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

XLVIII*. RETENTION BEHAVIOUR OF TRYPTIC PEPTIDES OF HUMAN GROWTH HORMONE ISOLATED BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A COMPARATIVE STUDY USING DIFFERENT CHROMATOGRAPHIC CONDITIONS AND PREDICTED ELUTION BEHAVIOUR BASED ON RETENTION COEFFICIENTS

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SUMMARY

The retention behaviour of the tryptic peptides of human growth hormone under a variety of reversed-phase chromatographic conditions has been investigated. By using water-acetonitrile gradients containing phosphate, bicarbonate, trifluoroacetate, or heptanesulphonate buffers, the influence of these ionic modifiers on peptide selectivity with several different alkylsilicas has been examined. Comparisons of observed and predicted retention behaviours for the various tryptic peptides in the different chrbmatographic systems are described. Application of these systems to the isolation of deamidated forms of human growth hormone is presented.

INTRODUCTION

Since their introduction several years ago, reversed-phase high-performance liquid chromatographic (RP-HPLC) methods have proved very versatile for the separation of peptides generated from the proteolytic digestion of polypeptides and proteins[1,2]. Generally, peptides derived from enzymatic fragmentation of proteins differ widely in amino acid composition and encompass an extensive range of polarities. As a consequence, gradient elution with water-organic solvent combinations is usually required, Depending on the complexity of the enzymatic digest, a single gradient elution protocol is rarely sufficient to allow complete resolution of all components. Differences in chromatographic selectivity for peptides separated on a specified microparticulate alkylsilica are known to arise from a variety of mobile phase effects but, to date, surprisingly few detailed comparative studies with enzymatic digests of proteins have been reported. In the present study, the relative retention behaviour of the tryptic peptides of human growth hormone (HGH) eluted with water-acetonitrile

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gradients containing phosphate, bicarbonate, trifluoroacetate or heptanesulphonate buffers from several different chemically bonded hydrocarbonaceous silicas has been determined. Criteria for the interconversion of retention data obtained with peptides eluted by different mobile phase systems from the same stationary phase have been established. Application of these methods to the isolation of peptides with minor structural differences is also discussed.

MATERIALS AND METHODS

Chemicals and reagents

HGH was isolated from extracts of frozen human pituitaries essentially as described by Chapman et *al.*[3]. For a final purification, a DEAE-cellulose (Whatman DE-52) column, pre-equilibrated with 50 m*M* ammonium acetate, pH *8.3,* was eluted with a 50-300 m*M* ammonium acetate, pH 8.3, linear gradient. Sigma type XI [Diphenylcarbamyl chloride (DPCC)-treated] trypsin was used for all the digests. The trypsin was dissolved in 1 m*M* hydrochloric acid, 2 m*M* calcium chloride at a concentration of 1 mg/ml. The enzymatic digestions were carried out at 37°C using an enzyme:protein ratio of 1:100. The HGH and its Cys-(CM) derivative were dissolved in 50 m*M* Tris–HCl, 2 m*M* calcium chloride, pH 7.8, at a concentration of 10 mg/ml immediately prior to digestion. Reactions were quenched by the addition of 1/10 volume of a solution of phenylmethylsulphonyl fluoride-methanol, and the samples were lyophilized. Reduction and carboxymethylation were carried out essentially as described previously by Hearn *et al.*[5]. Orthophosphoric acid and sodium dihydrogen phosphate were AristaR grade reagents obtained from BDH (Poole, Great Britain), trifluoroacetic acid was from Pierce (Rockford, IL, U.S.A.), sodium heptanesulphonate was obtained from Fluka (Buchs, Switzerland), ammonium bicarbonate was from Ajax Chemicals (Melbourne, Australia). Acetonitrile was from Waters Assoc. (Milford, MA, U.S.A.) or Burdick & Jackson (Muskegon, MI, U.S.A.).

Equipment

A Waters Model 224 liquid chromatograph equipped for gradient elution, a U6K sample injector and a Model 450 variable-wavelength detector were employed. Chromatographic separations were performed on μBondapak C_{18} and alkylphenyl columns (30 x 0.4 cm I.D., 10-μm particle size) and on Radial-Pak A cartridges (10 x 0.8 cm I.D., 10-μm particle size) in conjunction with a Waters RCM module. Sample injections were performed with Model 50A syringes from SGE (Melbourne, Australia). The pH measurements were performed with a Radiometer PHM64 pH meter equipped with a combination glass electrode.

Chromatographic conditions

All chromatographic separations were carried out at ambient temperature (*ca.* 18°C). Bulk solvents and mobile phases were filtered through 0.5-μm filters (Millipore Corp., Bedford, MA, U.S.A.) and degassed by sonication. Flow-rate was generally 1.0 ml/min through the stainless-steel analytical columns and 1.5 ml/min through the flexible-walled, polypropylene RCM cartridges. All samples were dissolved in the mobile phase corresponding to the initial elution condition, 350400 μg tryptic digest

being injected in 100-l 50-μl volumes. The eluted fractions were collected manually, and adjusted immediately to pH 7.0 with 15 m*M* sodium hydroxide, if necessary. The organic solvent was removed under vacuum and the residue was lyophilized. Amino acid analyses and N-terminal sequence assignments were carried out as described elsewhere[4,5]. The one-letter code for the protein amino acids is used as described by Dayhoff[6]. The apparent capacity factors, k'_{app}, for the gradient elution experiments were calculated by established procedures[7].

