Journal of Chromatography, 287 (1984) 29–44
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,432

INVESTIGATION OF THE PARAMETERS FOR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF COLLAGEN TYPES I AND III

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(Received October 7th, 1983)

SUMMARY

A detailed study was carried out on the behaviour of collagen and its subcomponents on large pore size reversed-phase high-performance liquid chromatographic columns. Investigation of laboratory-prepared C_{18} and CN 50-nm pore size supports using a simple solvent system showed that heat-denatured collagen chains could be retained and resolved and that the C_{18} packing had a greater selective capacity. However, because of inconsistencies in the chromatography obtained using laboratory-prepared supports commercial 33-nm pore size packings were subsequently studied with both pyridine-based and trifluoroacetic acid-based solvent systems. An optimised solvent was defined and used to check the resolving capacity of both C_{18} and CN commercial columns. These studies led to the description and interpretation of the effects of pH, counter-ion concentration, solvent strength, type of support and molecular weight of protein solutes on the chromatographic behaviour of this large protein and its CNBr peptides. An explanation of the major forces acting to bring about retention and resolution is presented and suggestions are made for the application of this methodology to other proteins.

INTRODUCTION

Collagen, the major connective tissue protein, exists as a multiplicity of genetic forms all having closely related structures and, in the fibrous types, equivalent molecular sizes[1]. In the past, separation and analysis of these molecules has relied almost exclusively on salt fractionation, conventional liquid chromatography and electrophoretic techniques[2,3], methods which are time consuming and, often, wasteful of sample. Consequently, much attention has recently been focused on the separation of collagens and collagen peptides by high-performance liquid chromatography

0021-9673/84/$03.00

(HPLC). The three main approaches which have been adopted are size exclusion HPLC, reversed-phase HPLC in high molarity pyridine-based solvents with post-column derivatization for detection and reversed-phase HPLC in low molarity, UV-compatible solvents.

Size exclusion HPLC has resulted in limited success in separations of fibrous collagens due to the similarity in molecular size of these species[4,5]. The technique has been applied to CNBr peptides of several collagen types with some success although no data was presented on resolution of peptides from mixtures of collagen types or from whole tissue[6]. Also, elution times were only slightly shorter than those reported for reversed-phase separations of similar CNBr peptides[7].

The potentially more powerful and versatile method of reversed-phase HPLC has been used to separate both native and denatured chains of several collagens in pyridine-based solvents[8,9]. Although a significant step in the understanding of reversed-phase HPLC of collagen, this method relied on an elaborate post-column derivatization for detection and used laboratory-prepared columns not generally available. The method also introduced large pore sized (30 nm, 50 nm) octyl (C_8) and cyanopropyl (CN) silicas for the resolution of proteins larger than 30 kilodalton.

A UV-compatible system, utilising perfluorinated carboxylic acids, has been developed for the separation of CNBr peptides of collagen types I, II and III as well as the tryptic peptides of type I collagen[7,10]. These systems also used large pore (30 nm) silicas with octadecyl (C_{18}) coatings and provided the advantage of low molarity and volatility for easy post-column analysis.

Recently we published a method for the separation of the native molecules of types I and III bovine skin collagens and also the resolution of the denatured chains of these collagens[11] using the commercially available 33-nm pore size Baker Bond C_{18} column. During the development of the simple, UV-compatible solvent system which we reported, several important factors influencing the behaviour of denatured type I chains on both CN and C_{18} Baker Bond packings were elucidated.

In this paper we present the data which led to the optimization of our system for the resolution of both denatured and native forms of type I and type III collagen and discuss the importance and significance of these findings to the approach needed in the evolution or adaptation of a solvent system for reversed-phase HPLC of proteins.

MATERIALS AND METHODS

Columns

The reversed-phase packings studied in this work were laboratory-prepared C_{18} and CN 50-nm pore size silicas (made as described earlier[8]) and Baker Bond C_{18} and CN 33-nm pore size silicas obtained through Linton Products (Harlow, Essex). All columns were 25 × 0.46 cm analytical size.

Reagents and equipment

For preparation of reversed-phase HPLC columns, Lichrosphere Si-500 was obtained from BDH Chemicals (Poole, U.K.) and γ-cyanopropylchlorosilane and octadecyltrichlorosilane from Aldrich (Gillingham, U.K.). All salts and acids used in this work were of highest analytical quality from BDH and all organic solvents

were HPLC grade from Rathburn Chemicals (Walkerburn, U.K.). Solvents were filtered and vacuum degassed before use except when containing ammonium bicarbonate when they were helium degassed continuously during chromatography. HPLC was carried out on a Du Pont Series 850 liquid chromatograph with a temperature-controlled column compartment and a Du Pont 860 UV detector.

