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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MOUSE EPIDERMAL GROWTH FACTOR AND ITS CONGENERS: MOBILE PHASE OPTIMIZATION WITH ION-PAIRING ADDITIVES

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SUMMARY

Optimization of the mobile phase in reversed-phase high-performance liquid chromatography has been examined with respect to the separation of multiple components from mouse epidermal growth factor preparations. Neither trifluoroacetic acid, nor heptafluorobutyric acid afforded optimal separations when added in various concentrations as potential ion-pairing agents, requiring low ionic strengths and yielding separations that were susceptible to changes in packings due to differences in stationary phase coverage and the presence of accessible silanol groups on the C-18 phases used. Pentadecafluorooctanoic acid, on the other hand, gave a reproducible, effective separation when combined with 0.155 *M* sodium chloride in the primary solvent, and eluted with a gradient of acetonitrile–1-propanol as the secondary solvent. Additional selectivities were conferred by the addition of triethylamine (0.015 *M*) to the mobile phase containing pentadecafluorooctanoic acid. These studies illustrate the value of such mixed mobile phases in achieving complex separations of closely eluted polypeptides.

INTRODUCTION

A recent review of protein–polypeptide separations by reversed-phase high-performance liquid chromatography (RP-HPLC) listed over 50 examples from different laboratories[1]. Over 30 individual aqueous mobile phases and some twenty individual ion-pairing agents at various concentrations were used, although there was little evidence that the mobile phase had been specifically optimized for individual separations in the majority of instances.

We, and others[2–7], have been interested in the separation, characterisation and identification of multiple protein components found in epidermal growth factor (EGF) preparations obtained by conventional methods. While apparently satisfactory resolution has been obtained using the ion-pairing mobile-phase additives trifluoroacetic acid (TFA) and heptafluorobutyric acid[3,4], some of these separations

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are not as reproducible as the literature might suggest, leading in extreme cases to a failure to resolve or recover EGF from some RP columns when the more hydrophobic ion-pairing reagent was used.

These problems prompted a systematic examination of mobile phase optimization, with particular reference to the type and concentration of ion-pairing reagent used, as well as the effect of column coverage. We have used, as a model system, the commercial preparations of mouse EGF containing multiple components because they exemplify the problems encountered in attempting to resolve closely related (and closely chromatographing) materials.

MATERIALS AND METHODS

Acetonitrile (S grade), methanol and 1-propanol (HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, U.K.). Single glass distilled water was prepared from Milli-Q low conductivity feedstock. The following halogenated carboxylic acids were used as ion-pairing additives: trifluoroacetic acid (TFA, sequencer grade, Rathburn Chemicals), trichloroacetic acid (TCA) and tribromoacetic acid (TBA) from Aldrich, Milwaukee, WI, U.S.A., perfluoropropionic acid (PFPA, Pfalz & Bauer, Stamford, CT, U.S.A.), heptafluorobutyric acid (HFBA, sequenal grade, Pierce, Rockford, IL, U.S.A.) and pentadecafluorooctanoic acid (PDFOA, Aldrich). Triethylamine (TEA) was SLR grade from Fisons (Loughborough, U.K.); other chemicals were AR grade, also from Fisons, except aldosterone, 18-hydroxycorticosterone and methyl red which were obtained from Sigma (Poole, U.K.).

Mouse submaxillary gland epidermal growth factor (mEGF) preparations were obtained from Collaborative Research (Lexington, MA, U.S.A.). Two batches of "culture grade" material (83-393 and 84-134) were used, for analytical and preparative purposes respectively.

RP-HPLC of mEGF was carried out using 250 × 4.6 mm I.D. stainless-steel columns slurry packed in the laboratory. Unless otherwise stated the following packings were used: Spherisorb S5 ODS1 (C_{18}, partially capped 7% carbon loading) or Spherisorb S5 ODS2 (C_{18}, fully end-capped, 12% carbon loading), both RP packings being based on the same 5-μm particle size, 8-nm pore-size silica with a specific surface area (S_{BET}) of 220 m^2/g. These two packings differ primarily in the extent to which they possess accessible silanol groups. Operating conditions were controlled using an Altex 340-40 chromatograph, with all separations carried out at 45°C and a constant flow-rate of 1 ml/min. Eluted proteins were detected by sequential UV absorbance (280 nm, LDC Spectromonitor III) and endogenous tryptophan fluorescence (254/340 nm, Schoeffel FS-970).

The extent of coverage by the alkylsilane-bonded stationary phase was determined before, during and after use of individual packed columns. Before and after use of columns accessible silanol groups were quantified by spectrophotometric determination of methyl red adsorbed by carefully washed and dried aliquots of the packings[8,9]. The extent of stationary phase coverage during use was evaluated by examining the behaviour of the corticosteroids 18-hydroxycorticosterone (18OH-B) and aldosterone with isocratic elution in methanol–water (40:60, v/v). With this solvent system the 18-hydroxysteroid (but not aldosterone) undergoes on-column methylation to its less polar 20-methoxy derivative if the RP packing is incompletely

covered[9], resulting in readily detectable asymmetrical broadening of this eluted steroid peak.

N-Terminal amino-acid sequences on 50–100 μg of HPLC-purified mEGF components were determined using a Beckman spinning-cup sequencer, by courtesy of Dr. G. Goodwin at the Chester Beatty Laboratories, Institute of Cancer Research, London, U.K.

RESULTS

In our original studies using ODS-type RP columns to separate hormonal polypeptides on the basis of their hydrophobicity[10,11], we opted for an aqueous solvent system that comprised 0.155 *M* sodium chloride with 0.01 *M* hydrochloric acid. Other mobile phases used primarily because of their volatility and UV transparency, notably 0.1% (v/v) TFA (0.013 *M*)[12] gave inferior results with much broader peaks of eluted polypeptides and small proteins[13].