RESULTS AND DISCUSSION

Comparative retention behaviour of tryptic peptides of HGH

General strategies for the isolation and analysis of peptides by reversed-phase and ion-exchange HPLC have been the subject of several recent publications from this laboratory[8–10]. In work on HGH and the 20-kdalton HGH variant as well as other pituitary protein hormone variants to be reported elsewhere[4,5,11,12] we have extensively utilised these HPLC procedures for the characterisation of both intact proteins and protein fragments arising from either enzymatic or chemical cleavages. The versatility of the RP-HPLC methods, in particular, has permitted the isolation in pure form of most of the tryptic peptides derived from these pituitary proteins and enabled complete primary structural analysis to be carried out on as little as 200 μg of starting material. Because complicated mixtures of tryptic peptides with large, as well as minor, structural differences often arose as part of these primary sequence determinations, it was necessary for the complete separations of the various peptides to employ several RP-HPLC elution programs, employing different co- and counter-ion systems in order to achieve adequate selectivities. The studies with the HGH tryptic peptides described in the present paper have permitted further extension of our earlier observations[8,13,14] on the role of ionic modifiers including surface-active ions, in peptide selectivity on alkylsilicas. The data allow detailed selectivity comparisons to be made between four mobile phase systems containing different ionic modifiers, all of which are now commonly used in RP-HPLC peptide mapping. In addition, the retention behaviour of these peptides has been examined with three different types of non-polar stationary phase.

Table I shows the comparative retention behaviour for the different chromatographic conditions examined and the complete sequence alignment for the HGH tryptic peptides, based on the NH,-terminal phenylalanine as Residue No. 1. As has been noted previously[5,15], native HGH was readily cleaved by trypsin at 18°C under the conditions that were used. Because of the disulphide linkage of cysteine 53 to cysteine 165 in intact HGH, only a single component corresponding to the tryptic peptides T-6 and T-16 was observed in all chromatograms, and similar behaviour occurred for tryptic peptides T-20 and T-21 due to the disulphide linkage of cysteine 182 to cysteine 189. However, both these composite two-chain peptides were replaced by the single-chain peptides T-6a, T-16a, T-20a and T-21a in the RP-HPLC separation of the tryptic digest of fully reduced and carboxymethylated HGH[5]. Also evident from Table I is the coincidental elution in all chromatographic systems examined of the tryptic peptide fragment corresponding to cleavage of HGH in the region of residues 169 to 178, *i.e.* the region encompassing the tryptic peptides T-18 and T-19. In fact, structural analysis of the tryptic fragment corresponding to this amino acid

TABLE I

COMPARATTVE RETENTION BEHAVIOUR OF HGH TRYPTIC PEPTIDES UNDER SEVERAL DIFFERENT CHROMATOGRAPHIC CONDITIONS

Condition 1: column, μBondapak C_{18}; flow-rate, 1.0 ml/min; solvent A, water-15 m*M* orthophosphoric acid, pH 2.3; solvent B, 15 m*M* orthophosphoric acid, water-acetonitrile (50:50); linear 60-min gradient from 0 to 100% B.
Condition 2: column, μBondapak C_{18}; flow-rate, 1.0 ml/min; solvent A, water- 100 m*M* ammonium bicarbonate; solvent B, 100 m*M* ammonium bicarbonate, water-acetonitrile (50:50); linear 60-min gradient from 0 to 100% B.
Condition 3: column, μBondapak C_{18}; flow-rate, 1.0 ml/min; solvent A, water-O.1 % TFA; solvent B, 0.1 % TFA, water–acetonitrile (50:50); linear 60-min gradient from 0 to 100% B.
Condition 4: column, μBondapak C_{18}; flow-rate, *1*.0 ml/min; solvent A, water–1 5 m*M* sodium heptanesulphonate–15 m*M* orthophosphoric acid; solvent B, 15m*M* sodium heptanesulphonate–15 m*M* orthophosphoric acid, water-acetonitrile (50:50); linear 60-min gradient from 0 lo 100 % B.
Condition 5: column, μBondapak alkylphenyl; flow-rate, 1.0 ml/min; solvent A, water-15 m*M* orthophosphoric acid; solvent B, 15 m*M* orthophosphoric acid, water-acetonitrile (50:50); linear 60-min gradient from 0 to 100% B.
Condition 6: column. RCM-Radial-Pak C_{18}; flow-rate. 1.5ml/min; solvent A. water 100 m*M* ammonium bicarbonate; solvent B. 100 m*M* ammonium bicarbonate. water acetonitrile (50:50); linear 180-min gradient from 0 to 100 % B.

