe-Met-NH_2, of the endogenous opiate peptide, Met-Enkephalin, Tyr-Gly-Gly-Phe-Met, and

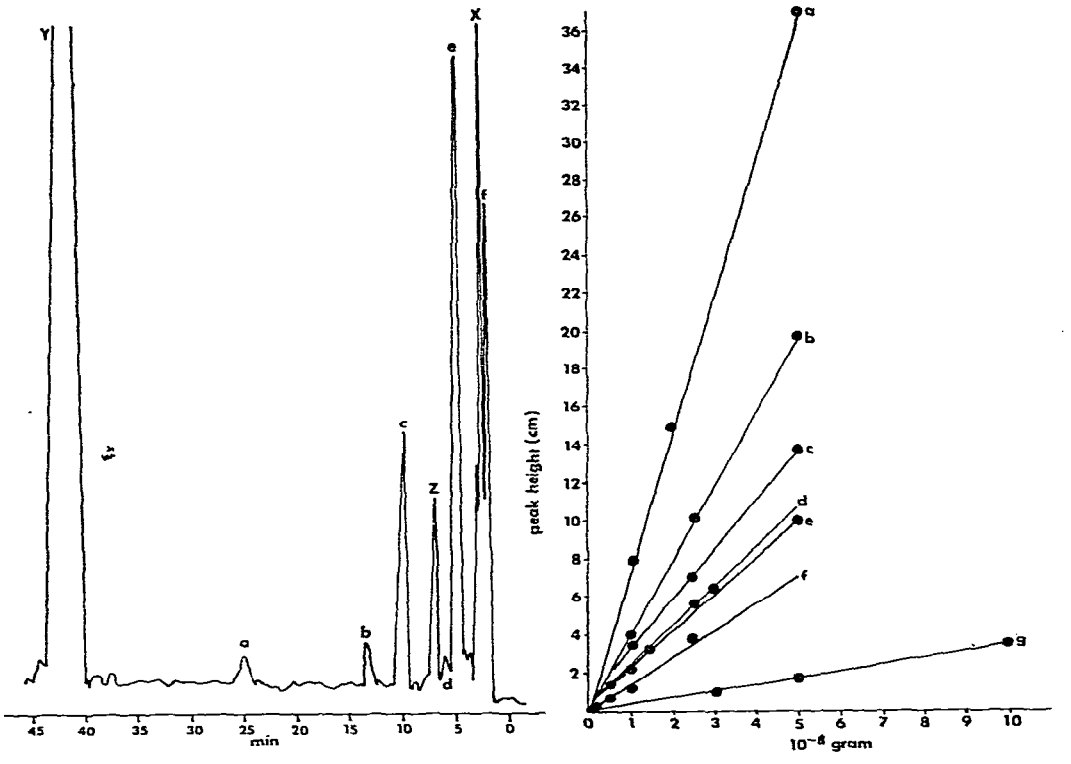

Fig. 1. Chromatogram of Dns-amino acids obtained from pGlu-Leu-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Column: Partisil PAC, 25 cm × 4.6 mm; solvent system: acetonitrile–water–acetic acid (50:50:1); flow-rate: .10 ml/min; chart speed: 0.1 in./min; detector: Model 440 at 254 mm, 0.005 a.u.f.s. a = Dns-Glu; b = Dns-Ser; c = Dns-Gly and Dns-Pro; d = Dns-Tyr; e = Dns-Leu and Dns-Arg; X = Dns-NH_2; Y = Dns-OH; Z = unknown.

Fig. 2. Standard curves for Dns-Arg (a), Dns-Leu (b), Dns-Gly (c), Dns-Pro (d), Dns-Ser (e),Dns-Tyr (f) and Dns-Glu (g). Conditions same as for Fig. 1.

a putative metabolite[21,22], <Glu-His-Pro-OH, of the thyrotropin releasing hormone, TRH, <Glu-His-Pro-NH_2 were also utilized.

The hydrolysis and dansylation reactions proceed in about a 75% combined yield on this scale. Some amino acids, *e.g.* serine and tyrosine, are partially degraded under these conditions[23]. Tryptophan is completely destroyed[23]. An aliquot equivalent to 58 ng (47 pmoles) of starting peptide gives the results shown in Fig. 1. The chromatographic patterns obtained after the hydrolysis and Dns derivatization of other peptide samples are shown in Figs. 2–4.

