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EFFECT OF STATIONARY AND MOBILE PHASES ON HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS AND PEPTIDES

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SUMMARY

A number of different stationary phases designed for hydrophobic interaction chromatography have been examined to assess their efficiency and resolving capability with respect to protein and peptide mixtures A packing with an ether-bonded phase was substantially less hydrophobic than those with propyl- or phenyl-bonded surface chemistry. While the overall efficiencies of most columns were broadly similar with respect to most proteins, some proteins did chromatograph with enhanced efficiency on specific packings. The elution order of individual proteins was, with one or two exceptions, similar for all columns tested using comparable mobile phases. It differed, however, substantially from orders obtained with conventional reversed-phase alkyl-bonded phases and from the elution orders obtained when the hydrophobic packings were used in a reversed-phase mode, i.e. with an organic modifier gradient. Varying the salt used in the mobile phase and its pH under hydrophobic interaction conditions (high ionic strength) changed overall retentivities and also altered specific retention orders, thus offering possibilities of selective resolution of some mixtures.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) was originally developed with soft-gel supports [1]. The first rigid matrices suitable for high-performance liquid chromatography (HPLC) were reported by Chang et al. [2], who

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used polyethylene oxide-derivatised controlled porosity mesoparticulate (30–70 μm) glass beads. In recent years a number of stationary phases have been designed specifically for the high-performance HIC [3–6] which are based on both microparticulate (5–10 μm) mesoporous (10–50 nm) silica and macroreticular polymer beads.

Because of their relatively low overall hydrophobicity, HIC systems do not necessitate the use of organic modifiers for elution, in contrast to the widely used conventional high-density reversed-phase (RP) packings [7]. Although the latter are now available with short alkyl chain-bonded phases (C_{3-4}) and wide pores (30 nm) for peptide and protein chromatography, they still require relatively harsh conditions for protein elution [8]. The shorter alkyl chain lengths result in some improvement in recovery, compared with longer-chain (C_{8-18}) packings [9], but changing the chain length does not generally afford useful selective effects with protein (as distinct from small molecule) chromatography [10].

Hydrophobic interaction (HI) phases with their sparsely distributed hydrophobic moieties apparently interact with only a limited number of hydrophobic residues or domains on proteins [11] in contrast to RP packings where most if not all such residues contribute to their overall retention [7]. Since HI phases have now been prepared with different covalently bonded ligands (e.g. alkyl, aryl, polyether) in different matrices (silica or polymer) there is a possibility of column-specific selective effects, which might be exploitable for specific protein separations.

Our present objective has been to compare some of these HI columns and packings and to attempt to define conditions under which column or mobile phase-specific effects can be used to enhance specific separations, either by differences in efficiency or altered selectivity.

EXPERIMENTAL

Materials

Solvents and mobile phase additives. Acetonitrile (S grade) was obtained from Rathburn (Walkerburn, U.K.); single glass-distilled water was prepared from Milli-Q feedstock. Salts were AR grade from Fisons (Loughborough, U.K.) or Analar grade (BDH, Poole, U.K.).

Solutes. Peptides were obtained from Universal Biologicals (Cambridge, U.K.) and proteins from Sigma (Poole, U.K.), except for human growth hormone (hGH) and its synthetic Met-hGH analogue which were a gift from Dr. D. Schulster (National Institute for Biological Standards, London, U.K.). Mouse epidermal growth factor (mEGF) was culture grade from Sigma. All proteins and peptides were dissolved in distilled water at 1 mg/ml, and 50 μg of each compound were injected. Their hydrophobic characteristics are listed in Table I.

Methods

Apparatus. Operating conditions were controlled using a Gilson Model 344 system, with eluted peptides and proteins monitored by UV absorbance (280 nm, LDC Spectromonitor III); bioassay of mEGF was carried out as described previously [12].