Samples

Bovine, chick and human types I and III collagens were purified by salt fractionation of pepsin-solubilised collagen from foetal bovine skin, chicken gizzard and human placenta as described before[12–14]. Pepsin-solubilised collagens from adult human skin, obtained at necropsy, were prepared as described by Lovell *et al.*[15]. CNBr peptides of foetal bovine skin type I collagen were prepared as previously described[3]. All collagen preparations were checked for purity by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis before use and were stored lyophilised at −20°C. Samples were dissolved at 1 mg/ml in either the starting solvent used for chromatography or 0.5 *M* acetic acid. Prior to loading, samples were centrifuged at 9000 *g* for 2 min or filtered through 0.45-μm pore size filters (Millipore, Bedford, MA, U.S.A.). In the studies of denatured collagen chains samples were heated to 60°C or 80°C for 1 min and then immediately loaded onto the chromatograph.

Reversed-phase HPLC

In these studies several different solvent systems were investigated. Parameters of molarity, pH and organic solvent were tested with both CN and C_{18} columns. In each case, the precise chromatographic conditions are indicated in the figure legends. Unless otherwise indicated a flow-rate of 1 ml/min was used. Native collagens were separated at ambient temperature and denatured chains of pure collagens were separated at 35°C, denatured chains of human type I and type III mixtures at 50°C and CNBr peptides at 50°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Analyses of purified collagens and post-HPLC fractions were carried out using 5.5% (w/v) acrylamide slab gels with the Tris–borate buffer system described by Sykes and Bailey[16]. Post-HPLC fractions (1 ml) in volatile solvents were dried at 44°C under vacuum in a Savant Speed Vac Concentrator Model RH 40-12 (Savant Inst., Hicksville, NY, U.S.A.). When necessary, peaks of protein were pooled and dialysed against water prior to freeze drying and analysis. CNBr peptides were collected and identified after HPLC by SDS–polyacrylamide gel electrophoresis on 10% (w/v) acrylamide gels as previously described[3].

RESULTS

Reversed-phase HPLC of collagen using laboratory-prepared columns

From the investigation of laboratory-prepared 50-nm C_{18} and CN columns used with UV-compatible solvent systems several characteristics of the chromatography of collagen chains became apparent. The C_{18} packing exhibited greater selective capacity than the CN packing and we were able to achieve a separation of

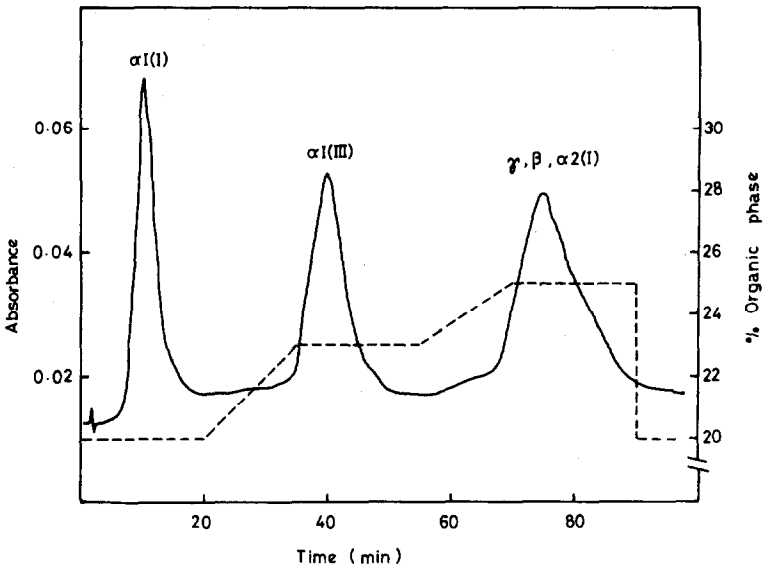


Fig. 1. Reversed-phase HPLC separation of denatured type I and III collagen chains. 500 μg of a mixture (1:1) of heat-denatured bovine types I and III collagen were loaded onto a 25 × 0.46 cm column of 50-nm pore size, C_{18}-coated laboratory-prepared silica. Flow-rate 1 ml/min, 35°C, solvent A = 0.1% (v/v) trifluoroacetic acid pH 2.2, solvent B = tetrahydrofuran. Gradient as shown was 20% B for 20 min, 20–23% B over 15 min, 23% B for 20 min, 23–25% B over 15 min, 25% for 20 min.

denatured αl chains of type I and type III collagen (Fig. 1). The elution order of the collagen chains was found to be dependent on the column packing and not the eluting solvent. However, the particular organic solvent used did have significant influence on the resolution of the denatured types I and III collagens. Tetrahydrofuran was demonstrated to give superior resolution to acetonitrile.

Though the packing characteristics (elution order and degree of resolution) were consistent between the columns tested, significant difficulties were also experienced. Exact reproducibility between runs with carefully controlled conditions was difficult to achieve and the columns appeared to deteriorate rapidly. The working lives of each of four columns were respectively 75, 79 and 119 runs for three separate C_{18} columns and 87 runs for the one CN column tested. Because of these inconsistencies the laboratory-prepared columns were abandoned in favour of commercial columns although the important characteristics of reversed-phase HPLC of collagen noted above were taken into account.

