ON THE ANALYSIS OF PHENYLTHIOHYDANTOIN AMINO ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

SVEN ERIK GODTFREDSSEN and RONALD W. A. OLIVER¹⁾

Department of Chemistry, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

¹⁾Present address:

Department of Biochemistry, University of Salford,
Salford 5, Lancs., U.K.

Keywords: Phenylthiohydantoin amino acids, high performance liquid chromatography, high speed liquid chromatography, micro-bore columns

The key experimental variables and the corresponding separations of synthetic mixtures of phenylthiohydantoin amino acids achieved by high performance liquid chromatography have been abstracted from the literature and are listed in tabular form. From this table it is seen that the large majority of separations reported since 1977 have been performed utilizing stainless steel tubing of large internal diameter (i.d. ≥ 3.9 mm). The reason for the widespread adoption of this particular practice is that a number of earlier studies have indicated that use of small diameter columns leads to significant loss of chromatographic resolution. An experimental study, employing two commercial chromatographic systems and tubings of differing internal diameter (i.d. 4.5 to 1.5 mm), leads to the conclusion that columns of reverse phase support material packed in stainless steel micro-bore tubing (i.d. ≤ 2 mm) can effect separation of phenylthiohydantoin amino acids with similar efficiency as larger diameter columns. The practical advantages of employing micro-bore columns in general are discussed and adoption of micro-bore columns for the routine separation of this particularly important group of amino acid derivatives is recommended.

Abbreviations: HPLC = high performance liquid chromatography, PTH = phenylthiohydantoin, PC = paper chromatography, TLC = thin layer chromatography, GLC = gas liquid chromatography, IEC = ion exchange chromatography, s.s. = stainless steel, i.d. = internal diameter.

1. INTRODUCTION

Methods for the identification of PTH-amino acids obtained by degradation of peptides and proteins according to the procedure developed by EDMAN (2) are of considerable importance since elucidation of the primary structure of peptides and proteins rely almost exclusively on application of the Edman procedure. Accordingly, a wide variety of chromatographic techniques, such as paper chromatography, thin layer chromatography, gas liquid chromatography and ion exchange chromatography have been adapted to this important analytical problem with the aim of providing unambiguous, rapid, reproducible, sensitive and economical methods for the analysis of PTH-amino acids and closely related amino acid derivatives (for a review, see ref. 6). The developments in liquid chromatography in the 1960's have led to the modern practice of high performance liquid chromatography which would be expected to be particularly well suited to fulfil these analytical requirements. Thus, PTH-amino acids being UV absorbing at 254 nm are amenable to detection and accurate quantitation by UV detector systems commonly used in most HPLC equipment. Moreover, identification of the individual components in a mixture of the amino acid derivatives can be made by reference to the chromatographic behaviour of authentic specimens of PTH-amino acids, and, furthermore, it is reasonable to expect that the time required for analysis of a mixture of PTH-amino acids would be comparable to the 20 minute time period necessary to handle the daily volume of samples generated by the currently available automated peptide sequencers.

The extent to which these expectations have been realized in practice can be demonstrated by reference to Table I which lists the HPLC separations of PTH-amino acids published since 1973. A detailed study of this table leads to the following observations:

1. The majority (28) of the 39 separations reported in the 35 papers abstracted have been performed using columns of reverse phase support material. Only seven separations (paper 1, 3, 5, 7, 8, 9, and 21) were performed on silica support material whilst three papers (nos. 18, 19 and 23) report separations on non-commercial tripeptides bonded to silica supports.

2. One paper (No 21) reports an efficient separation on a silica support. However, this method has not been widely adopted probably because of the poor reproducibility of separations obtained using this support.

3. The whole range of commercial reverse phase supports have been employed. The following list details the number of the reported applications of each of the hydrocarbon support materials:

μ Bondapak C18	Paper Nos. 8, 10, 14, 16, 22, 25, 27, 28, 30, 33, 34
Corasil C18	Paper Nos. 2, 4, 6
Spherisorb ODS	Paper Nos. 31, 35
Partisil ODS C18	Paper No. 12
ODS -18	Paper No. 26
Zorbax ODS	Paper Nos. 11, 17, 20, 24
Lichrosorb C8	Paper Nos. 29, 32
Lichrosorb C18	Paper No. 13
Hypersil ODS	Paper No. 31

In addition, two papers (Nos. 6 and 11) report separations involving bonded ether phases, Permaphase ETH, and one paper (No. 15) reports the use of Micropak CN.

4. Whilst hydrocarbon C18 and C8 bonded support material has been used for 25 of the 28 separations on reverse phase columns, no detailed comparative studies of the efficiencies of the separations obtained with the various commercial materials utilising these particular bonded phases has yet been published. A detailed comparison of the separations abstracted in Table I leads to the conclusion that reproducible, rapid and efficient separation of mixtures of PTH-amino acids can be carried out on any of the commercially available C18 or C8 reverse phase support materials packed in stainless steel columns of diameters ≥ 3.9 mm and used in conjunction with gradient elution involving mixtures of aqueous sodium acetate buffers and either acetonitrile or methanol at elevated temperatures.

5. Since 1977 all separations reported to be carried out on reverse phase support material packed in stainless steel tubing employed wide bore tubing (i.d. ≥ 3.9 mm). One paper (No. 26) published since 1977 reports the use of a small diameter column which, however, was packed in teflon tubing (i.d. 0.5 mm).

