Comparison of buffers and detection systems for high-pressure liquid chromatography of peptide mixtures

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(Received 18 February 1981/Accepted 19 June 1981)

The chromatographic properties of peptides ranging in length from 2 to 65 residues have been compared on reverse-phase packings in three buffer systems at low pH. Of the buffers examined, two are widely used in connection with u.v. detection [(i) phosphate/acetonitrile or (ii) phosphate/propan-2-ol] and the third for fluorescence detection [(iii) pyridine/formate–pyridine/acetate/propan-1-ol]. The addition of a chaotropic salt, NaClO₄, to the phosphate buffers, as first described by Meek (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1632–1636, is shown to significantly improve the chromatographic behaviour of more hydrophobic peptides. The two most commonly used detection systems, u.v. and fluorescence, are compared in terms of ease of use and sensitivity.

In any peptide/protein-sequence determination the most tedious and time-consuming part has been the isolation of a sufficient number of fragments to account for the entire length of the polypeptide chain. This often requires numerous enzymic hydrolyses and chemical cleavages either of the intact protein and/or of large fragments. The 'standard' preparative methods used are usually a combination of gel filtration, ion-exchange chromatography and either paper electrophoresis or chromatography. The shortcomings of these methods are limited resolution, poor yields and the time required to perform these operations. For larger fragments, e.g. from cleavage with CNBr, yields are often so low that large quantities of starting material are necessary. Similarly, peptides differing only slightly in length, by perhaps one or two residues, are notoriously difficult to separate, and separation of those which are heterogeneous at a specific position, especially when the amino acids are similar (e.g. glycine/alanine), has been essentially impossible.

Recent advances in h.p.l.c. applications have made it possible to circumvent some of these problems. To date, h.p.l.c. has been most widely used in the identification of thiohydantoin arising from Edman-type degradations [Godtfredsen & Oliver (1980) have summarized the experimental conditions and separations reported for amino acid

phenylthiohydantoin derivatives in 35 different papers that have appeared since 1972]. Its usefulness as an analytical tool in synthetic-peptide chemistry has long been recognized. However, only since recent improvements in reverse-phase packings, instrument design, and buffer systems, has the preparative isolation of peptides by h.p.l.c. become practical (Fullmer & Wasserman, 1979; Hughes *et al.*, 1979; Rubinstein, 1979; Meek, 1980; Honegger *et al.*, 1981; Hughes *et al.*, 1981*a,b*; Wilson *et al.*, 1981*b*; some of these papers accompany the present one). Attention has been directed toward the use of RP-18 packings and elution with a gradient system at low pH. The most widely used organic eluant has been acetonitrile, and the detection systems have, for the most part, been limited to measuring the absorbance of the peptide bond in the u.v. region. Automated fluorescence-detection systems (Böhlen *et al.*, 1975) have enabled the use of buffers that, owing to their high-absorption properties, cannot be utilized in conjunction with detectors measuring at low wavelengths.

The purpose of the present study was to compare the adequacy of a series of buffer systems to resolve peptide mixtures, ranging from 2 to 65 amino acid residues in length, and to determine the yields of individual fragments with respect to the organic eluant used and the peptide's hydrophobicity. In addition, the two most widely employed detection systems, u.v. and fluorescence, are evaluated in terms of ease of use and sensitivity. The substitution of propan-2-ol for acetonitrile in the eluant is shown

Abbreviations used: h.p.l.c., high-pressure ('performance') liquid chromatography; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK').

to significantly improve chromatographic behaviour of large hydrophobic peptides without loss of either sensitivity or, in most cases, chromatographic resolution.