Tryptic peptide	*Sequence*	*Condition I*		*Condition 2*		*Condition 3*		*Condition 4*		*Condition 5*		*Condition 6*	
		k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*	k'_{app}	*Elution order*
T1	FPTIPLSR	14.3	15	19.2	13	*17.6*	15	*22.5*	11	*17.6*	*16*	*54.0*	*14*
T2	LFDNAMLR	12.7	12	17.5	12	*16.0*	*14*	*22.5*	11	*16.3*	*14*	*48.0*	13
T3	AHR	0.2	3	3.3	4	*1.9*	*4*	15.8	*6*	*0.1*	1	8.0	*4*

T4	LHQLAFDTYQEFEEAYIPK	19.8	17	10.7	14	19.2	17	27.2	17	23.9	20	58.2	15
T5	EQK	0.1	1	0.05	1	0.1	1	x.9	1	0.1	1	0.3	1
T6*	YSFLQNPQTSLCFSESIPTPSNR	22.5	19	24.0	16	21.1	19	28.4	18	20.6	18	68.5	17
T7	EETQQK	0.3	4	0.1	2	0.1	1	10.4	3	0.4	4	0.3	1
T8	SN LQLLR	10.7	11	41.7	21	14.8	11	37.0	21	12.5	11	83.5	19
T9	ISLLLIQSWLEPVQFLR	28.7	21	31.6	20	27.2	21	33.4	20	22.0	19	91.8	21
T10	SVFANSLVYGASNSDVYDLLK	20.7	18	21.2	15	20.3	18	26.4	16	a	21	61.2	16
T11	DLEEGIQTLMGR	18.2	16	29.1	18	18.3	16	26.0	15	16.3	14	88.0	20
T12	LEDGSPR	3.7	6	4.6	5	7.2	6	14.2	5	3.4	6	10.2	5
T13	TGQIFK	6.1	7	9.7	7	11.4	7	18.6	7	6.4	7	28.3	8
T14	QTYSK	3.4	5	5.2	7	5.3	5	13.1	4	0.9	5	11.3	6
T15	FDTNSHNDDALLK	9.4	8	31.0	19	13.2	10	20.8	8	11.1	8	25.2	7
T16*	NYGLLYCFR	22.5	19	24.0	16	21.1	19	28.4	18	18.9	17	68.5	17
T17	K	0.1	1	0.1	2	0.1	1	8.9	1	0.1	1	0.7	3
T18**	DMDK	13.1	13	15.2	10	15.7	12	23.6	13	15.6	12	41.0	II
T19**	VETFLR	13.1	13	15.2	10	15.7	12	23.4	13	15.6	12	41.0	II
T20***	IVQCR	9.4	8	12.2	8	12.5	8	20.8	8	11.4	Y	32.1	9
T21***	SVEGSCGF	9.4	8	12.2	8	12.5	8	20.8	8	11.4	9	32.1	9

* Not present in the Cm-(Cys)-HGH digest but replaced by peptide T-6a (YSFLQNPQTSL(Cm)CFSESIPTPSNR) and peptide T-16a (NYGLLY(Cm)CFR).

** Corresponds to peptide T-18 + 19 (DMDKVETFLR). Under different digest conditions, the peptide KDMDKVETFLR can also be isolated from these RP-HPLC separations.

*** Not present in the Cm-(Cys)-HGH digest but replaced by peptide T-20a [IVQ(Cm)CR] and peptide T-21a [SVEGS(Cm)CGF].

sequence region, following isolation of the component from the appropriate zone of the various chromatographic profiles, is in accord[5,15] with this peptide having the primary structure DMDKVETFLR, i.e. an elongated peptide T-18 plus T-19. This indicates that only minor cleavage occurred at lysine 172 under the proteolytic digestion conditions used.

Fig. 1 illustrates the retention behaviour of the HGH tryptic peptides on μBondapak C_{18} columns with the four different mobile phase systems, namely (a) 15 m*M* orthophosphoric acid, pH 2.3; (b) 100 m*M* ammonium bicarbonate, pH 7.8; (c) 0.1% trifluoroacetic acid; and (d) 15 m*M* sodium heptanesulphonate-15 m*M* orthophosphoric acid, pH 3.0, using in each case linear water-acetonitrile gradients with a final acetonitrile volume fraction of φ_s 0.5. Also shown in Fig. 1 is the retention behaviour under similar gradient conditions of the HGH tryptic peptides on a RCM-Radial-Pak C_{18} (RCM-C,,) column with a primary 100 m*M* ammonium bicarbonate eluent.

Several salient points relevant to peptide selectivity in RP-HPLC are clearly evident from Fig. 1.

Firstly, as expected on the basis of column dimensions and stationary-phase characteristics, the k'_{app} values obtained for the HGH tryptic peptides with the RCM-C_{18} column were uniformly larger (generally by a factor of 2) than the k'_{app} values obtained with the μBondapak C_{18} column under similar gradient conditions.

