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HYDROPHOBIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HORMONAL POLYPEPTIDES AND PROTEINS ON ALKYLSILANE-BONDED SILICA

M. J. O'HARE and E. C. NICE

Unit of Human Cancer Biology, Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Sutton, Surrey SM2 5PX (Great Britain)

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SUMMARY

Thirty-two hormonal polypeptides and nine proteins (8–65 kD) have been used to evaluate the potential of high-performance liquid chromatography on alkylsilane-bonded silica for separating and recovering biologically active compounds of this type. The basic method used was gradient elution with acetonitrile in an acid phosphate buffer. Variation of key chromatographic parameters demonstrated that low pH (<4.0) and high buffer molarity (>0.1 *M*) are mandatory for reproducible high efficiency polypeptide chromatography. Simple NaCl–HCl mixtures of appropriate acidity and molarity could be substituted for the acid phosphate buffer, with the advantage of minimising non-physiological ion contributions to eluted materials. Minor selective effects were noted with different organic modifiers, but variation of other parameters, including choice of specific alkylsilane packings, did not materially influence separations. Under optimal conditions all of the polypeptides tested could be efficiently chromatographed, and many simultaneously resolved, as could most of the proteins tested. Three of the more hydrophobic proteins could not, however, be eluted from the alkylsilane packings. Retention orders of smaller compounds (<15 residues) generally correlated with the sum of the Rekker fragmental constants of their strongly hydrophobic residues. Larger polypeptides showed numerous anomalies when ranked by this means, however, limiting its predictive value. The separation of at least eighteen discrete components from a partially-purified posterior pituitary extract has demonstrated the capability of alkylsilane-type reversed-phase packings for the hydrophobic high-performance liquid chromatography of complex biological mixtures.

INTRODUCTION

We have recently published a method whereby a number of members of the adrenocorticotrophin (ACTH)-endorphin "family" of hormones can be simultaneously separated using reversed-phase high-performance liquid chromatography (HPLC) by gradient elution from an alkylsilane-bonded silica packing with acetonitrile in an acid–salt buffer[1]. These conditions, which were adapted from a system described by

Molnár and Horváth[2] for small peptides, are also compatible with the retention of both immuno- and bioactivity of ACTH, suggesting that the method might be of general use in the separation, purification and identification of polypeptide and protein hormones.

Although increasing numbers of individual polypeptide and some protein separations are now being reported with a variety of different HPLC methods[3–11], with the notably exception of a recent report by Rivier[12], systematic examinations of the limits of individual systems and their optimization in respect of complex mixtures have been rare. In this study we have, therefore, examined the behaviour on alkylsilane-bonded microparticulate (5 μm) porous silica packings, of a total of 32 polypeptide hormones and fragments thereof, and nine proteins ranging from 8000 to 65,000 daltons, using as a basic method that developed for the separation of the ACTH-endorphin compounds[1].

From the retention orders and separations achieved with this system, it has been possible to infer that the basic mechanism governing chromatography is hydrophobic interaction with the stationary phase and to conclude that it is applicable to a wide range of polypeptide hormones and to some, but not all, proteins when using alkylsilane-bonded packings. Variation of key chromatographic parameters has enabled those which can be used to facilitate and optimize specific separations to be identified.

MATERIALS AND METHODS

Chromatography was carried out at constant pressure using a DuPont Model 830 high-pressure liquid chromatograph, equipped with a Model 837 variable wavelength spectrophotometer and a Schoeffel Model 970 fluorimeter connected in series.

Organic solvents used were HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). Buffers and salt solutions were made up with AnalaR or Aristar (BDH, Poole, Great Britain) reagents using double-glass distilled water further purified by pumping it through 50 × 5 mm I.D. stainless-steel columns packed with Partisil-ODS (Whatman, Maidstone, Great Britain).

Sources of some of the polypeptides tested have been given previously[1], the remainder were obtained from Bachem (Torrance, Calif., U.S.A.), Serva (Heidelberg, G.F.R.) and Calbiochem-Behring Corp. (La Jolla, Calif., U.S.A.). Unlabelled proteins were obtained from Sigma (Poole, Great Britain, Grade I) and Calbiochem (A grade), and ^{14}C-methylated proteins from New England Nuclear (Dreieich, G.F.R.). Pitressin was obtained from Parke-Davis (Pontypool, Great Britain).

Compounds for chromatography were freshly dissolved in the primary aqueous solvent (see Results) and 250 ng–10 μg of each injected via a Rheodyne septumless valve with 175-μl loop onto a 100 × 5 mm I.D. stainless-steel column slurry-packed with one of the following alkysilane-bonded porous microparticulate (5 μm) silica packings: Hypersil-ODS (Shandon, Runcorn, Great Britain), Nucleosil 5-C_{18} (Macherey-Nagel, Düren, G.F.R.), Spherisorb-ODS (Phase Separations, Queensferry, Great Britain) and LiChrosorb RP-18 and RP-8 (Merck, Darmstadt, G.F.R.), all of which were obtained via Chrompack UK (London, Great Britain). Zorbax-C_8 monolayer (DuPont, Hitchin, Great Britain) was tested as a pre-packed 250 × 5 mm I.D. column. All columns gave comparable efficiencies of 20,000–30,000 theoretical

plates (TP)/m as determined by isocratic polypeptide elution at flow-rates of 1–1.5 ml/min (800–1200 p.s.i.).

Eluted polypeptides and proteins were sequentially detected by UV-absorption and endogenous fluorescence (275 nm activation; emission filter 370 nm), the latter being usable only with tryptophan-containing compounds. The UV-absorption wavelength used (225 nm) was as close to the isosbestic point (215 nm), at which molar absorption of polypeptides is independent of conformation[10], as was compatible with the use of our batches of acetonitrile (minimum transmission 50% at 205 nm) under conditions of gradient elution. Typical limits of sensitivity under these conditions were >100 ng per peak (UV) and >10 ng per peak (fluorescence)[1].

RESULTS

HPLC of polypeptides on Hypersil-ODS

The 32 polypeptides listed in Table I were injected onto Hypersil-ODS columns in 0.1 *M* NaH_2PO_4–H_3PO_4 buffer (pH 2.1, total phosphate concentration 0.2 *M*) at a flow-rate of 1–1.5 ml/min. After a loading phase of 3 min, during which polypeptides were concentrated on the head of the column while most amino acids, and other small compounds such as cyclic nucleotides and bioamines were eluted rapidly by the primary solvent, the peptides were sequentially eluted at ambient temperature using acetonitrile as the secondary solvent. The binary gradient profile was composed of three linear segments, lasted 50 min, and was terminated when the acetonitrile concentration reached 60% (v/v) (Fig. 1), this being the highest concentration of this organic modifier compatible with solubility of phosphate buffer salts after mixing. This system has been shown previously to effectively separate the lipotrophin-ACTH-endorphin-enkephalin compounds[1].

