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USE OF HYDROPHOBIC INTERACTION METHODS IN THE ISOLATION OF PROTEINS FROM ENDOCRINE AND PARAENDOCRINE TISSUES AND CELLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have recently described the separation of a large number of polypeptide hormones, related peptides and some protein standards by hydrophobic interaction high-performance liquid chromatography (HPLC). This paper reports the practical application of these methods to the reproducible isolation and separation of components of a mixture of immunoreactive calcitonin-like proteins (<25 kD) synthesised and secreted by human tumour cells *in vitro*. Using hydrophobic interaction HPLC on ODS-silica for both preliminary bulk fractionation and subsequent analytical separation >80% recoveries of small (ng) quantities of immunoreactive proteins were obtained from samples containing <100 mg total protein, and characteristic profiles of synthesised and secreted materials were established. Using a partially purified hypothalamic extract, containing a number of small proteins (12–25 kD), we have also examined the effects of varying chromatographic conditions in an attempt to modify the separations obtained with ODS-silica using an acid-saline–acetonitrile gradient elution system at ambient temperature, and achieve further resolution of its components. No useful selective effects were observed when temperature, organic modifier, gradient profile or hydrophobic stationary phase were altered. These techniques may not therefore be inherently capable of completely resolving all components of natural protein mixtures. They do, however, offer an adjunct to and in certain cases a substitute for conventional methods of protein separation.

INTRODUCTION

We report here the high-performance liquid chromatography (HPLC) of some proteins in complex biological mixtures, using methods based on our previous studies of proteins and hormonal polypeptide standards[1,2]. The objective of this study was to evaluate the practical role of this technique in the isolation and separation of these materials and we have successfully applied it to two separate problems, which have involved the resolution and recovery of soluble proteins ranging from *ca.* 12 to 25 kD in weight.

Hydrophobic interaction chromatography of proteins was originally developed as a low-pressure technique using non-ionic and ionisable amphiphilic gel supports[3,4]. Both alkyl- and aryl-substituted agaroses have been employed in the separation of large proteins[5,6], with ionisable supports such as aminoalkylagarose[4,7–9] theoretically enabling both hydrophobic and electrostatic interactions to be utilised for improved selectivity. These techniques have not, however, been widely deployed, in spite of their unique capability for separating proteins by interaction with their accessible hydrophobic domains, irrespective of size, charge, solubility or shape[10]. Their limited use has probably been due to their inherently low efficiencies, slowness and the possibility of protein denaturation caused by either the eluting solvents or the longer alkyl-chain substituent groups themselves[11], as well as a tendency of the agarose-based supports to irreversibly adsorb some proteins[12].

Some of these problems of protein hydrophobic interaction chromatography may be overcome with high-performance systems. HPLC methods capable of resolving a wide range of polypeptides and some proteins have been recently described[1,2], based on the use of reversed-phase packings pioneered in this role by Rivier[13,14] and depending primarily on hydrophobic interactions[15] to achieve separations. Under optimal conditions a large number of polypeptides can be simultaneously resolved with retention of biological activity by larger compounds such as $ACTH_{1-39}$[1]. Similar methods have recently been used in the isolation of some smaller naturally occurring peptides[16–18], the analysis of polypeptide hormone degradation *in vivo*[19,20], and *in vitro*[21], peptide mapping of protein digests[22,23], purification of synthesised polypeptides[24–27] and analysis of pharmaceutical peptide preparations[28].

The use of the longer alkyl-chain chemically-bonded stationary phases (*e.g.*, C_{18} and C_8) for hydrophobic separation and recovery of undegraded proteins, as compared with polypeptides, has not appeared as promising. Apparently irreversible adsorbtion of a number of small protein standards tested was noted[1] and the pore size of these supports would also appear to limit the access of larger proteins. In spite of these drawbacks we have nevertheless successfully used hydrophobic HPLC to isolate proteins from two separate endocrine sources: calcitonin-like immunoreactive materials produced by a human tumour cell-line[29], and porcine hypothalamic proteins from partially purified tissue extracts[30]. In an attempt to extend hydrophobic HPLC methods to an even wider range of proteins and escape from the relatively inflexible selectivity obtained with the longer chain alkyl-substituted supports we have also explored the potential of short alkyl-chain and aminoalkyl-substituted reversed-phase packings.

MATERIALS AND METHODS

Preparation of biological extracts

Immunoreactive calcitonin-like materials were obtained from the BEN human lung tumour cell-line, the origin, characteristics and ectopic hormone secretion of which have been described by Ellison *et al.*[29]. Tissue culture medium, containing 5% foetal calf serum, was collected from cultures, acidified and directly fractionated by HPLC without further preparation (see *Pre-analytical HPLC fractionation*). Cell extracts were then prepared by scraping the remaining cells from the culture flasks and sonicating them on ice in 0.1 *N* HCl solution containing 0.1% 2-mercapto-

ethanol, 20 μg/ml *p*-methylsulphonyl fluoride and 20 μg/ml pepstatin A. The sonicate was diluted with three volumes of 0.9% (w/v) NaCl solution (0.155 *M*) which had been adjusted to pH 2.1 with HCl, and centrifuged at 105,000 *g* for 1 h at 4°. [^{3}H]Substance P undecapeptide (12 ng, specific activity 19.6 Ci/mmole; Radiochemical Centre, Amersham, Great Britain) was added to the supernatant as an internal recovery and chromatography standard. Sources of other chromatographic standards used have been given previously[1].