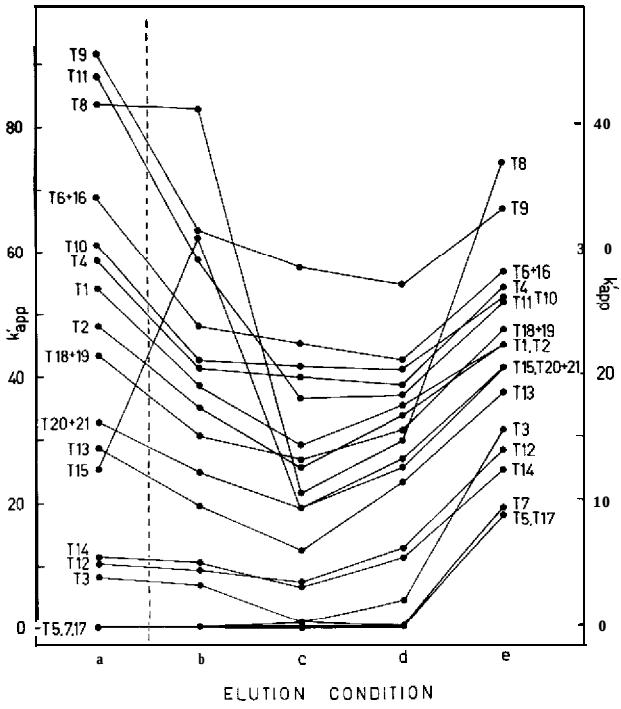


Fig. 1. Comparative retention behaviour of the HGH tryptic peptides under different chromatographic conditions. The set of data shown in the left-hand panel was obtained on a Waters RCM-C_{18} column and the four sets of data shown in the right-hand panel were obtained on μBondapak C_{18} columns. The keys to the peptide structures and elution conditions are listed in Table I. Elution conditions: (a), condition 6; (b), condition 2; (c), condition 1; (d). condition 3: (e), condition 4.

Secondly, the small acidic peptides such as EQK (T-5) and EETQQK (T-7) showed only limited retention on either the μBondapak-C_{18} or the RCM-C_{18} stationary phase at pH 7.8 with the 100 m*M* ammonium bicarbonate systems, a result anticipated on the basis of amino acid end group and side-chain ionization considerations[2,10]. The elution order of the HGH tryptic peptides chromatographed on the RCM-C_{18} stationary phase and the μBondapak C_{18} stationary phase were generally similar under these ammonium bicarbonate conditions, the only significant selectivity changes being associated with tryptic peptides T-8 and T-15. With low-pH eluents, peptides, particularly basic argininyl peptides, often interact more strongly with the RCM-C, $_8$ stationary phase compared to other capped alkylsilica packing materials. It has been proposed"." that the retention behaviour of peptides on the RCM-C_{18} support is due to differences in carbon loading, and this has led to the assumption that specific electrostatic interactions arise between silanol groups on the stationary-phase surface and the protonated peptide sample. However, with low-pH mobile phases, accessible silanol groups will be essentially non-ionized. As a consequence, electrostatic interactions between accessible silanol anionic groups and protonated cationic amino groups are not expected to play a dominant role in the retention process. In contrast, it can be argued that this retention behaviour is due to hydrogen bonding associated with the interaction of the solvated ionized solute with solvent multi-layers at the surface of the alkylsilica matrix[10,19]. A further indication that multi-layer solvation processes are important with the RCM-C,, stationary phases (and in general with many other alkylsilicas) is the improvement in chromatographic behaviour observed[16,18] with solvents which can engage in hydrogen bonding. It also follows from these considerations that acidic peptides should exhibit attenuated retention as the pH is increased to *ca.* pH 8.0 due to a combination of changes in the extent of ionization of both the solute and free silanol groups and this retention behaviour has been observed experimentally. Variations in the strong hydrogen bonding characteristics of the silanol groups, which permit solvent multi-layers to be formed at the matrix surface, are also expected to occur in a similar pH-dependent fashion. The retention behaviour of the relatively non-polar peptide T-15 on the μBondapak C_{18} column with the bicarbonate-based eluent appears anomalous (compared to, for example peptide T-7 on the same stationary phase) despite the fact that the β-carboxylic acid groups of the aspartic acid residue of peptide T-15 (FDTNSHNDDALLK) would be anticipated to be fully ionized at pH 7.8. However, this chromatographic behaviour is consistent with other observations[2,20] on the RP-HPLC of peptides with aspartic acid or glutamic acid residues and relatively hydrophobic amino- or carboxyl-terminal sequence regions. It is worth noting that the peak shape of the various HGH tryptic peptides on the μBondapak C_{18} columns was, however, generally superior when compared to the RCM-C, $_8$ columns eluted with the same 100 m*M* ammonium bicarbonate eluent conditions.

Thirdly, peptide retention was noticably shorter with the low pH phosphate-based systems when compared to the bicarbonate system, dramatic selectivity changes being associated with peptides T-8, T-l 1 and T-15. other minor selectivity reversals between these two ionic modifiers were also evident, e.g. T-12/T-14 and T-18 + T-19/T-2. The general decrease in k'_{app} values, observed when the HGH tryptic peptides were eluted with the low pH phosphate-based mobile phase, parallels our earlier experiences[2,7,8,14] and presumably reflects the composite effect on the distri-

bution equilibrium of each individual peptide as phosphate-mediated changes in ionization state and pairing-ion interactions occur.

Fourthly, the elution order for the various HGH tryptic peptides was very similar with both the 15 m*M* orthophosphoric acid system and the 0.1% trifluoroacetic acid (TFA) system. In fact, except for the small, early emerging peptides, such as peptide T-3 (AHR) the relative retention order was essentially coincidental.