The value of collating chromatographic separations of a complex mixture lies in the

Table I

Summary of the experimental conditions and separations reported for synthetic mixtures of phenylthiohydantoin amino acids by high performance liquid chromatography.

Ref. No.	COLUMN				ELUTION		SEPARATION		
	Support	l cm	i.d. m.m.	θ °C	Eluant Composition (v/v)	Gradient	t mins	N	Assignment (in order of elution)
1.	Silica (15-20 μ m)	100	1.8	25	C ₇ H ₁₆ /CHCl ₃ 50:50	isocratic	16	6	P,L,I,V,F,M.
					CHCl ₃ 100	isocratic	28	4	A,G,Y,T.
					CH ₃ OH/CHCl ₃ 3:97	isocratic	12	3	S,Q,N.
2.	Corasil C ₁₈	91.4	2	Am	(I)NaOAc, 0.01M pH 3.8	linear	30	14	D/S,N,T,G,Q,E,A,H, Y,R,P/M/V,L,I,K.
					CH ₃ CN 95:5 (II)CH ₃ CN				
3.	Silica (5 μ m)	50	3	N.G.	CH ₂ Cl ₂ /DMSO/t-BuOH 1000 : 0.8 : 8	isocratic	7	9	P,L,I,V,F,M,A,W,G.
	Merckosorb SI 60				CH ₂ Cl ₂ /DMSO/H ₂ O 80 : 15 : 2	isocratic	7	7	K,Y,T,E,Q,N,D.
4.	Corasil C ₁₈ (37-50 μ m)	100	2	23	(I)H ₂ O/CH ₃ CN/i-PrOH 100 : 1.5 : 1	stepped (I) to (II)	40	14	D,E,N,S/T,G,Q,A, Y,P,V,M/H,I,L,F.
					(II)H ₂ O/CH ₃ CN/i-PrOH 15 : 1.5 : 1				
5.	Silica (5 μ m) Merckosorb SI 60	50	2	20	C ₂ H ₄ Cl ₂ +0.8% i-PrOH	isocratic	17	9	P,L,I,V,F,M,W,A,G.
6.	Permaphase ETH	50	2.1	N.G.	(I)H ₂ O (II)CH ₃ CN	linear (I) to 35%(II)	25	12	T,G,A,P,V,M,Y,L,I, F,K,W.
	Corasil C ₁₈	50	2.1	N.G.	(I)H ₂ O (II)CH ₃ CN	linear (I) to 40%(II)	20	4	D,N,Q,E.
7.	Zorbax Silica	25	2.1	40	(I)C ₆ H ₁₄ /CH ₃ OH/PrOH 3980 : 9 : 1 (II)CH ₃ OH/PrOH 9 : 11	concave (I) to (II)	40	13	I/L,V,F,M,A,T/G,W, K/Y,S,Q,N,E,D.
8.	Bondapak C ₁₈ (1 μ m)	61	4	N.G.	(I)NaOAc, 0.01M pH 4 (II)CH ₃ CN	concave (I) to 50% (II)	30	15	D,C,N,S,Q,T,E,G,H, A,Y,R,V/M,I,I,K.
	Silica gel (5 μ m)	30	3.2	N.G.	(I)C ₇ H ₁₆ /CHCl ₃ 75:25 (II)i-PrOH with 1% 0.1M perchloric acid	concave (I) + 1% (II) to 40% (II)	40	17	P,L,I, Δ T,V,F,M,A,G, W,D,E/Y,K,T,S,Q,N.
9.	Partisil 5	30	2.8	N.G.	(I)C ₇ H ₁₆ /CHCl ₃ 3 : 1 (II) i-PrOH	stepped (II) added to (I)	40	14	P,L,I, Δ T,V,F,M,A, G/W,Y,K,T,S,Q.
10.	μ Bondapak C ₁₈	60	4	Am	(I)NaOAc, 0.01M pH 4 CH ₃ CN 9 : 1	concave 8%(II) to 90%(II)	40	15	H,N,D,S,T,Q/R,G,C,E, A,Y,V/M/P,F/W/I,L,K.
					(II)NaOAc, 0.01M pH 4.0 CH ₃ CN 1 : 9				
11.	Zorbax ODS	25	2	N.G.	(I)NaOAc, 0.01M pH 3.8 CH ₃ CN 95 : 5 (II) CH ₃ CN	linear (I) to 52% (II)	40	15	H,N,D,S,T,Q/R,G,C, Y,M/V,P,W,F,I,L,K.
	Permaphase ETH 100		2	N.G.	(III)NaOAc 0.01M (IV) CH ₃ CN	concave (III)to 57%(IV)	40	15	S/T/H/R,Q,N,G,D,A,E, P,V,Y/M,I,L,F,W,K.