TABLE I

HYDROPHOBIC PARAMETERS OF PROTEINS AND PEPTIDES

Compound[a]	Abbreviation	Hydrophobicity	
		Summated Rekker constant[b]	Mol.-% hydrophobic residues[c]
Liver growth factor	GHK	0 (0)	
Angiotensin II	Ang	3.27 (2.84)	-
Bombesin	Bomb	3.68 (3.20)	-
Substance P	Sub P	3.43 (3.38)	-
Tyr8-substance P	Tyr-P	2.89 (2.84)	-
Neurotensin	Neur	4.22 (4.22)	-
ACTH 1-24	ACTH	5.17 (3.85)	-
Epidermal growth factor (mouse)	mEGF	-	30.2
Cytochrome c	Cyt c	-	25.0
Ribonuclease	RNase	-	21.8
Lysozyme	Lys	-	26.4
Growth hormone (22 kD) (human)	hGH	-	34.0
Growth hormone (synthetic)	Met-hGH	-	34.05
Bovine serum albumin	BSA	-	28.4

[a]In order of size.
[b]Sum of fragmental Rekker constants for W, F, L, I, Y, V, C-C and M (brackets = W+F+L+I+Y) (see ref. 7).
[c]F+W+Y+V+M+L+I.

TABLE II

HYDROPHOBIC INTERACTION COLUMNS

All columns were prepacked except SynChropak-Propyl which was slurry packed in the laboratory.

Type	Source	Dimensions (length × I.D.) (mm)	Particle size (μm)	Pore size (nm)	Bonded phase	Matrix	Ref.
Spherogel CAA-HIC	Beckman	100×4.6	5	30	Polyether	Silica	4
TSK-Phenyl 5PW	Anachem	75×7.5	10	>100	Phenyl	Polymer	3
SynChropak-Propyl	Alltech	75×7.5	6.5	50	Propyl	Silica	5
Phenyl-Superose HR5/5	Pharmacia	50×5	10	>100	Phenyl	Polymer	

Reversed-phase chromatography. RP-HPLC was carried out using a Beckman–Altex Ultrapore RPSC (C_3-bonded phase, 5 μm particle size, 30 nm pore size) column (75 mm×4.6 mm I.D.) at 40°C with a flow-rate of 1.0 ml/min and a gradient of acetonitrile in 0.15 *M* NaCl–0.01 *M* HCl (pH 2.1).

Hydrophobic interaction chromatography. HI-HPLC was carried out using the columns listed in Table II, at room temperature with a flow-rate of 1.0 ml/min, using linear gradients of decreasing ionic strength.

RESULTS

Relative hydrophobicity of different HI columns

To assess the relative hydrophobicity of the different columns, a series of peptides and proteins was eluted using an inverse gradient of ammonium sulphate in sodium phosphate buffer. Results are shown in Table III. Both phenyl and propyl functionalities gave similar overall results in terms of the salt con-

TABLE III

EFFECT OF STATIONARY PHASE ON HI-HPLC OF PROTEINS AND PEPTIDES

Compounds were eluted with a linear inverse gradient from 3 *M* $(NH_4)_2SO_4$ to 0 *M* over 30 min in 0.1 *M* sodium phosphate (pH 8.0). Other conditions as described in Experimental Results are expressed as the molarity of ammonium sulphate at which elution of a given compound was observed.

Compound[a]	Apparent molarity of elution[b]		
	TSK-Phenyl	SynChropak-Propyl	CAA-HIC
GHK	2.8	2.8	2.8
Cyt *c*[c]	1.61	1.18	1.72
RNase[d]	0.73	0.53	1.20
Neur	0.54	0.50	1.05
BSA	0.39	0.14	1.00
Ang	0.34	0.70	-
Lys[e]	0.33	0.24	1.06
Tyr-P	0.24	0.22	0.79
Sub P	0.23	-	-
ACTH	0.10	-	0.55
Bomb	0 (+0.5 min)	0 (+2.1 min)	0.46
hGH	N.D.	N.D.	0.41
Met-hGH	N.D.	N.D.	0.31
mEGF	0 (+5–20 min)	0 (+5–20 min)	0 (+1.1 min)

[a]See Table I for abbreviations.