Reversed-phase HPLC of collagen using commercial columns

When the commercial Baker Bond reversed-phase columns became available and were examined using bovine type I collagen with the trifluoroacetic acid–organic solvent system[7,10] no collagenous material was recovered from these columns although their packings carried the same derivative as those in our laboratory-prepared columns. In other words, columns from different sources with the same bonded derivative exhibited significantly different results.

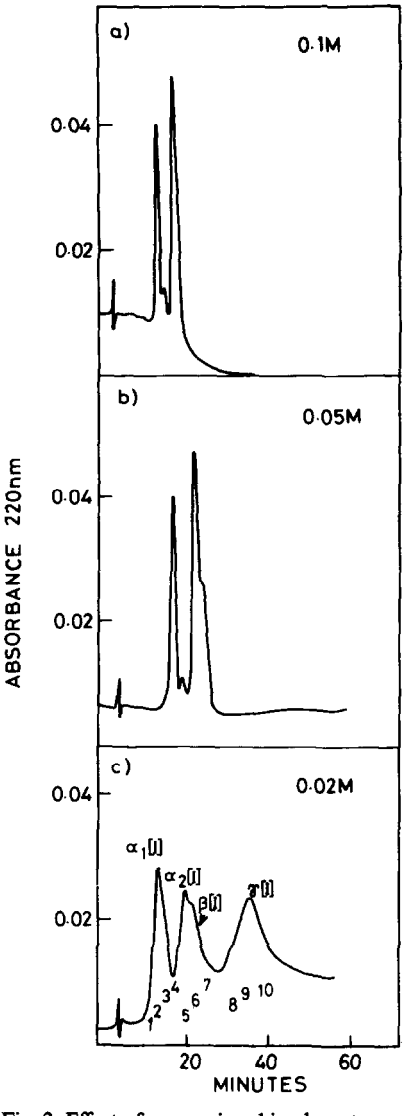


Fig. 2. Effect of ammonium bicarbonate concentration on the elution and resolution of denatured bovine type I collagen chains. The figure shows the effect of adding 0.1 *M* (a), 0.05 *M* (b) and 0.02 *M* (c) ammonium bicarbonate to the TFA–water phase (solvent A) of the UV-transparent system. Solvent B was acetonitrile and the gradient was from 20 to 30% B over 60 min.

Pyridine-based solvent system

The behaviour of the Baker Bond 33-nm CN and C_{18} columns was examined using the pyridine–formate–propanol method as outlined in the literature accompanying the columns[17]. Fractions (6 ml) were collected and visualised by SDS-PAGE analysis. The type I collagen chains were retained but not resolved with the programme used even when 2-min fractions were collected. This was consistent with the provided literature.

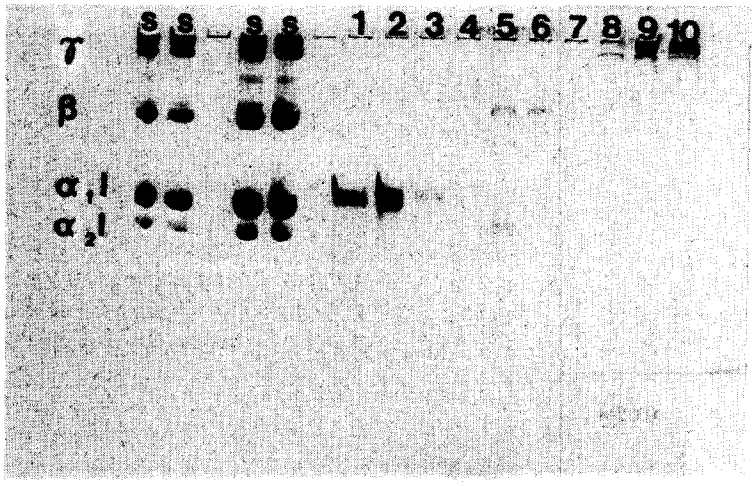


Fig. 3. SDS–polyacrylamide gel analysis of fractions collected after HPLC of denatured bovine type I collagen chains. HPLC was carried out with 0.02 *M* ammonium bicarbonate in solvent A (TFA–water), see Fig. 2c. S = Standard type I collagen. Note that α1 chains appear in fractions 1–3, α2 chains and β components in fractions 5 and 6 and γ components in fractions 8–10.

UV-compatible solvent system

Apart from the chemical nature of pyridine and its contribution to the elution/resolution of collagens, the most striking aspect of the pyridine-based solvent systems was their high molarity. As the ionic strength of the mobile phase increases so does the extent of interprotein and protein–stationary phase hydrophobic interactions[18], but the presence of a suitable counter-ion in the solvent can also overcome any ionic interactions caused by free, uncapped silanols. As mentioned above, the trifluoroacetic acid–organic solvent system alone did not elute any collagen from Baker Bond CN or C_{18} columns. Following the rationale that this effect could be due to excessive protein–support ionic interactions, the molarity of the mobile phase was increased. Ammonium bicarbonate was investigated as a volatile salt with low UV absorbance to increase the molarity of the trifluoroacetic acid–organic solvent system. Ammonium bicarbonate (0.5 *M*) with hydrochloric acid and 2-propanol was found to elute bovine skin type I collagen chains from the Baker Bond 33-nm CN packing (not shown).