Finally, in the sodium heptanesulphonate system, peptide retention was invariably increased, the most noticable changes being associated with peptides T-3, T-8 and T-18 + T-19. These changes in chromatographic retention behaviour can be rationalized in terms of pairing-ion interactions between the protonated peptides and the surface-active heptanesulphonate counter-ion[2,13,14]. In addition, asymmetric peak shapes were noted with peptides T-7 and T-14, eluted early by the heptanesulphonate system. Similarly decreased chromatographic efficiencies have been observed previously for peptides eluted under gradient conditions when surface-active ions are present in the mobile phase. Although the bandwidth of a zone in gradient elution RP-HPLC of peptides is usually found[7] to be constant across the gradient with inorganic ionic modifiers, the elution behaviour noted above with sodium heptanesulphonate (and related hydrophobic surfactants) presumably reflects changes in the distribution isotherm and binding kinetics of the surface-active hydrophobic heptanesulphonate anion as the organic solvent concentration in the mobile phase increases during gradient development.

In earlier studies with non-polar peptides, improved chromatographic behaviour has been observed, including reduced retention on μBondapak alkylphenyl columns compared to μBondapak C_{18} columns[1,21]. Fig. 2 illustrates the retention behaviour of the various HGH tryptic peptides on these two non-polar stationary phases when a low-pH phosphate eluent is used in conjunction with a linear O-50 % acetonitrile gradient. Although both non-polar peptides, T-9 and T-l 1, showed significant decreases in retention on the μBondapak alkylphenyl column, in most cases retention was comparable or increased over that found with the μBondapak C_{18} column. Nevertheless, for non-polar peptides the μBondapak alkylphenyl column has other advantages over the μBondapak C_{18} column, notably higher recoveries for peptides which are eluted under gradient conditions with k'_{app} above 15 (refs. l 1 and 21).

Relationship between observed and predicted elution behaviour

It is now well recognized that with structurally diverse peptides which are not subject to conformational constraints, the chromatographic behaviour of a specific peptide under a particular set of RP-HPLC conditions can predominantly be equated with its amino-acid composition, and in particular, the summated relative hydrophobic contribution of each amino acid residue in the peptide sequence. Based on a variety of retention-structure observations, empirical relationships and group coefficients for the prediction of retention of peptides subjected to regular reversed-phase isocratic and gradient elution have been reported[6,7,22,23]. Germane to the validation of these relationships have been theoretical treatments which relate selectivity for ionized peptides on alkylsilicas to differences in the effective molecular hydrophobic areas of the peptides in contact with the stationary phase. It is assumed that differences in selectivity can be linearly equated with differences in the overall standard

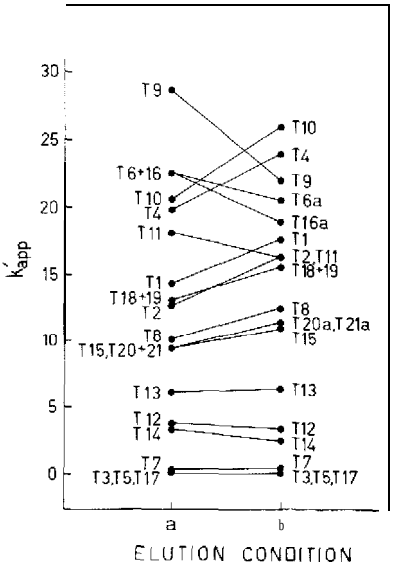
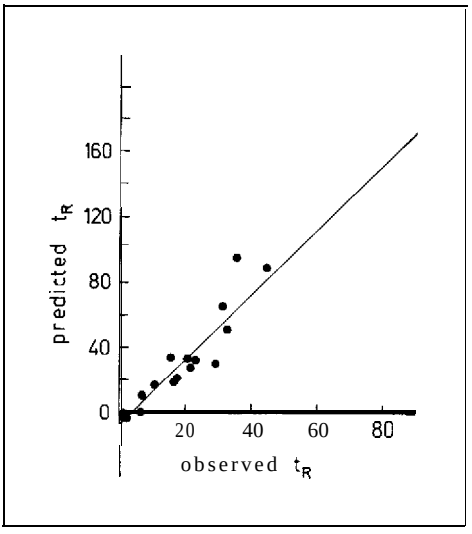


Fig. 2. Comparative retention behaviour of the HGH tryptic peptides on μBondapak C_{18} (a) and μBondapak alkylphenyl (b) columns, eluted with a linear 60-min gradient from 15 m*M* orthophosphoric acid–water to 15 m*M* orthophosphoric acid, water–acetonitrile (50:50) at equivalent flow-rates. The peptide key is in Table 1.

free energy changes in which the electrostatic terms remain constant. Clearly, such predictive approaches are useful not only for peak identification but also for optimization of resolution provided, of course, that data accumulated with one chromatographic system can be translated to another. The experimental results obtained for the HGH tryptic peptides with mobile phases of different composition provide a useful test to validate these predictive approaches and confirm the reliability of the derived retention coefficients.

