The behaviour of peptides on reverse-phase supports during high-pressure liquid chromatography

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High-pressure ('performance') liquid chromatography has been used to investigate the reverse-phase chromatographic behaviour of peptides, ranging in length from 2 to 65 amino acid residues, which have originated from primary-sequence determinations or solution/solid-phase syntheses. By using a pyridine/formate-pyridine/acetate/propan-1-ol buffer system, as previously described [Hughes, Winterhalter & Wilson (1979) *FEBS Lett.* **108**, 81–86], the influence of various experimental parameters were examined. (a) Peptide retention was observed to be temperature-independent between 25 and 55°C. (b) The dependence of chromatographic retention on pH decreases with increasing peptide hydrophobicity. (c) Chromatographic results from C₈- and C₁₈-chain-length, as well as from 5 µm- and 10 µm-particle-size, supports were comparable. (d) The hydrophobic strength of the organic solvent in the mobile phase was observed to decrease: propan-1-ol $\approx$ propan-2-ol > acetonitrile $\gg$ methanol. (e) When gradient rates (% of buffer B/unit time) were systematically decreased, peptide retention decreased in a hyperbolic manner. Comparisons of the peptides chromatographed with respect to their measured retention properties and calculated hydrophobicities were performed by computer analysis. Deviation of peptide chromatographic behaviour was observed to be essentially independent of hydrophobicity, chain length and charge. On the basis of the measured retention properties of the chromatographed peptides, hydrophobic constants for the various amino acid side chains were determined and compared with similar constants available from the literature.

In all its various forms, liquid chromatography, in contrast with gas chromatography, has been, and will continue to be, used in almost all isolations of biological molecules. The method has been subdivided into different, although to some extent overlapping, categories: (1) liquid-liquid chromatography, (2) liquid-solid chromatography, (3) bonded-phase chromatography, (4) ion-pair or reverse-phase chromatography, (5) ion-exchange chromatography and (6) size-exclusion chromatography. In peptide/protein chemistry the most widely used forms have been ion-exchange and size-exclusion chromatographies for purification and characterization studies. Similarly, the liquid-solid and ion-exchange methods are often used to isolate, as well as check the purity of, peptides arising from solid- and/or solution-phase synthesis.

Abbreviations used: h.p.l.c., high-pressure ('performance') liquid chromatography; the single-letter code for amino acids is as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [see *Biochem. J.* (1969) **113**, 1–4].

During the past decade the number of papers describing the use of h.p.l.c. in peptide and, to some extent, in protein separations has dramatically increased. In part this has been due to improved chromatographic equipment, the availability of improved packings and columns, and the necessity to search for methods that would allow the investigator to work on smaller amounts of material. Of the various categories of liquid chromatography, the most widely used (to date) appears to be reverse phase for both analytical and preparative h.p.l.c. of smaller proteins mol.wt. (below <10 000–15 000), large peptide fragments, e.g. from cleavages with CNBr, and smaller peptide mixtures [for general papers and related literature, see Goodman & Meienhofer (1977), Hawk (1979), Gross & Meienhofer (1979) and Lottspeich *et al.* (1981)].

In a series of recent publications concerning microsequence methodology (Hughes *et al.*, 1979b; Wilson *et al.*, 1979a,b; Wilson & Hughes, 1980), we illustrated the use of reverse-phase h.p.l.c. in both peptide isolation (Hughes *et al.*, 1979a) and in the

identification of degradation products from Edman-type sequencing (Wilson *et al.*, 1979a). The elution of fragments arising from either chemical (CNBr) or enzymic (trypsin) cleavages was monitored by allowing pmol (μg) amounts (which were removed by a stream-splitting device) to react with fluorescamine or *o*-phthalaldehyde and measuring the ensuing relative fluorescence in a fluorometer equipped with a flow-through cell. Although peptides from complicated mixtures were isolated to homogeneity in the studies, as well as in those described by Hughes *et al.* (1981) in an accompanying paper, it was not apparent, nor was any attempt made to correlate the chromatographic properties of a specific peptide to its primary sequence, secondary structure or the hydrophobicity of its constituent amino acid side chains.