A systematic study was then initiated to establish the stability and reproducibility of this packing with ammonium bicarbonate-containing solvents and to determine what factors influenced the resolution.

The effect of changing ammonium bicarbonate concentration on resolution

Since ammonium bicarbonate was introduced into the system to increase the ionic strength of the solvent, it was interesting to note that decreasing the concentration of ammonium bicarbonate significantly improved the resolution as shown in Fig. 2. It is apparent that there is an optimal concentration of ammonium bicarbonate for resolution which lies between 0.02 and 0.05 *M*. The concentration of 0.05 *M* was selected for the optimised solvent system to prevent the peak broadening effects ob-

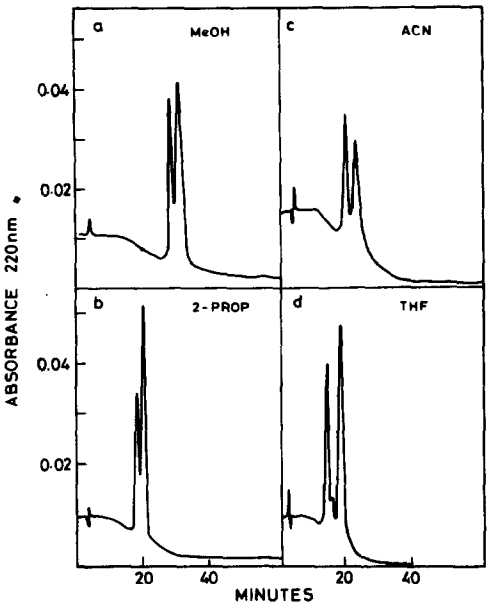


Fig. 4. Effect of organic solvent on retention and resolution of denatured bovine type I collagen chains during HPLC. Bovine type I collagen denatured chains were separated on a 25 × 0.46 cm Baker Bond CN column (33 nm) in 0.05 *M* ammonium bicarbonate–TFA–water pH 3.2 (solvent A) with a gradient formed with methanol (a), 2-propanol (b), acetonitrile (c) and tetrahydrofuran (d) as solvent B, 1 ml/min, 35°C. Gradient as in Fig. 2.

served at lower molarity. Fig. 3 identifies the material eluting in each fraction and illustrates the order of elution of collagen type I chains from the Baker Bond 33-nm CN column using 0.02 *M* ammonium bicarbonate.

The effect of organic solvents on resolution

As previously experienced with the laboratory-prepared columns and as proposed by the theory of Snyder[20], the organic solvent used has a significant influence on selectivity. Fig. 4 illustrates that tetrahydrofuran gave the best selectivity for the type I chains on the baker Bond 33-nm CN packing under the conditions described.

The resolution equation suggests that, within limits, increasing retention time leads to improved resolution. However, Fig. 4 clearly shows that defining organic solvents in terms of hydrophobicity alone does not reflect on the ability of that solvent to improve resolution (that is, its selective capacity) of the collagen chains. This is consistent with our observation (not shown) that the collagen components elute at a very specific organic phase concentration.

The effect of pH on resolution

Fallon *et al.*[9] noted a critical dependence of the separation of native collagens on the pH of their solvent. Fig. 5 shows that the pH also affects selectivity when ammonium bicarbonate (0.05 *M*), trifluoroacetic acid and tetrahydrofuran are used as the eluting solvent system. As the pH of the solvent was decreased, the resolution of the denatured chains of the type I collagen was improved.