In preliminary studies with mEGF, the hydrophobic ion-pairing additive HFBA was found to give the better separations, yielding three major peaks plus several minor components from "culture grade" preparations purified from submaxillary glands by Bio-Gel P-10 chromatography (see also ref. 3). The identities of these components and their biological relationship and significance will be described subsequently. This system, however, revealed a lack of reproducibility. Thus, repeated runs with HFBA (particularly at relatively low ionic strengths) sometimes resulted in a rapid deterioration with loss of efficiency and compromised separations. A major cause of such a lack of reproducibility in systems which use solvents of low ionic strength, is the presence of accessible silanol groups found on RP columns which are incompletely covered, or become so during use.

Effects of column coverage

To examine the effects of column coverage on mEGF chromatography with hydrophobic ion-pairing additives the performance of two RP packings with different coverage but based on the same silica, *viz.* Spherisorb ODS1 and ODS2, was examined. Levels of accessible silanol groups in the batches of Spherisorb ODS1 and ODS2 used here were equivalent to 55 μg/100 mg and 5 μg/100 mg, respectively, of methyl red adsorbed, and were confirmed by the chromatographic behaviour of 18-hydroxycorticosterone in the presence of methanol (Fig. 1a and 1b inserts). The substantial deterioration in polypeptide chromatography seen when an incompletely covered packing is used with 0.2% (v/v) HFBA (0.015 *M*) and acetonitrile gradient elution is evident (compare Fig. 1a and 1b). Subsequent experiments were all carried out (unless otherwise specified) with the fully capped ODS2.

Effects of HFBA concentration

To optimize the concentration of HFBA for resolving mEGF components, we compared 0.1% (v/v) (0.0076 *M*), 0.2% (v/v) (0.015 *M*) and 0.4% (v/v) (0.03 *M*). The pH of these mobile phases ranged from 2.0 to 2.5. Particular care was taken to standardise the washing and re-equilibration procedures to obtain reproducible performance on multiple consecutive runs, and to obviate "memory" effects. At 0.1% (v/v) HFBA (Fig. 1c) the EGF components were substantially retarded on the gra-

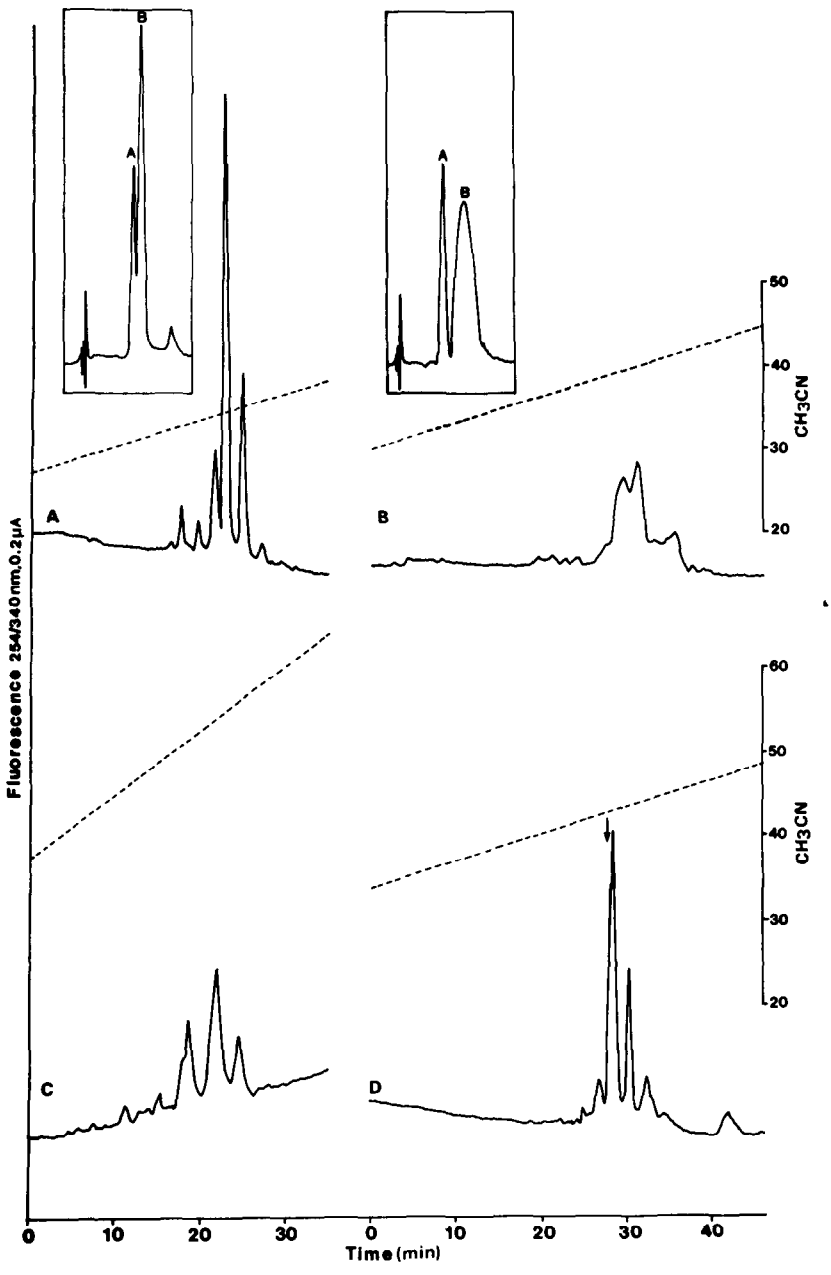

Fig. 1. Effect of HFBA concentration on elution profiles of mEGF (5 μg, culture grade batch 83-393) from 25 cm C_{18} RP columns using HFBA as the sole mobile phase additive chromatographed on (a) Spherisorb ODS2 (fully end-capped) and (b) Spherisorb ODS1 (partially capped) with 0.2% (v/v) (0.015 *M*) HFBA and acetonitrile gradient elution (0.3%/min, dashed line). Insets show UV absorption (240 nm) profiles of aldosterone (A) and 18OH-B (B) chromatographed isocratically with methanol–water on these columns. In (c) and (d) the chromatograms of mEGF were developed using 0.1% (v/v) (0.0076 *M*) and 0.4% (v/v) (0.03 *M*) HFBA, respectively, with a fully end-capped C_{18} column. Note in the former the loss of efficiency even with 0.75%/min gradient and in the latter case the shoulder on the main peak (arrow) and reduction in height of the immediately preceding peak indicative of a change in selectivity compared with (a). Other chromatographic conditions in this and subsequent figures were as described in the Materials and Methods.

dient and peaks were considerably broader than with 0.2% (v/v). With 0.4% (v/v) (0.03 *M*) peak widths were correspondingly reduced (Fig. 1d). However, separation of one of the major peaks was lost (Fig. 1d), a change of selectivity that cautions against arbitrary increases in mobile phase additive concentration to compensate for progressive column deterioration.