Materials and methods

An h.p.l.c. unit consisting of two Altex pumps (model 110), a Rheodyne injector (model 7125, with a 20 μ l sample loop), a Kontron microprocessor (model 200) and an Uvikon 725 spectrophotometer equipped with an 8 μ l flow-through cell was used for u.v. detection. Chromatography on LiChrosorb RP-18 columns (4.6 mm $\times$ 250 mm, 10 μ m particle size, Brownlee Laboratories, Santa Clara, CA 95050, U.S.A.) at room temperature was performed at a flow rate of 1 ml/min with the following buffer systems: (i) buffer A, 0.01 M-NaClO₄ in 0.1% H₃PO₄, pH 2.1; B, as A except 60% (v/v) in acetonitrile; or (ii), as in (i), except 60% (v/v) propan-2-ol was used. For h.p.l.c. using fluorescence detection, a similar combination of instruments was employed; however, the post-column system consisted of an automatic sampling unit and a fluorometer for monitoring a fluorecamine reaction [see the preceding paper (Wilson *et al.*, 1981a) and Hughes *et al.* (1979a) for more detailed descriptions of this system]. Peptide recoveries were determined either by comparing peak areas or by amino acid analysis. In the latter case, fractions corresponding to the individual peaks were collected, vortex-evaporated, hydrolysed in 6 M-HCl under vacuum and analysed as described in the preceding paper (Wilson *et al.*, 1981a).

Basic pancreatic trypsin inhibitor was reduced and carboxymethylated with iodo[³H]acetic acid (187 mCi/mmol, New England Nuclear) as described by Hirs (1967), desalted by Sephadex G-25 chromatography in 5% (v/v) acetic acid and recovered by freeze-drying. For tryptic digestion, the carboxymethylated protein was suspended in 0.2 M-

N-ethylmorpholine/acetate buffer, pH 8.0, Tos-Phe-CH₂Cl-treated trypsin was added [2% (w/w); Worthington] for 2 h at 37°C and then freeze-dried. CNBr-cleavage fragments of α -chain of human haemoglobin were prepared as previously described (Hughes *et al.*, 1979). Haemoglobin from the lanceolate fluke *Dicrocoelium dendriticum* was isolated as described previously (Tuchschnid *et al.*, 1978), cleaved with CNBr (Gross, 1967) and freeze-dried. The fragments were subsequently separated by gel filtration and the C-terminal fragment, which by amino acid composition appeared heterogeneous (see Table 1), was used in the present study. Similarly, the C-terminal CNBr-cleavage peptide from *Aeromonas proteolytica* aminopeptidase (Prescott *et al.*, 1971) was isolated, and heterogeneity of this peptide was also suggested by its amino acid composition (Table 1). The amino acid composition of the tryptic overlap peptide, including the entire CNBr fragment, also suggested heterogeneity (results not shown).

Buffers for the fluorecamine-detection system were prepared from chemicals freshly distilled over ninhydrin (1 g/litre), and acetic anhydride was added to react with primary amines present (Hughes *et al.*, 1979). H.p.l.c.-quality acetonitrile and propan-2-ol were purchased from J. T. Baker Chemicals B.V., 7400 AA Deventer, The Netherlands; water was twice-distilled from quartz apparatus. All other chemicals were of reagent-grade quality from Fluka or Merck and used without further purification.

Results and discussion

During the last few years the use of h.p.l.c. in its various forms, isocratic and gradient, has found numerous applications in biochemistry. The technique offers, in comparison with other chromatographic methods (e.g. ion-exchange or gel filtration), the advantages of higher yields and resolution, as well as shorter run times. Its adaptation to peptide,

Table 1. Common buffer systems for gradient reverse-phase h.p.l.c. on RP-8 or -18 packings of peptides and proteins
Under the column headed 'Buffers', A and B represent initial and final buffers respectively.