All of the other polypeptides tested that are listed in Table I were also successfully chromatographed on Hypersil-ODS using this system. Fig. 1 illustrates some of the separations achieved, with the characteristic absorption–fluorescence profiles of these mixtures aiding in the identification of individual polypeptides. The separation of members of the ACTH-endorphin group has been illustrated previously[1]. The retention times of all polypeptides tested, which ranged from 8.5 to 46 min, are given in Table II. Effective resolution of many compounds was possible in a single run (Fig. 1) due to the notably small volume (0.5–1 ml) of solvent in which each was recovered, irrespective of the time elapsed from injection to elution. Resolution was further enhanced by the reproducibility of retention times for all the polypeptides (coefficient of variation <1.0%). Experiments with radiolabelled polypeptides (^{125}I-ACTH and ^{125}I-calcitonin) gave recoveries of >95% and no loading effects were noted on fresh columns.

HPLC of proteins on Hypersil-ODS

Although the polypeptides successfully chromatographed included compounds such as insulin (MW 5800) and β-lipotrophin (MW 11,600)[1] the general use of this method for separating both polypeptide and protein hormones and hormone precursors of biological interest is obviously contingent on a capacity to chromatograph much larger compounds.

The pore size of Hypersil silica support (100 Å) is theoretically compatible

TABLE I

POLYPEPTIDE HORMONE SEQUENCE, USING AMINO ACID ABBREVIATIONS GIVEN IN DAYHOFF[28]

Compounds marked (*) were tested as isolated from natural sources, the remainder as synthetic polypeptides.

Peptide	*Amino-acid residues*	*Sequence*
Met-enkephalin	5	Y G G F M
Leu-enkephalin	5	Y G G F L
$ACTH_{5-10}$	6	E H F R W G
$ACTH_{34-39}$	6	A F P L E F
β-LPH_{39-45} (ovine)	7	K K D S E P Y
$ACTH_{4-10}$	7	M E H F R W G
Angiotensin II	8	D R V Y I H P F
Substance P_{6-11}	8	P Q Q F F G L M
Oxytocin	9	C Y I Q N C P L G
Arginine vasopressin	9	C Y F Q N C P R G
Lysine vasopressin	9	C Y F Q N C P K G
Arginine vasotocin	9	C Y I Q N C P R G
Substance P	11	R P K P Q Q F F G L M
α-Melanotrophin (MSH)	13	S Y S M E H F R W G K P V
Neurotensin	13	Z L Y E N K P R R P Y I L
Somatostatin	14	A G C K N F F W K T F T S C
Bombesin	14	Z Q R L G N Q W A V G H L M
α-Endorphin	16	Y G G F M T S E K S Q T P L V T
Gastrin I (human)	17	Z G P W L E E E E E A Y G W M D F
$ACTH_{1-18}$	18	S Y S M E H F R W G K P V G K K K K
*Insulin A (bovine)	21	G I V E Q C C A S V C S L Y Q L E N Y C N
*$ACTH_{18-39}$ (CLIP)	22	R P V K V Y P N G A E D E S A E A F P L E F
$ACTH_{1-24}$ (Synacthen)	24	S Y S M E H F R W G K P V G K K R R P V K V Y P
*Melittin	25	G I G A V L K V L T T G L P A L I S W I K R K R Q
*Glucagon (porcine)	29	H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T
*Insulin B (bovine)	30	F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A
β-Endorphin (ovine)	31	Y G G F M T S E K S Q T P L V T L F K N A I I K N A H K K G Q
Calcitonin 1 (salmon)	32	C S N L S T C V L G K L S Q E L H K L Q T Y P R T N T G S G T P
Calcitonin (human)	32	C G N L S T C M L G T Y T Q D F N K F H T F P Q T A I Q V G A P
$ACTH_{1-39}$ (human)	39	S Y S M E H F R W G K P V G K K R R P V K V Y P N G A E D E S A E A F P L E F
*$ACTH_{1-39}$ (porcine)	39	S Y S M E H F R W G K P V G K K R R P V K V Y P N G A E D E L A E A F P L E F
*β-Lipotrophin (human)	91	E L A G A P P E P A R D P E A P A E G A A A R A E L E Y G L V A E A Q A A E K K D E G P Y K M E H F R W G S P P K D K R Y G G F M T S E K S Q T P L V T L F K N A I V K N A H K K G Q

with the access of globular proteins of up to 40,000–50,000 daltons[13]. The evident lack of direct correlation between molecular weight and retention times of many of the polypeptides tested (Table II) indicated, however, that the behaviour of larger