Effects of varying sodium chloride concentration in the presence of HFBA

If packing coverage is low resulting in compromised polypeptide chromatography (Fig. 1a *versus* 1b), increased ionic strength of the eluent does, in general, compensate for the resultant peak broadening.

We attempted, therefore, to determine whether the high efficiency associated with high molarity sodium chloride-based systems could be combined with the selectivity associated with addition of the hydrophobic ion-pairing additive HFBA at lower concentrations, but without the change in selectivity that results when the concentration of HFBA itself is increased (Fig. 1d).

The use of a sodium chloride (0.155 *M*)–HFBA (0.015 *M*) mobile phase did substantially improve the performance of the partially covered Spherisorb ODS1 which was used to simulate the performance of potentially degraded columns (compare Figs. 1b and 2b). However, this aqueous mobile phase did not improve overall separations of EGF and its congeners when the fully covered Spherisorb ODS2 was used (Fig. 2a). Thus, increasing the sodium chloride concentration from 0.03 and 0.465 *M* with HFBA (0.015 *M*) did improve overall efficiency, but the separation of two of the component peaks which coeluted with higher concentrations of HFBA alone was again lost (compare Figs. 2c and 2d with Fig. 1d).

Effects of varying HFBA concentration in the presence of sodium chloride

The results of chromatographing culture-grade mEGF with an aqueous mobile phase of 0.155 *M* sodium chloride to which various concentrations of HFBA between 0.025% (v/v) (0.0019 *M*) and 0.4% (0.03 *M*) had been added are illustrated in Fig. 3. At the higher ionic strengths the separation of some components was compromised. The presence of the salt did, however, permit the concentration of HFBA to be reduced to at least 0.0019 *M* (0.025%, v/v) without further loss of separations and without the substantial loss of efficiency using HFBA concentrations of 0.1% (v/v) or lower in the absence of added sodium chloride.

Effects of other ion-pairing additives

The limitation of HFBA as an ion-pairing additive for mEGF separations was that optimal selectivity (*i.e.*, resolution of the maximum number of components) was obtained over a relatively narrow concentration range at a low ionic strength (0.015 *M*). Attempting to compensate for changes or differences in column performance due to differences in alkylsilane coverage either by adding sodium chloride or increasing HFBA concentrations resulted in changed selectivity, with a failure to resolve some known components of the mixture. Following Bennett *et al.*[14] and Schaaper *et al.*[15], the effects of higher perfluoroalkanoic acids and other halogenated carboxylic acids were examined.

TCA, TBA and PFPA were tested at comparable concentrations (0.010–0.018 *M*) as the sole mobile phase additive in both primary and secondary solvents with