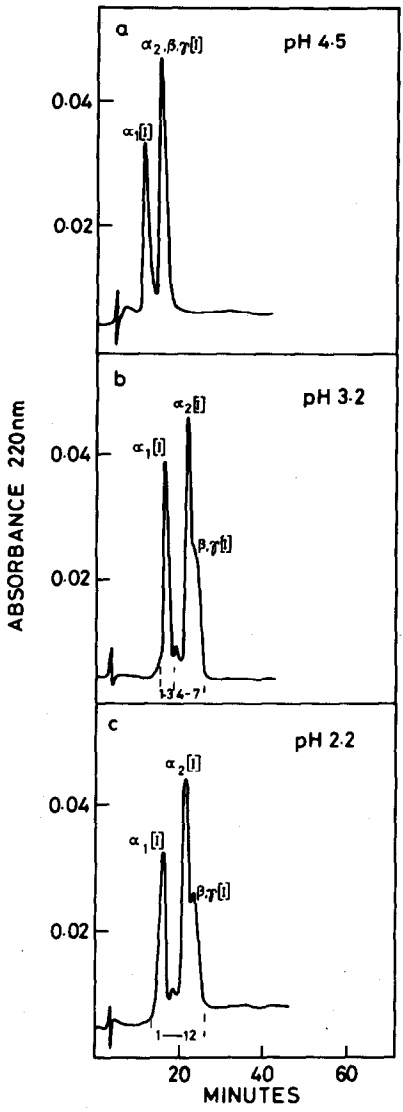


Fig. 5. Effect of pH on retention and resolution of denatured bovine type I collagen chains during HPLC. Denatured bovine type I collagen chains were separated by HPLC on a 33-nm Baker Bond CN column (25 × 0.46 cm) in 0.05 *M* ammonium bicarbonate–TFA–water (solvent A) with a gradient of THF. Solvent A was adjusted, by addition of TFA, to pH 4.5 (a), pH 3.2 (b) and pH 2.2 (c); 1 ml/min, 35°C, gradient as in Fig. 2.

The amount of trifluoroacetic acid added to 0.05 *M* ammonium bicarbonate to give pH 3.2 was 0.4%. Only very small differences in this amount of trifluoroacetic acid were required to give the different pH values examined.

The effect of stationary phase on resolution

In our systematic study of the parameters needed to resolve denatured collagen

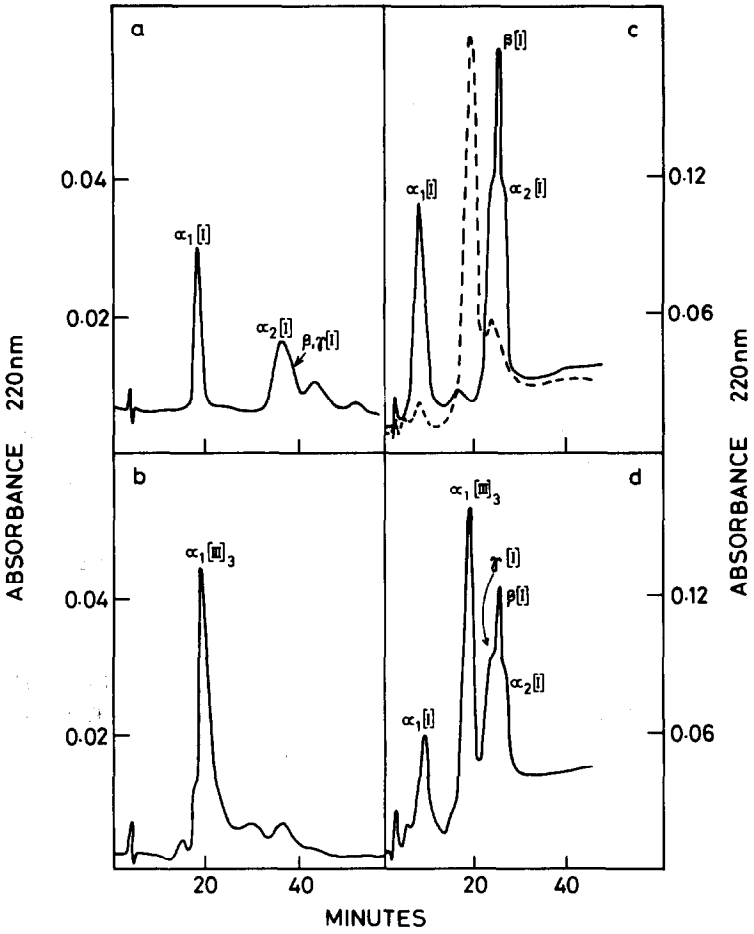


Fig. 6. Effect of column type on resolution of denatured bovine collagen chains by HPLC on 33 nm-pore-size columns. Chains of type I, type III or a mixture of type I and III collagens were separated by HPLC in the optimised solvent system on a CN column (a and b) and a C_{18} column (c and d). a, Denatured bovine type I collagen; b, denatured bovine type III collagen; c, denatured bovine type I collagen with position of separately chromatography denatured type III collagen α1 (III) chains shown by dotted line; d, mixture of denatured bovine types I and III collagen. Flow-rate 1 ml/min, 35°C, solvent A = 0.05 *M* NH_4HCO_3, 0.4% (v/v) trifluoroacetic acid pH 3.2, solvent B = tetrahydrofuran. Gradient in each case 20% B for 10 min, 20–30% B over 30 min, 30% B for 15 min.

chains we used the Baker Bond 33-nm CN column. Although this packing gave resolution of type I collagen chains it could not resolve the denatured chains of type III collagen from those of type I when eluted with the optimised solvent system (Fig. 6a, b). However, when mixtures of denatured chains of type I and type III collagen were chromatographed on Baker Bond 33-nm C_{18} columns, good resolution could be achieved using the same optimised solvent system (Fig. 6c, d). It is interesting to note that the elution order of the type I components was different from the CN packing when compared to the C_{18} packing. The reproducibility of the separation of denatured types I and III chains, which takes only 35 min, was extremely good