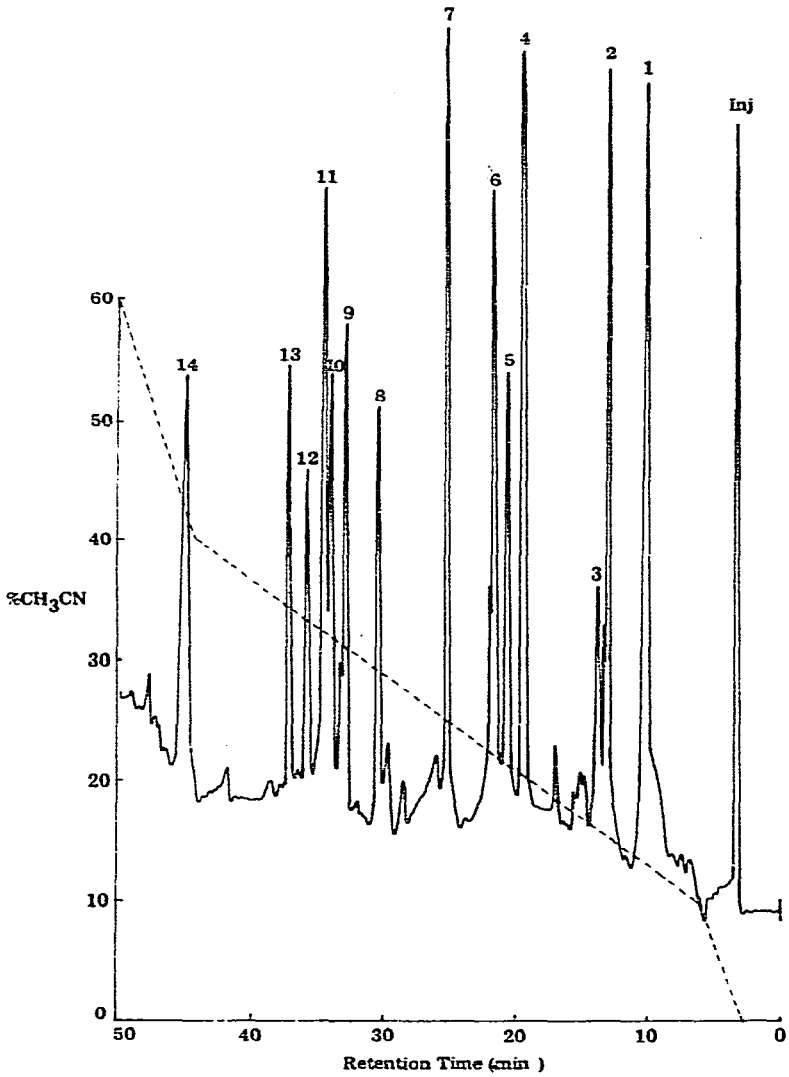

Fig. 1. Chromatogram of polypeptide standards separated at ambient temperature on Hypersil-ODS (100 × 5 mm I.D.). Primary solvent: 0.1 *M* NaH_2PO_4–H_3PO_4 (pH 2.1 total phosphate concentration 0.2 *M*); secondary solvent: acetonitrile. The gradient profile is given by the dotted line. 1–10 μg of each polypeptide was injected in primary solvent with 500 ng of tryptophan as an internal standard and eluted compounds were detected at 225 nm (0.16 a.u.f.s.). Inj.: injection artifact; 1 = tryptophan; 2 = lysine vasopressin; 3 = arginine vasopressin; 4 = oxytocin; 5 = $ACTH_{1-24}$; 6 = insulin A; 7 = bombesin; 8 = substance P; 9 = somatostatin; 10 = insulin B; 11 = human calcitonin; 12 = glucagon; 13 = salmon calcitonin; 14 = melittin. Polypeptide sequences are given in Table I.

proteins might be unpredictable with this system. A series of proteins ranging in size from 8000 to 65,000 daltons, and including several ^{14}C-methylated compounds, was therefore tested on Hypersil-ODS using the system illustrated in Fig. 1. They included cobra neurotoxin 3 (*Naja naja siamensis*, MW 7800), horse ^{14}C-cytochrome *c* (11,700), porcine pancreatic ribonuclease A (13,700), chicken lysozyme (14,300), bovine lactal-

TABLE II

RETENTION TIMES OF POLYPEPTIDE AND PROTEIN STANDARDS CHROMATOGRAPHED ON HYPERSIL-ODS WITH 0–60% ACETONITRILE GRADIENT IN pH 2.1 0.1 *M* NaH_2PO_4–H_3PO_4 BUFFER AT AMBIENT TEMPERATURE AND AN INITIAL FLOW-RATE OF 1 ml/min

Gradient profile is illustrated in Fig. 1.

Standard	*Retention time (min)*	*Hydrophobicity**	
		<15 residues	*>16 residues*
β-LPH_{34-39} (ovine)	8.5	0.67 (0.67)	
L-Tryptophan**	10.0	—	
Arginine vasotocin	12.0	1.71 (1.63)	
Lysine vasopressin	13.0	1.96 (1.88)	
Arginine vasopressin	14.0	1.96 (1.88)	
$ACTH_{5-10}$	17.0	2.49 (2.49)	
Phenylalanylphenyalanine (Phe 2)	18.0	2.42 (2.42)	
$ACTH_{1-18}$	18.5		3.64 (3.16)
Met-enkephalin	19.0	1.93 (1.88)	
Oxytocin	19.5	2.67 (2.59)	
$ACTH_{4-10}$	20.5	2.54 (2.49)	
$ACTH_{1-24}$	21.5		5.17 (3.83)
α-Endorphin	22.0		3.32 (2.84)
Leu-enkephalin	22.0	2.84 (2.84)	
Insulin A (bovine)	22.0		5.08 (4.22)
Angiotensin II	23.0	3.27 (2.84)	
Cobra neurotoxin 3	24.0		—
Neurotensin	24.5	4.22 (4.22)	
α-Melanotrophin	26.0	3.64 (3.16)	
Bombesin	26.0	3.68 (3.20)	
RNAse	27.5		—
Phe 3	28.0	3.63 (3.63)	
Gastrin I	28.5		5.45 (5.40)
Substance P	29.0	3.43 (3.38)	
Substance P_{4-11}	30.0	3.43 (3.38)	
$ACTH_{1-39}$ (human)	30.5		8.55 (7.21)
$ACTH_{18-39}$	30.5		4.81 (4.05)
$ACTH_{34-39}$	31.0	3.38 (3.38)	
Somatostatin	32.0	5.01 (4.91)	
Insulin (bovine)	32.0		15.18 (13.03)
$ACTH_{1-39}$ (porcine)	33.0		9.51 (8.17)
Insulin B (bovine)	33.5		10.1 (8.81)
β-Endorphin (ovine)	34.0		7.41 (6.93)
β-Lipotrophin (human)	34.5		14.07 (12.68)
Calcitonin (human)	34.5		7.66 (7.18)
Cytochrome *c*	35.0		—
Glucagon	36.0		7.44 (6.96)
Phe 4	36.5	4.84 (4.84)	
Calcitonin (salmon)	37.0		5.9 (5.47)
Lysozyme	37.5		—
Bovine serum albumin (BSA)	43.0		—
Myoglobin	45.0		—
Melittin	46.0		8.86 (8.0)

* Sum of fragmental constants (Rekker) for hydrophobic amino acids (W + F + L + I + Y + V + C-C + M). Value for strongly hydrophobic residues only (W + F + L + I + Y) given in parentheses. No correction for terminally located residues has been made.

** Internal standard.

bumin (14,200), whale myoglobin (17,200), porcine pancreatic elastase (25,000), ^{14}C-ovalbumin (42,000) and ^{14}C-bovine albumin (65,000).

These proteins were used as test compounds instead of hormonal proteins because of their known sequences and structures in most cases, and the heterogeneity encountered in some preparations of the hormones. A number of them, including neurotoxin, myoglobin, cytochrome *c* and lysozyme, could be reproducibly chromatographed and separated on Hypersil-ODS (Fig. 2). Lactalbumin, elastase and ovalbumin, however, were apparently irreversibly bound to the column packings when injected in small (1–10 μg) amounts.