In the present paper an attempt has been made to correlate the chromatographic behaviour of 'underivatized' peptides on a commercially available reverse-phase packing. The peptides used, from commercial sources or isolated during sequence investigations, have ranged from 2 to 65 amino acid residues in length. In addition to yielding information concerning the elution characteristics of a peptide (s), this chromatographic system, when combined with fluorescence detection, provides a rapid and highly sensitive method for the analytical (purity check) or preparative separation of closely related analogues (isomeric oligomers).

Materials and methods

The h.p.l.c. instrument used consisted of two Altex pumps (model 110), controlled by an Altex microprocessor (model 420) and a Rheodyne syringe injector (model 7125 with a 100 μl sample loop). Detection was based on the reaction of a predetermined amount of the effluent with fluorescamine and measurement of the resultant fluorescence. The detection system has been described in detail elsewhere (Hughes *et al.*, 1979a; Böhlen *et al.*, 1975).

All experiments were performed on columns (4.6 mm internal diameter $\times$ 250 mm) of either LiChrosorb RP-8 or RP-18 (5 μm or 10 μm particle sizes), which were purchased from Brownlee Laboratories, Santa Clara, CA 95050, U.S.A. Unless otherwise noted, chromatographic buffers were A (0.125 M-pyridine/formate, pH 3.0) and B [1.0 M-pyridine/acetate (pH 5.5)/60% (v/v) propan-1-ol] (final concentrations based on pyridine). A flow rate of 0.70 ml/min at ambient temperature resulted in pressures between 1.38 and 4.83 MPa (20–700 lbf/in², 13–48 bar) during a chromatographic separation. After a 5–10 min column equilibration in buffer A, injections were made with sample volumes of less than 20 μl at peptide concentrations of ~0.1–0.2 nmol/ μl of buffer A. In most cases,

approx. 100 pmol of material was diverted for detection. The remainder was either discarded or, in those cases where peak identification was necessary, collected and dried by vortex evaporation. Amino acid analysis, after hydrolysis in 6 M-HCl under vacuum, was then performed on a Durrum D-500 analyser or by a similar procedure on a h.p.l.c. apparatus (Kontron) to which had been connected an interface and an eight-port motorized buffer-selection valve (G. J. Hughes, K. H. Winterhalter and K. J. Wilson, unpublished work). Chemicals used for buffer preparation, and distilled from ninhydrin (Merck, 'zur Analyse' grade, 1 g/litre) just before use, were pyridine and propan-1-ol from Fluka (puriss grade); formic and acetic acids from Merck ('zur Analyse' grade). Acetonitrile (Rathburn Chemicals; h.p.l.c. grade) and methanol (LKB, sequencer grade) were used directly. Those for the detection system, and not requiring further purification, were boric acid ('puriss' grade) and LiOH ('purum' grade) from Fluka; acetone ('reinst' grade) from Merck; and fluorescamine from Sigma. The peptides used and their sources are given in Table 1. Those which were not from commercial sources were isolated during the sequence investigations of *Aeromonas proteolytica* aminopeptidase (K. J. Wilson, unpublished work) and mitochondrial aspartate aminotransferase (U. Hausner, P. Christen, K. J. Wilson, unpublished work).

Results and discussion

Typical results from the chromatography of peptides using the described h.p.l.c. system are shown in Fig. 1 for two linear gradients (0–50% of buffer B in the A + B buffer mixture) of durations 36 min (Fig. 1a) and 132 min (Fig. 1b). After sample injection, a 12 min elution with buffer A preceded the start of the gradient. Failure to include the 'wash' period before the application of a relatively steep gradient resulted in the co-elution of peptides B and C during the earlier portion of the gradient. The elution points of peptides A (unretained), C and D were independent of the presence or absence of the wash step. Additionally, one can observe how the elution points are dependent on the steepness of the gradient employed during separation. This point is further discussed in connection with Fig. 6 (below). As expected with reverse-phase chromatography, and in contrast with isocratic elution, peak width and height is rather well maintained during the duration of the gradient and peak tailing is minimal. These factors are important when maximal sensitivity at the low-picomolar range is desired. Here the order of peak elution is in agreement with the calculated hydrophobicities.