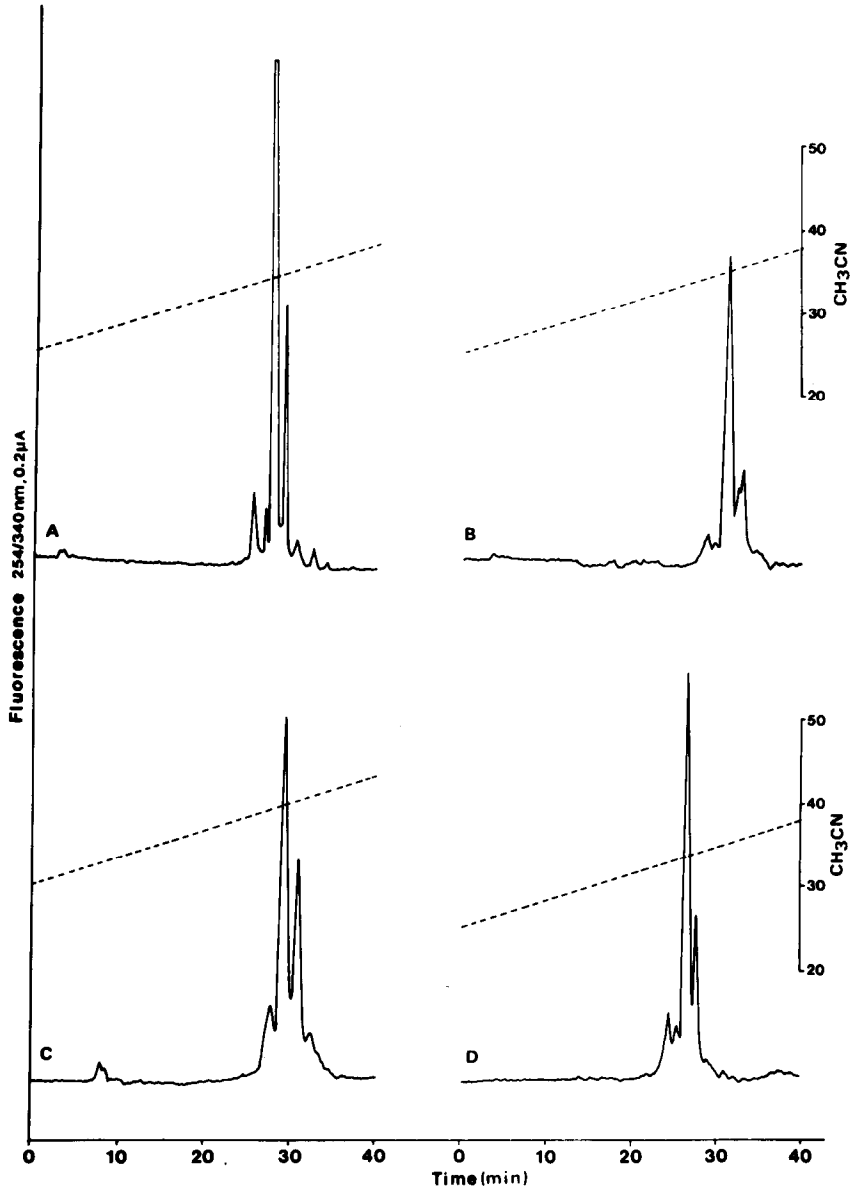

Fig. 2. Effect of sodium chloride on elution profiles of mEGF from C_{18} RP columns using HFBA. Chromatograms (a) and (b) were obtained with 0.2% (v/v) (0.015 *M*) HFBA and 0.155 *M* sodium chloride on Spherisorb ODS2 and ODS1 respectively. Note improvement in peak shape in the latter compared with Fig. 1b, but loss of selectivity in both cases compared with Fig. 1a. Chromatograms (c) and (d) show mEGF separated with 0.2% (v/v) HFBA in the presence of 0.03 *M* sodium chloride (c) and 0.465 *M* (d) on a fully end-capped column.

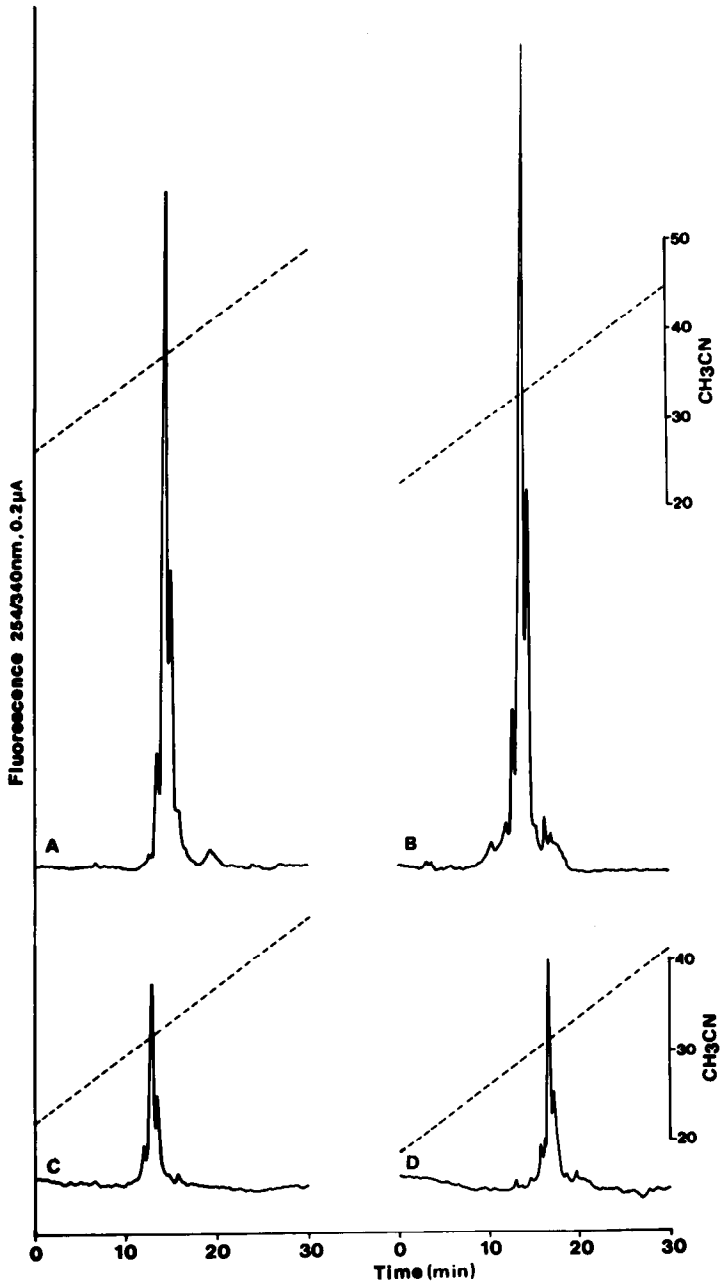

Fig. 3. Effect of HFBA concentration on elution profiles of mEGF from C_{18} RP columns at a constant sodium chloride concentration (0.155 *M*). Fully end-capped columns were used for all separations. In (a) the concentration of HFBA was 0.4% (v/v) (0.03 *M*), in (b) 0.1% (v/v) (0.0076 *M*), in (c) 0.05% (v/v) (0.0038 *M*) and (d) 0.025% (v/v) (0.002 *M*). Note that despite preservation of efficiency in no case was selectivity adequate for complete resolution of mEGF components.

TABLE I

EFFECT OF THE AQUEOUS MOBILE PHASE ON mEGF CHROMATOGRAPHY

All separations were performed with 5 μg of culture-grade mEGF (batch 83-393) eluted with a 0.33%/min acetonitrile gradient from a 25-cm Spherisorb ODS2 column at a flow-rate of 1 ml/min and 45°C. With the exception of the acetic acid–TEA, all mobile phases were less than pH 2.9.

*Mobile phase**	*Retention of EGF (% acetonitrile)*	*Number of major peaks resolved*	*Separation of α- and β-EGF (% acetonitrile)***
0.013 *M* TFA (0.1%)	36.5	2	0.66
0.018 *M* PFPA (0.2%)	39.2	2	0.60
0.015 *M* HFBA (0.2%)	42.5	3	0.75
0.015 *M* HFBA (0.2%) plus 0.155 *M* NaCl	34.1	2	0.48
0.0048 *M* PDFOA (0.2%) plus 0.155 *M* NaCl	41.8	3	0.48
0.012 *M* TCA (0.2%)	41.0	2	1.0
0.013 *M* TBA (0.4%)	42.0	2	0.80
0.05 *M* CH_3COOH–TEA pH 5.6	27.3	2	0.46
0.155 *M* NaCl–0.01 *M* HCl	30.1	1***	0.33

* All mobile phase additives expressed as % (v/v), except PDFOA (%, w/v).