Lyophilised porcine hypothalamic extracts, which had been partially purified by the procedure of Bristow *et al.*[30] with CMC-cellulose chromatography, were dissolved in the primary HPLC solvent and injected directly onto the analytical columns (see *Analytical HPLC*).

Pre-analytical HPLC fractionation

Immunoreactive calcitonin-containing cell extracts and culture medium were first fractionated in bulk using an HPLC "mini-column" system. Sonicate supernatants, or culture medium samples adjusted to pH 2.1 with HCl, were pumped directly onto 80 × 4.6 mm I.D. stainless-steel columns, tap-packed with Partisil 10-ODS (10 μm particle size, 55–60 Å pore size; Whatman, Maidstone, Great Britain) using an AE 10-4 high-pressure microdiaphragm pump (Orlita, Giessen, G.F.R.). Columns were washed with 5 ml of 0.155 *M* NaCl at pH 2.1, and retained proteins and polypeptides were then eluted, in order of hydrophobicity, with individual 5-ml batches of acid saline containing acetonitrile (HPLC S grade; Rathburn Chemicals, Walkerburn, Great Britain) at concentrations ranging stepwise from 10 to 60% (v/v). Acetonitrile was evaporated under nitrogen from such eluate fractions and aliquots of the resultant aqueous solutions were taken to check recoveries of total protein by Lowry's method, the tritiated peptide internal recovery standard by liquid scintillation counting and calcitonin-like materials by radioimmunoassay. The remainder of the aqueous samples were subjected to analytical HPLC.

Analytical HPLC

High-resolution chromatography of samples and standards was carried out on 100 × 5 mm I.D. stainless-steel columns slurry-packed in the laboratory with either Hypersil-ODS (octadecylsilyl), Hypersil-SAS (trimethylsilyl) or Hypersil-APS (aminopropylsilyl) spherical porous (100 Å) microparticulate (5 μm) chemically-bonded silica packings (Shandon Southern, Runcorn, Great Britain). The characteristics of these materials have been described by Knox and Pryde[31]. The chromatographs used were a DuPont 830 (DuPont, Hitchin, Great Britain) with 838 programmable gradient module, operated at constant pressure (1 ml/min starting flow-rate), and a microprocessor-controlled Spectra-Physics SP 8000 (Spectra-Physics, Santa Clara, Calif., U.S.A.), at constant flow of 1 ml/min. Samples were loaded via a septumless valve fitted with a 2-ml injection loop to accommodate mini-column fractions, and proteins and polypeptides therein were separated at ambient temperature using the 50-min tripartite linear gradient of acetonitrile in primary solvent of 0.155 *M* NaCl (pH 2.1) described previously[1], unless otherwise specified. Retention times of eluted standards were lengthened by *ca.* 2 min in comparison with previously reported values[1,2] because the inclusion of the large volume injection loop delayed the arrival of the gradient at the top of the column, but chromatographic efficiencies were not impaired. Eluted compounds were detected by

sequential UV-absorbance at 225 nm (DuPont 837 or Schoeffel 770 spectrophotometers) and by endogenous fluorescence (Schoeffel 970, activation wavelength 275 nm, emission filter 370 nm) and radioimmunoassay. Detection limits of these systems for polypeptides have been given previously[2].

To prevent possible cross-contamination of samples by incompletely-eluted large-molecular-weight components, a problem observed with some standards[1], all extracts of a single type were processed on dedicated columns, with retention times of calcitonin standards determined only after all samples had been run. Similar precautions were also applied to Partisil 10-ODS minicolumns.

Assay of eluted proteins

Calcitonin-like materials in 100-μl aliquots of 1 ml or 1 min column eluate fractions were measured after evaporation of acetonitrile, using an antiserum raised against synthetic human sequence calcitonin (Ciba, Basle, Switzerland), and [^{125}I]calcitonin as the radioligand. A polyethylene glycol precipitation radioimmunoassay procedure was used with an intra-assay coefficient of variation of 3.1%, as determined by application of Snedcor's formula to duplicate sample determinations ($n = 29$). The minimum detection limit was *ca.* 100 pg monomeric calcitonin per ml of column eluate, and all results have been expressed in terms of monomeric human sequence calcitonin equivalents. The tritiated peptide internal standard did not cross-react with the antiserum and did not interfere with γ-counting for the assay; its position and profile on the analytical chromatography runs were determined by liquid scintillation counting of eluate aliquots.

The molecular weights of proteins in the hypothalamic extracts before and after HPLC were determined by SDS-polyacrylamide gel electrophoresis with Coomassie blue staining.