S. E. GODTFREDSEN & R. W. A. OLIVER: HPLC of PTH-amino acids

Ref. No.	COLUMN			ELUTION		SEPARATION			
	Support	l cm	i.d. m.m.	ø °C	Eluant Composition (v/v)	Gradient	t mins	N	Assignment (in order of elution)
12.	Partisil ODS C ₁₈ (10 µm)	25	4.6		(I)NaOAc 0.01M pH 4 10% CH ₃ CN	isocratic	6	8	C,D,N,T,S,G,E,Q.
					(II)NaOAc 0.01M pH 4 20% CH ₃ CN	isocratic	8	4	A,Y,V,M.
					(III)NaOAc 0.01M pH 4 30% CH ₃ CN	isocratic	11	8	P,W,I,L,F,K,H,R.
13.	Lichrosorb C ₁₈	25	4.6	Am	(I)NaOAc 0.01M pH 4.6 (II) CH ₃ CN	linear from 5% (II) to 40% (II)	25	11	D,N,E,Q,G,A,Y, V/M/P,I/F/W,L,K.
14.	µBondapak C ₁₈	60	4	N.G.	(I)NaOAc 0.01M pH 4 CH ₃ CN 95 : 5 (II)NaOAc 0.01M pH 4 CH ₃ CN 5 : 95	linear from 5% (II) to 60% (II)	60	12	D,N,S,T,Q,G,E,A,Y, P,L,K.
15.	Micropak CN (10 µm)	25	2.1	N.G.	(I)C ₆ H ₁₄ (II)CH ₂ Cl ₂ , i-PrOH 8:2	mixed linear and isocratic from (I) to (II)	3	16	P,L/I,V,F,M,A,T,G, Y,K,S,W,Q,N,E,D.
16.	µBondapak C ₁₈	N.G.	N.G.	N.G.	(I)H ₂ O,CH ₃ OH,HOAc pH 4.1 90:10:0.25 (II)H ₂ O,CH ₃ OH,HOAc 10:90:0.025	linear 95%(I) + 5%(II) to 40%(I) + 60%(II)	N.G.	N.G.	N.G.
17.	Zorbax ODS	25	4.6	62	(I)NaOAc 0.01M pH 4.5 (II)CH ₃ CN	mixed linear and isocratic 24%(II) to 45%(II)	20	19	D,E,N,S,Q,T,G,A,Y,H, V,M,P,W,K,F,I,L,R.
					NaOAc 0.01M pH 4.5 CH ₃ CN 58 : 42	isocratic	13	19	As above except S/Q and P follows W
18.	Bonded tripeptide L-Val-L-Ala-L-Ser to SiO ₂ gel CT	25	3.8	20	1% citric acid-water pH 2.5	isocratic	70	18*	H,R,S,T,N,G,Q,A,E, D,V,M,P,Y,L,I,F,W.
19.	Bonded tripeptide L-Val-L-Phe-L-Val to Partisil 10	25	2.1	N.G.	1% citric acid-water pH 2.5	isocratic	N.G.	18*	H,R,T,S,N,Q,G,A,E, V,P,D,M,Y,I,L,F,W.
20.	Zorbax ODS	25	4.6	62	(I)NaOAc 0.01M pH 4.18 20% CH ₃ CN (II)NaOAc 0.01M pH 4.18 30% CH ₃ CN (III)NaOAc 0.01M pH 4.18 40% CH ₃ CN	three step isocratic programme	25	14	D,N,E/S,Q,T,G,A,Y, M,V,P/W,F/K,I,L.
21.	µPorasil	30	3.9	N.G.	(I) i-C ₈ H ₁₈ (II)CH ₃ OH, i-PrOH 9:11 (III) i-C ₈ H ₁₈ CHCl ₃ 7:3 (IV) i-C ₈ H ₁₈ CH ₃ OH, CHCl ₃ 15 : 42.5 : 42.5	concave 5%(II) to 70%(II) in (I)	25	14	L/I,V,P,F/M,A,G,T, W,S,Y,K,N,Q,D/E.
						concave 1%(IV) to 100%(IV) in (III)	25	15	P,L,I,V,F,M,A,G,W/K, Y,T,S,P/E,Q,N.