The relationships between observed and predicted retention behaviour are illustrated in Figs. 3-5, which correlate the k'_{app} (observed) or t_R (observed) with the corresponding predicted values, calculated from summated hydrophobic group retention coefficients of the constituent amino acid residues (Table II). The latter were derived from data obtained for peptides separated on μBondapak C_{18} columns with a phosphate-based water-acetonitrile linear gradient system by Meek and Rosetti[22] and Su *et al.*[23] and with a TFA system by Browne et *al.*[24]. The correlation between the observed and predicted retention ($r^2 = 0.87$, 0.96 and 0.79, respectively) for the two phosphate-based systems and the TFA-based system support the hypothesis that hydrophobic group contributions of the amino acid residues have an essentially additive influence on peptide retention with alkylsilicas. Listed in Table III are the correlations between the observed retention times for the HGH tryptic peptides separated by either the orthophosphoric acid or the TFA elution system and the predicted retention times calculated from retention coefficients derived for peptides separated by phosphate, TFA or pyridine formate elution.

The data in Table III illustrate the difficulties in predicting peptide retention values for a particular chromatographic system using retention coefficients derived



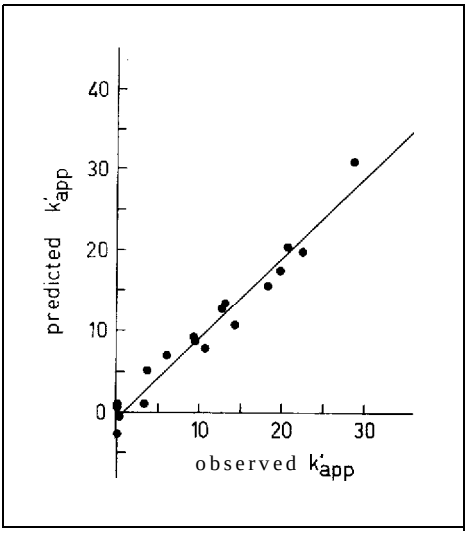


Fig. 3. Correlation between the observed retention times (t_R) of the HGH tryptic peptides and the predicted retention times of the same peptides calculated according to the retention coefficients of Meek and Rosetti[22]. The data were obtained with the phosphate-based chromatographic condition 1 (Table 1).

Fig. 4. Correlation between the observed k'_{app} of the HGH tryptic peptides and the predicted k'_{app} of the same peptides, calculated with the retention coefficients of Su *et al.*[23]. The data were obtained with the phosphate-based chromatographic condition 1 (Table I).

from a different chromatographic system. Comparisons between observed and predicted retention for elution systems of similar selectivities, e.g., for phosphate with TFA-based mobile phases, result in modest to good correlation but this becomes considerably less reliable as the overall selectivity of the chromatographic systems diverge, as can be seen, e.g., in the comparison of the phosphate with pyridine formate systems. The data further indicate that comparisons between buffer effects can be made reliably only when both of the following chromatographic criteria are satisfied: firstly, the same stationary phase must be employed throughout; secondly, the organic solvent modifier must be common to all elution systems and the linear gradient compositional limits must be identical. Factors other than the hydrophobicity of the amino acid side-chain are known to affect retention behaviour and both specific ionization and solvation effects are implicated. The participation of these effects may account for the data scatter evident in Figs. 3-5. The retention coefficients derived by Browne ***et al.***[21] for the TFA system express the predicted retention in terms of acetonitrile concentration, as indicated by the gradient former. A similar method has been used by Wilson et al.[25] for a pyridine formate-pyridine acetate–1-propanol elution system. Because neither method takes into account the actual eluent composition of the peptide zone as it leaves the column, the larger divergencies found between the observed retention and the predicted values based on these retention coefficients are not completely unexpected.

In order to permit data interconversion between two chromatographic systems, comparability in selectivity must exist. This requirement can be assessed for various peptides e.g. from plots of observed retention times obtained with different

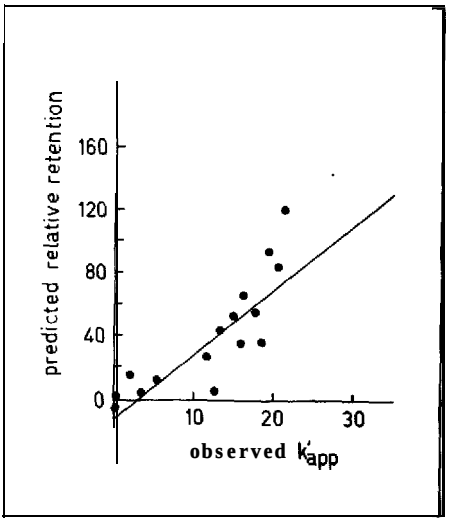


Fig. 5. Correlation between the observed retention times of the HGH tryptic peptides and the predicted elution positions of the same peptides, based on the retention coefficients derived by Browne et *al.*[24]. The data were obtained with the trifluoroacetic acid-based chromatographic condition 3 (Table I).