RESULTS

HPLC of immunoreactive calcitonin-like materials from human tumour cells

Analytical HPLC column performance deteriorates rapidly when complex biological mixtures, such as tissue culture medium or whole cell extracts, are injected *in toto*. It is, however, possible to inject <1 ml of tissue culture medium with foetal calf serum (6 mg total protein) onto a fresh 100 $\times$ 5 mm I.D. column of Hypersil-ODS and obtain a single high-resolution chromatogram. In the sample illustrated (Fig. 1), immunoreactive "eutopic" calcitonin secreted by a freshly disaggregated human medullary thyroid carcinoma (C-cell tumour) was resolved and recovered using a tripartite linear gradient of acetonitrile in 0.155 *M* NaCl at pH 2.1. The retention time of the greater part of the immunoreactive calcitonin was identical with that of synthetic human calcitonin (mol. wt. 3.5 kD), the sequence of which was originally determined from hormone extracted from similar tumours.

This sample, and standards added to tissue culture medium, showed that the retention times and chromatographic efficiencies of individual components were not altered by the presence, in the same sample, of large amounts of other materials, although a serious loss of efficiency was noted in all subsequent chromatograms obtained with the same column. Irreversible adsorption of serum components was probably the cause of this deterioration and a simple procedure for their removal,

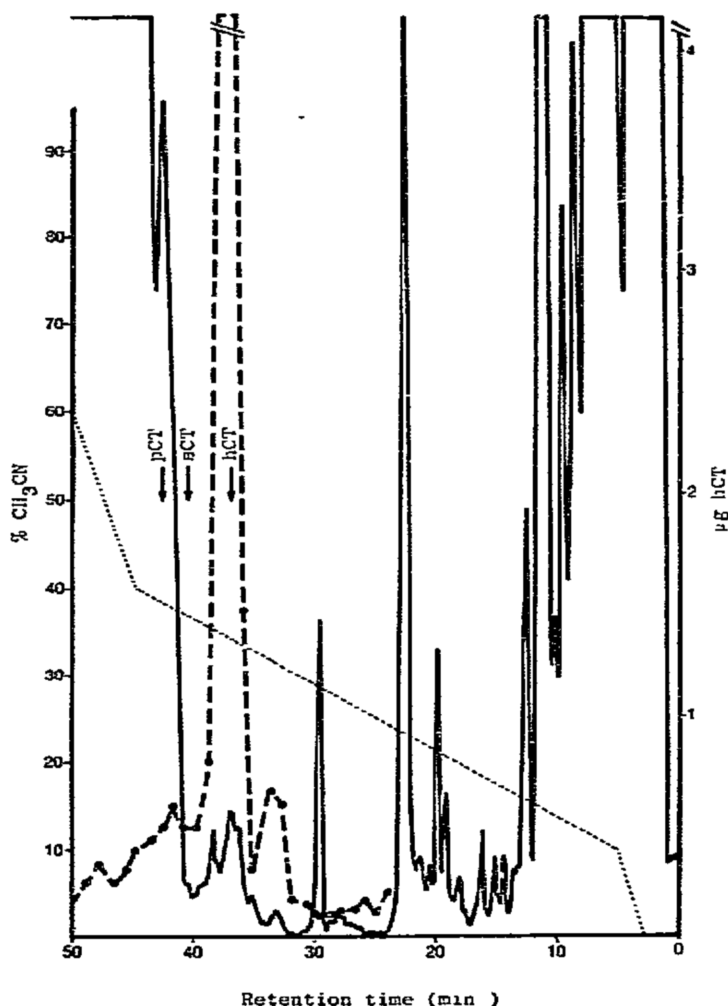

Fig. 1. Hydrophobic HPLC of calcitonin secreted in tissue culture medium by a human medullary thyroid carcinoma. The separation was performed at ambient temperature on Hypersil-ODS (100 × 5 mm I.D.) with a primary solvent of 0.155 *M* NaCl (pH 2.1) and a secondary solvent of acetonitrile. The dotted line indicates the gradient profile, the dashed line the recovery of immunoreactive calcitonin in 1 ml eluate fractions and the solid line the UV absorption trace of 1 ml of culture medium (225 nm, 0.16 a.u.f.s.). hCT, sCT and pCT indicate the retention times of human, salmon and porcine sequence monomeric calcitonins.

and the concentration of elutable materials, has been devised. Although acid conditions tend to favour the oxidation of methionine residues, checks with standards indicated that no significant conversion (<2%) of human calcitonin monomer to its methionine sulphoxide form occurred during any of the extraction or chromatography procedures used in this study. The oxidised polypeptide eluted 6 min earlier than the native hormone using the system illustrated in Fig. 1.

Culture medium and cell extracts were fractionated, prior to analytical chro-

matography, on readily-repacked "minicolumns" of Partisil 10-ODS, as described under *Pre-analytical HPLC fractionation*. The result of applying this procedure to culture medium from a human tumour cell-line (BEN) producing ectopic calcitonin-like materials[29] is illustrated in Fig. 2. Virtually all of the immunoreactive compounds were adsorbed from acidified medium and they were subsequently recovered, and partially fractionated, using a stepwise gradient of acetonitrile in acid-saline, while the bulk of the serum proteins were eluted during loading. A 100-mg amount of total protein could be processed in this manner in a single run without compromising the recovery of active components, and the acetonitrile concentrations used could be adjusted so that specific compounds were completely resolved (Fig. 2). Commercial separation cartridges, or pellicular ODS-silica packings were not capable of handling these amounts or of reproducing the fractionations obtained with the high surface area (400 m²/g) porous minicolumn packings. Fractions from the Partisil 10-ODS columns could, furthermore, be injected repeatedly onto Hypersil-ODS analytical columns without detriment to the performance of the latter.