S. E. GODTFREDSEN & R. W. A. OLIVER: HPLC of PTH-amino acids

Ref. No.	COLUMN				ELUTION		SEPARATION		
	Support	1 cm	i.d. m.m.	θ °C	Eluant Composition (v/v)	Gradient	t mins	N	Assignment (in order of elution)
22.	μ Bondapak C ₁₈	30	3.9	N.G.	(I)NaOAc 0.01M pH 6 (II)CH ₃ CN	exponential from 21%(II) to 38%(II)	20	15	D,E,C,N,S,T,Q/H,G,R, A,Y,V/P/M,I/F,L/W,K.
23.	Bonded tripeptide L-Val-L-Ala-L-Pro to Partisil 10	30	2.1	N.G.	1% citric acid-water pH 2.5	isocratic	40	9	H,T,G,E,V,P,Y,F,W.
24.	Zorbax ODS	25	4.6	55	(I)NaOAc 0.02M pH 5 (II)CH ₃ CN	multi linear isocratic (I) to 90%(II)	20	20	D,E,N,S,Q,T,G,H/S, A,Y,C,M,V,P,W,K,F, I,L,R.
25.	μ Bondapak C ₁₈	30	3.9	26	(I)NaOAc 0.005M pH 5.3 23% CH ₃ OH 20 μ l acetone (II)CH ₃ OH-water 44:56	mixed exponential isocratic programme (I) to (II)	16	17	D,E,N,S,T,G,Q, Δ T,A, Y,P,V,W,F,I,L,K.
26.	ODS-18 (5 μ m)	9	0.5	N.G.	(I)NaOAc 0.005M pH 5.3 23% CH ₃ OH (II)H ₂ O,CH ₃ CN,i-PrOH 7.5 : 1.5 : 1	isocratic isocratic	25 40	7 6	D,E,N,S,T,G,Q. A,Y,P/M/V,W,I/L/F,K.
27.	μ Bondapak	30	4	20-23	(I)NaOAc 0.01M + 10% CH ₃ CN (II)NaOAc 0.01M + 90% CH ₃ CN Both buffers pH 4	25%(II) to 50%(II) 2%(II) to 30%(II)	20 15	9 7	A,Y,V,P/M,I,W/F,L,K. D,N,S,T,G,Q,E.
28.	μ Bondapak C ₁₈	30	4	Am	(I)H ₂ O,CH ₃ OH,HOAc, (CH ₃ CO) ₂ O, pH 4.1 900:100:2.5:0.05 (II)H ₂ O,CH ₃ OH,HOAc 100:900:0.25	linear 5%(II) to 45%(II)	20	18	D,N,S,T,G,H,Q,E,A,R, Y,P,V/M,W,I,F,L,K.
29.	Lichrosorb RP8 (5 μ m)	30	3.9	50	NaOAc 0.01M, CH ₃ CN pH 4.6 75 : 25	isocratic	60	17	D,N,S,Q,E,T,G,H,A,R, Y,M/P,V,W,I,F,L.
30.	μ Bondapak C ₁₈	30	4	N.G.	(I)NaOAc 0.01M, CH ₃ OH pH 4.0 9 : 1 (II)NaOAc 0.01M, CH ₃ OH pH 4.0 1 : 9	concave plus isocratic 5%(II) to 40%(II)	32	18	N,D,T,H,G,Q,E,A,R,T, Δ S,P,V/M,W,F,I,K,L.
31.	Hypersil ODS (5 μ m) Spherisorb ODS (5 μ m)	23 23	4.6 4.6	Am Am	(I)NaOAc 0.01M in H ₂ O (II)NaOAc 0.01M in CH ₃ OH	linear 27%(II) to 65%(II)	45	18	D,E,N,S,T,G,Q,A,Y,C, P,M,V,W,F,I,K,L. V/M not resolved on Spherisorb
32.	Lichrosorb RP 8 (10 μ m)	25	4.6	35	(I)NaOAc 0.0022M pH 4.9 (II)CH ₃ OH	double linear 5%(II) to 48%(II)	25	16	D,N,E,S,T,G,Q,A,Y,H, P,M,V,R,I/K/F,L.
33.	μ Bondapak C ₁₈	30	3.9	23	(I)NaOAc 0.005M pH 5.6 20% CH ₃ OH (II) 38% CH ₃ OH in H ₂ O	two step isocratic programme (I) then (II)	45	15	D,E,N,S,T,G,Q,A,Y,P, V,W,F,L,K.

Ref. No.	COLUMN				ELUTION		SEPARATION		
	Support	l cm	i.d. m.m.	θ °C	Eluant Composition (v/v)	Gradient	t mins	N	Assignment (in order of elution)
34.	μ Bondapak C ₁₈	30	4	20	(I) 8.0 mM DETA 20 mM TCA pH 4.2 (II) as for (I) plus 60% CH ₃ CN	concave 5%(II) to 75%(II)	36	17	D,N,S,T,G,H,N,E,A, R,Y,P/V,M,I,L.
35.	Spherisorb ODS (5 μ m)	25	4.6	37	(I) LiOAc 0.02M pH 5.2 (II) LiOAc 0.1M 20% CH ₃ CN 80% pH 5.2	mixed linear plus isocratic 25%(II) to 95%(II)	18	19	D,G,N,S,T,Q,G,H,A,Y, R,V,M,P,I,W,F,L,K.

l is column length, i.d. is internal diameter, θ is temperature of the column, N is number of peaks, Am is ambient, N.G. is not given, t is time required for the separation, * calculated from retention data given for PTH-amino acids, DETA is diethylenetriamine, TCA is trichloroacetic acid.