Buffers	Detection	Reference
A: 0.1% H ₃ PO ₄ ; B: acetonitrile	U.v.	Fullmer & Wasserman (1979)
A: 0.1% Trifluoroacetic acid; B: propan-1-ol, 0.1% in A	U.v.	Mahoney & Hermodson (1980)
A: 5% Formic acid; B: methanol, 5% in A	U.v.	Takagaki <i>et al.</i> (1980)
A: 0.1% H ₃ PO ₄ , 0.1 M-NaClO ₄ ; B: 60% acetonitrile in A	U.v.	Meek (1980)
A: 0.01 M-Ammonium acetate, pH 4.0, 5% in propan-2-ol; B: 0.01 M-ammonium acetate, pH 4.0, 50% in propan-2-ol	U.v.	Chang <i>et al.</i> (1980)
A: 0.01 M-Heptafluorobutyric acid, 12.8% acetonitrile; B: 0.01 M-heptafluorobutyric acid, 44.8% acetonitrile	U.v.	van der Rest <i>et al.</i> (1980)
A: 1 M-Pyridine/0.5 M-acetic acid; B: 40% propan-1-ol in A	Fluorescence	Rubinstein <i>et al.</i> (1977)
A: 1 M-Sodium acetate, pH 7.5; B: 40%-propan-1-ol in A	Fluorescence	Rubinstein <i>et al.</i> (1979)
A: 0.5 M-Pyridine formate, pH 3.0, or 1.0 M-pyridine acetate, pH 5.5; B: 1.0 M-pyridine acetate, pH 5.5, 60% propan-1-ol	Fluorescence	Hughes <i>et al.</i> (1979)
A: 0.26 M-Trifluoroacetic acid/0.21 M-pyridine, pH 1.5; B: 60% propan-1-ol in A	Fluorescence	Hughes <i>et al.</i> (1981a)

and in some cases to protein, chromatography has, and is becoming, more widespread. Table 1 lists some of the more commonly used buffer systems for reverse-phase h.p.l.c. of samples that have arisen from such widely diverse sources as enzymic digestions (Fullmer & Wasserman, 1979; Takagaki *et al.*, 1980; Hughes *et al.*, 1979; Hughes *et al.*, 1981a), chemical fragmentations (Mahoney & Hermodson, 1980; van der Rest *et al.*, 1980) and tissue extracts (Chang *et al.*, 1980; Rubinstein *et al.*, 1977, 1979). Recent literature and the communications presented at a recent symposium concerning the use of h.p.l.c. in protein and peptide chemistry (Lottspeich *et al.*, 1981), have indicated that preparative isolations of peptides are most conveniently performed on RP-8 or -18 packings with elution by a gradient at low pH. When possible, volatile buffer systems have been utilized in order to avoid multiple sample desaltings. As is shown in Table 1, there are basically two different means for monitoring peptide elution. The use of each is dictated by the chemical compositions of the buffers: those containing solvents which strongly absorb in the u.v. (e.g. pyridine, acetic or formic acids) employ fluorescence detection, whereas the others allow direct measurement of the absorption properties of a peptide's amino acids and the peptide bond.

The use of a methanol or acetonitrile gradient in 0.1% H_3PO_4 (Hearn & Hancock, 1979), or the same buffer containing 0.1 M- NaClO_4 (Meek, 1980), is a convenient system to employ with u.v. detection. Fig. 1 compares peptide yields from separations performed in the presence and absence of 10 mM- ClO_4^- and with the organic eluants propan-2-ol and acetonitrile. As peak areas indicate (identical amounts were injected for each chromatogram), the inclusion of ClO_4^- results in a sharpening of the peaks and increases peptide retention. The use of propan-2-ol rather than acetonitrile significantly improves the chromatographic behaviour of the more hydrophobic fragments [note the increased area of the third major peak in Fig. 1(a), upper versus lower chromatograms, which is the C-terminal 65-residue CNBr fragment from human haemoglobin α -chain].

The chromatography of smaller tryptic peptides from ^3H -labelled carboxymethyl-basic pancreatic trypsin inhibitor with $\text{PO}_4^{3-}/\text{ClO}_4^-$ buffers and eluting with acetonitrile or propan-2-ol are contrasted in Fig. 2. As expected, the peptides elute at lower concentrations, and are therefore more compressed, when a propan-2-ol-containing B buffer is employed. The amino acid compositions of the peptides chromatographed by using the acetonitrile-containing B buffer indicated minimal cross-contamination and recoveries in excess of 75% for most fragments (Table 2). A comparison of the calculated hydrophobicity values (Table 2) with the

Fig. 1. H.p.l.c. analyses of the CNBr fragments from aminoethylated α -chain of human haemoglobin

The starting buffers in (a) and (b) were 0.1% H_3PO_4 (lower chromatograms) or 0.1% $\text{H}_3\text{PO}_4/0.01\text{ M- NaClO}_4$ (upper chromatograms). The limiting buffers (B) had the same compositional arrangement, but contained 60% (v/v) propan-2-ol in (a) or acetonitrile in (b). For each separation, 6.25 nmol of material was injected. In this Figure and similar succeeding ones, injection points are marked with arrows and the continuous straight line represents the developed gradient.