The degree of resolution, as well as yield, is

Table 1. *Elution properties of peptides investigated by reverse-phase chromatography*

Peptide hydrophobicity (Σf) was calculated by using the values of Rekker (1977), with the exceptions of arginine (−0.82), pyroglutamic acid (1.00), C-terminal amide (−1.00) and for the other amino acid forms, assuming that the Rekker values apply. Actual elution points are measured values expressed as percentage of buffer *B* in *A* + *B* buffer mixture and calculated values are derived from the linear-regression analysis of all peptides that gave a slope of 1.07 (see Fig. 6). Deviation is the difference between actual and calculated elution points, whereas percentage deviation compares the deviation from the calculated value. The symbols *, ** and *** represent percentage deviations of <10, >10 <20 and >20 respectively. Peptide sources are represented by superscript letters: ^a, *Aeromonas* aminopeptidase; ^b, *m*-aspartate aminotransferase; ^c, aminoethyl-(α -chain of human haemoglobin); ^d, Bachem; ^e, Fluka; ^f, Mann; ^g, Sigma. Non-standard abbreviations used: Nle, norleucine; iso-D, isoaspartic acid; pyro-E, pyroglutamic acid.

Peptide	Σf	Elution point		Deviation	Percentage deviation
		Actual	Calculated		
A. 1 AF ^d	2.77	27.70	31.30	−3.60	−11.49**
2 FL ^d	4.23	38.00	34.06	−3.94	11.58**
3 GF ^e	2.24	28.50	30.29	−1.79	−5.92*
4 GW ^e	2.31	31.00	30.43	0.57	1.89*
5 GY ^d	1.70	8.50	29.27	−20.77	−70.96***
6 LL ^d	3.98	33.50	33.58	−0.08	−0.25*
7 LM ^d	3.07	27.70	31.86	−4.16	−13.07**
8 VF ^g	3.70	31.00	33.05	−2.05	−6.22*
9 ETY ^d	1.37	28.50	28.65	−0.15	−0.52*
10 EWE ^f	2.17	28.50	30.16	−1.66	−5.51*
11 KWK ^f	3.35	17.00	32.39	−15.39	−47.52***
12 LWL ^f	6.29	42.50	37.95	4.55	11.98**
13 LWM ^f	5.38	43.00	36.23	6.77	18.68**
14 KD VY ^d	3.66	28.00	32.98	−4.98	−15.10**
15 YPF ^d	5.96	41.00	37.33	3.67	9.84*
16 FDASV ^f	3.65	31.50	32.96	−1.46	−4.43*
17 HPPGF ^d	4.03	35.00	33.68	1.32	3.92*
18 IISMR ^b	3.68	32.50	33.02	−0.52	−1.56*
19 KPPGF ^d	4.78	34.50	35.10	−0.60	−1.70*
20 RKDVY ^d	2.84	29.00	31.43	−2.43	−7.73*
21 RPPGF ^d	3.44	34.50	32.56	1.94	5.95*
22 TKPPR ^d	1.46	11.50	28.82	−17.32	−60.09***
23 YGGFL ^d	5.93	44.00	37.27	6.73	18.05**
24 YGGFM ^d	5.02	39.50	35.55	3.95	11.11**
25 DAWALR ^b	4.52	39.50	34.61	4.89	14.14**
26 EVIRVL ^a	6.01	44.50	37.42	7.08	18.91**
27 FFKFSR ^b	5.86	41.00	37.14	3.86	10.40**
28 HSEGT ^f	1.12	27.80	28.18	−0.38	−1.33*
29 LAHAH ^b	4.58	27.00	34.72	−7.72	−22.23***
30 MKRPPG ^d	2.80	28.00	31.35	−3.35	−10.69**
31 NDYNPR ^a	−0.23	26.00	25.62	0.38	1.47*
32 YGGFLR ^d	5.11	39.50	35.72	3.78	10.58**
33 AAEEVGL ^a	4.37	33.00	34.32	−1.32	−3.85*
34 AIHQVIK ^b	2.92	26.00	31.58	−5.58	−17.67**
35 RVYIHPF ^d	7.35	49.00	39.96	9.04	22.63***
36 YPFPGPI ^d	8.96	47.00	43.00	4.00	9.30*
37 DRVYIHPF ^d	7.33	41.70	39.92	1.78	4.46*
38 RPPGFSPF ^d	6.13	44.00	37.65	6.35	16.87**
39 SENNFQPK ^a	−0.05	27.80	25.96	1.84	7.08*
40 VGANFLQR ^b	3.26	38.10	32.22	5.88	18.24**
41 MKRPPGFSPF ^d	5.49	36.50	36.44	0.06	0.17*
42 RPPGFSPFR ^d	5.31	42.00	36.10	5.90	16.35**
43 RPPGFSPYR ^d	4.77	35.00	35.08	−0.08	−0.22*
44 WAGGNASGE ^d	1.69	29.50	29.25	0.25	0.85*
45 DAGLELQA YR ^b	4.74	40.00	35.02	4.98	14.22**
46 DRVYIHPFHL ^d	9.09	47.00	43.25	3.75	8.68*
47 GSQDLANQYK ^a	0.93	30.00	27.82	2.18	7.85*
48 MKRPPGFSPF ^d	7.73	43.00	40.68	2.32	5.71*
49 RGSQDLANQY ^a	−0.41	29.50	25.28	4.22	16.68**