[b]N.D. = not determined under conditions given but see Table IV Compounds marked - could not be detected, either because they eluted in the void volume or because peaks were extremely broad.

[c]Elutes at 1.59 *M* with Phenyl-Superose column.

[d]Elutes at 0.28 *M* with Phenyl-Superose column.

[e]Elutes at 0 *M* (+1.9 min) with Phenyl-Superose column.

centration at which a given protein or peptide was eluted. The silica- and polymer-based columns were also similar in this respect to the Phenyl-Superose column, whose structure resembles that of the original soft-gel-type HI matrices [1]. The polyether silica column (CAA-HIC), on the other hand, required substantially higher salt concentrations in order to retain these compounds, indicating that its surface was significantly less hydrophobic than the others, as with ether-derivatized polymer matrices [6].

HI column efficiencies

Using a standard test compound (lysozyme), the efficiencies of all the HI columns tested were similar and generally lower than RP columns used under gradient elution conditions. Some components of the protein test mixtures, such as bovine serum albumin (BSA), ran poorly on all the HI columns tested here, giving broad peaks approximately three or four times as wide as that, for instance, of lysozyme shown in Fig. 1. Other compounds showed very different peak shapes on different columns. For example, mEGF chromatographed efficiently on the CAA-HIC column, eluting in a sharp peak (3–4 min) at the end of the ammonium sulphate gradient. Its efficiency with both the TSK-Phenyl and SynChropak-Propyl columns was, however, so low that it was vir-

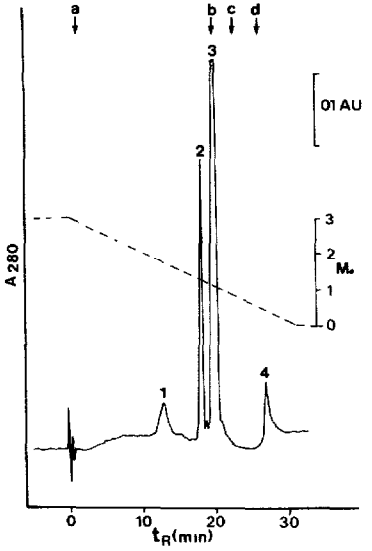

Fig. 1. Hydrophobic interaction chromatography of proteins and peptides using a CAA-HIC (Beckman-Altex) column. Compounds were eluted with an inverse gradient of $(NH_4)_2SO_4$ in 0.1 *M* sodium phosphate (pH 8.0) at room temperature and a flow-rate of 1.0 ml/min. Eluted peaks (5–10 μg): 1 = cytochrome c; 2 = RNase; 3 = lysozyme; 4 = synthetic hGH. The elution positions of the peptides GHK (a), neurotensin (b), Tyr8-substance P (c) and bombesin (d) are show for comparison.

TABLE IV

EFFECT OF STATIONARY PHASE ON RESOLUTION OF GROWTH HORMONES BY HI-HPLC

Compounds were eluted using a primary solvent of 1.8 *M* ammonium sulphate and a secondary solvent of 0.1 *M* sodium phosphate buffer (pH 8.0) over a 120-min linear gradient.

Column	Retention time (min)		Peak width at half height (min)	Separation/ peak width half height
	hGH	Met-hGH		
CAA-HIC	31.0	35.0	4.5	0.89
SynChropak-Propyl	93.7	96.8	2.2	1.41
TSK-Phenyl	108.0	111.5	2.1	1.67

tually undetectable by absorbance, although bioassay showed it to be present but spread out over about 20 ml of eluate.

The practical significance of such differences in peak width was apparent when attempting to resolve hGH and its synthetic Met-hGH analogue. The differences in retention times for this pair were similar on all columns tested (Table IV). When the polyether column was used, however, the peak widths were so great that the two peaks merged into one, precluding separation. With TSK-Phenyl and SynChropak-Propyl columns, on the other hand, virtually complete separation of the two proteins was achieved (Table IV; see also ref. 13).