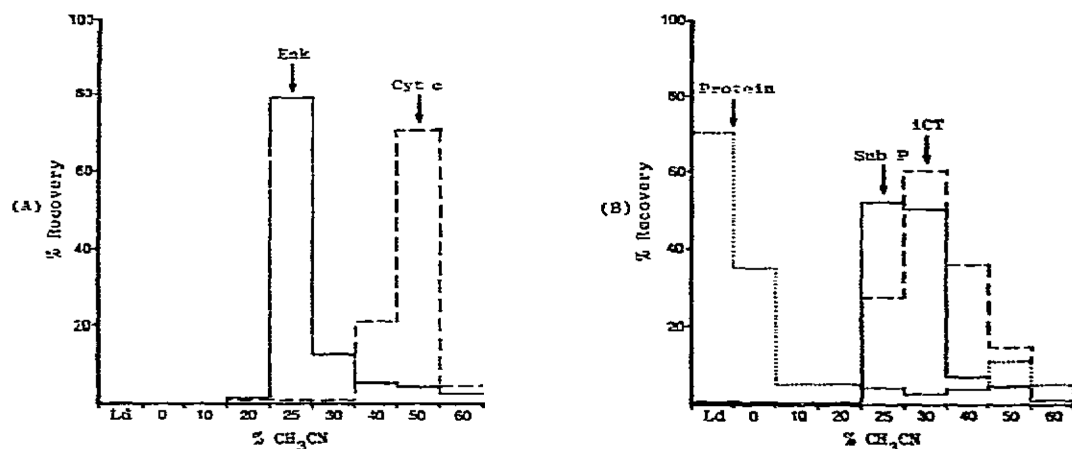

Fig. 2. HPLC minicolumn separation and recovery on Partisil 10-ODS (see *Pre-analytical HPLC fractionation*) of [³H]leu-enkephalin and [¹⁴C]cytochrome *c* standards (A) and of high-molecular-weight immunoreactive calcitonin-like materials from 40 ml of tissue culture medium (100 mg total protein) from a human lung tumour cell line (BEN) (B). [³H]Substance P was added as an internal recovery and chromatography standard. 5-ml fractions of acetonitrile in 0.155 *M* NaCl (pH 2.1) were used to elute adsorbed materials (Ld = load fraction).

It has been shown in a separate study[32] using size exclusion chromatography that the bulk of the immunoreactive calcitonin-like material from BEN cells is of relatively high molecular weight (20–25 kD) compared with monomeric calcitonin (3.5 kD). This, therefore, forms the greater part of the material directly fractionated here by HPLC. Minicolumn fractions of BEN culture medium containing immunoreactive calcitonin (20–50% acetonitrile) were pooled and further resolved by high-performance hydrophobic chromatography on Hypersil-ODS analytical columns, as described under *Analytical HPLC*. Multiple peaks of immunoreactive calcitonin were obtained (Fig. 3), whose retention times could be correlated with that of the internal standard, [³H]Substance P undecapeptide, which was simultaneously chromatographed. Little or no material corresponding to monomeric human sequence calci-