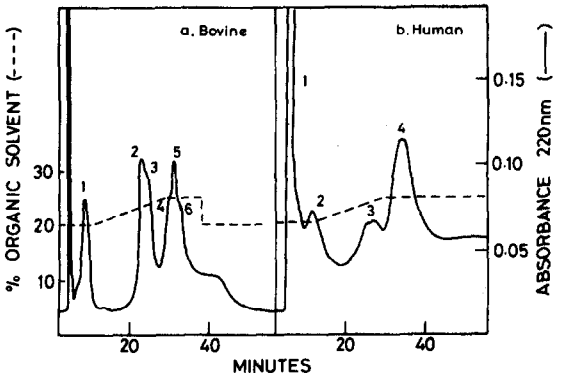


Fig. 7. Species differences in HPLC of collagen chains. The optimised solvent system (see Fig. 6) was used to separate the denatured chains of bovine type I and III collagens (a) and human type I and III collagens (b). Peaks in (a) contained α1 (I) chains (1), α1 (III) chains (2, 3), and α2 (I) chains, β and γ components of type I (4–6). In (b) peaks contained α1 (I) chains (2), α1 (III) chains (3) and α2 (I), β and γ components of type I (4).

and 89 and 65 consecutive runs on two separate C_{18} columns led to no observable column deterioration or decrease in resolution.

Species variability

All of the data presented above were obtained using bovine types I and III collagen. The same reversed-phase HPLC system, already shown to resolve fully the mixed chains of denatured bovine types I and III, when used with human type I and

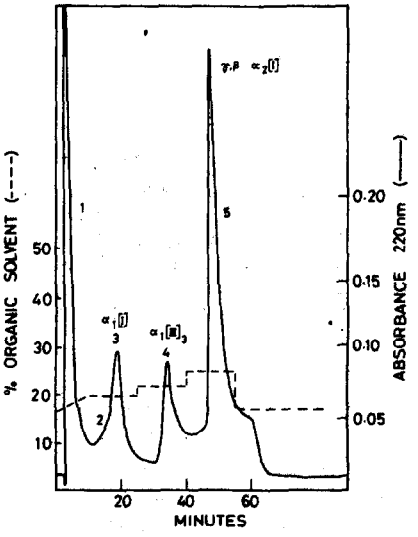
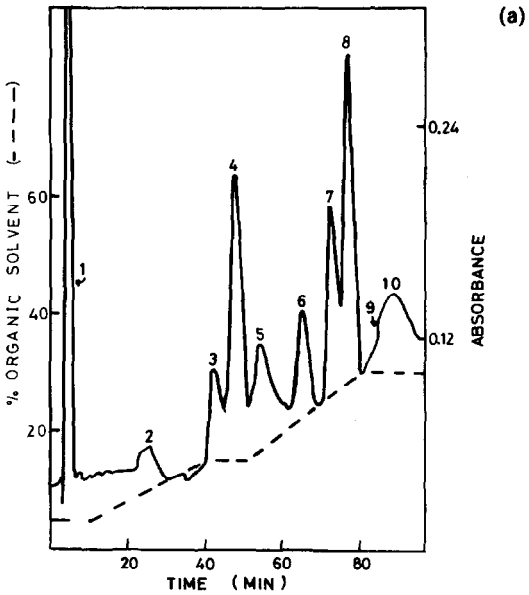


Fig. 8. Manipulation of gradient to achieve resolution of denatured human types I and III collagen chains. Full resolution of human α1 (I), α1 (III), and γ (I), β (I) and α2 (I) components was achieved by subtle alteration of the gradient elution as shown in the figure. The column was a 25 × 0.46 cm Baker Bond (33-nm pore size) C_{18} and was eluted with 0.05 *M* ammonium bicarbonate–TFA pH 3.2 (solvent A) and THF (solvent B). Flow-rate 1 ml/min, 35°C.



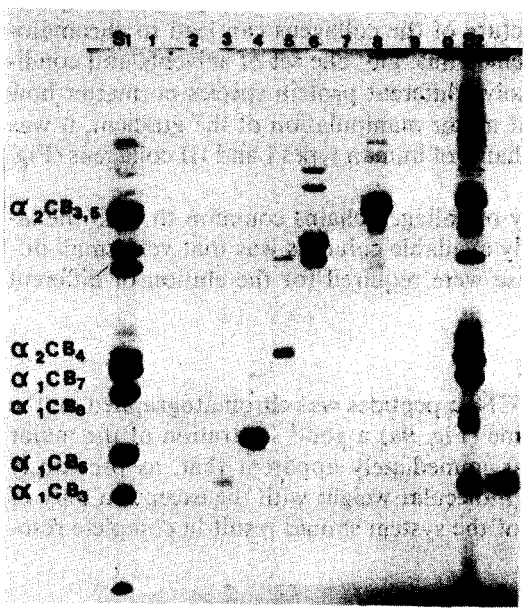


Fig. 9. Resolution of CNBr peptides of bovine type I collagen. (a) HPLC of bovine type I CNBr peptides on C_{18} 33-nm column in the optimised solvent system (as in Fig. 6). Flow-rate 1 ml/min, 50°C. (b) SDS-polyacrylamide gel analysis of fractions from section a. S1 and S2 are two standard mixtures of bovine type I CNBr peptides.