REFERENCES TO TABLE

- GRAFFEO, A. P., A. HAAG & B. L. KARGER: Anal. Letters 6, 505-511 (1973)
- ZIMMERMAN, C. L., J. J. PISANO & E. APPELLA: Biochem. Biophys. Res. Comm. 55, 1220-1224 (1973)
- FRANK, G. & W. STRUBERT: Chromatographia 6, 522-4 (1973)
- HAAG, A. & K. LANGER: Chromatographia 7, 659-622 (1974)
- FANKHAUSER, P., P. FRIES, P. STAHALA & M. BRENNER: Helv. Chim. Acta 57, 271-277 (1974)
- DE VRIES, J. X., R. FRANK & C. BIRR: F. E. B. S. Letts. 55, 65-67 (1975)
- MATTHEWS, E. W., P. G. H. BYFIELD & I. MACINTYRE: J. Chromatog. 110, 369-373 (1975)
- BRIDGEN, J., A. P. GRAFFEO, B. L. KARGER & M. D. WATERFIELD: Article on page 140 in »Instrumentation in Amino Acid Sequence Analysis«. Edited by R. N. Perham. Academic Press. London 1975
- NIECE, R. L.: P. 233-240, Proc. 1st Int. Conf. in Solid Phase Methods Protein Sequence Analysis. Pierce Chem. Co. Rockford Ill., 1 (1975)
- DOWNING, M. R. & K. G. MANN: Anal. Biochem. 74, 298-319 (1976)
- ZIMMERMAN, C. L., E. APPELLA & J. J. PISANO: Anal. Biochem. 75, 77-85 (1976)
- LOMINAC, G. D. & H. S. KINGDON: Archiv. Biochem. Biophys. 173, 320-325 (1976)
- RODKEY, J. A. & C. D. BENNETT: Biochem. Biophys. Res. Commun. 72, 1407-1413 (1976)
- MOO-PEN, W. F., D. L. JUE, K. C. BECHTEL, M. H. JOHNSON & R. M. SCHMIDT: J. Biol. Chem. 251, 7557-7562 (1976)
- BOLLET, C. & M. CAUDE: J. Chromatog. 121, 323-328 (1976)
- BRIDGEN, P., G. A. M. CROSS & J. BRIDGEN: Nature 263, 613-614 (1976)
- ZIMMERMAN, C. L., E. APPELLA & J. J. PISANO: Anal. Biochem. 77, 569-573 (1977)
- KIKTA, E. J. & E. GRUSHKA: J. Chromatog. 135, 367-376 (1977)
- FONG, G. W. K. & E. GRUSHKA: J. Chromatog. 142, 299-309 (1977)
- ERICSSON, L. H., R. D. WADE, J. GRAGNON, R. M. McDONALD, R. GRANBERG & K. A. WALSH: Solid Phase Methods in Protein Sequence Analysis. Insem. Symposium 5. Ed. A. Previero and M. A. Coletti-Previero 137-142. Elsevier (1977)
- DEVILLERS-THIERY, A. & G. BLOBEL: Anal. Biochem. 87, 66-70 (1978)
- KLAPPER, D. G., C. E. WILDE & J. D. CAPRA: Anal. Biochem. 85, 126-131 (1978)
- FONG, G. W. & E. GRUSHKA: Anal. Chem. 50, 1154-1161 (1978)
- HANKAPILLER, M. W. & L. E. HOOD: Biochemistry 17, 2124-2133 (1978)
- ZEEUWS, R. & A. D. STROSBURG: F. E. B. S. Letts. 85, 68-72 (1978)
- VAN BEEUMAN, J., J. VAN DAMME & J. DELEY: F. E. B. S. Letts. 93, 373-376 (1978)
- MARGOLIES, M. N. & A. BRAUER: J. Chromatog. 148, 429-439 (1978)
- BHOWN, A. J., J. E. MOLE, A. WEISSENGER & J. C. BENNETT: J. Chromatog. 148, 532-535 (1978)
- ABRAHAMSSON, M., K. GRÖNINGSSON & S. CASTENSSON: J. Chromatog. 154, 313-317 (1978)
- ELION, J., M. R. DOWNING & K. G. MANN: J. Chromatog. 155, 436-438 (1978)

31. OESTVOLD, G., E. JENSEN & T. GREIBROKK: *Medd. Norsk Farm. Selsk.* 40, 173–179 (1978)
32. STRICKLAND, M., W. N. STRICKLAND, W. F. BRANDT, C. VAN HOLT, B. WITTMANN-LIEBOLD & A. LEHMANN: *Eur. J. Biochem.* 89, 443–452 (1978)
33. MOO-PEN, W. F., M. H. JOHNSON, K. C. BECHTEL & D. L. JUE: *J. Chromatog.* 172, 476–480 (1979)
34. ANNAN, W. D.: *J. Chromatog.* 173, 194–197 (1979)
35. MOSER, P. W. & E. E. RICKLE: *J. Chromatog.* 176, 451–455 (1979)

application of any general observations drawn from the exercise to the understanding and further improvement of the current analytical situation.

Thus, the conclusion included in the fourth observation above can be readily understood in view of the diverse physico-chemical properties of the PTH-amino acids and the mechanism of the chromatographic processes involved when reverse phase support materials are employed. However, the final observation is difficult to understand since current chromatographic theories (7) leads to the conclusion that micro-bore columns (i.d. ≤ 2 mm) should be equally as efficient as large diameter columns. Indeed, these theories form the basis of the design and successful development of at least one commercial micro-bore chromatographic system (5). Further, some of the separations reported before 1977 actually employed reverse phase support material packed in micro-bore stainless steel tubing (e.g. Table I, paper 2). A closer study of the listed papers published prior to 1977, indicate that the probable origins of the current experimental practice is the claim of WOLF (9) and DE STEFANO (1, 8) that use of larger diameter columns improved HPLC separations because »wall effects« were eliminated. In view of these opposing theoretical and practical claims, and the fact that one reverse phase column separations of the PTH-amino acids employing micro-bore Teflon tubing has been reported (Table I, paper 26), it was decided to reinvestigate whether or not efficient separation of PTH-amino acids can

be achieved using columns of reverse phase support material packed in micro-bore stainless steel tubing.

2. MATERIALS AND METHODS

2.1. PTH-amino acids

A mixture of the PTH derivatives of the following amino acids was dissolved in methanol and kept tightly stopped in a freezer before use. PTH derivatives of alanine (A), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), isoleucine (I), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), serine (S), threonine (T), valine (V), tryptophan (W), tyrosine (Y), and PTH-(ϵ -phenylthiocarbamoyl)-L-lysine (K) were obtained from the Pierce Chemical Co., Rockford, Illinois, USA. The concentration of each amino acid derivative in the stock solution was 0.2 mg·ml⁻¹.

2.2. Support materials

Two batches of reverse phase support material of differing particle size, LiChrosorb RP-18.5 μ m and 10 μ m, obtained from the Merck Company, Darmstadt, were employed.

2.3. Stainless steel tubing

Stainless steel tubing of four different internal diameters, 4.5, 3.0, 2.1 and 1.5 mm, in 25 cm lengths was obtained from Mikrolaboratoriet, Aarhus, fitted with stainless steel frits and was filled with the support material by the slurry packing technique using a mixture of isopropanol and methanol (7:3) as the solvent phase and a slurry packing apparatus manufactured by Magnus Scientific Instrumentation Ltd., Cheshire, U.K.