Table 1—continued

	Peptide	Σf	Elution point		Deviation	Percentage deviation
			Actual	Calculated		
50	SASLPNASVK ^a	3.31	29.70	32.32	-2.62	-8.10*
51	HNAGYPAAMPF ^a	7.35	42.50	39.96	2.54	6.36*
52	KSEGKNVVSAL ^a	4.24	34.50	34.08	0.42	1.25*
53	TITGSEAPNEW ^a	3.57	24.50	32.81	-8.31	-25.32***
54	EEQWKELASVVK ^b	6.93	45.00	39.16	5.84	14.91***
55	IASLILNTPELR ^b	8.73	48.50	42.57	5.93	13.94**
56	YVMGHFRWDRFG ^d	9.14	48.00	43.34	4.66	10.75**
57	KEWLVEVKGMADR ^b	8.89	45.00	42.87	2.13	4.97*
58	SLMKRPPGFSPFR ^d	8.34	40.70	41.83	-1.13	-2.70*
59	YGGFLRRIRPKLK ^d	9.50	41.00	44.02	-3.02	-6.87*
60	AGCKNFFWKTFTSC ^d	10.69	9.50	46.27	-36.77	-79.47***
61	NVVSALQLDMTNYK ^a	6.70	45.70	38.73	6.97	18.00**
62	DTNSKKMNLGVGAYR ^b	4.04	34.80	33.70	1.10	3.27*
63	GGFMTSEKSQTPLVTL ^d	7.23	46.60	39.73	6.87	17.29**
64	YGGFMTSEKSQTPLVT ^d	6.94	40.50	39.18	1.32	3.36*
65	DVYLPKPSWGNHTPIFR ^b	12.30	45.50	49.32	-3.82	-7.74*
66	YADSGEGDFLAEGGGVR ^d	6.89	43.50	39.09	4.41	11.29**
67	YGGFMTSEKSQTPLVTL ^d	8.93	48.00	42.95	5.05	11.77**
68	ASAELALGENSEAFKSGR ^b	5.10	39.00	35.70	3.30	9.24*
69	HTTQDTLANSDPTGSHAK ^a	-0.22	28.00	25.64	2.36	9.20*
70	SDHASWHNAGYPAAMPFESK ^a	8.71	37.70	42.53	-4.83	-11.35**
71	SYSMEHFRWGKPVGKKRRPVKVYP ^d	14.64	39.00	53.74	-14.74	-27.43***
72	HFIEQGIDVVLSSQSYAKNMGLYGER ^b	13.09	51.00	50.81	0.19	0.37*
73	YGGFMTSEKSQTPLVTLFKNAIKNAYKKGE ^d	17.82	50.50	59.76	-9.26	-15.49**
74	CNBr peptide 1-32 ^c	16.58	43.00	57.41	-14.41	-25.10***
75	CNBr peptide 33-76 ^c	24.44	51.50	72.28	-20.78	-28.75***
76	CNBr peptide 77-141 ^c	44.89	62.50	110.95	-48.45	-43.67***
B. 77	G-D,L-Nle ^f	1.90	12.50	29.82	-17.32	-58.08***
78	FLamide ^d	3.23	33.00	32.17	0.83	2.59*
79	LMamide ^d	2.07	18.40	29.97	-11.57	-38.61***
80	Y-D-AGF-D-L ^d	6.46	50.20	38.27	11.93	31.16***
81	YFPFamide ^d	4.96	38.00	35.44	2.56	7.23*
82	Y-D-AGF-D-Lamide ^d	5.46	51.00	36.38	14.62	40.18***
83	Y-D-AGFMamide ^d	4.55	39.00	34.66	4.34	12.52**
84	GEGFLG-D-FL ^d	8.39	59.50	41.92	17.58	41.92***
85	HPFHL-D-LVY ^d	9.93	55.50	44.84	10.66	23.78***
86	AGAFTVICR ^b	5.67	43.00	36.78	6.22	16.91**
CM						
87	DYMGWMDFamide ^d	7.37	49.50	39.99	9.51	23.77***
88	RPPG- <i>p</i> -chloro-FSP- <i>p</i> -chloro-FR ^d	5.31	51.30	36.10	15.20	42.11***
89	CYFQNCPRGamide ^d	3.21	35.50	32.13	3.37	10.50**
90	CYIQNCPRGamide ^d	2.96	32.50	31.66	0.84	2.67*
91	WAGG-iso-D-NASGE ^d	0.64	29.40	27.27	2.13	7.82*
92	WAGG- β -D-DASGE ^d	2.70	29.50	31.16	-1.66	-5.34*
93	pyro-EGLPPRPKIPP ^d	9.66	39.50	44.33	-4.83	-10.89**
94	RPKPQFFGLMamide ^d	6.09	49.00	37.57	11.43	30.41***
95	pyro-EADPNKFYGLMamide ^d	7.93	46.10	41.05	5.05	12.29**
96	pyro-EPKDAFIGLMamide ^d	8.71	51.00	42.53	8.47	19.92**