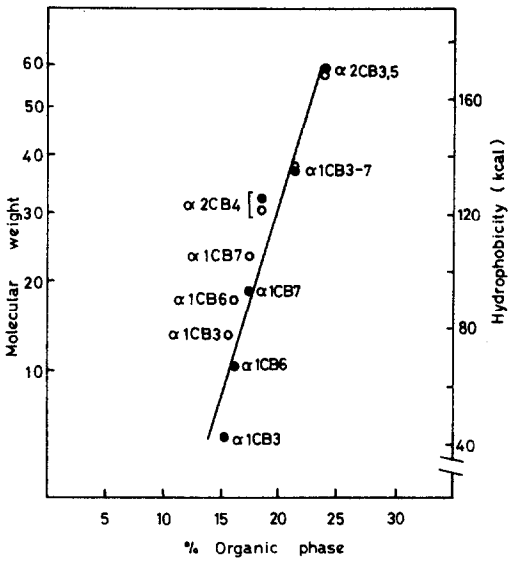


Fig. 10. Graph of molecular weight and hydrophobicity of CNBr peptides of type I collagen *vs.* % solvent B in which they were eluted. The hydrophobicity of each peptide was calculated from data in ref. 25. ●, Values of hydrophobicity; ○, values of molecular weight.

III gave poor resolution of denatured chains (Fig. 7). This result highlights the point that species variability in primary structure of the collagens can lead to chromatographic differences using the same system. Thus, any one set of solvents and conditions cannot be expected always to resolve different protein species no matter how closely related. However, by careful but minor manipulation of the gradient, it was possible to resolve fully the denatured chains of human types I and III collagens (Fig. 8).

A feature of the chromatography of collagen chains common to both the laboratory-prepared and the commercially available columns was that very small differences in the percentage organic phase were required for the elution of different components.

Collagen CNBr peptides

When a mixture of bovine type I CNBr peptides was chromatographed on the C_{18} column using a modified programme (Fig. 9a) a good separation of the major peptides was achieved (Fig. 9b). It was immediately apparent that, as previously shown[7], the peptides eluted in order of molecular weight with the exception only of α2CB4 (Fig. 10). Further modification of the system should result in complete resolution of all the type I CNBr peptides.

DISCUSSION

Recent interest in the development of HPLC methods for the separation of proteins larger than 30 kilodalton has led to the manufacture of wide pore reversed-phase supports. It has been suggested that the improved efficiency obtained with

these supports is due to the increased surface area available to proteins which can penetrate the particles. Pearson *et al.*[21] have also shown that the silica particles alone may lead to differences in protein chromatography. In the present report we have studied the reversed-phase HPLC of collagen and its subcomponents on both laboratory-prepared and commercially obtained supports and we have found considerable variation in chromatographic behaviour due to different stationary phase characteristics. Using these supports we have also developed a solvent system superior to those reported earlier[9,10].

The systematic studies carried out in our investigations led to the optimization of a UV-compatible system at pH 3.2 in 0.05 *M* ammonium bicarbonate with tetrahydrofuran as the eluting organic solvent. Manipulation of the gradients allowed resolution of all the denatured chains of type I and III bovine collagen as well as human type I and III collagens, native bovine types I and III collagen[11] and the CNBr peptides of bovine type I collagen. In each case the pH of the aqueous solvent was adjusted to 3.2 with trifluoroacetic acid.

When heptafluorobutyric acid was used as an alternative to trifluoroacetic acid, higher concentrations of organic solvent were required for elution than when trifluoroacetic acid was used (results not shown) with no improvement in resolution. This is not an unexpected result since the theory of ion-pairing predicts that the bulkier four-carbon butyric acid would make the protein more hydrophobic than the smaller acetic acid molecule. Further, the background absorbance from this reagent was significantly higher introducing the practical difficulties of large baseline changes during gradient elution[10].

Meek and Rossetti[22] found that the retention of small peptides (20 amino acid residues or less) on reversed-phase columns could be predicted by summing the contribution of each amino acid and end group. The retention coefficients used describe the hydrophobic contribution to retention of each amino acid and end group. Their results suggest that conformation and primary sequence have minor effects on retention. Other groups have shown that when different solvents are used, the retention coefficients have different values[22–24]. Since the hydrophobic contributions determine the chromatographic behaviour of the peptides, this suggests that partitioning between the hydrophobic stationary phase and the mobile solvent is the main event in the column contributing to resolution.

However, mechanisms involved in the separation of large peptides and proteins by reversed-phase chromatography are not so clearly defined. The experiences reported by researchers investigating the separation of large proteins suggest that each protein requires its own specific conditions for elution. As yet these specific conditions cannot be predicted and must be experimentally determined.