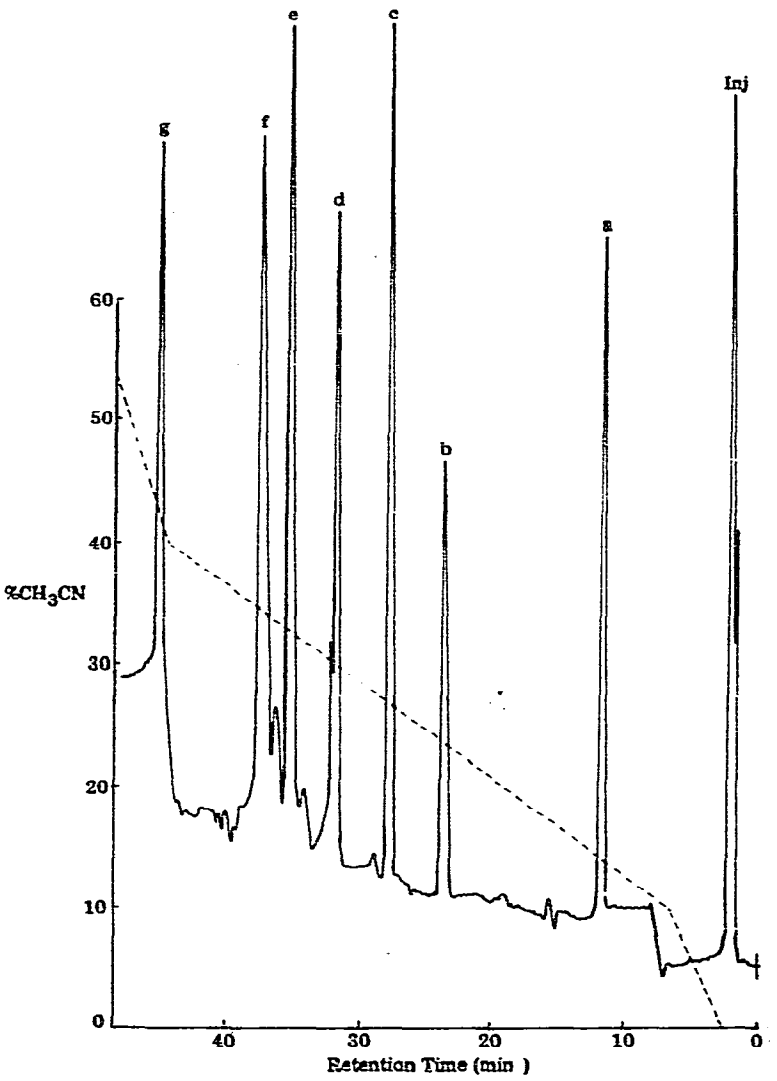

Fig. 2. Chromatogram of protein standards separated at ambient temperature on Hypersil-ODS with chromatographic conditions as given in Fig. 1. a = Tryptophan; b = cobra neurotoxin 3; c = ribonuclease A; d = insulin (complete molecule); e = cytochrome *c*; f = lysozyme; g = myoglobin.

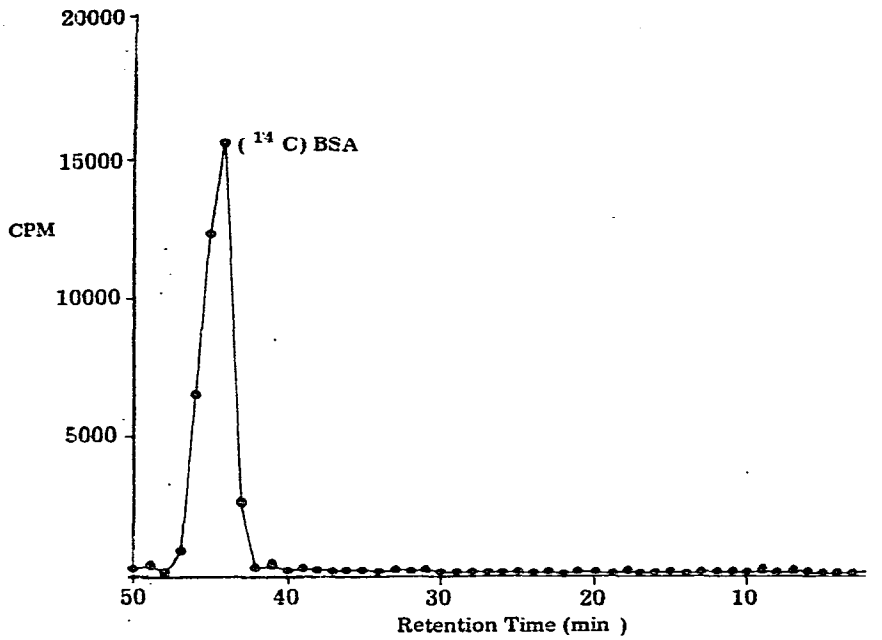

Fig. 3. Radiochromatogram of 3 μg ^{14}C-methylated bovine serum albumin chromatographed under gradient elution conditions given in Fig. 1.

The much larger bovine serum albumin (MW 65,000) could, however, be reproducibly chromatographed. It gave a rather broad peak (Fig. 3), with recoveries of only 30–40% when small amounts (1–5 μg) of radiolabelled protein were used. The remaining activity eluted in the same place on the gradient on a subsequent blank run. Injection of larger quantities gave better recoveries (<80%) with proportionately less "bleed-off" on subsequent runs, indicating that a loading effect may be observed with some larger proteins. This problem was also encountered, albeit to a lesser degree, with some of the smaller late-eluting proteins such as lysozyme and myoglobin, but not with late-eluting small polypeptides such as melittin (MW 3150) (Fig. 1). Retention times of the proteins were notably more sensitive to small changes in solvent flow-rate than those of the polypeptides, with typical variances of <5% compared with <1% for the latter.

HPLC of "Pitressin" on Hypersil-ODS

Pitressin is a pharmaceutical posterior pituitary extract containing the majority of the pressor activity of the gland[14]. It also contains corticotrophic-hormone-releasing activity, some, but not all of which, can be accounted for by its content of vasopressin[15]. It has been used, therefore, to examine the capability of the ODS-reversed-phase system described above for separating natural mixtures of biologically active compounds. Because of the problems encountered with incomplete elution of protein standards (see above) it is essential for biological work that such mixtures be separated using a "dedicated" column, with standards run separately. The precision with which retention times are reproduced, even on separate columns, makes this practically feasible.

Fig. 4 shows the range of compounds obtained from Pitressin. A series of at least five early eluting UV-absorbing, but non-fluorescent peaks corresponded in retention time to vasopressin standards (Fig. 1, Table II). The remainder of the peaks, most of which were fluorescent and therefore not degradation products of the vasopressins have not yet been identified. Several show demonstrable biological activity after chromatography, however, and they may also include neurophysins (MW $\approx$ 10,000), which have been recently shown to be chromatographable on reversed-phase supports[10].

Mechanism of polypeptide–protein HPLC on alkylsilane-type packings

It is evident from Table II and Figs. 1–3 that separation of polypeptides and proteins on ODS-silica is not determined directly by size. To ascertain which factors influence these separations, a systematic examination of the effect of changing key chromatographic parameters was undertaken. This served both to elucidate the mechanism(s) of chromatography and to identify the variables that could be most effectively used to optimize specific separations.

Column packing type. In previous studies on the separation of steroid hormones by reversed-phase HPLC[16] we have shown that considerable selectivity resides in the precise nature of the column packing, (with the accessible silanol groups of otherwise nominally identical ODS-type packings correlating with their resolving power in respect of some closely related compounds). In the present studies of polypeptide chromatography no such obvious differences were noted.