directly related to the elutropic property of the organic solvent used to elute the absorbed peptides. Fig. 2(a) illustrates that the solvent 'strength' (its ability to elute a given peptide at a given buffer percentage in comparison with another solvent) for elution decreases propan-1-ol > acetonitrile > methanol. Large peptide fragments or stron-

gly hydrophobic mixtures, are optimally separated with propan-1-ol [Rubinstein *et al.*, 1977; Hughes *et al.*, 1979a, 1981] (an accompanying paper)]. The absorbance in the far-u.v. (≤ 210 nm) of both isomers is, however, relatively high, and, as a result, to date neither have acquired widespread use. The high resolving power for both large and small peptides,

Fig. 1. Separation of a peptide mixture under different gradient conditions

(a) 36 min and (b) 132 min gradients from 0 to 50%. The percentage of buffer B in the A + B mixture is indicated on each chromatogram by the series of straight lines. Amounts of peptides (nmol) injected were: A, 1.62; B, 1.70; C, 2.20 and D, 3.61; 6.7% of the effluent was removed for detection. The peptide sequences and their hydrophobicity values [calculated as described by Rekker (1977) and assuming a value of -0.82 for arginine (see Table 2)] are: A, TKR (-0.56); B, TKPPR (1.46); C, AIHQVTK (2.92); D, DVYLPKPSWGNTPIFR (12.30). The peak marked 'Z' represent contamination arising during sample preparation. In this Figure and in

ease of purification (relatively low cost) and low toxicity make their use inevitable [Mahoney & Hermodson, 1980; Wilson *et al.*, 1981 (an accompanying paper)].

Elution differences from C_8 - and C_{18} -chain-length supports have been found to be minimal with the described propan-1-ol solvent system (Fig. 3). However, hydrophilic peptides, which are only slightly adsorbed or elute during the prewash, are sometimes affected (peak B, Fig. 3B). These results are in general agreement with those of Blevins *et al.* (1980), where the elution characteristics of neurohypophyseal peptide hormones on different reverse-phase packings were investigated. They observed, however, slightly larger variation in retention times between the C_8 - and C_{18} -chain-length stationary phases. Due to the poor selectivity of C_2 -chain-length supports, presumably due to the polar character of the stationary phase (Blevins *et al.*, 1980), no attempt was made to characterize such a packing.