2.4. Solvents

Acetonitrile, HPLC Grade S, was obtained from Rathburn Chemicals, Peeblesshire, Scotland and was filtered through a micro-pore filter (pore-size 0.5 μ m) prior to use.

2.5. Chromatographic systems

A Waters chromatographic system equipped with a variable UV-detector Model 450, a solvent programmer Model 660, a solvent delivery system Model 6000A, an injector system Model U6K and a Hewlett Packard integrator Model 3380 A was used in the first series of experiments.

A Hewlett Packard liquid chromatograph Model 1074 A equipped with a fixed wavelength detector Model 1036 A ($\lambda = 254$ nm) was employed in the second series of experiments.

3. RESULTS AND DISCUSSION

The operating conditions and the average (mean of three consecutive runs) time of appearance for each of the thirteen peaks forming the chromatogram obtained when a mixture of the 17 PTH-amino acids was separated using LiChrosorb RP-18 (10 μ m), slurry packed in stainless steel tubing of four

different diameters and the Waters chromatographic system, is given in Table II.

Before proceeding to discuss the experimental results given in Table II it should be noted that the conditions adopted for the separation of the PTH-amino acids on this support material (LiChrosorb RP-18) were based on those reported in the literature (Table I). The initial separation of the PTH-amino acid mixture was performed on the column of the largest diameter (4.5 mm) and once satisfactory reproducibility of the peak elution times had been achieved [$\frac{dt}{t} \times 100 \leq 1.5\%$] no further studies were made in order to improve the separation by altering the eluant gradient programme or the operating temperature since the aim of the experiments was to determine the effect of reducing the diameter of the chromatographic columns. In the subsequent experiments (as detailed in Table II) with the smaller diameter columns only those variables which would be expected to be linearly related to the volume of the column, namely the sample

Table II

Summary of the elution times and operating conditions for the HPLC separation of a synthetic mixture of seventeen PTH-amino acids performed on a Waters liquid chromatograph employing columns of LiChrosorb RP-18 (10 mm) packed in 25 cm lengths of stainless steel tubing of differing internal diameters at ambient temperature.

Column diameter (mm)	4.5	3.0	2.1	1.5
Sample volume (μ l)	5.0	2.2	1.0	0.5*
Flow rate (ml/min)	3.0	1.3	0.9	0.4
Photometer attenuation	64	32	24	16

Peak no.	Retention time (min)			
1	3.93	3.11	2.46	2.23
2	5.01	3.54	2.94	2.72
3	5.76	3.94	3.36	3.13
4	6.22	4.88	4.07	3.76
5	7.32	5.80	4.60	4.23
6	7.82	6.18	5.15	4.67
7	9.14	7.27	7.72	7.08
8	13.23	10.98	12.51	11.93
9	14.80	16.09	15.40	14.60
10	17.04	18.18	17.86	17.60
11	19.07	20.41	20.24	21.40
12	19.66	20.78	20.24 sh	21.70 sh
13	20.25	21.37	21.12	22.40

Solvent A was 0.01M NaOAc, pH 4.3. Solvent B was 50% CH_3CN + 50% A. Eluent programme: 35% B isocratic for 10 mins. (8 mins. when 1.5 mm column was used), gradient (programme 3) to 92% B for 16 mins. sh is shoulder. * 5 μ l of a 10 fold dilution in solvent B of the stock solution applied.

loading and eluant flow rate, together with the derived variable – the spectrophotometer attenuation – were altered in accordance to that calculated from the corresponding changes in the dimensions of the columns employed.

It should also be noted that some separate initial experiments were performed on the larger diameter column (4.5 mm) in order to ascertain the effect of reducing the pre-column volume and the post-column-pre-detector volume in the Water's system employed. For the former, the Water's 2 ml sample loop in the injection system was replaced by a short (≈ 10 cm) length of small bore (0.15 mm) s.s. tubing, a modification which led to the appearance of regularly spaced baseline solvent »peaks« on the chart output. These were eventually traced to the pulsation effects of the piston pumps employed in the eluant system. Clearly the large (2 ml) sample loop in the injection system had been designed to buffer these effects and therefore the chromatographic data given in Table II were obtained using the un-modified sample injection system. However, improved chromatographic resolution was obtained when the normal long (≈ 40 cm) piece of s.s. tubing of large i.d. (≈ 0.5 mm) from the column to the photometer was replaced by a short (5 cm) piece of small diameter (0.15 mm) s.s. tubing. This simple modification was the only one made to the Water's apparatus in these series of experiments. A study of the chromatographic data given in Table II shows that practically identical separation of the PTH-amino acid mixture is obtained using columns of Lichrosorb RP-18 packed in s.s. tubing of differing internal diameters under the conditions specified in the table. Indeed, the only significant change observed when the micro-bore columns were used is that peaks Nos. 11 and 12, which were partially resolved when larger diameter columns are used, merged to form a single peak with a distinct shoulder. It may thus be concluded from this series of experiments that only a very small loss in chromatographic resolution occurs when separations are performed on columns packed in micro-bore s.s. tubing. Further, it should perhaps be emphasised that in this initial series of experiments little or no attempt was made to improve the separations obtained with the micro-bore columns by »fine-tuning« the operating conditions.