Retention times constant to $\pm 5\%$ and no changes in retention orders were noted with a series of ACTH-related compounds (including $ACTH_{4-10}$, $ACTH_{1-24}$ and $ACTH_{1-39}$) using both spherical (Nucleosil, Hypersil and Spherisorb) and irregular (LiChrosorb RP) packings in conjunction with the acetonitrile–acid-phosphate gradient system described above. Furthermore, polypeptides could be chromatographed with virtually identical retention times ($\pm 2\%$) on both C_8 alkylsilane-bonded packings (*e.g.* RP-8) and C_{18} packings (RP-18).

Organic modifier. Previous studies have also shown that the nature of the organic modifier can confer useful selective effects on the chromatography of steroid hormones in biological samples[17]. To ascertain whether similar effects were applicable to polypeptide separations a series of water-miscible UV-transparent solvents ranging in polarity from methanol (ε^0 $Al_2O_3 = 0.95$) to tetrahydrofuran (0.45) have been compared with acetonitrile (0.65), in conjunction with the acid-phosphate primary solvent in a single linear gradient system (Table III).

Retention times of all polypeptides tested were shifted in accord with the polarity of the solvent being used. Of more practical significance, however, was the fact that some reversals in retention order were apparent with certain solvents (*e.g.* insulin and $ACTH_{1-39}$ in methanol), with corresponding shifts in relative retention times (Table III). Thus the nature of the organic modifier affords a limited degree of selectivity.

Isocratic chromatography of polypeptides revealed that the majority of compounds could only be efficiently chromatographed over a very narrow range of organic modifier concentration, different for each polypeptide and above which they were very rapidly eluted and below which they were strongly bound to the column. This is illustrated in Fig. 5 which shows the capacity factor (k') for two closely eluted

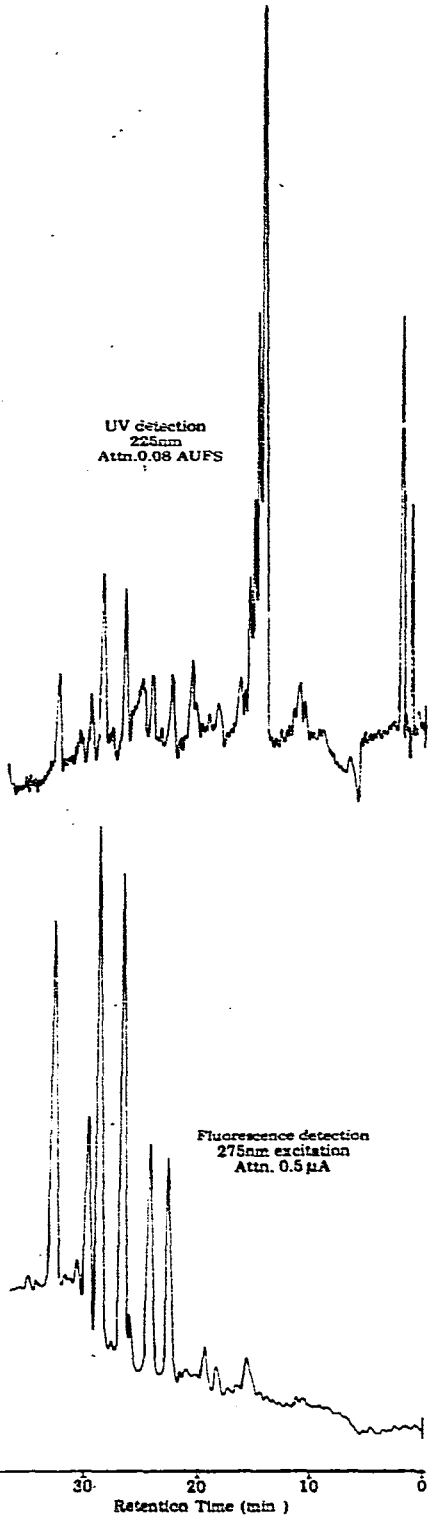

Fig. 4. Simultaneous UV- and fluorescence chromatograms of "Pitressin" chromatographed under gradient elution conditions given in Fig. 1. An aliquot of this posterior pituitary extract equivalent to 2 pressor units was injected.

TABLE III

EFFECT OF THE ORGANIC MODIFIER ON POLYPEPTIDE HPLC

Peptide	*Acetonitrile*	*Tetrahydrofuran*	*Dioxane*	*Methanol*
L-Tryptophan	8.0*	7.3	8.8	13.2
AVP	10.0	8.5	10.2	18.6
Met-enkephalin	13.2	14.3	15.2	23.6
$ACTH_{1-24}$	13.6	13.0	15.4	26.0
Leu-enkephalin	14.8	16.6	18.0	34.8
α-MSH	15.2	15.4	18.0	28.4
Insulin	12.6	19.0	23.6	40.4
$pACTH_{1-39}$	18.5	20.6	25.4	35.4

* Retention times (min) of the compounds listed chromatographed on Hypersil-ODS (100 × 5 mm I.D.) with 0.1 *M* NaH_2PO_4–H_3PO_4 as the primary solvent are given. The organic modifiers listed were employed as the secondary solvent in a single linear gradient elution system of 50 min duration, giving a constant rate of change of 2% per min. Retention times are, therefore, not directly comparable to those illustrated in Fig. 1, in which a tripartite linear gradient was used.

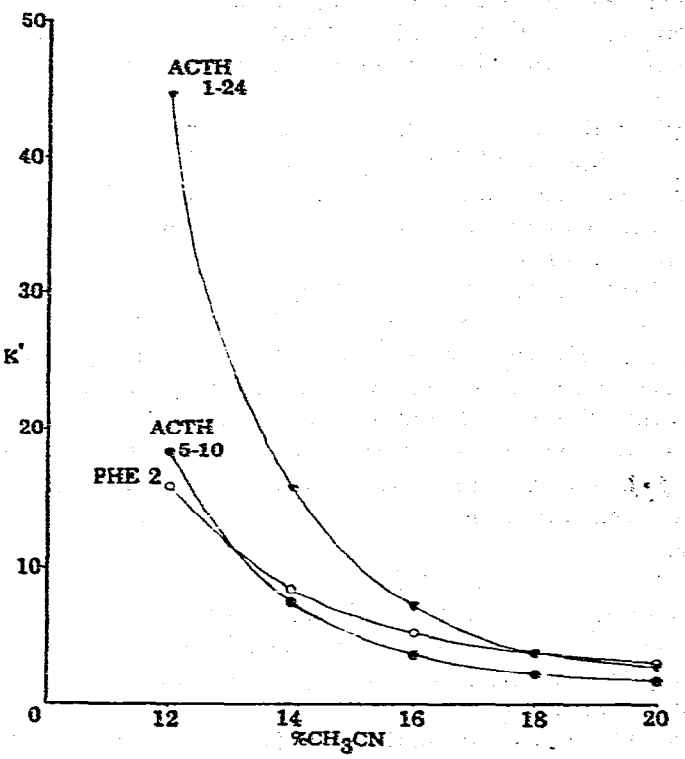

Fig. 5. Effect of organic modifier (acetonitrile) concentration on polypeptide retention times using Hypersil-ODS with 0.1 *M* NaH_2PO_4–H_3PO_4 as the primary solvent under isocratic conditions.