Inasmuch as the aim of the present investigation was to correlate a peptide's chromatographic properties with its hydrophobicity, the consideration of other experimental parameters was necessary. As Fig. 4(a) illustrates, peptides are eluted essentially independent of temperature between 25 and 55°C with the described solvent-detection system. Other authors (Mahoney & Hermodson, 1980) have observed, however, that peptides elute somewhat earlier and with decreased peak resolution. As will be shown in the following paper (Wilson *et al.*, 1981), such small temperature-dependent changes would not be detected with the presently used detection system, owing to post-column mixing. Differences in the pH of the initial buffer can lead to significant changes in the elution properties of some peptides (Fig. 4b). This effect has also been noted with numerous larger peptides which, because of their solubility properties, were chromatographed with both buffers at pH 5.5 (Hughes *et al.*, 1979a). The chromatography of those peptides that elute during the first part of the gradient, i.e. the more polar peptides, is most strongly effected. This characteristic has in fact been used by Kratzin *et al.* (1980) as a means to separate polar tryptic peptides where initial separation was at pH 6.0 followed by the reinjection of impure fractions at pH 3.0.

Peptide chromatographic behaviour was found to be identical when 5 μ m- or 10 μ m-particle-size packings were tested (results not shown). The use of 3 μ m-particle-size packings has been reported (Kratzin & Yang, 1981) to improve separations of the earlier-eluting peptides (<10% buffer B in A + B buffer mixture). Since gradient elution is functioning

similar succeeding ones, injection points are marked by arrows and the continuous straight line represents the developed gradient.

Fig. 2. Effects of different mobile-phase solvents on peptide elution

(a) Chromatography using 60% (v/v) propan-1-ol (upper), acetonitrile (middle) and methanol (lower) in buffer *B*. CNBr fragments of aminoethylated α -chain of human haemoglobin were chromatographed (4.2 nmol injected, 9.0% of effluent removed for detection). Peaks marked as 1, 2 and 3 represent the peptides corresponding to residues 1–32, 33–76 and 77–141, with hydrophobicity values of 16.58, 24.44 and 44.89 respectively. (b) Chromatography, using 60% (v/v) propan-2-ol and propan-1-ol in buffer *B* (as above), of the same peptide mixture, with identical amounts being injected as in Fig. 1.

essentially as an isocratic system at each extreme, this observation would be expected. Thus any improvements in the chromatographic properties of a packing should result in enhanced resolution.

The results in Fig. 5 illustrate the effect on the elution of peptides B, C and D when gradients of

decreasing steepness in percentage of buffer *B* are used. As expected, those less strongly retained components, i.e. peptides A (not shown) and B, elute essentially independent of the gradient. Conversely, separation of the more strongly interacting peptides is not only dependent on the steepness of the

Fig. 3. Comparison of peptide separations on C_8 (upper) and C_{18} (lower) supports. The peptides, amounts thereof and percentages of effluent removed for detection, are identical with Fig. 2 [e.g. in (a), the CNBr fragments, and in (b), the peptide mixture].

gradient, but it is also apparent that their points of elution (in terms of percentage of buffer B) decrease with decreasing steepness in a hyperbolic manner.

Results from the chromatography (Fig. 1) of the four-peptide mixture suggested that the elution order approximates a direct function of hydrophobicity. This is further illustrated in Fig. 6, which correlates the elution points of the peptides listed in Table 1 with the summation of the hydrophobicities of their constituent amino acids, calculated by using the values given by Rekker (1977; also see Table 2). Although significant point scatter is apparent, the peptides do tend to elute as expected, i.e., dependent

on their respective hydrophobicities. A closer examination, however, indicates that factors other than side-chain hydrophobicity alone, are responsible. For example, included are the slopes arising from linear-regression analyses of those peptides with Σf (peptide hydrophobicity) < 15 (slope = 1.89, < 26 residues), and including the four fragments with $\Sigma f > 15$ (slope = 1.07, > 31 residues). One possible explanation for this difference might be the presence of secondary structure in the larger peptides (see Fig. 8b below and discussion thereof). Other separations, notably of diastereoisomers and of position isomers of the same peptide, have not been

Fig. 4. Effect of temperature and pH of initial buffer on chromatography

Portions of the peptide mixture, identical with those used for Figs. 1–3, were injected at the indicated temperatures and eluted, after a 12 min pre-wash, with a gradient from 0 to 70% buffer B in A + B buffer mixture over 36 min. Similarly, the effect of pH was investigated by equilibrating the column with a freshly prepared buffer A solution at the indicated pH values, injection and elution being as described above. Distances (cm) were measured from injection point to the peak centre.