TABLE 11

HYDROPHOBIC GROUP RETENTION CONTRIBUTION FOR AMINO ACID RESIDUES

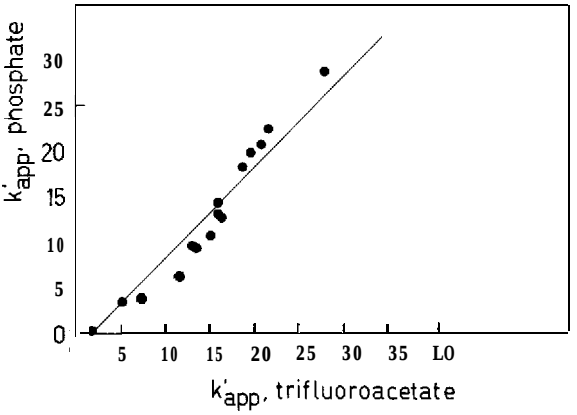
*Amino acid**	*Ref.* 22	*Ref.* 23	*Ref.* 24	*Ref.* 25
D	-1.6	1.92	−2.9	−1.4
N	-4.2	−1.76	-5.7	-0.2
T	1.7	-0.79	0.8	-2.2
E	-3.2	0.66	-4.1	-0.6
Q	2.0 1.1	-0.76 0.44	-7.1 -0.3	-0.2 0.0
P	3.1	−0.40	5.1	2.2
G	0.2	-0.82	−1.2	1.2
A	1.0	-0.12	7.3	-0.3
C	4.6	−1.25	-9.2	6.3
V	4.6	1.08	3.5	5.9
M	4.0	3.56	5.6	2.5
I	7.0	5.80	6.6	4.3
L	9.6	3.16	20.0	6.6
Y	6.7	-0.79	5.9	7.1
F	12.6	2.52	19.2	7.5
H	-2.2	−2.67	-2.1	-1.3
K	-3.0	-0.53	−3.7	-3.6
R	-2.0	−1.37	−3.6	-1.1
W	15.1	-0.28	16.3	1.9
end groups	2.5	1.54	6.4	−

* One-letter code for the protein amino acids as in ref. 6.

TABLE III

RELATIONSHIP BETWEEN OBSERVED RETENTION AND PREDICTED RETENTION OF THE HGH TRYPTIC PEPTIDES, BASED ON RETENTION COEFFICIENTS IN DIFFERENT ELUTION SYSTEMS

Retention observed in		*Retention predicted according to*			
		Ref. 22	*Ref. 23*	*Ref. 24*	*Ref. 25*
Orthophosphoric acid system	r^2	0.87	0.96	0.85	0.77
	y intercept	2.76	0.68	2.72	4.50
	Slope	0.27	0.98	0.18	0.31
Trifluoroacetic acid system	r^2	0.78	0.90	0.79	0.68
	y intercept	5.27	3.56	− 11.77	6.86
	Slope	0.24	0.89	4.37	0.27



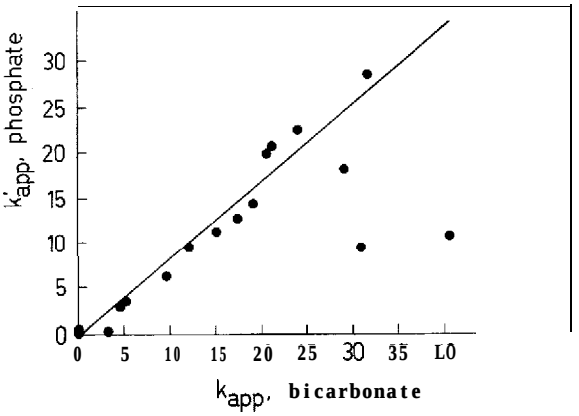


Fig. 6. Correlation between the observed retention times of the HGH tryptic peptides separated on a μBondapak C_{18} column by the phosphate-based and TFA-based mobile phases.

Fig. 7. Correlation between the observed retention times of the HGH tryptic peptides separated on μBondapak C_{18} columns by the phosphate-based and bicarbonate-based elution systems.

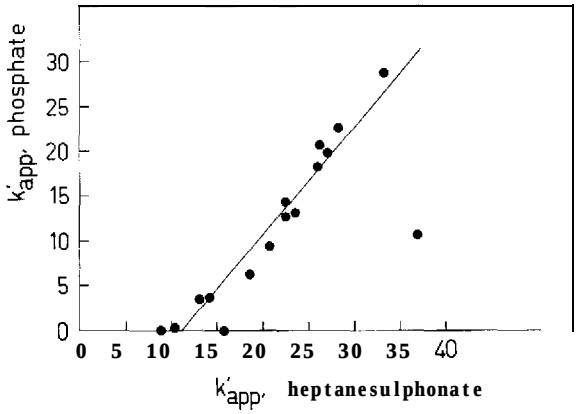


Fig. 8. Correlation between the observed retention times of the HGH tryptic peptides separated on μBondapak C_{18} columns by the phosphate-based and heptanesulphonate-based mobile phases.

eluents on the same stationary phase. The simplest form which this interdependency can take for a gradient systems of different buffer composition but with a fixed rate of change of the common organic modifier, may be expressed as

$$k'_{\mathrm{app},i} = Mk'_{\mathrm{app},j} + C$$

where $k'_{\mathrm{app}\,i}$ and $k'_{\mathrm{app},j}$ are the apparent capacity factors of the same peptide eluted by the two'different gradient elution systems, M is the relative eluotropic strength difference due to the buffer components and C is a system constant. Figs. 6–8 show that there are essentially linear relationships between the observed retention of the HGH tryptic peptides for the four elution systems examined in the present study, despite the obvious peptide retention divergences discussed earlier. Table IV itemizes the M and C values together with the appropriate correlation coefficients. Inherent to the derivation of these parameters was the assumption that peptide retention on