Since the above conclusion is contrary to the claims which ZIMMERMANN et al. (Table I, paper 17) put forward based on separations performed at elevated temperatures and on columns containing smaller particle size support material, a second series of experiments were performed using an un-modified Hewlett-Packard liquid chromatograph operated at 48 °C and columns of Lichrosorb RP-18 of particle size 5 μ m. The advantages to be gained from the use of this particular chromatograph, in addition to the ability to operate readily at elevated temperatures, include the ease whereby the instrument may be automatically operated so as to vary the gradient elution programme and hence to »fine-tune« the separation conditions. As before, the initial separation of the PTH-amino acid mixture, was performed on the largest diameter column (4.5 mm), (c.f. Figure 1A).

When the separation was performed, using essential identical conditions of elution, on the smaller diameter column (3.0 mm) the chromatogram shown in Figure 1B was obtained.

Comparison of the two chromatograms shown in Figures 1A and 1B indicates that a distinctly improved separation of the PTH-amino acid mixture was obtained on the smaller diameter column. Thus, the peaks numbered 5 and 6, and the final peak, in both chromatograms are completely resolved on the 3 mm column and the shoulder of peak number 13 (Figure 1A) is partially resolved in Figure 1B (peak 13). A total of 15 and 16 peaks are apparent in the chromatograms obtained when using the 4.5 and 3.0 mm column respectively.

Further, whilst the resolution of the separations were reduced when the two micro-bore columns of i.d. 2.1 and 1.5 mm were used under operating conditions essentially identical to those employed with the wide-bore columns – compare the chromatograms shown in Figures 1A and 1B with those shown in Figures 1C and 1D – it was again found that the chromatographic resolution achieved by use of the smaller of the two columns (1.5 mm) was better than that obtained when using the 2.1 mm diameter column. Thus, comparison of the chromatograms shown in Figure 1C and 1D shows that an extra chromatographic peak (peak No. 13, Figure 1D) is well resolved on the smaller of these two columns. A total of 12 and 13 peaks

Figure 1A. HPLC Chromatogram of PTH-amino acids obtained on a 4.5 mm (i.d.) column of LiChrosorb RP-18, 5 μ m.

10 μ l of the PTH-amino acids dissolved in methanol was injected onto the column. Solvent A was 0.01 M-NaOAc, pH 4.3 and solvent B acetonitrile. Eluent programme: 9.2% B isocratic for 8 mins, linear gradient to 15% B for 2 mins, linear gradient to 30% B for 15 mins, linear gradient to 35% B for 1 min, linear gradient to 40% B for 10 mins. The temperature of the column was 48 $^{\circ}$ C, the flow was 3.0 ml $\cdot$ min $^{-1}$ and the chromatogram was monitored at 254 nm. By co-chromatography with authentic specimens the following assignment of the peaks was made: 1 = D, 2 = N, 3 = S, 4 = T, 5 = Q, 6 = G, 7 = E, 8 = A, 9 = Y, 10 = P, 11 = M and V, 12 = W, 13 = I and F, 14 = L, 15 = K. Ordinate in all chromatograms shown is absorbance at 254 nm. The photometer attenuation was 64.

were observed when using the 2.1 and 1.5 mm columns respectively.

These interesting findings provided the rationale for the final series of experiments which were again performed on the un-modified Hewlett-Packard Liquid Chromatograph. In these, a systematic study was made of the effect of controlled changes in the elution conditions on the chromatogram obtained earlier when the 1.5 mm diameter column had been used to separate the PTH-amino acids (Figure 1D). Visual comparison of the chromatogram corresponding to the optimal separation obtained as a result of these »fine tuning« studies (c.f. Figure 2) with that obtained in the previous experiments on the

Figure 1B. HPLC Chromatogram of PTH-amino acids obtained on a 3.0 mm column of LiChrosorb RP-18, 5 μ m.

Conditions were as specified by Figure 1A except that 5 μ l of the solution of PTH-amino acids was applied onto the column which was operated at a flow of 1.5 ml $\cdot$ min $^{-1}$.

same column (Figure 1D) shows that considerable improvements in the chromatographic resolution had been achieved. Thus, peak number 10 in Figure 1D appears as two peaks in

Figure 1C. HPLC Chromatogram of PTH-amino acids obtained on a 2.1 mm column of LiChrosorb RP-18, 5 μ m.

Conditions were as specified by Figure 1A except that the injection volume was 0.5 μ l and the flow 0.6 ml $\cdot$ min $^{-1}$.

Figure 1D. HPLC Chromatogram of PTH-amino acids obtained on a 1.5 mm column of LiChrosorb RP-18, 5 μ m.

Conditions were as specified by Figure 1A except that the injection volume was 0.2 μ l and the flow 0.5 ml·min⁻¹.

Figure 2. HPLC Chromatogram of PTH-amino acids obtained on a 1.5 mm column of LiChrosorb RP-18, 5 μ m.

Eluent programme: 10% B for 2 mins, linear gradient to 15% B for 4 mins, linear gradient to 30% B for 15 mins, linear gradient to 35% B for 1 min, linear gradient to 40% B for 10 mins, isocratic at 40% B for 10 mins. Injection volume was 1 μ l. Otherwise conditions were as specified under Figure 1D.

Figure 2, while the last peak of the chromatograms is only partially resolved in Figure 1D, but completely resolved in Figure 2. However, the shoulder on peak number 5 in Figure 1D is not apparent in Figure 2.