Fig. 5. Relationship between gradient steepness and peptide elution

Samples from the peptide mixture, as used in Figs. 1–4, were eluted, after a 12 min pre-wash, with gradients from 0 to 50% buffer B in A + B buffer mixture which extended over 25, 36, 48, 72, 96 and 132 min. Elution times are plotted for peptides B, C and D (see Fig. 1) with respect to buffer concentration at point of elution, i.e. peak maximum.

Fig. 6. Plot of peptide hydrophobicity (Σf) against elution point (in percentage of buffer B in A + B buffer mixture)

After a 12 min pre-wash, chromatography was performed with a 36 min gradient from 0 to 70% buffer B in A + B buffer mixture. The symbol * is used to represent peptides that contained only L-amino acids and were not blocked at either the N- or C-terminus (see Table 1a). Modified peptides (see Table 1b) are represented by the symbol O. For the unmodified peptides (*) with $\Sigma f < 15$, a slope of 1.89 was found by linear-regression analysis. Similarly, a value of 1.07 when those unmodified peptides with $\Sigma f > 15$ were also considered.

satisfactorily explained. However, differences in their molecular hydrophobicities, rather than the hydrophobic properties of the peptides constituent amino acids, have been implicated (Terabe *et al.*, 1979).

Deviation from the expected chromatographic behaviour seems to be independent of hydrophobicity (Fig. 7a) and of propan-1-ol concentration (Fig. 7b). In the latter case, those peptides eluting at the beginning of the gradient are affected to some degree. However, their sequences [e.g. GY, KWK, TKPPR and AGCKNFFWKTFTSC (single-letter code), see Table 1a] have little in common. The

differences between observed and predicted peptide retentions (Fig. 8c) agree rather well. As indicated for Fig. 7b, peptides that are eluted early are strongly affected. Similarly, most of the peptide derivatives (see Table 1b), which elute at the higher end of the gradient (>47% buffer B in the A + B buffer mixture), and which elute more rapidly than expected, contain D-amino acids or *p*-chlorotyrosine

Fig. 7. Deviation of peptide elution with respect to peptide hydrophobicity (a), percentage of buffer B in A + B mixture (b), and the correlation between predicted and actual elution points of the peptides (c)

Percentage deviation was calculated from the linear-regression analysis by using a slope value of 1.89 (see Fig. 6). Peptide hydrophobicities were calculated by using the side-chain constants of Rekker (1977); predicted elution values were obtained by assuming that expected chromatographic behaviour is represented by the best-fit line with slope = 1.89. For symbol definition, see Fig. 6.

Fig. 8. Effect of peptide charge (a) and chain length (b) on elution

The percentage deviation was calculated from the linear-regression analysis, where slope = 1.89 (see Fig. 6). Peptide charge, considered to be constant throughout the gradient, was calculated by assuming total protonation of aspartic acid, glutamic acid, histidine, lysine and arginine residues; N- and C-termini were protonated and deprotonated respectively. For the dependence on chain length only, results are given for peptides up to 24 residues long. For symbol definition, see Fig. 6.

residues. Since the hydrophobic constants of Rekker (1977) have been used without correction, discrepancies in their predicted retention times are expected. For most of the remaining examples, the hydrophobic summations have been adjusted for blocked N- and/or C-termini (see Table 1).

Correlations between percentage deviation from predicted elution and charge, which was assumed to have remained constant throughout the gradient, as

Table 2. *Amino acid hydrophobicity constants and their relevance in calculation of peptide chromatographic retention times*

The arginine constant (*) under 'Rekker (1977)' was calculated by minimization of the deviation for the arginine-containing peptides from the linear regression for those peptides lacking arginine. Constants determined in the present study were calculated by multivariate regression analysis. The correlation coefficients are calculated for all peptides in Table 1 for which hydrophobic constants for the individual amino acids are available (see below).