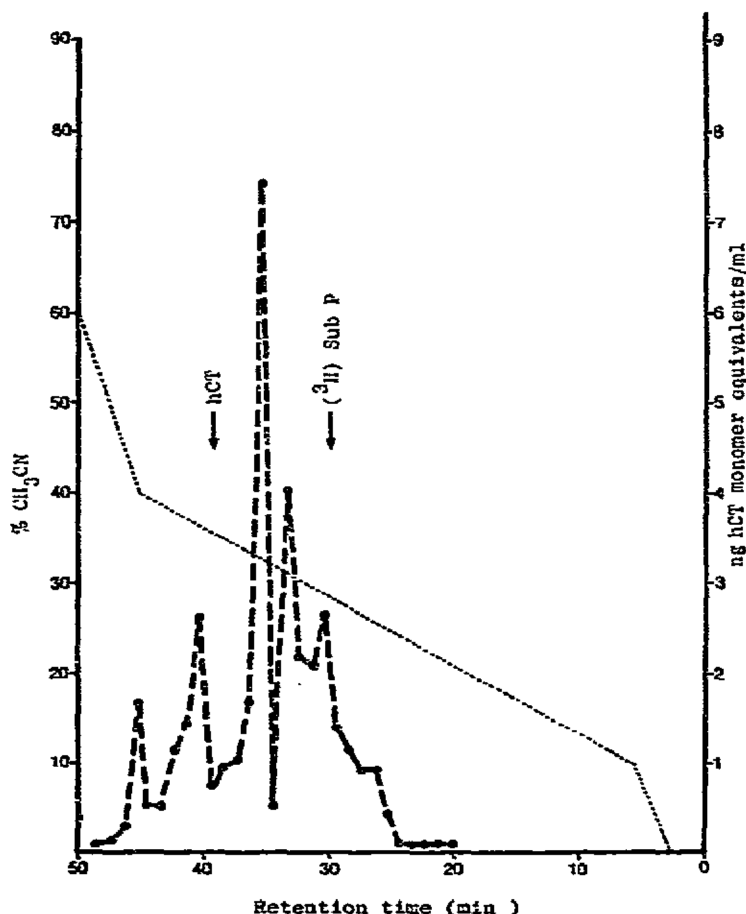

Fig. 3. Hydrophobic HPLC on Hypersil-ODS of high-molecular-weight immunoreactive calcitonin(s) in BEN cell culture medium after minicolumn fractionation as shown in Fig. 2B. Chromatographic conditions were as described in the legend to Fig. 1. The dashed line represents the immunoreactive calcitonin detected in 1 ml eluate fractions. Aliquots were also used to determine the retention time of [^{3}H]Substance P internal chromatography standard. The retention time of human sequence calcitonin monomer (hCT) was determined on a consecutive chromatogram using the same column.

tonin was recovered from culture medium but a number of earlier eluting peaks were detected. These corresponded to the large-molecular-weight material separated by size exclusion methods (20–25 kD), notably a large peak of activity with a retention time of 36 min preceded by at least two smaller peaks (Fig. 3).

Immunoreactive calcitonin(s) in BEN cell extracts, as distinct from secreted materials in culture medium, were also directly analysed by HPLC. Up to 6 mg of total soluble protein, prepared as described under *Preparation of biological extracts*, and corresponding to *ca.* $5 \cdot 10^7$ cells, could be processed in a single run by minicolumn fractionation and subsequent Hypersil-ODS analytical chromatography. A pattern of immunoreactive material very similar to, but not identical with that of secreted material was seen (Fig. 4). Activity corresponding to high-molecular-weight

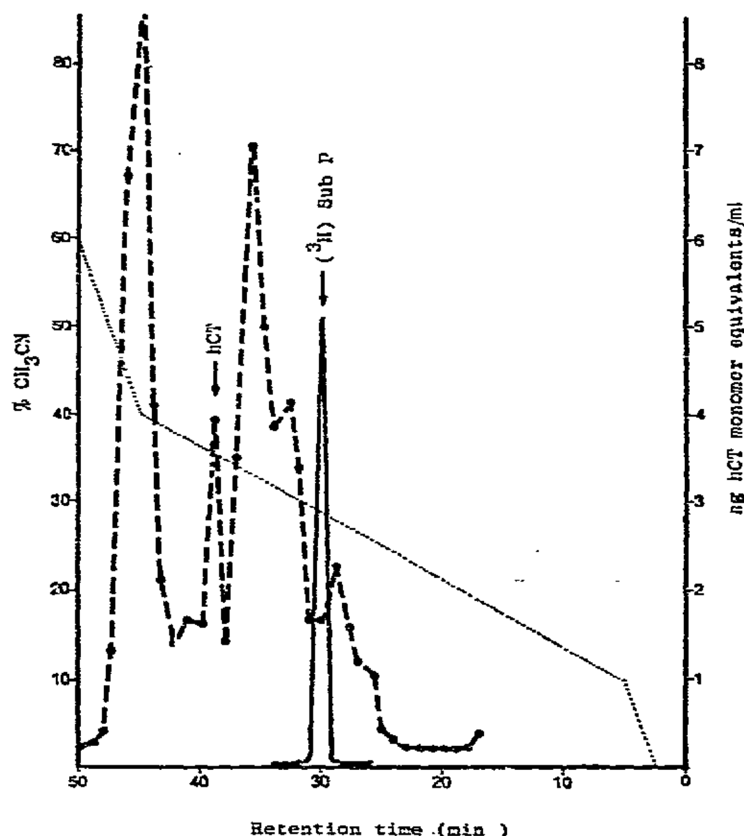

Fig. 4. Hydrophobic HPLC on Hypersil-ODS of high-molecular-weight immunoreactive calcitonin(s) extracted from BEN cells (*cf.*, Fig. 3) as detailed under *Preparation of biological extracts*. Chromatographic conditions as in Fig. 3. The solid line represents the elution profile of [^{3}H]Substance P as determined by liquid scintillation counting of aliquots of eluted fractions.

(20–25 kD) calcitonin(s) was again detected in at least three early-eluting peaks, and while a peak corresponding in retention time to monomeric human calcitonin was seen, it was small in comparison with the other materials.

Although large amounts of serum-containing culture medium or soluble proteins in BEN cell extracts could, therefore, be reproducibly chromatographed by the procedures described here, the relatively small (ng) amounts of immunoreactive calcitonin(s) produced precluded their direct detection by spectophotometric methods in the presence of the other UV-absorbing materials still present in these samples. To establish the upper, preparative, limits of these HPLC systems, and to examine more easily the potential selective effects of varying key chromatographic parameters with proteins, we have used a partially-purified porcine hypothalamic extract[30].