TABLE IV

CALCULATED COEFFICIENTS* FOR THE INTERCONVERSION OF RETENTION DATA BETWEEN DIFFERENT ELUTION SYSTEMS

Ionic species	*M value*	*C value*	r^2
0.1 % TFA**	1.04***	−2.05***	0.94***
100 m*M* Ammonium bicarbonate**	0.53***	2.38***	0.56***
	0.86§	-0.63 §	0.94§
15 m*M* Sodium heptanesulphonate**	0.9***	−8.34***	0.71***
	1.2§§	-13.3""	0.94§§

* Calculated according to the relationship $k'_{\mathrm{app},i} = Mk'_{\mathrm{app},j} + C$.

** Relative to the standard 15 m*M* orthophosphoric acid-acetonitrile gradient system on a μBondapak C_{18} column, for which $M = 1$ and C = 0.

*** Including data for all HGH tryptic peptides.

§ Excluding data for the tryptic peptides T-8 and T-15.

§§ Excluding data for the tryptic peptide T-8.

alkylsilicas in binary water-organic solvent gradient combinations of different buffer composition is governed by solvophobic effects. The enhanced retention in the heptanesulphonate system compared to the phosphate system is clearly evident. Good inter system correlation is seen for all HGH tryptic peptides in the phosphate and TFA systems ($r^2 = 0.94$) The correlation between the phosphate and bicarbonate systems or the phosphate and heptanesulphonate systems is low when the retention data for all peptides are included, but it significantly increases when the data for the anomalous peptides T-8 and T-15 are excluded. It thus appears that interconversion of data from one elution system to another ---even when the two essential criteria of selectivity comparability are satisfied— will require some caution, at least for high-pH buffer systems or eluents containing surface-active hydrophobic ions, and certainly until the origin of anomalous peptide behaviour similar to that noted above for peptide T- 15 becomes more clearly understood. Nevertheless, useful information can be obtained from the above results. For example, approximate elution positions can be estimated for peptides in both low-pH elution systems now commonly used in RP-HPLC separations. For other buffer ions, an indication is provided of the initial organic solvent concentration to be selected if comparable retention is to be achieved under gradient conditions. Following further extension and refinement of the data base on solvent and buffer effects, it should prove possible to predict more reliably elution positions of peptides based on a knowledge of their molecular and solution properties and thus provide a very powerful approach to the isolation of peptides from biological extracts or from protein fragmentation.

Isolation **of** *minor deamidated peptides*

Deamidation of proteins frequently occurs during post-translation modification or during isolation. Incomplete deamidation will give rise to protein microheterogeneity which will be revealed during structural studies. particular in tryptic and

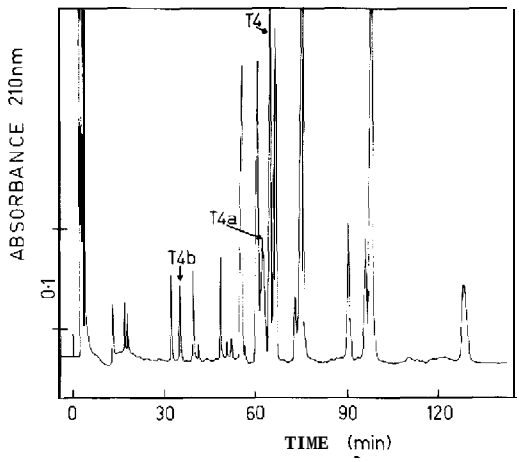


Fig. 9. Chromatographic profile of a HGH tryptic digest after incubation of HGH with 15 m*M* orthophosphoric acid. Elution conditions: column, μBondapak C_{18}, flow-rate I.0 ml/min, mobile phase, (A) 100 m*M* ammonium bicarbonate–water, (B) 100 m*M* ammonium bicarbonate, water–acetonitrile (50:50), linear 60 min gradient from 0 to 100% B. The elution positions of the tryptic peptides, T-4, T-4a and T-4b are indicated by arrows.

other enzymatic peptide maps. Fig. 9 illustrates an example of this type of structural variability by the resolution of structurally related peptides in the tryptic digest of an HGH preparation. The indicated chromatographic peaks in Fig. 9 correspond to the tryptic peptide T-4, the peptide T-4a ($^{22}Gln \rightarrow {}^{22}Glu$ deamidation modification) and the peptide T4b ($^{22,29}Gln \rightarrow {}^{22,29}Glu$ deamidation modification). Both deamidated T-4-peptides arise by hydrolysis on storage of the protein in 15 m*M* orthophosphoric acid. The elution order for these deamidated T-4-peptides is consistent with increased side-chain ionization. The ability of RP-HPLC procedures to resolve in good yield peptides with such minor structural differences is a very important attribute of these techniques compared to conventional methods of peptide purification. The application of similar methods to the characterisation of other deamidated protein hormone variants will be described elsewhere[4,11].

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