Amino acid (one-letter symbol in parentheses)	Source ...	Constants (Σf) according to:					The present study
		Bigelow & Channon (1976)	Meek (1980)	Pliška & Fauchère (1979)	Rekker (1977)	Segrest & Feldmann (1974)	
Tryptophan (W)		3400	18.1	1.87	2.31	6.5	7.9
Phenylalanine (F)		2500	13.9	1.80	2.24	5.0	7.5
Leucine (L)		1800	10.0	1.66	1.99	3.5	6.6
Isoleucine (I)		2950	11.8	1.56	1.99	5.0	4.3
Tyrosine (Y)		2300	8.2	1.70	1.70	4.5	7.1
Valine (V)		1500	3.3	1.06	1.46	3.0	5.9
Cystine		—	-2.2	—	1.11	0	6.3
Methionine (M)		1300	7.1	1.39	1.08	2.5	2.5
Proline (P)		2600	8.0	0.56	1.01	1.5	2.2
Cysteine (C)		1000	—	0.69	0.93	—	—
Arginine (R)		750	-4.5	-1.23	-0.82*	—	-1.1
Alanine (A)		500	-0.1	0.38	0.53	1.0	-0.3
Lysine (K)		1500	-3.2	-0.93	0.52	—	-3.6
Glycine (G)		0	-0.5	0	0	0	1.2
Aspartic Acid (D)		0	-2.8	-1.23	-0.02	—	-1.4
Glutamic Acid (E)		0	-7.5	-1.20	-0.07	—	0
Histidine (H)		500	0.8	-1.30	-0.23	1.0	-1.3
Threonine (T)		400	1.5	0.33	-0.26	0.5	-2.2
Serine (S)		-300	-3.7	0.04	-0.56	-0.5	-0.6
Asparagine (N)		—	-1.6	-0.27	-1.05	-1.5	-0.2
Glutamine (Q)		—	-2.5	-0.09	-1.09	-1.0	-0.2
Correlation coefficient		0.536	0.681	0.713	0.693	0.826	0.831

well as length, are illustrated in Fig. 8. The behaviour of the peptides with aspartic acid and glutamic acid residues (Table 1), those side chains effected by the pH gradient (pH 3.0–5.5), and those without is essentially the same, i.e., differences in terms of percentage deviation are equally effected. Peptide lengths up to about 18 residues have little or no effect on retention deviation. For those larger fragments, however, it was observed that each tends to elute more rapidly than predicted from hydrophobic considerations alone. The use of a 30nm (300 Å)-pore-size RP-18 packing for the chromatography of CNBr fragments of collagens (mol.wts. between 3000 and 62000) has been reported (van der Rest *et al.*, 1980). In contrast with what would be expected with the smaller-pore-size packings, i.e. poor resolution and yields, their high recoveries of the larger fragments suggest that such packings can be used to investigate how configuration influences chromatographic behaviour.

The hydrophobic constants for the individual amino acids determined in the present study, as well as those available from the literature, are given in

Table 2. Correlation coefficients are given and have been calculated only for those peptides for which the respective hydrophobic constants are available for all of their constituent amino acids. Since the low correlations observed are independent of the source of the amino acid hydrophobicity constants, it appears that predicting exact peptide retention, at least for this particular chromatographic system, is difficult. Graphically this is illustrated in Fig. 9, where the constants from Meek (1980) and those from the present study (Table 2) have been used to predict elution times. Considering that during gradient development not only the percentage of the organic eluant changes, but also the pH and ionic strength, this might be expected. However, from these results some information can be obtained: (i) approximate elution times can be established, i.e. for a prediction of 30% the peptides elute between 28 and 35%, for 40% between 34 and 44% and for 50% between 42 and 51%; (ii) for an indication of when a less apolar solvent (acetonitrile, methanol) should be used; in this case, the utilization of a solvent lower in the elutotropic series results in a spreading-out of the

Fig. 9. Comparison of predicted peptide retentions calculated by using various hydrophobic constants

The constants (see Table 2) from Meek (1980) were used to predict elution points in (a), and from the present study in (b). Only those peptides listed in Table 1(a) for which constants were available for all of its constituent amino acids were considered.

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peaks eluted [an accompanying paper (Wilson *et al.*, 1981)]; (iii) the predicted value also allows for an estimation of where, in terms of organic solvent concentration, a gradient should be initiated.

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