Fig. 2. Chromatography of the tryptic peptides from [³H]carboxymethylated basic pancreatic trypsin inhibitor Buffer A for both chromatograms was 0.1% H₃PO₄/0.01 M-NaClO₄; buffer B was identical, except that it contained 60% (v/v) acetonitrile in (a) and propan-2-ol in (b). The peptide quantity injected was 16.0 nmol, containing 185 000 d.p.m. distributed between the six [³H]carboxymethylcysteine residues.

Table 2. Amino acid compositions, recoveries and hydrophobicities of the tryptic peptides from [³H]carboxymethylated basic pancreatic trypsin inhibitor

Pools 1–10 refer to pooled peaks from Fig. 2(a). Threonine (9%) and serine (7%) values have been corrected for loss during hydrolysis. Hydrophobicity values have been calculated as described by Rekker (1977), assuming –0.82 (from Wilson *et al.*, 1981a) and 0.35 (calculated as described in Wilson *et al.*, 1981a) for arginine and carboxymethylcysteine respectively. The complete amino acid sequence of the basic pancreatic trypsin inhibitor is RPDFCLEPPYTGPKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA (Dayhoff, 1972).

			Composition (mol/mol)										
Amino acid	Pool	...	1	2a	2b	3	4	5	6	7	8	9	10
Lysine			1.00	—	1.00	—	—	0.92	—	—	0.88	—	0.73
Arginine			—	1.00	1.02	—	—	—	1.00	1.10	—	0.99	0.94
Aspartic acid			—	—	—	—	—	2.00	—	1.00	1.00	—	0.83
Threonine			—	—	—	—	1.02	—	—	—	—	1.08	0.88
Serine			—	—	—	—	—	—	—	1.01	—	—	—
Glutamic acid			—	—	—	—	—	—	—	0.90	—	1.00	0.83
Proline			—	—	—	—	—	—	—	—	—	—	2.88
Glycine			—	—	—	—	2.00	—	—	—	—	2.83	1.01
Alanine			1.28	1.08	1.08	—	0.92	—	—	0.88	0.90	0.91	—
Carboxymethylcysteine			—	—	—	—	0.72	—	—	0.70	—	1.00	1.33
Valine			—	—	—	—	—	—	—	—	—	0.85	—
Methionine			—	—	—	—	—	—	—	0.98	—	—	—
Isoleucine			—	—	—	1.00	—	—	1.79	—	—	—	—
Leucine			—	—	—	—	—	—	—	—	—	1.17	1.00
Tyrosine			—	—	—	—	—	—	—	—	1.85	1.03	1.01
Phenylalanine			—	—	—	—	—	1.23	—	—	1.26	1.27	1.09
Recovery (%)			33.1	96.9	70.0	—	77.5	78.1	76.9	78.8	85.6	72.5	99.4
Residues			40–41	16–17	40–42	—	54–58	43–46	18–20	47–53	21–26	27–39	1–15
Radioactivity yield													
(% of that applied)													
Acetonitrile			1.4	0.25	0.25	2.0	13.5	0.2	1.5	13.5	0.2	23.9	29.2
Propan-2-ol			1.1	—	—	0.2	16.0	0.7	1.7	13.7	0.1	52.5	—
Hydrophobicity (Σf)			1.05	−0.29	0.23	—	0.62	2.72	1.17	1.07	6.67	7.61	11.18