To summarize the experimental results, comparison of the final chromatogram obtained with the 1.5 mm i.d. micro-bore column (Figure 2) with the best resolved chromatogram obtained from a wide-bore column (Figure 1B) shows that it is indeed possible to obtain equivalent separations of a complex mixture of PTH-amino acids using columns of LiChrosorb RP-18 (5 μ m) packed in either wide-bore or micro-bore s.s. tubing. However, before proceeding to comment on the general implications of this particular experimental finding we should like to draw attention to the fact that the precise chromatographic operating conditions for the equivalent separations differ according to the diameter of the column employed (see legends to Figures 1D and 2). This series of experiments demonstrate, therefore, that when the resolution of a chromatographic separation originally performed on a wide-bore column, is reduced when a micro-bore column is used under equivalent conditions, then »fine tuning« of the gradient elution programme may restore the original resolution. This difficulty in directly transferring chromatographic conditions to columns of differing diameter is probably due to the existence of »wall effects«.

The ability of columns of suitable support material packed in micro-bore s.s. tubing to separate mixtures of PTH-amino acids is of practical importance primarily because smaller samples may be analysed. In all of the present experiments the sample volume per mm² cross-sectional area of the column employed was constant, and since the flow rate was correspondingly reduced an effective ten-fold increase in sensitivity of the analytical procedure was achieved by transferring the separation from a 4.5 column to a 1.5 mm column. Accordingly, the chromatograms shown in the figures were all obtained using the same photometer attenuation despite of the fact that varying sample volumes were applied. Two further advantages would also accrue from the adoption of micro-bore columns for this particular separation. The first would be that one of the major running costs of the assay, that of the organic solvents acetonitrile

or methanol commonly employed, could be significantly reduced because of the reduced flow rates employed. Secondly, the amount of expensive column support material would also be significantly reduced; by a factor of nine if the column diameters employed were changed from 4.5 mm to 1.5 mm as in these studies.

The adoption of micro-bore columns could theoretically also lead to an economical chromatographic system in which complete separation of each of the 17 components in the mixture of PTH-amino acids was achieved by using longer lengths of s.s. tubing to form longer columns possessing correspondingly greater resolving power. More importantly, the significant reduction in eluant flow rates resulting from the use of micro-bore columns should greatly simplify the as yet unresolved general problem of »in-line« coupling of liquid chromatographs to mass spectrometers – a solution to which is necessary before unambiguous identification of the eluted components can be made routinely (3).

Clearly, all of the above arguments in favour of adopting micro-bore S.S. columns for the analysis of PTH-amino acids, and more generally for analyses involving liquid chromatography, apply with even more force to separations performed on columns with even smaller diameter than those employed in these studies. At present time no chromatographic separations of PTH-amino acids performed on sub micro-bore (i.d. $\leq$ 0.2 mm) diameter s.s. columns have been reported but these may be confidently predicted now that several pumps capable of operating at very low flow rates are commercially available and details of a sub micro-litre detector cell has been published (4). To cover the interim period before the results of such studies are published it is recommended, to those using HPLC methods in sequence studies, that serious consideration should be given to the advantages to be gained from the adoption and implementation of the experimental findings presented above.

ACKNOWLEDGEMENTS

The authors would like to thank Professor M. OTTESEN for his sustained interest and encouragement throughout these studies. In addition we would like to thank Dr. I. SVENDSEN for his practical assistance and for the assignment of the peaks in the chromatogram shown in Figure 1A as well as Mrs. H. SØRENSEN for preparing the drawings. We would also like to acknowledge Mrs. E. JENSEN of the library of the Carlsberg Research Center for advice obtained during the course of the bibliographic studies involved in the completion of Table I. Finally, one of us (R.W.A.O.) acknowledges the hospitality of the laboratory during the period in which this work was performed.

REFERENCES

1. BEACHELL, H. C. & J. J. DE STEFANO: Effect of CSP support particle size on the performance of larger diameter liquid partition columns. *J. Chromatogr. Sci.* 10, 480–486 (1972)
2. EDMAN, P.: Determination of the amino acid sequence in peptides. *Arch. Biochem. Biophys.* 22, 475–476 (1949)
3. GUICHON, G. & P. J. ARPINO: Liquid chromatography – mass spectrometry coupling. *Anal. Chem.* 51, 682A–701A (1979)
4. HERSHBERGER, L. W., J. B. CALLIS & G. D. CHRISTIAN: Sub-microliter flow-through cuvette for fluorescence monitoring of high performance liquid chromatographic. *Anal. Chem.* 51, 1444–1446 (1979)
5. ISHII, D., K. ASAI, K. HIBI, T. JONOKUCHI & M. NAGAYA: A study of micro-high-performance liquid chromatography. *J. Chromatog.* 144, 157–168 (1977)
6. ROSMUS, J. & Z. DEYL: Methods for the identification of N-terminal amino acids in peptides and proteins; part B – PTH-amino acids. *J. Chromatog.* 70, 292–339 (1972)
7. SCOTT, R. P. W. & P. KUCERA: Mode of operation and performance characteristics of micro-bore columns for use in liquid chromatography. *J. Chromatog.* 169, 51–72 (1979)
8. STEFANO, J. J. DE & H. C. BEACHELL: Performance of large diameter liquid partition analytical columns packed with controlled surface porosity support. *J. Chromatogr. Sci.* 8, 434–438 (1970)
9. WOLF, J. P.: Large diameter columns for preparative scale high speed liquid chromatography. *Anal. Chem.* 45, 1248–1250 (1973)