** Absolute peak separation (expressed here as % difference in acetonitrile concentration as peaks elute) is not the only criterion of mobile phase optimisation, as is evident from Figs. 1a, 1c and 6a.

*** Partial resolution of α- and β-EGF was obtained with 25-cm columns and "slow" gradients (compare Fig. 4c).

acetonitrile gradient elution. In no case was an improved resolution of mouse EGF components obtained, compared with HFBA alone (Table I).

Pentadecafluorooctanoic acid (PDFOA) gave the results illustrated in Fig. 4a, b and d. When it was included as the sole additive in both primary and secondary solvents no components of mEGF were eluted at acetonitrile concentrations up to 60% v/v. In the presence of 0.155 *M* sodium chloride and 0.2% w/v PDFOA (0.0048 *M*) all three major peaks were resolved from commercial Biogel P-10 prepared material as well as several minor peaks (Fig. 4a). This compares with the two components recognised by Matrisian *et al.*[2] under isocratic conditions with TEA. Not only was the separation of all of the components seen under the best conditions with 0.2% v/v HFBA realised, but chromatographic efficiency was considerably improved with sharper peaks that facilitated the collection of individual components in a minimum volume of eluent. Furthermore, the increased ionic strength due to the presence of the salt tended to protect the separation against any gradual deterioration of the RP packing (Fig. 4b). Furthermore, despite caveats concerning the stability of alkylsilane-bonded packings with higher perfluoroalkanoic acids[16], there were no significant changes in performance in this study when using the maximum-coverage ODS2-packed columns with PDFOA–sodium chloride for extended periods of time (at least 40 h), provided that the columns were regularly washed with additive-free acetonitrile. Methyl red adsorption tests on such used packings did eventually (100 h) show some, but by no means complete, loss of stationary phase coverage.

Similar effective and reproducible mEGF separations with PDFOA–sodium chloride were obtained when a maximum-coverage large pore-size short alkyl chain

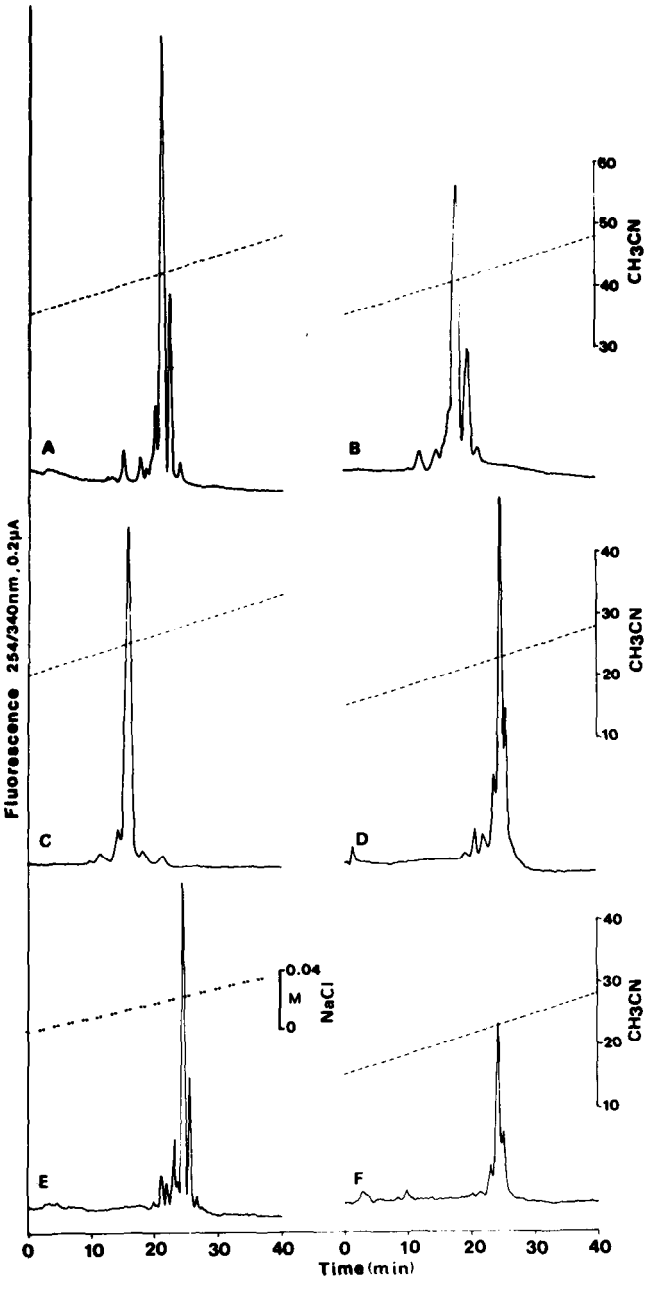

Fig. 4. Elution profiles of mEGF from RP columns using PDFOA as the mobile phase additive. In (a) and (b) the proteins were eluted from a Spherisorb ODS2 and ODS1 25-cm column, respectively, using gradient elution with acetonitrile–0.2% (w/v) (0.0048 *M*) PDFOA from an aqueous primary solvent of 0.2% (w/v) PDFOA with 0.155 *M* sodium chloride. In (c) and (d) mEGF was chromatographed on a 7.5-cm Ultrapore-RPSC (C_3) column with acetonitrile gradient elution and 0.155 *M* sodium chloride–0.01 *M* hydrochloric acid in (c), contrasting with the enhanced resolution obtained with 0.2% (w/v) PDFOA–0.155 *M* sodium chloride in (d), also evident in (f) where the main peak from (c) was trapped and rechromatographed with the latter system. In (e) mEGF components were separated with a 0 to 0.04 *M* sodium chloride gradient (dotted line) in the presence of 0.2% (w/v) PDFOA and a constant acetonitrile concentration of 50% (v/v), showing a profile very similar to that obtained with conventional organic modifier gradient elution on this Spherisorb ODS2 column (a).