Fig. 3. Fluorescence detection of eluted peptides. For (a), 2.5 nmol of the tryptic peptides from [^3H]carboxymethylated basic pancreatic trypsin inhibitor were injected and 9.8% (240 pmol) of the column effluent was removed for fluorescence detection (100 mV recorder range). In (b), 9.4 nmol of the CNBr fragments of aminoethylated human haemoglobin α -chain were injected and 6.0% (560 pmol) was removed for detection (500 mV recorder range). Recorder chart speeds were 6 min/cm; buffers: A, 0.125 M-pyridine/formate, pH 3.0; B, 1.0 M-pyridine/acetate, pH 5.5, 60% propan-1-ol (concentrations based on pyridine).

elution points in terms of percentage of buffer B (Fig. 2) for each peptide demonstrates, as has been shown in the preceding paper (Wilson *et al.*, 1981a), that there is only a limited correspondence between the two; i.e., peptides 8–10 appear to be eluted as a function of their hydrophobicities, whereas peptides 1–7 are not. The co-elution of peaks 9 and 10 in the propan-2-ol buffer system suggests that the same peak resolution cannot be expected when one changes from one organic eluant to another. Attempts to separate the two peptides with a less steep gradient (results not shown) failed to resolve the mixture.

Fig. 3 illustrates the chromatography of the tryptic [^3H]carboxymethyl-basic pancreatic trypsin inhibitor peptides (Fig. 3a) and the CNBr haemoglobin α -chain fragments (Fig. 3b) when using the pyridine-based buffers, propan-1-ol as organic eluant, and fluorescence detection. In both cases, peptide resolution has decreased, owing to post-column mixing (cf. Figs. 1 and 2). This can be overcome by employing a different gradient (less steep and using isocratic holds), but then run times are extended. The sensitivities of the two detection

systems are also apparent by comparing the quantities of material chromatographed, and, in the case of fluorescamine, the amounts removed for the detection reaction. In the latter case, we have consistently found that between 50–200 pmol of peptide is sufficient for detection (see Hughes *et al.*, 1979, 1981b), which for most separations represent a small percentage (<5–10%) of the chromatographed material. For absorbance, the detector response could theoretically be increased to 0.01 full-scale deflection at 220 nm to achieve the separation on 300 pmol per component as shown in Fig. 2. However, the increase in the baseline during gradient development would be prohibitive. Conversely, by increasing the fluorescence sensitivity 5–10-fold, chromatograms at the picomolar level are obtained, which are not significantly altered in appearance.

During the determination of primary structure it is frequently found that a given fraction appears to contain more than one peptide fragment. This is usually concluded from non-integral ratios of the constituent amino acids. It is the degree of contamination, as well as the results from N- and C-terminal analyses, that determine if further purification is necessary. In those instances where another purification step(s) does not change the composition, peptide heterogeneity, e.g. more than a single amino acid at a specific position within the polypeptide chain, is often implicated. Determining the amino acid sequences of such peptides is often hindered by the methods used for identification of the thiohydantoin derivatives arising from Edman-type sequencing (t.l.c., Bridgen *et al.*, 1975; g.l.c., Niall, 1973; mass spectrometry, Morris & Dell, 1975; and h.p.l.c., Godtfredsen & Oliver, 1980). These methods would be hard-pressed to detect, much less quantify, heterogeneity at <20%, especially as 'overlap' accumulates during an extended degradation. In addition, certain thiohydantoin derivatives are more labile than others, and thus the nature of the amino acids at the heterogeneous position are critical.

Since reverse-phase h.p.l.c. has proven to be readily applicable to peptide isolation it was considered that it should also be useful in confirming microheterogeneity. Previous attempts with ion-exchange and paper-chromatographic methods had failed to separate the heterogeneous C-terminal CNBr-cleavage fragment of *Dicrocoelium* haemoglobin. The results from carboxypeptidase digestions and Edman degradations had suggested heterogeneity at position 9:

(K. J. Wilson, R. Stötzl, P. A. Kunz & G. J. Honegger, unpublished work). Analysis by h.p.l.c.,

with detection by absorbance [Fig. 4(a) (i)], clearly illustrated the presence of two peptides. The lack of fine detail obtained from fluorescence derivatization is apparent in Fig. 4(b) (i); however, as discussed above, the peptides are perfectly separated by a modified gradient [Fig. 4(c) (i)]. It should be noted that since the post-column derivatization only affects

the portion of the eluant going for detection, collected fractions are better separated than the chromatogram would indicate. Composition data for the two peptides, given in Table 3, confirm the presence at position 9 of glycine for pool 1 and alanine in pool 2. The C-terminal CNBr fragments of *Aeromonas* aminopeptidase, which also appeared

Fig. 4. H.p.l.c. separation of the CNBr C-terminal fragments from *Dicrocoelium haemoglobin* (i) and those from *Aeromonas aminopeptidase* (ii)

(i): (a) 2.6 nmol of material was injected; detection was at 220 nm; (b) 2.6 nmol was injected and 650 pmol was removed for fluorescence detection (100 mV recorder range); (c) preparative isolation from 11.6 nmol of peptide; 2.0 nmol was removed for fluorescence detection (500 mV recorder range). (ii): (a) 2 μ l of peptide mixture was injected; detection was at 220 nm; (b) preparative isolation from 50 μ l of mixture; 25% was removed for fluorescence detection (500 mV recorder range); (c) rechromatography of the 4.8 nmol of material from the main peak [eluting at 16% B in A + B mixture; see (b)(ii) above]; 25% was removed for fluorescence detection (100 mV recorder range). Buffer systems for u.v. detection were the same as those described in Fig. 2, containing acetonitrile, and for fluorescence detection they were as in Fig. 3.

Table 3. Amino acid compositions, yields and hydrophobicities of the C-terminal CNBr fragments from (a) *Dicrocoelium* haemoglobin and (b) *Aeromonas aminopeptidase*

Pools 1 and 2 under (a) and (b) refer to the pooled peaks in Fig. 4. Threonine and serine values have been corrected for loss during hydrolysis (9% and 7% respectively). Peptide hydrophobicities have been calculated as described by Rekker (1977). Values in parentheses are nearest integers.

	Composition (mol/mol)					
	(a)			(b)		
	Mixture	Pool 1	Pool 2	Mixture	Pool 1	Pool 2
Lysine	1.00 (1)	1.00	1.00	—	—	—
Histidine	2.36 (3)	2.60	2.75	—	—	—
Arginine	—	—	—	—	—	—
Aspartic acid	—	—	—	1.55	1.09 (1)	1.87 (2)
Threonine	—	—	—	3.07 (3)	2.81	2.90
Serine	—	—	—	1.04 (1)	1.03	1.00
Glutamic acid	1.15 (1)	0.91	0.96	0.60	—	0.98 (1)
Proline	0.95 (1)	0.89	0.96	2.00 (2)	2.00	2.00
Glycine	0.64	1.02 (1)	—	2.83 (3)	2.60	2.55
Alanine	1.52	0.97 (1)	1.88 (2)	0.93 (1)	0.86	0.84
Valine	—	—	—	—	—	—
Isoleucine	1.82 (2)	2.05	2.10	—	—	—
Leucine	2.71 (3)	3.02	3.11	—	—	—
Phenylalanine	0.94 (1)	1.15	1.15	—	—	—
Recovery (%)	—	—	84.1	—	—	63.2
Hydrophobicity (Σf)	—	+13.49	+14.02	—	+0.16	-1.98

to be heterogeneous by amino acid composition, were likewise successfully separated by using h.p.l.c. [Figs. 4(a) (ii), 4(b) (ii) and 4(c) (ii)]. Edman degradations performed on the peptide mixture had given the sequence:

Gly-Ser-Ala-Thr-Gly-Asp-Thr-Pro-Thr-Pro-
(Gly,Asn,Gln)

(K. J. Wilson, unpublished work). Amino acid compositions (Table 3) indicate that glycine is in position 11 and that the peptide from pool 2 has an extra asparagine and an extra glutamine residue over that from pool 1. The differences in the calculated hydrophobicities of the peptides, 0.53 Rekker units for the *Dicrocoelium* and 1.82 for the *Aeromonas* peptides, and the high yields, 84 and 63% respectively, indicate the performance that can be expected from reverse-phase h.p.l.c.