HPLC of porcine hypothalamic extracts

The material used in this study contained at least three major proteins, whose molecular weights ranged from *ca.* 12 to 25 kD as determined by SDS-polyacrylamide gel electrophoresis (Fig. 5). Direct injection of this partially purified material onto

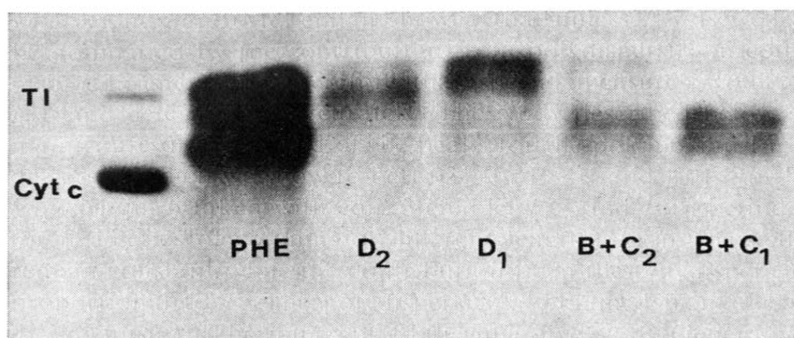

Fig. 5. SDS-polyacrylamide gel electrophoresis of a partially purified porcine hypothalamic extract (PHE), and the eluted peaks obtained by preparative hydrophobic HPLC (see Fig. 6). Molecular-weight standards used were cytochrome *c* (Cytc 11.7 kD) and trypsin inhibitor (TI 21 kD). Samples of 1 ml HPLC eluate fractions collected across the major peaks illustrated in Fig. 6 (B + C and D) were analysed. No protein band corresponding to HPLC peak A was seen on the SDS gels.

Hypersil-ODS analytical columns and elution with the gradient of acetonitrile in acid-saline described under *Analytical HPLC* resolved several of its components (Fig. 6). Simultaneously recorded UV and fluorescence chromatograms (not illustrated) revealed that peaks B + C and D, but not A, were endogenously fluorescent (275 nm activation wavelength for tryptophan-containing compounds). All of these fluorescent peaks were proteins, as was evident from their behaviour on SDS-polyacrylamide gel electrophoresis (Fig. 5), which also demonstrated that they were recovered from HPLC unchanged in size. Up to 10 mg of porcine hypothalamic extract has been directly chromatographed without apparent loss of efficiency or resolution and the injection of these amounts enabled a number of minor components well separated from the major proteins to be detected.

As, however, only partial separation of some of these major components was achieved with the acid-saline–acetonitrile gradient system, the potential selective effects of other chromatographic conditions were examined. Neither increasing the temperature of chromatography from ambient to 45° or 65°, nor substituting dioxane, tetrahydrofuran or methanol for acetonitrile materially improved separations, although a slight increase in resolution of peaks A, B + C and D was noted with methanol, confirming the minor selective effects noted with this, but not the other organic modifiers in our previous study of polypeptide standards[1]. Substitution of a phosphate buffer system[2] for acid-saline at the same molarity did not alter the profile at all.

Further attempts at complete resolution of the hypothalamic proteins were made using different chemically-bonded stationary phases. In particular it was hoped that there would be specific selective effects of shorter alkyl-substituted supports, analogous to those obtained with different chain lengths on low-pressure alkylagarose systems[5]. Freshly packed Hypersil-SAS (short alkyl-chain) gave, however identical results to octadecylsilane- and octylsilane-bonded supports when hypothalamic extracts and a range of polypeptide and protein standards were tested using the acid-saline–acetonitrile gradient system. Its performance deteriorated rapidly, over 24–48 h however, with loss of retentivity for polypeptides. An increase

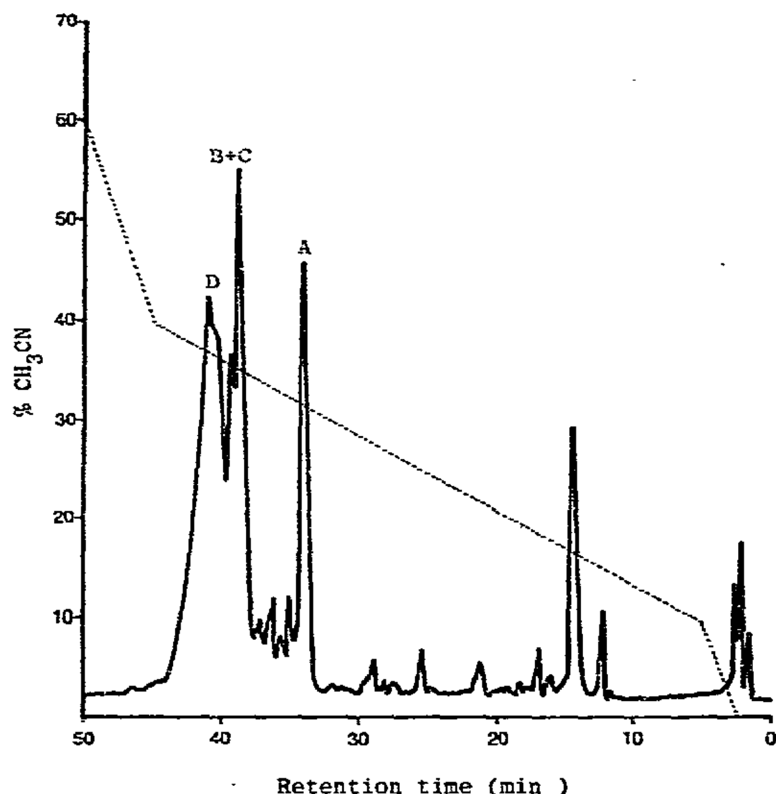

Fig. 6. Hydrophobic HPLC of 10 mg of partially purified hypothalamic extract on Hypersil-ODS. Conditions of chromatography were as detailed in Fig. 1, with the gradient profile indicated by the dotted line. The UV absorption trace of this material (225 nm, 2.56 a.u.f.s.) is illustrated.