Dns-histidine is not eluted from Partisil PAC under the conditions needed for the other derivatives. A column of Vydac polar phase was used as an alternative to analyze the derivatives obtained from the tripeptide, <Glu-His-Pro-OH, (Fig. 5).

Total chromatography time is 45 min or less to achieve a separation that can be used for qualitative identification of the amino acid derivatives as well as quantitative determination of relative quantities based on peak height or area. Since the chromatography is done under isocratic conditions there is no need for column regeneration.

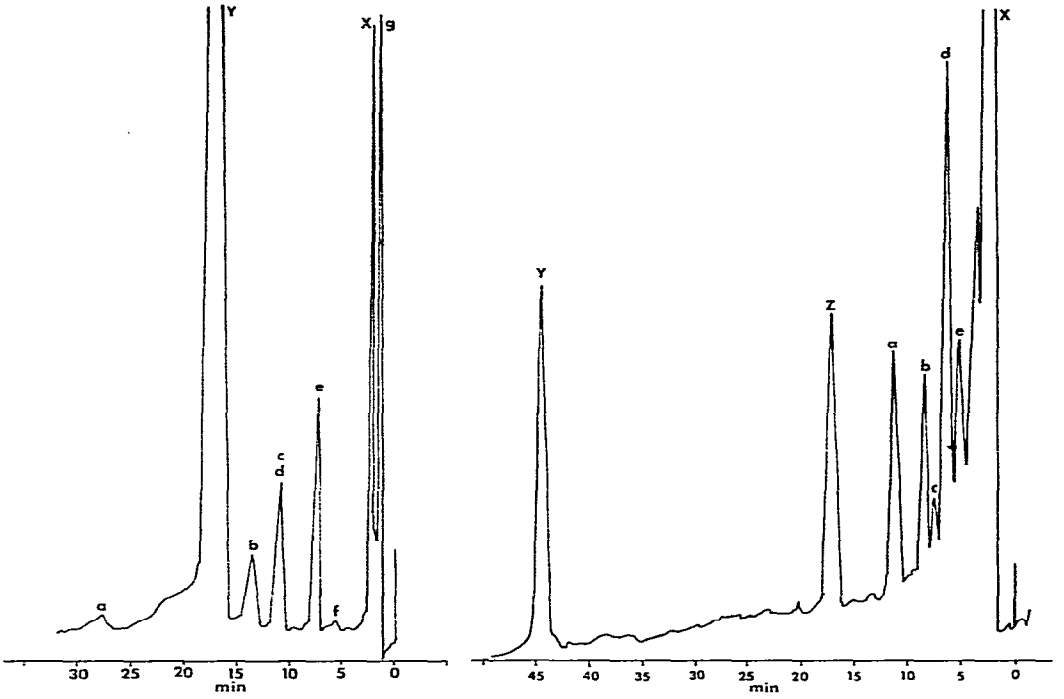

Fig. 3. Chromatogram of Dns-amino acids obtained from pGlu-Leu-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Column: Partisil PAC, 25 cm × 4.6 mm; solvent system: acetonitrile–water–acetic acid (30:70:1); flow-rate: 1.0 ml/min; chart speed: 0.1 in./min; detector: Model 440 at 254 nm, 0.005 a.u.f.s. a = Dns-Glu; b = Dns-Pro; c = Dns-Gly; d = Dns-Ser; e = Dns-Leu; f = Dns-Tyr; g = Dns-Arg; X = Dns-NH_2; Y = Dns-OH.

Fig. 4. Chromatogram of Dns-amino acids obtained from Tyr-D-Ala-Gly-Phe-Met-NH_2. Column: Partisil PAC, 25 cm × 4.6 mm; solvent system: acetonitrile–water–acetic acid (50:50:1); flow-rate: 1.0 ml/min; chart speed; 0.1 in./min; detector: Model 440 at 254 nm, 0.005 a.u.f.s. a = Dns-Gly; b = Dns-Ala; c = Dns-Met; d = Dns-Phe; e = Dns-Tyr; X = Dns-NH_2; Y = Dns-OH; Z = unknown.