Using the HI columns for the separation of smaller compounds showed that most peptides tested could be successfully eluted (Table III). However, they gave much broader peaks than with RP columns, comparable in efficiency to those of typical proteins under HI conditions.

HI column reproducibility

The behaviour of all HI columns and the reproducibility of retention times remained constant for extended periods of time (up to 100 h or 150 runs) when tested as described above. Under standard gradient elution conditions, replicate elution times did not differ by more than 0.3 min (equivalent to 0.03 *M* or ± 2%) with re-equilibration times of 20 min prior to each analysis.

Selective effects with HI columns

In the light of the differences in overall hydrophobicities noted above, it is difficult to make accurate comparisons of selectivity. In general terms, however, the different HI packings did not show marked individual selective effects with the protein standards tested here (Table III). However, one or two changes in retention order were seen between silica- and polymer-based packings (e.g. BSA and lysozyme). Elution orders did, however, differ markedly from those

TABLE V

ELUTION ORDERS OF PEPTIDES AND PROTEINS BY RP-HPLC COMPARED WITH HI-HPLC

Compound[a]	RP-HPLC[b]	HI-HPLC[c]
GHK	1	1
Ang	2	6
ACTH	3	9
Tyr-P	4	8
Neur	5	4
Bomb	6	10
RNase	7	3
Cyt c	8	2
Lys	9	7[d]
BSA	10	5[d]
hGH	11	11

[a]See Table I for abbreviations.
[b]RPSC (C_3) column; conditions as in Experimental.
[c]TSK-Phenyl; conditions as in Table III.
[d]Retention order reversed with SynChropak-Propyl and CAA-HIC (see also ref. 12).

TABLE VI

EFFECT OF SALT CONCENTRATION ON ELUTION OF PROTEINS FROM AN HI COLUMN USED IN RP MODE

Proteins were eluted from a TSK-Phenyl column with primary solvent of 0.1% (v/v) trifluoroacetic acid (pH 2.1) with indicated concentrations of NaCl; the secondary solvent was acetonitrile with a 2%/min gradient.

Compound	Retention time (min)		
	0 *M*	0.15 *M*	0.6 *M*
RNase	0	0	13.2
Cyt c	14.8	17.2	19.3
Lys	16.5	18.0	19.7
BSA	19.5	21.1	22.1

observed with reversed-phase chromatography (Table V), as noted in other studies [11]. The effective hydrophobicity of the proteins in aqueous solvents clearly differs from their molar hydrophobic amino acid contents (Table I) which do, however, correlate with their behaviour in RP systems [7].

The comparisons in Table V were made using different RP and HI columns. When a typical HI column (TSK-Phenyl) was used in an RP mode, i.e. under acid conditions with an organic modifier, it gave retention orders (and efficiencies) for proteins which matched those obtained with conventional RP

columns (Table VI), indicating that the differences were due to the mechanism of interaction rather than the column itself. High salt concentrations were, however, required in the primary solvent to obtain loading of some proteins (notably RNase) onto the HI column, prior to their elution with an organic modifier, unlike conventional high-density alkyl-bonded RP packings to which proteins bind under conditions of low ionic strength [8].

Effect of pH on HIC of proteins

The interactions of proteins with HI columns were strongly influenced by pH. Thus protein elution orders were altered at different acid pH values (Table VII) compared with mildly alkaline conditions (Table III). Under strongly acid conditions proteins were strongly adsorbed and BSA, for example, could not be eluted. By contrast, at pH 6 neither RNase nor cytochrome c could be loaded onto the column, even in the presence of 3.0 *M* NaCl. Further selectivity was obtained by the addition of an organic modifier to the secondary solvent under acid conditions. This reduced the elution time of lysozyme to a greater extent than that of BSA.

Effect of salt type on HIC of peptides

The smaller peptides did not interact with the HI columns operated under acidic conditions with salt gradients. At mildly alkaline pH different selectivities were obtained by varying the nature of the salt used and substituting Mg, Na or Li for ammonium ions. Marked differences were observed in effective resolution of different peptide pairs, under these conditions (Table VIII).