(C_3) packing was used, a column which also fails to resolve the major components of "culture grade" mEGF preparations with acetonitrile gradient elution using 0.155 *M* sodium chloride–0.01 *M* hydrochloric acid as the primary aqueous mobile phase (Fig. 4c).

Comparison of separations using either anionic or cationic ion-pairing additives

The selectivities and retentivities of mEGF components were compared using cationic as well as anionic additives, to determine (a) whether any reversals in retention order of mEGF components occurred or additional selectivities were conferred, as a result of potentially pairing negatively, as opposed to positively charged amino acids with hydrophobic moieties, and (b) whether the alpha and beta mEGF components separately designated by Matrisian *et al.*[2] using TEA and Burgess *et al.*[3] using HFBA were, in fact, the same.

Using a TEA-containing primary aqueous mobile phase (0.05 *M* acetic acid buffered to pH 5.6 with TEA) in conjunction with acetonitrile gradient elution and the same batch of culture-grade mEGF (83-393) it was evident that the major component eluted first on both systems and its identity was confirmed by trapping and separate rechromatography.

Use of RP columns in ion-exchange mode

Fig. 4e shows the separation of mEGF components obtained solely with the use of a salt gradient on a C_{18} column, in the presence of a constant acetonitrile

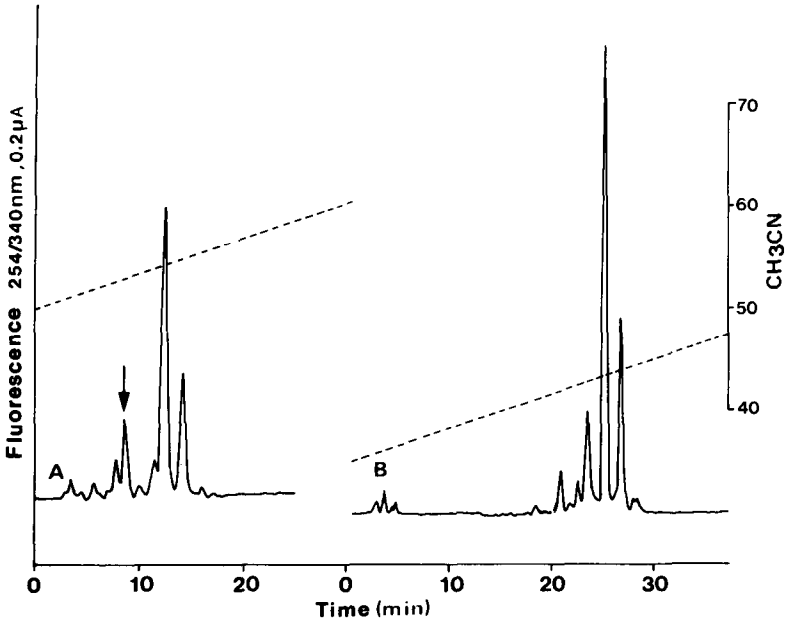

Fig. 5. Effect of simultaneous presence of anionic and cationic mobile phase additives on elution profiles of mEGF from a Spherisorb ODS2 column. In (a) the mobile phase consisted of 0.2% (w/v) (0.0048 *M*) PDFOA plus 0.2% (v/v) (0.015 *M*) TEA with acetonitrile gradient elution. Note the enhanced selectivity with respect to early-eluting peaks (arrow), which is reduced when 0.155 *M* sodium chloride is included in the aqueous mobile phase (b).

concentration, and (0.2%, w/v) PDFOA. The chromatographic profile so obtained is similar to but not identical with that obtained using a gradient of acetonitrile, and constant sodium chloride concentration.

Effects of addition of both anionic and cationic ion-pairing additives

A further attempt at enhanced selectivity was made by combining both anionic and cationic additives in the same mobile phase. In the presence of 0.015 *M* TEA it was possible to chromatograph mEGF at pH 2.9 with 0.2% (w/v) PDFOA without the greatly extended retentions seen with the latter alone (greater than 60% acetonitrile) (Fig. 5a). Although the growth factor was retained until *ca.* 50% acetonitrile on the gradient (compared with 25% with TEA–acetic acid at pH 5.6), the early-eluting component was now well-separated from the remainder. Addition of sodium chloride reduced this selective effect (Fig. 5b).

Effects of different organic modifiers

Further specific selective effects were obtained when a binary secondary solvent, acetonitrile–1-propanol (50:50), was used (Fig. 6) in conjunction with the 0.0048 *M* PDFOA–0.155 *M* sodium chloride aqueous solvent under gradient elution conditions. In this case there was a clear separation of at least two components from the early eluting single peak obtained with acetonitrile as the sole organic modifier.

Identity of major components

We have recently shown that EGF prepared from mouse submaxillary glands using only RP-HPLC techniques can yield essentially a single product, provided that appropriate precautions against proteolysis are employed[7], when such material is chromatographed using the isocratic system of Matrisian *et al.*[2] with which the alpha and beta forms were first defined. Fig. 6b shows the same preparation, originally purified using the sodium chloride–hydrochloric acid system (in which alpha and beta forms are not separated)[7] chromatographed with 0.0048 *M* PDFOA–0.155 *M* sodium chloride and an acetonitrile–propanol secondary solvent, demonstrating again its essential homogeneity and lack of the beta form by this more stringent criterion. To elucidate further the nature of the beta form, the same system was used to purify both alpha and beta mEGF from conventional commercial preparations (Fig. 6a). Following removal of the PDFOA–sodium chloride from each peak by separate re-chromatography using 0.1% (v/v) TFA under gradient elution conditions with acetonitrile, their N-terminal amino-acid sequences were determined. Sequencing studies showed that the β component has an N-terminal amino acid different from that of the α form, *viz.* serine in contrast to asparagine, when separated using this protocol. The first twelve residues of the β component corresponded to residues 2–13 of α EGF.