The Dns acid byproduct is easily separated from the Dns derivatives and does not interfere. Mold growth has not been observed in the aqueous methanol and acetonitrile mixtures utilized in these procedures, which avoids the problem sometimes encountered during amino acid analysis by ion-exchange methods.

ACKNOWLEDGEMENTS

The authors express their appreciation to the Campus Research Board of the University of Illinois at the Medical Center for partial support of this project. The gift of the peptide samples provided by Dr. Karl Folkers of the Institute for Biomedical Research is gratefully acknowledged. The assistance of Ms. Rachel Hilton in the preparation of the manuscript is appreciated.

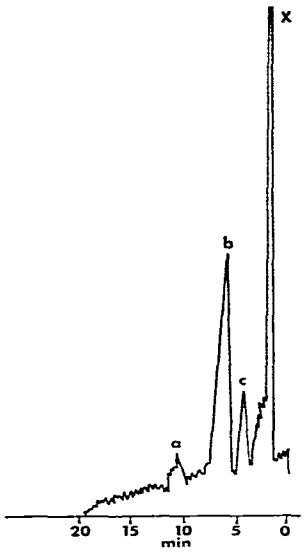

Fig. 5. Chromatogram of Dns-amino acids obtained from pGlu-His-Pro-OH. Column: Vydac polar phase, 3 ft. × 1/8 in.; solvent system: acetonitrile–water–acetic acid (10:90:1); flow-rate: 1.0 ml/min; chart speed: 0.1 in./min; detector: Model LC-25 at 250 nm, 0.1 a.u.f.s. a = Dns-His; b = Dns-Pro; c = Dns-Glu; X = Dns-NH_2 and Dns-OH.

REFERENCES

1 T. Griebrokk, B. L. Currie, K. N. G. Johansson, J. J. Hansen, K. Folkers and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, 59 (1974) 704.
2 T. Greibrokk, J. Hansen, R. Knudsen, Y.-K. Lam, K. Folkers and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, 67 (1974) 338.
3 W. R. Gray and B. S. Hartley, *Biochem. J.*, 89 (1963) 379.
4 W. R. Gray, *Methods Enzymol.*, 11 (1967) 469.
5 W. R. Gray and J. F. Smith, *Anal. Biochem.*, 33 (1970) 36.
6 B. S. Hartley, *Biochem. J.*, 119 (1970) 805.
7 W. R. Gray, *Methods Enzymol.*, 25B (1972) 121, 333.
8 C. L. Zimmerman, J. J. Pisano and E. Apella, *Biochem. Biophys. Res. Commun.*, 55 (1973) 1220.
9 A. P. Graffeo, A. Haag and B. L. Karger, *Anal. Lett.*, 6 (1973) 505.
10 E. W. Matthews, P. G. H. Byfield and I. MacIntyre, *J. Chromatogr.*, 110 (1975) 369.
11 P. Edman, *Acta. Chem. Scand.*, 4 (1950) 283.
12 P. Edman and G. Begg, *Eur. J. Biochem.*, 1 (1967) 80.
13 Z. Deyl and J. Rosmus, *J. Chromatogr.*, 69 (1972) 129.
14 H. Engelhardt, J. Asshauer, U. Neue and N. Weigand, *Anal. Chem.*, 46 (1974) 336.
15 T. Yamabe, N. Takai and H. Nakamura, *J. Chromatogr.*, 104 (1975) 359.
16 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, *Anal. Chem.*, 48 (1976) 1106.
17 M. S. F. Ross, *J. Chromatogr.*, 141 (1977) 107.
18 J. Humphries, G. Fisher, Y.-P. Wan, K. Folkers and C. Y. Bowers, *J. Med. Chem.*, 17 (1974) 569.
19 J. Humphries, Y.-P. Wan, G. Fisher, K. Folkers and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, 57 (1974) 675.
20 C. B. Pert, A. Pert, J.-K. Chang and B. T. W. Fong, *Science*, 194 (1976) 330.
21 R. M. G. Nair, T. W. Redding and A. V. Schally, *Biochemistry*, 10 (1971) 3621.
22 K. M. Knigge and D. Schock, *Neuroendocrinology*, 19 (1975) 277.
23 T.-Y. Lin, *Methods Enzymol.*, 25B (1972) 44.