TABLE VII

EFFECT OF pH AND ORGANIC MODIFIER ON PROTEIN HI-HPLC

Compounds were eluted from a TSK-Phenyl column using a primary solvent of 3 *M* NaCl in 0.5 *M* ammonium acetate at the indicated pH and a secondary solvent of 0.5 *M* ammonium acetate with or without acetonitrile with a 30-min gradient. NA = not adsorbed, NE = not eluted.

Compound	Retention time (min)			
	pH 4.0	pH 6.0	pH 6.0	
			10% Acetonitrile	20% Acetonitrile
RNase	9.0	NA	NA	NA
Cytochrome c	29.0	NA	NA	NA
Lysozyme	25.0	23.3	21.5	17.2
BSA	NE	19.5	17.7	15.8

TABLE VIII

EFFECT OF SALT TYPE ON PEPTIDE HI-PHLC

Compounds were eluted from a CAA-HIC column at pH 7.5 using a 30-min gradient from 3 to 0 *M* $(NH_4)_2SO_4$, 2 to 0 *M* $MgSO_4$, 5 to 0 *M* NaCl or 10.4 to 0 *M* LiCl in 0.1 *M* sodium phosphate (ammonium and sodium salts) or 0.01 *M* Tris–HCl (magnesium and lithium salts). Values in parentheses are retention orders. N.D. = not determined.

Compound	Retention time (min)			
	$(NH_4)_2SO_4$	$MgSO_4$	NaCl	LiCl
Angiotensin II	17.4 (1)	13.0 (2)	5.1 (1)	6.0 (1)
Neurotensin	19.4 (2)	16.0 (3)	6.2 (2)	14.0 (2)
Tyr8-substance P	22.1 (3)	18.1 (4)	15.5 (3)	N.D.
ACTH 1–24	24.5 (4)	23.6 (5)	20.1 (5)	22.2 (3)
Bombesin	25.4 (5)	12.1 (1)	15.7 (4)	N.D.

DISCUSSION

HPLC is an indispensible tool in isolation, purification and characterization of proteins. Separations of proteins and peptides using RP packings with hydrophobic ion-pairing systems have been widely exploited. This method is suited to a wide range of smaller proteins [7]. Organic modifiers must, however, be used to elute these compounds from the highly hydrophobic high-density alkyl-bonded phases. These conditions may compromise bioactivity of larger proteins, either as a result of solvent denaturation or because of conformational changes caused by the stationary phase itself [14]. HIC proper is characterised by a low ligand density, permitting the use of aqueous mobile phases and minimising on-column denaturation and loss of activity [5,11,15]. Under conditions of moderate ionic strength ($\sim$0.5 *M*) weakly hydrophobic neutral non-ionic HI phases can also be operated in a size exclusion mode [4].

HI systems do, however, have certain limitations. In general, efficiencies are lower than with RP systems, and this can compromise some separations. Some differences in efficiency between polymer- and silica-based polyether-derivatized HI columns have been reported when they are operated in the size exclusion mode [16]; when aryl- and ether-derivatised polymer-based HI packings were compared they showed similar efficiencies [6]. Peak widths of individual components can also differ significantly with the same column, as seen here and noted by others [5]. This effect is due in part to the fact that both early- and late-eluting proteins are being chromatographed under isocratic conditions and are thus subject to extra peak broadening [11]. This is, however, not the whole explanation as the behaviour of some proteins (e.g. growth hormone, mEGF) seems to be determined by specific interactions with a particular type of packing. Other proteins show consistently poor efficiencies on different col-

umns. In the present study BSA invariably gave broad peaks with poor recoveries, including the ether-bonded CAA-HIC column. In other studies, methanol [17] or ether-bonded polymer weak hydrophobic phase [6] have been used to improve the efficiency with which it chromatographs under HI conditions. This poor efficiency is due to the specific type of chromatographic interaction involved because the same HI column (TSK-Phenyl) can be used in an RP mode to chromatograph BSA with much improved efficiency, provided that high salt concentrations are used to promote interaction with the bonded phase (Table VI). Longer-chain ether-bonded phases can also be operated in the RP mode, but without this additional constraint [4].