in methyl red adsorption by used SAS-packings indicated that a loss of stationary phase and/or an increase in free silanol groups was caused by these chromatographic conditions (pH 2.1).

Hypersil-APS, on the other hand, was apparently stable at this pH but gave much lower chromatographic efficiencies when protein mixtures were tested (Fig. 7). No significant selective effects were observed, although some proteins (*e.g.*, lactalbumin) which were irreversible bound to ODS-silica could be eluted from Hypersil-APS at pH 2.1, as might be expected from the less hydrophobic nature of the shorter alkyl-chain substituent of this packing. The presence of ionisable groups on this packing raises the possibility of selective effects at different pH values due to potential electrostatic interactions reducing the strength of hydrophobic bonding, as has been observed with analogous low-pressure systems[7–9]. This possibility is currently being explored.

DISCUSSION

HPLC techniques, based for the most part on alkylsilane-bonded hydrophobic stationary phases, have now been used successfully to separate a large number of

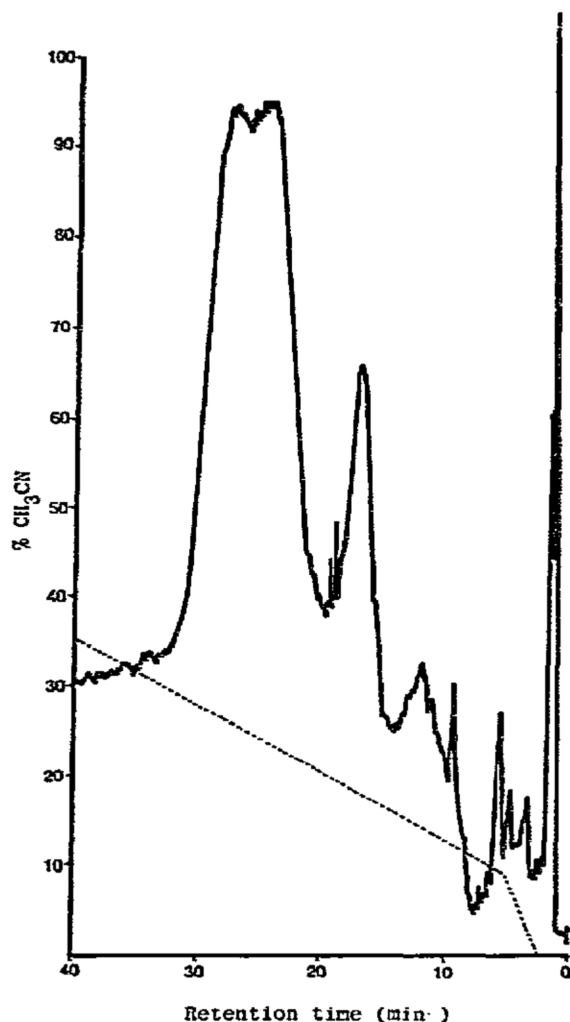

Fig. 7. Hydrophobic HPLC of 200 μg of partially purified hypothalamic extract on Hypersil-APS. Other chromatographic conditions, including gradient (dotted line), were as described in Fig. 1. The UV-absorption trace (225 nm, 0.16 a.u.f.s.) is shown by the solid line (*cf.*, Fig. 6).

diverse peptides of natural and synthetic origin[1,2,13–28]. The resolving power of these systems under optimised conditions[1,2,14] is such that they must be the method of choice for both analytical and preparative peptide chromatography. The behaviour of smaller compounds (<15 residues) is largely predictable on the basis of their specific hydrophobic amino-acid content[1], and the method is ideally suited to the separation of closely related and potentially cross-reacting compounds prior to radioimmunoassay[2,33]. Comparable HPLC studies of proteins, however, have been relatively few in number as yet[1,14,34–36].

In this paper we have explored some practical aspects of the hydrophobic interaction chromatography of proteins by HPLC. As these studies show methods developed primarily for polypeptide hormone separation can also be used to resolve

the mixtures of small soluble proteins (12–25 kD) that we have tested, and used directly to isolate some of them from bulk samples of complex biological mixtures. Reproducible patterns of immunoreactive proteins were obtained from cell extracts (Fig. 3) and from much larger ($<$100 mg total protein) quantities of extraneous materials in tissue culture medium (Fig. 2), notably with high recoveries ($>$80%) and effective resolution of several calcitonin-like materials. The direct preparative capabilities of this technique were demonstrated by resolution of milligram ($<$10 mg) quantities of partially-purified hypothalamic tissue extracts (Figs. 5 and 6).