Our data indicate that a predominant interaction in the chromatography studied here is ionic or electrostatic in nature as trifluoroacetic acid (TFA)–organic solvents alone would not elute any collagen from either CN or C_{18} columns. With addition of low amounts of salt these interactions were masked and other non-ionic associations became predominant. 50 m*M* ammonium bicarbonate did not fully mask the ionic interactions, however, since at pH 4.5, where free silanols would be expected to carry low net negative charges and collagen would carry a net negative charge (assuming full ion-pairing of charged lysine and hydroxylysine side chains with TFA), some electrostatic repulsion was in evidence causing early elution of collagen chains

and, consequently, less resolution. At pH 3.2, where the collagen molecule, if fully ion-paired, would carry no net negative charge and free silanols are protonated the retention times increased and resolution was improved. At pH 2.2, this effect was even more marked indicating the minimal rôle of charge in this chromatography and the consequent importance of hydrophobic interactions. Similarly, use of heptafluorobutyric acid further increased retention times confirming that hydrophobic interactions are of prime importance in these separations.

This effect is highlighted if the total hydrophobicity of the collagen molecules separated is considered. As the molecules have a uniform Gly-Pro-X sequence (where X can be any amino acid except glycine) any fragment or polymer of the parent α chain will contain roughly the same number of hydrophobic residues per 1000 residues as the α chain. This means that the total hydrophobicity of any collagen peptide or polypeptide will be directly related to its molecular weight. Thus, if hydrophobicity is the major force in affecting retention times we would expect that the separation of molecules would correlate with molecular weight. This is indeed what we found with plots of molecular weights or hydrophobicity of CNBr peptides against eluting solvent concentration (Fig. 10). Van der Rest *et al.*[7] have also shown a molecular weight-dependent elution of collagen CNBr peptides, with small peptides eluting first. A similar effect has been found using the pyridine–formate system (Fallon, unpublished results).

The apparent anomaly of the high retention times for $\alpha 2$ chains (which may be expected to be the same as those for $\alpha 1$ chains) may be explained in terms of a higher net hydrophobicity of these chains[25]. Similarly, the apparently anomalous elution characteristics of the structurally more complex collagen dimers (β) and trimers (γ) is probably due to the effect of conformational restrictions on availability of hydrophobic sites.

An important observation of the present work is that collagen exhibits very specific chromatographic characteristics on reversed-phase HPLC columns. It was found that the collagen components studied, which included native molecules[11], denatured chains, β and γ components and CNBr peptides, all elute under specific conditions which are very similar. The elution of each of the collagen components at a particular per cent organic phase meant that gradient manipulation had to be precisely controlled. Baseline resolution could be achieved for some components when the difference in organic elution phase varied by as little as 2%. Therefore a short linear gradient up to a plateau at the precise percentage organic phase required to elute one component can be followed by a linear gradient and plateau to elute the next component and this can be carried out in a repetitive sequence to elute a mixture of collagen components. The optimum gradient was established for the collagens at 1/3% change in organic phase per minute. A shallower gradient resulted in peak broadening with an adverse effect on resolution.

These data suggest that an "on–off" type mechanism is acting in the elution characteristic of these large proteins. A very small increase in organic phase concentration elicits a large change in interaction sufficient to elute the protein.

A useful analogy is that of ion-exchange chromatography of proteins wherein the charged protein binds to the support and at a specific salt concentration or pH is desorbed *in toto*. In this case there is no partition of the protein between stationary and mobile phase and column length has little or no effect on chromatography char-

acteristics except in determining the capacity of binding. In the case of collagen reversed-phase HPLC under the conditions which we have used, we suggest that an absorption–desorption effect is involved wherein the predominant binding forces are hydrophobic. Thus, at the critical organic phase concentration the molecules of one species are fully desorbed. With other large proteins a similar independence of chromatographic characteristics on column length was observed[19] further supporting our suggestion of an "on–off" mechanism.

This has profound implications for the practice of testing column integrity with small molecules such as benzene and acetophenone which are separated by classical partition chromatography during HPLC. We found that we had to assess the column integrity with the type of protein we were investigating, *i.e.*, collagen, and routinely used the elution of type I collagen to test the column stability over a long period of time.

The importance of collagen in the initiation and course of many diseases is still ill defined. Similarly, the analysis of minor forms of collagen such as type M[26] and type IV[1] is often hampered merely by the lack of suitable analytical techniques. HPLC now offers a totally new approach to the problem of biochemical analysis of the various genetic forms of collagen in normal and pathological tissues. Because of the versatility of the method procollagens, whole molecules, denatured chains, CNBr peptides and proteolytic fragments may all, potentially, be prepared and analysed. The present investigations and recent reports by other investigators in the field of HPLC of large proteins show that HPLC can fulfil the need for rapid, more efficient separation techniques in the field of collagen research.

ACKNOWLEDGEMENTS

We are indebted to Mr. T. J. Sims and Mr. N. C. Avery for excellent technical assistance. K.A.S. was in receipt of a postgraduate grant from the Medical Research Council of New Zealand.

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