DISCUSSION

Approaches to protein purification using ion-pairing RP-HPLC

The purity of a protein can only be established by the criterion that it remains homogeneous when tested with all available systems under conditions of maximum potential selectivity and operational resolving power. It is evident from this study that the use of appropriate ion-pairing additives and mixed organic modifiers can

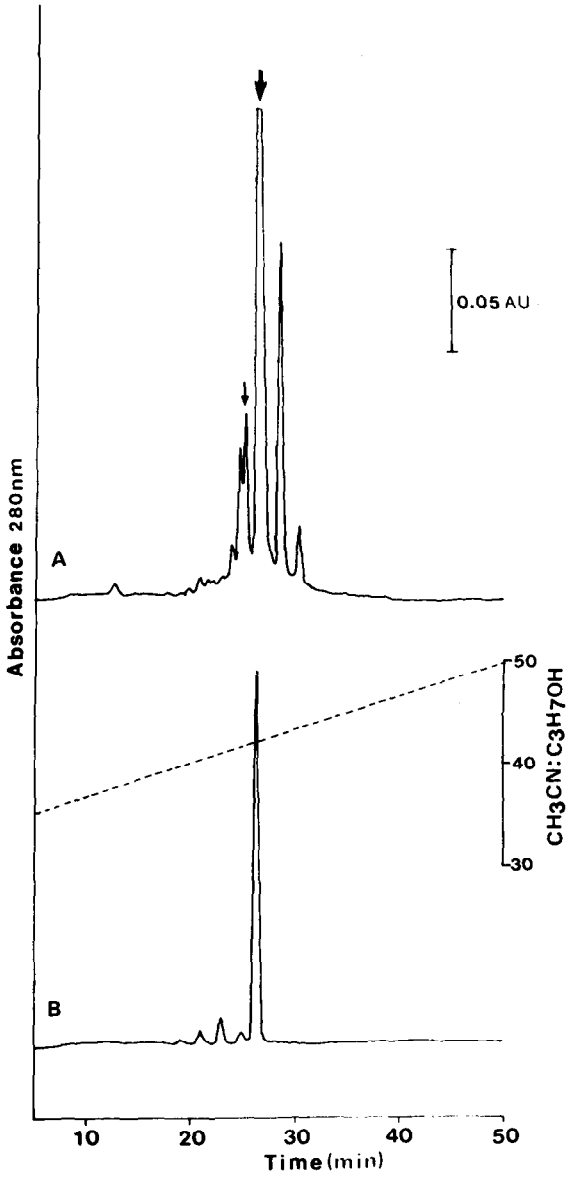

Fig. 6. Elution profiles of mEGF from Spherisorb ODS2 columns using mixed organic modifiers. In (a) 300 μg of Bio-Gel P-10 prepared mEGF (batch 84-134) was chromatographed with 0.2% (w/v) (0.0048 *M*) PDFOA–0.155 *M* sodium chloride as the primary solvent and a 1:1 (v/v) mixture of acetonitrile–1-propanol and 0.2% (w/v) PDFOA as the secondary solvent. Note the splitting of the first peak (small arrow) into two components (the off-scale peak corresponds to EGF-α (large arrow), followed by EGF-β). In (b) the same solvent system has been used to chromatograph RP-HPLC-purified EGF and demonstrates its essential homogeneity, despite having been originally separated using a sodium chloride–hydrochloric acid system[7], as in Fig. 4c, in which α- and β-forms are not resolved.

significantly enhance RP-HPLC selectivities with respect to closely chromatographing protein–polypeptide mixtures of natural origin. Our general approach to optimized separations would involve at least four steps and can be compared (and contrasted) with that recently outlined by Bennett[17], which involves different anionic and cationic ion-pairing agents used sequentially at low ionic strengths. First, a fully covered RP stationary phase should be selected with a chain length and pore size appropriate to the materials under consideration. Second, the aqueous mobile phase for the first analytical gradient elution separation (and for batch-wise elution for preliminary trace-enrichment if required) should be of high ionic strength (*e.g.* 0.155 *M* sodium chloride) so as to protect the separation against inadvertent column degradation and so as to obtain maximal efficiencies provided that the yield of more hydrophobic materials is not compromised. Third, the selective effects of ion-pairing additives of both charges should be explored, preferably using reagents of sufficient intrinsic hydrophobicity to preserve effective separations in the presence of salt. Lastly, the type (*e.g.* hydroxylated *versus* approtic) and proportion of the organic modifiers used for gradient elution should be varied so as to attain maximal selectivity. Gradient elution systems are preferable for the type of separation exemplified by EGF and its congeners, in our experience, because of the greater precision with which retention times of individual peaks can be defined and therefore correlated from run to run, as compared with isocratic polypeptide RP-HPLC. The gradient profile should be adjusted for maximum effective separation without loss of precision or greatly increased eluent volumes for each peak (greater than 0.3%/min).

Reproducibility of protein chromatography with ion-pairing mobile phase additives

The choice of individual ion-pairing reagents must also be considered with care. Thus, although HFBA gave superior resolution to TFA, after as few as four or five consecutive gradient elution runs (*i.e.* a total of only 6–7 h use) we noted a loss of efficiency and compromised separation with several types of column. This problem was not encountered with TFA-containing mobile phases. In addition, when HFBA-exposed columns were used subsequently with TFA- or sodium chloride-based gradient elution systems the expected separations were modified in a manner than indicated a "memory" of HFBA-type separations. Thus, reproducible chromatography with HFBA and other hydrophobic additive-containing solvents could only be obtained if the columns were exhaustively washed in additive-free solvents between runs or at regular intervals.

The choice of a maximum-coverage column packing is also an important parameter in ensuring reproducibly high efficiency chromatography of peptides and proteins, particularly if low ionic strength mobile phases are being used. Gradual degradation of even the best-covered RP packings cannot, however, be indefinitely prevented or avoided when using potentially corrosive mobile phases, and this may also contribute to further loss of reproducible chromatography.