It would, however, be premature and inaccurate to claim that systems of the type described here can serve as universal tools for the separation of proteins. Although chromatography of large proteins, aldolase (158 kD) catalase (240 kD) and ferritin (450 kD), has been reported in another recent study[36], it seems unlikely that materials of this size would gain access to the interior of the porous silica microparticles with pore sizes[37] of 50–100 Å. If this is the case then the loading capacity for such large materials must be severely limited, as is suggested by the results of total serum protein fractionation illustrated in Fig. 2B. The irreversible adsorption of some proteins to ODS-silica[1] may also pose problems, although in the two cases studied here all components of the protein mixtures could be successfully eluted.

One of the specific advantages of the hydrophobic HPLC of polypeptides and proteins is the remarkable precision with which individual retention times can be reproduced[1]. In studies of complex mixtures, such as those illustrated here, this enables the identity (or non-identity) of eluted materials in relation to retention times of known standards to be established with some certainty. This precision does, however, entail a potential practical disadvantage in the relative inflexibility of the separations. Thus we have been unable to significantly modify by altering a wide variety of chromatographic parameters the resolution of components of the complex mixtures. Use of alternative stationary phases to octadecylsilane and octylsilane for this purpose has been thus far disappointing. Selective effects of short (C_2–C_4) as opposed to long ($>C_8$) alkyl-substituted supports have been obtained with low-pressure hydrophobic interaction chromatography systems[4–11]. These effects are possibly dependent on the depth of hydrophobic domains or clefts in proteins[5] many of which are at or near the surface[38]. No selective effects were obtained here with HPLC with a short alkyl-chain silica (Hypersil-SAS), but the analogies between the low and high pressure systems can only be partial in view of differences that can exist in the degree of coverage by the hydrophobic substituents[39], differences that can probably modify the number of such groups involved in binding a single protein molecule. It is also worth noting that the number of hydrophobic domains in an individual protein can vary from one[40] to eight or more in the case of serum albumin[41]. Hofstee[9] has already noted that the presence of such multiple hydrophobic binding sites may cause peak broadening or even false inhomogeneity in low-pressure systems. It remains to be determined whether such effects are responsible for the variations in protein peak widths noted in hydrophobic HPLC systems[1]. One lesson to be drawn from these studies, however, is that multiple peaks of eluted compounds may not invariably imply molecular heterogeneity, although in at least one of the cases we have studied (Fig. 5) genuine resolution of different proteins has been obtained.

It has been suggested[42] that polypeptides and proteins chromatograph on

hydrophobic packings in the presence of acid anions as ion pairs and the alternative term ion-pairing chromatography has been used to describe these systems. If true, the nature of the available counterion could afford potential selectivity[43]. The choice of the aqueous primary solvent is, however, limited by the requirements of subsequent assays of eluted compounds and our use of isotonic (0.155 *M*) acid-saline was prompted by this consideration. Many other neutral salts with different chaotropic effects on hydrophobic bonding are available[44] but few recommend themselves for biological purposes. Under the conditions of gradient elution used here the organic modifier concentration essentially dictates separation of polypeptides and proteins, and the effective chromatography of individual compounds is limited to a small segment of the gradient profile[1]. Selective effects of different salt anions may be of limited use under these conditions. The use of detergents[45] or deforming agents such as imidazole[4,5] to facilitate protein elution also poses problems of their subsequent removal from eluted materials.

Having obtained resolution of major components of any complex protein mixture by hydrophobic HPLC the key question of whether the proteins have been degraded, denatured or otherwise rendered biologically inactive remains to be answered. Three possible causes of protein denaturation in these HPLC systems can be identified, the salts in the aqueous primary solvent, the organic modifier and the hydrophobic stationary phase itself. The concentrations of salt required for optimal HPLC of peptides and proteins (0.1–1 *M*) are probably not high enough to cause problems, although higher concentrations may result in conformational changes[44]. Organic solvents can cause conformational changes at certain concentrations[46] but these need not necessarily be of an irreversible nature. The stationary hydrophobic phase itself may be the most likely cause of denaturation. In low-pressure systems short (C_3) alkyl-chain substituents are compatible with catalytic activity of adsorbed and eluted proteins but longer substituents may cause denaturation[4–11]. One might anticipate therefore that the C_8 and C_{18} packings used in HPLC systems are unlikely to be compatible with bioactivity of large proteins, particularly if the alkyl substituents disrupt the internal hydrophobic interactions on which the structure of such molecules may partially depend[47]. Large polypeptides, however, such as $ACTH_{1-39}$[2] and β-endorphin[18] can be chromatographed on ODS-silica and retain their bioactivity. The situation with small proteins is not yet clear and we are not aware of any studies involving estimation of their biological activity after hydrophobic HPLC. Our present studies do, however, show retention of immunoreactivity by larger materials (12–25 kD) in one case, and no change in size after chromatography in the other.

In summary, therefore, these results show that hydrophobic interaction HPLC methods can be directly applied to the resolution of some small proteins in complex natural mixtures, thus significantly extending the potential range of this technique in respect of these biopolymers.

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