Column-specific selective effects can be obtained with HI systems, but are largely confined to the marked difference between aryl- and alkyl-bonded phases on the one hand, and the much less hydrophobic ether-bonded silica phase on the other (Table III). Ether-bonded polymer phases are also less hydrophobic than their aryl-bonded counter-parts [6]. In HI phases constructed from homologous series of alkyl substituents no specific selective effects were noted, although overall retentivity was increased from methyl to pentyl substituents [18]. Nevertheless, some column-specific selective effects were noted in the present study with the more strongly hydrophobic aryl- and alkyl-bonded phases, for example BSA and lysozyme (Table III), although in general elution orders and resolution were comparable. Likewise elution orders were broadly comparable between strong and weak silica-based hydrophobic phases, as reported for equivalent polymer phases [6], although again some anomalies were apparent (Table III). The resolving capabilities of the weaker phases are, however, compromised to some extent by the narrower range of ionic strengths over which most proteins are eluted.

The substantial differences in overall hydrophobicity between different HI phases lend themselves to trace enrichment procedures. Thus selective retention of the more hydrophobic proteins can be achieved while less retained compounds are rapidly eluted [19]. The value of different HI phases in this context lies in the ability to select one according to the retentivity of the desired protein, as no one phase affords effective resolution over quite such a wide range as classical RP systems.

Selectivity in HIC can also be altered by pH, as noted by Fausnaugh et al. [11] and by Gooding et al. [18]. These effects can be particularly dramatic, as in the case of cytochrome c when a substantial increase in apparent hydrophobicity was noted when the pH was reduced to 4.0 (Table VII). At even lower pH (2.0) this protein was completely adsorbed to the column, even in the absence of salt when other proteins tested were only partly adsorbed, or did not interact with the column at all. In other studies [18] selectivity changes in HIC have been observed over quite narrow pH ranges (6.0–6.8).

Selectivity can also be modified by different salts. The strength of interaction between proteins and HI stationary phases in the presence of different

salts has been related to their the molal surface tension [20]. Thus salts with higher molal surface tension increments produce higher protein retentions at equivalent concentrations. Generally it follows the lyotropic (Hofmeister) series [21]. However, in the presence of magnesium [21,22] and calcium [23] salts actual retention times for individual proteins deviated from those predicted, indicating that other interactions may occur, including interactions between the salt ions and the protein and effects on its hydration. The effect of detergents on HIC also indicates that retention is not simply a function of surface tension [24]. The considerable variations in peptide elution order noted in the present study with different salts (Table VIII) are consistent with multiple effects. Because of their small size, alterations in tertiary conformation seem unlikely to be involved in this instance. Barford et al. [25] have discussed the possibility of anomalous elution of proteins being caused by interaction of sulphate ions not only with proteins but also with the column packings, raising the possibility of mixed mode chromatography being observed.

Despite its lower efficiency compared with RP-HPLC, the present results together with the other studies referred to show that HIC is potentially a very versatile technique. There is ample opportunity for exploiting selective effects by changing the mobile phase conditions, notable in the choice of pH and salt. These effects in conjunction with the intrinsically milder conditions required make it a useful addition to the other modes of protein HPLC.

Conventional HIC has been used previously in the preparation of human prolactin [26], but the resolving power was insufficient to separate completely the prolactin from hGC. The HPLC packings, however, substantially extend the resolution which can be achieved, such that even differences as small as a single additional amino acid may be sufficient to allow separation as in that of native from recombinant hGH (ref. 13; Table IV). This resolving power may be of general importance in the purification of recombinant proteins such as hGH for therapeutic purposes, where mistranslated, improperly folded and thus inactive protein is often found in addition to the active product.

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