Prediction of peptide retention times for the acetonitrile (Figs. 5a and 5c) and propan-2-ol (Figs. 5b and 5d) systems have been evaluated in a manner analogous to those in the preceding paper (Wilson *et al.*, 1981a). In addition to the identified tryptic fragments from [^3H]carboxymethyl-basic pancreatic trypsin inhibitor (Fig. 2 and Table 2), various synthetic peptides from Bachem AG (see Table 2 in Wilson *et al.*, 1981a) were chromatographed. By using the retention coefficients for the amino acid

residues at pH 2.1 given by Meek (1980) and those determined for the pyridine-based propan-1-ol system (Wilson *et al.*, 1981a), correlation coefficients of 0.752 and 0.566 were calculated for these peptides chromatographed with acetonitrile as the organic eluant. The number of peptides injected on the propan-2-ol system has not been sufficient to allow a similar comparison. However, the discrepancies between our values for the acetonitrile buffer system and those of >0.99 as determined by Meek (1980) deserve comment. In both studies, the column packings were RP-18, which were eluted with buffer flow rates of 1.0 ml/min. The acetonitrile gradient was significantly 'faster' in this investigation, 2.2%/min as against 0.75%/min, and the NaClO_4 concentrations in buffers A and B lower, 10 mM as against 100 mM. To date we have been unable to account for these observed chromatographic differences.

Since the chromatography of all peptide mixtures were performed with both detection systems, some observations concerning their respective uses can be made. (i) There is considerable sample dilution occurring with the fluorescamine-detection system which is not seen when the absorbance of the effluent is directly measured. For this reason the 'fine' detail of the acetonitrile/ or propan-2-ol/ NaClO_4 systems are more obvious. For fluorescence detection the necessity of employing additional pumps to deliver

Fig. 5. Correlation between predicted and actual elution point of peptides in the acetonitrile (a and b) and 2-propanol (c and d) buffer systems

Peptide hydrophobicities in (a) and (c) were calculated by using the constants of Meek (1980) and in (b) and (d) from those determined for the pyridine-based buffers with propan-1-ol as organic eluant (Wilson *et al.*, 1981a). Predicted elution values were then calculated by assuming that the expected chromatographic behaviour is represented by the slope of the best-fit line through the actual elution points and the peptide's calculated hydrophobicities.

an *o*-phthalaldehyde/buffer solution and fluorescamine in acetone (Hughes *et al.*, 1979) can be problematic owing to pump maintenance and performance. (ii) The sensitivity of either system is theoretically high enough for detection at the picomolar level. Obviously both are dependent on the purity of the solvents used. For fluorescence detection, all that is required is for the removal of primary-amine impurities, which is readily performed by distillation over ninhydrin. U.v. absorbance, however, requires the removal of virtually all impurities, a far-from-easy task for solvents such as acetonitrile. Practically, the sensitivity of the

fluorescence detection is 5–10-fold greater than that of absorbance. Additionally, samples having been through several manipulations acquire quantities of extraneous material that also have u.v. absorbance. In these cases the fractions containing peptides are far more readily determined by the use of fluorescence, which is virtually restricted to the measurement of primary amines. (iii) For preparative purposes the pyridine-based buffers are extremely convenient, since their volatility is sufficiently high to allow removal by evaporation. The salt remaining after evaporation of a NaClO_4 -containing buffer often makes desalting necessary.

However, when only amino acid compositions are desired, it is possible to hydrolyse in the presence of the salts arising from 0.5 ml or less buffer without significant losses of any amino acids other than tryptophan. Similarly, peptides can be attached to glass supports for solid-phase sequencing with 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate ('DABITC') (Hughes *et al.*, 1979; Wilson & Hughes, 1980) without prior desalting.

We thank the Sandoz-Stiftung, Sandoz AG, for the grant through which one of the h.p.l.c. units used in this study was purchased.

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