ACTH peptides of different size plotted against the organic modifier concentartion under isocratic conditions.

It is noteworthy that this stringency was not as acute in the case of very small peptides, leading to reversals in retention order in respect of phenylalanyl–phenylalanine and the ACTH peptides, but not the hormonal polypeptides themselves, as

the solvent concentration was increased and decreased in isocratic chromatography (Fig. 5). The narrow range of organic modifier concentrations over which individual polypeptides can be chromatographed results in sharp peaks and small elution volumes irrespective of absolute retention times (Figs. 1 and 2). It is also responsible for the precision with which individual retention times can be repeated[1] under conditions of gradient elution, thus contributing to both the resolving power and the reproducibility of the method.

Temperature and flow-rate. Although some polypeptide separations could be enhanced by elevated temperatures[1] using the system described here, there was in general not a great deal to be gained in terms of additional efficiency or resolution by this means. Thus, isocratic elution of $ACTH_{1-24}$ at a flow-rate 1 ml/min gave efficiencies of 15,000 TP/m at ambient temperature, rising to 26,800 TP/m at 40°, but slowly declining to 20,300 TP/m at 70° on Hypersil-ODS. The enhanced efficiency at 40° must, however, be balanced against the possibility of degradation of compounds in biological samples.

Under conditions of gradient elution, small temperature changes make little difference to the absolute retention times by which peak identities are assigned. Thus, raising the temperature from 40° to 70° reduced the retention time of $ACTH_{1-24}$ by only 15%.

Similarly, flow-rate changes made only a small difference to gradient elution times. At the extremes, the retention time of $ACTH_{1-24}$ was shortened by only 18% when the starting flow-rate was doubled using the system illustrated in Fig. 1, and it was lengthened by only 6% when the flow-rate was halved from 1 ml/min to 0.5 ml/min. Similar results were obtained with other polypeptides.

pH. The choice of a low pH for gradient elution of polypeptides and proteins on alkylsilane-bonded packings was determined originally by adaption of the system of Molnár and Horváth[2] shown to be effective for small peptides and subsequently applied successfully to polypeptides of the ACTH-endorphin series[1]. Although this system is compatible with retention of bio- and immunoactivity by $ACTH_{1-39}$, it does represent an unphysiological environment for active hormones. We have, therefore, examined the behaviour of selected compounds over a range of pH values while maintaining a total phosphate concentration of 0.1 *M* and using the acetonitrile gradient system illustrated in Fig. 1.

Compounds tested included human calcitonin, porcine $ACTH_{1-39}$ and $ACTH_{1-24}$, all of which chromatograph efficiently and reproducibly at pH 2.1 (NaH_2PO_4–H_3PO_4, 0.2 *M* total phosphate) and pH 2.3 (NaH_2PO_4–H_3PO_4, 0.1 *M* total phosphate) (Fig. 6A). Raising the pH did not change gradient retention times (34.5, 33 and 21.5 min, respectively) but at pH 4.5 (0.1 *M* NaH_2PO_4) there was a perceptible broadening of the ACTH peaks although the efficiency of calcitonin chromatography was preserved. At pH 6.2 (NaH_2PO_4–Na_2HPO_4, 0.1 *M* total phosphate) ACTHs were eluted as very broad peaks (Fig. 6B) and with further reduction in acidity of the aqueous buffer they were lost entirely. Calcitonin, unlike the ACTHs, was chromatographed efficiently at both pH 6.2 and 8.5. Returning to the original conditions (*i.e.* pH 2.1), using the same column, restored the optimum efficiencies for the ACTHs. Thus for the most efficient chromatography and resolution of all polypeptides on alkylsilane packings it would appear that a low pH (<4.0) is necessary, although less extreme conditions may be used with certain compounds.