The use of high molarity aqueous mobile phases (*e.g.* 0.155 *M* sodium chloride) is, therefore, desirable as they will tend to suppress ionic interactions between protonated basic solutes and any exposed silanol groups. The deliberate use of "mixed-mode" chromatography for such separations[18–20] is, in our opinion, less desirable as the limited selective effects that can be obtained (confined largely to smaller peptides) are offset by the loss of efficiency and reproducibility.

The combination of high molarity with ion-pairing selectivity can, however, permit reproducible high resolution chromatography of specific hydrophobic closely eluted proteins (*e.g.* mEGF and its congeners) and we have demonstrated here that the combination of PDFOA and sodium chloride fulfils this role.

Ion-pairing selectivities

It might be anticipated that further selectivities in protein separations would be afforded by the use of cationic additives at higher pH values[3–5] when most peptide carboxyl groups are unprotonated[14] as opposed to anionic additives at low pH[2,3]. The general similarity of mEGF eluted profiles with both TEA and perfluorocarboxylic acid indicates, however, that there are probably insufficient charge differences between the various components to effect radical changes in the separations. This is, of course, more likely to be the case with closely related groups of larger polypeptides than with smaller peptides where cationic and anionic ion-pairing can afford useful differential selectivities[21].

Mechanisms of action of ion-pairing additives

It has long been known that hydrophobic ion-pairing reagents can directly modify the alkylsilane stationary phase itself, associating with it to form a dynamic ion-exchange situation[22], and that this may be the predominant effect dictating separations, as distinct from the "ion-pair" partition mechanism often invoked[23]. The observation that such reagents can reduce capacity factors for ions of like sign has been taken as evidence of a predominant ion-exchange mechanism. Thus in the case of TEA at pH 5.6 it would appear that the repulsive effects of such a dynamically loaded TEA-rich stationary phase outweigh any increase in net hydrophobicity associated with ion-pairing with negatively charged residues present on the EGF at this pH (Table I). In the case of the carboxylic acids at low pH (2–2.5) it is not possible to distinguish specifically between ion-pair and ion-exchange effects because their net effect is the same.

In practice, of course, it is likely that both ion-exchange and ion-pairing mechanisms will operate to varying degrees at different pHs and ionic strengths with different additives and packings with different alkyl chain lengths[1,18,21]. The difficulty lies in predicting the net effect on selectivity and thus effective separations. The inclusion of an ion such as Cl^- in the mobile phase may alter the relative dominance of ion-exchange *versus* ion-pairing mechanisms in a given separation in the presence of hydrophobic additives. It might first compete with the free hydrophobic anion to suppress ion-pair formation and only at higher concentrations compete with the potentially multiple ionic interactions between each multiple positively charged protein molecule and the cation-exchanger created by dynamic loading of perfluorocarboxylic acids such as PDFOA into the stationary phase. In the latter situation cooperativity effects would tend to increase the strength of interaction, compared with association in free solution (*i.e.* as ion-pairs in the mobile phase). Thus, some separations in complex mixtures may be particularly sensitive to ionic strength in the presence of a hydrophobic additive if an ion-pair mechanism tends to dictate their retention, while others in which ion-exchange is the dominant mode may be less affected. In the case of crude mEGF preparations one component is indeed particularly sensitive to the overall ionic strength of the eluent. The remainder, by contrast,

are less affected, indicating that ion-exchange may be the main mechanism responsible for their separation.

Organic solvent-dependent selective effects

The use of mixed organic solvents also provided additional selectivities in the present study. Again the difficulty lies in predicting the effects. As noted by Tarr and Crabb[24] the use of mixed organic modifiers gives apparently anomalous (*i.e.* disproportionately extended) retentions with polypeptides. These effects are well known in reversed-phase systems using mixed organic modifiers with other solutes, and are, for example, encountered when attempting to optimize ternary solvent compositions by computer-aided methods[25]. They have been extensively considered in a recent review by Snyder[26].

Nature of mEGF components

In this study a crude preparation of mEGF was used to simulate the likely spectrum of related products that might also be found when other less well-defined growth factors are extracted from tissues[27,28]. Evaluation of the significance of the selectivity changes obtained in these experiments does, of course, relate to the identity of the mEGF components.

Some microheterogeneity of proteins can be caused by alterations to amino acids during isolation and handling, including exposure to certain solvents. These include oxidations, deamidations, esterifications and amino group changes. In the case of mEGF, however, some of the observed heterogeneity appears to be due to sequence differences.

The observation that the α and β forms do not share the same N-terminal amino acid contrasts with a previous report[3], and indicates that the β congener is probably a proteolytic product ($mEGF_{2\text{-}53}$) of the α, *i.e.* true mEGF. This explains why it is possible under some conditions to isolate the α form directly from the submaxillary glands, essentially free from other components (see Fig. 6b). The other major early-eluting peak in the Biogel P10 preparations used here probably contains so-called EGF-2 (EGF_{1-51}), this is another product of limited proteolysis originally described by Savage and Cohen[29], and is largely removed by conventional DEAE-column chromatography *e.g.* when the receptor grade EGF is prepared.

Irrespective of whether the other EGF congeners are a consequence of artifactual proteolytic degradation during extraction, or intracellular processing of a quasi-physiological type as has been suggested by others[3,5], their biological significance must be evaluated in the light of the observation[3,7] that the alpha and beta forms share comparable biological activities, in contradistinction to the original report by Matrisian *et al.*[2]. So too do the alpha 1 and alpha 2 moieties recently separated by Burgess *et al.*[6] using anion-exchange HPLC with MonoQ (Pharmacia). Further studies on the minor components resolved with the PDFOA–sodium chloride system, as illustrated in Fig. 5, indicate that these are also generally bioactive with equivalent potencies[30] and are, therefore, all likely to share major sequence homologies with α/β EGF.

Despite the apparently rather rigidly determined elution orders of proteins under RP-HPLC conditions, it is clear that useful selective effects can be obtained with a systematic approach to mobile phase composition and with the use of more

complex mixtures than the simple binary solvents that have tended to predominate up to now.

It is hoped that these approaches will prove of value in the further purification of other growth factors such as fibroblast growth factor (FGF)[27], and other pituitary-derived materials[28].

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