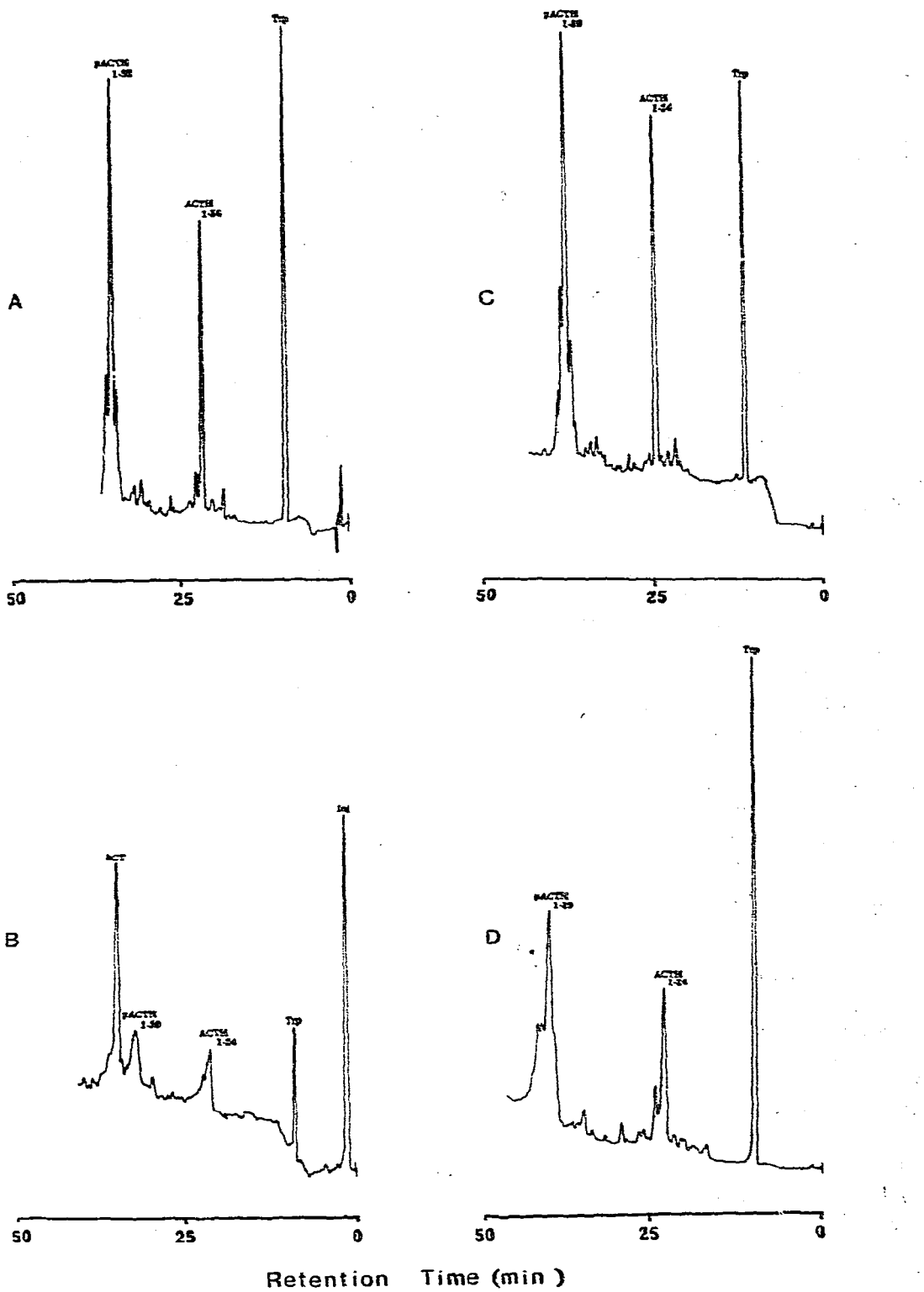

Fig. 6. Effect of primary solvent composition on polypeptide chromatography with acetonitrile gradient elution as illustrated in Fig. 1. (A) NaH_2PO_4–H_3PO_4 with 0.1 *M* total phosphate concentration (pH 2.3); (B) NaH_2PO_4–Na_2HPO_4 with 0.1 *M* total phosphate concentration (pH 6.2); (C) NaCl–HCl with 0.16 *M* total chloride concentration (pH 2.1); (D) 0.1 *M* H_3PO_4 (pH 1.9). Compounds tested were 500 ng tryptophan (Trp), 5 μg Synacthen ($ACTH_{1-24}$), 10 μg of an impure porcine pituitary ACTH preparation ($pACTH_{1-39}$) and 10 μg synthetic human calcitonin (hCT) (0.16 a.u.f.s., 225 nm). Note loss of resolution of components of $pACTH_{1-39}$, and reduced efficiency of other compounds chromatographed under sub-optimal conditions (B,D).

Both the phosphate and the potassium in the original buffer system of Molnár and Horváth[2] can interfere when eluted compounds are returned to some bioassay systems. Substitution of sodium (NaH_2PO_4), as in the present study, for potassium (KH_2PO_4)[1] does not influence separations. The effect of substituting HCl for H_3PO_4 and 0.9% (0.16 *M*) NaCl for NaH_2PO_4 has been examined, giving an unbuffered acid–salt solution at pH 2.1 as the primary solvent to minimise further the effects of extraneous ions on bioassay and immunoassay systems. Fig. 6C shows the separations obtained with this primary solvent in the acetonitrile gradient. No difference from the NaH_2PO_4–H_3PO_4 system was noted, even with the separation of complex mixtures such as Pitressin, although some corrosion problems may be encountered with long-term use of unbuffered acid–salt solutions.

Primary solvent molarity. Lastly, the specific requirement for salt as well as acid in the aqueous primary solvent when separating polypeptides on Hypersil-ODS has been examined. A pH of approximately 2.0 was maintained and primary aqueous solvents modified while using the acetonitrile gradient system illustrated in Fig. 1.

Reduction of the total phosphate concentration of the NaH_2PO_4–H_3PO_4 buffer much below 0.1 *M* while maintaining a pH of approximately 2 was not possible owing to the contribution of the acid itself. Increasing the salt concentration to give a total phosphate of 1.0 *M* slightly shortened retention times for compounds such as $ACTH_{1-24}$ (21.5 to 19 minutes) and $pACTH_{1-39}$ (34.5 to 27 minutes), but did not significantly enhance resolution or efficiency. Problems of precipitation were encountered, however, with high salt concentrations under conditions of gradient elution with organic modifiers.

Deletion of the salt from the NaH_2PO_4–H_3PO_4 primary solvent had a deleterious effect on polypeptide chromatography, even when the total phosphate concentrations was maintained. Thus 0.1 *M* H_3PO_4 alone (pH 1.9) resulted in significant lengthening of retention times for larger polypeptides such as $ACTH_{1-39}$ and, more important, in marked loss of resolution (Fig. 6D) compared with NaH_2PO_4–H_3PO_4 at a total phosphate concentration of 0.1 *M* (pH 2.3), when both were used in conjunction with the acetonitrile gradient system (see Fig. 1). Increasing the H_3PO_4 concentration to 0.2 *M*, when the acid was used alone, restored some of the lost efficiency, but the low pH of this primary solvent (pH 1.75) renders it unsuited for prolonged use with chemically-bonded silica packings, as indeed does that of 0.1 *M* H_3PO_4 (pH 1.9).

Acetic acid commends itself as a potential component of a polypeptide HPLC system because of its volatility, which facilitates processing of samples for subsequent radioimmuno- and bioassay. When used alone in conjunction with the acetonitrile gradient, however, it resulted in very poor chromatography of a variety of polypeptide standards and Pitressin at 1.0 *M* (pH 2.3) and at pH 2.1 (1.26 *M*), thus negating this potential advantage. Furthermore, its intrinsic UV-absorbance also precludes detection of polypeptides and proteins at their isosbestic point (215 nm), and the use of acetic–pyridine mixtures[11] strongly quenches endogeneous tryptophan fluorescence, precluding detection by this method.

DISCUSSION

The object of this study has been the development of HPLC systems capable

of separating a wide range of hormonal polypeptides and their protein precursors, similar to that we have reported elsewhere[1] for the separation of members of the lipotrophin-endorphin-ACTH-enkephalin "family". A wider range of polypeptides, and proteins of known sequence and structure have now been examined using reversed-phase HPLC packings of the alkylsilane type. Optimization of their chromatography and resolution has been attempted and efforts made to determine whether correlations between chromatographic behaviour, sequence and structure occur. Specifically we have sought a system which can separate with high recoveries both large and small proteins and polypeptides under conditions compatible with retention of biological activities.

Similar criteria have been recently specified by Rivier[12] who has developed several systems based on novel tetraethylammonium and trialkylammonium phosphate buffers, using a variety of alkylsilane-, nitrile- and alkylphenyl-bonded silica HPLC packings, which are capable of separating some proteins and polypeptides similar to those tested here, with varying efficiencies. All 32 polypeptides (Table I) tested in the present study were chromatographed with high efficiency, reproducibility recovery and resolution using a single system of gradient elution with an organic modifier in a simple acid–salt solution of low pH and high molarity using only alkylsilane-type RP packings.

Since the original observations of Burgus and Rivier[3] on separation of hypothalamic peptides by HPLC, a number of studies of individual small peptides, some polypeptides and a few proteins chromatographed on reversed-phase type supports have been reported[2–12]. Few systems have, however, been specifically optimized for separation of complex biological mixtures, and most studies have been confined to a small number of compounds. Various approaches to peptide HPLC have been recently reviewed by Stein[11] with specific attention to detection of peptide fluorophors, but a general method has yet to emerge. The high resolving potential of HPLC has not yet, in our opinion, been fully exploited in most studies, where it has usually been used in the final stages of peptide purification[3,11].

The use of RP-HPLC systems for separating biopolymers has been given impetus from the relative lack of success with systems based on steric exclusion. Controlled porosity glasses (CPG) and silicas can be used for biopolymer HPLC[18], but the resolution and recoveries afforded by these supports with polypeptides and proteins in aqueous media are, in our experience, substantially inferior to those obtained with synthetic polymers in non-aqueous eluting solvents[13]. Irreversible adsorption of biopolymers is a major problem even when buffers designed specifically to minimise this problem are used[19]. The ion-exchange HPLC of proteins on derivatised glasses (glycophase–CPG) pioneered by Chang *et al.*[20] has suffered a setback from current non-availability of microparticulate (5–10 μm) controlled porosity glasses.

Optimization of reversed-phase HPLC for the larger polypeptides and proteins by systematic variation of all chromatographic parameters has seldom been reported in previous studies and selection of appropriate conditions for specific problems has been complicated by the widely different conditions that have been used, sometimes within the compass of a single study[7]. The choice of optimal chromatographic conditions can also be compromised by the requirements imposed by different detection systems utilising UV-absorbance, UV-fluorescence or visible fluorescence, and the

quest for ultimate limits of sensitivity. The present study has made it abundantly clear that low pH and high molarity of the aqueous solvent used are mandatory for efficient chromatography and resolution of these compounds. These requirements are probably due to a necessity to minimise ionic and absorptive interactions with the reversed-phase support[3,6,21,22] and to suppress ionisation of ampholyte acidic moeities which can cause peak doubling as different charged entities are formed from single peptides at high pH-values[22]. As we have shown elsewhere[1] these conditions need not compromise the recovery of bioactive materials. Optimal conditions are not obtained with the use of dilute acids alone (*e.g.* 0.1 % H_3PO_4) as used in some studies[7,8] and salt plus acid mixtures are required to obtain the necessary anion concentration without exceeding the pH limits of the columns (>pH 2).

The present study has also shown that simple unbuffered mixtures of NaCl and HCl can be substituted for phosphate without materially altering polypeptide chromatography. This has the advantage of minimising the contribution of extraneous non-physiological ions to subsequent assays, but stainless steel components of the chromatograph may require passivation to prevent corrosion. No detectable breakdown of polypeptides during chromatography at ambient temperature has been noted. Elevated temperatures, as used by Molnár and Horváth in their original system[2], are neither desirable for recovery of bioactive materials, nor necessary as loss of polypeptide chromatographic efficiency has been noted above 45° in this and another study[12].

Although other studies have shown that alkylsilane supports give superior resolution and recovery of small peptides, compared with the amine and nitrile-type derivatised silica[22] it is, however, by no means certain that this is also true for proteins. The failure of some proteins to elute from alkylsilane supports using the present system is clearly a major limitation to its general use for the larger compounds, although the largest compound tested (BSA) could be recovered (Fig. 3).

This selective retention highlights the question of the mechanism of chromatography. Most workers are agreed that separations of peptides on reversed-phase supports are dictated primarily by hydrophobicity[2,3,6,9,12,23,24]. The formation of ion pairs with the buffer acid anion as the counter-ion has also been suggested[7]. Under gradient elution conditions, as in the present study, overall hydrophobicity undoubtedly predominates as the relevant structural feature determining retention orders (Table III). The theory of hydrophobic interaction chromatography has been detailed by Horváth *et al.*[24] and applied to the behaviour of small peptides during HPLC by Molnár and Horváth[2] who have related the retention of individual amino acids and their oligomers with their lipophilicity, expressed in terms of the fragmental hydrophobic constant of Rekker[25].

We have extended this numerical analysis to the sequence of the polypeptide standards used in this study. The contributions of all individual hydrophobic residues, in terms of their absolute fragmental constants[25], have been summed for each compound (Table II). This procedure predicts with reasonable accuracy the retention order of smaller polypeptides (<15 residues), but it is empirically improved by confining the calculation to the five most strongly hydrophobic residues (Table II). Analogous calculations based on the hydrophobicity scale of Nozaki and Tanford[26] were less accurate in predicting retention orders.

For larger polypeptides (>16 residues) the predictive value of hydrophobic

fragmental constant summation was diminished, and many anomalies were noted (Table II). Some pairs of related larger compounds (*e.g.* porcine and human ACTH) were eluted in the order predicted by their hydrophobic amino-acid content (Table I) but even this limited relationship did not always hold true (*e.g.* human and salmon calcitonin). Discrepancies of this sort are not unexpected, since secondary and tertiary structure will modify the number of exposed hydrophobic residues in the larger compounds. It is, however, interesting that anomalies occur even with relatively small polypeptide hormones in which stable secondary structures are not anticipated[27].

With the proteins proper, a general correlation of retention order and hydrophobicity (as mole % of hydrophobic residues) can be discerned (Table IV) in spite of the limited number of hydrophobic residues exposed in aqueous solution. No correlation of retention order of proteins with size or basic amino-acid content is evident in the present study (Table IV). The retention orders of the proteins tested recently in a similar HPLC system by Mönch and Dehnen[9] appear, therefore, to have coincided with their molecular weights by chance.

TABLE IV

AMINO ACID CONTENT OF PROTEIN STANDARDS

	Protein amino acids (mole%)						
	Lactalbumin	*Elastase*	*Myoglobin*	*Lysozyme*	*Cytochrome c*	*RNAse*	*Neurotoxin*
Acidic (D + E)	13.8*	4.2	13.1	7.75	11.5	6.5	7.0
Basic (K + H + R)	13.0	8.75	22.9	13.9	23.1	14.5	15.5
Hydrophobic (F + W + Y + V + M + L + I)	35.8	32.5	31.3	26.4	25.0	21.8	21.1

* Figures for acidic, basic and hydrophobic residues were calculated from sequences given by Dayhoff[28] for the specific proteins used.

The potential of reversed-phase HPLC for large compounds, is presently limited by the partial recoveries and occasional irreversible binding of certain proteins. This phenomenon may be due to the presence of residual free silanol groups on the derivatised packings. In this case the problem may be minimised by selection of alkylsilane-type RP supports with the least number of adsorptive sites[16]. Alternatively, less strongly hydrophobic column packings may be used. Rivier[12] has, however, shown that these have a reduced resolving capability for some large polypeptides in comparison with the strongly hydrophobic packings of the alkylsilane type. The use of ternary gradient elution systems with other solvents such as methoxyethanol, as used by Mönch and Dehnen[9] may be of value in recovering larger proteins. On the basis of the results obtained in this study, reversed-phase HPLC will, however, remain the method of choice for most polypeptide separations, and will serve to separate some proteins until efficient, high recovery, steric exclusion HPLC methods are devised for biopolymers.

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