Journal of Chromatography, 212 (1981) 295–304
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,836

SEPARATION OF CYTOCHROMES *c* BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received April 1st, 1981)

SUMMARY

Six kinds of cytochrome *c* of different origin, *i.e.*, bovine, chicken, dog, horse, rabbit and tuna, were subjected to separation by reversed-phase high-performance liquid chromatography on three commercial packing materials; octadecyl-, octyl- and cyanoalkyl-silicas. The effects of reversed-phase material, mobile phase and temperature on the separation of cytochromes *c* were examined. The parameters of the mobile phase were the organic modifier, the pH, the salt concentration and additives. Under optimal conditions, five of the six cytochromes *c* were resolved in 10 min. The relative retention values cannot be explained in terms of the relative lipophilicities of the side-chains of the amino acid residues.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is now a well-established method for the separation of native and synthetic peptides[1,2]. Several papers[3–12] have described the success of RP-HPLC in separating closely related peptides. Some detailed investigations[4,6,13–16] have been reported on the experimental conditions and parameters affecting the separation of such peptides. On the other hand, recent advances in aqueous gel permeation chromatography have permitted rapid analysis of a mixture of proteins of a wide range of molecular weights by size exclusion[2,17–19]. However, proteins of comparable sizes cannot be separated by this method.

We have already shown[8–10] that RP-HPLC is extremely useful for the separation of peptides having closely related structures, such as those that differ only in (i) the number of constituent amino acid residues by one or more, (ii) the kind of amino acid residues, including optical isomers, (iii) the sequence of amino acid residues, or (iv) the structure of the acyl groups attached to the N-terminal groups. The molecular weights of the peptides in our previous work[8] are below 6000.

In the present study, cytochromes *c* were chosen as a group of closely related proteins in order to explore the possibility of the fine separation of larger peptides by RP-HPLC. Cytochrome *c* is a hemoprotein found in the cells of all aerobic or-

0021-9673/81/0000–0000/$02.50

TABLE I

DIFFERENCES IN AMINO ACID SEQUENCES IN SIX CYTOCHROMES c[20]

The differences between the sequence of amino acid residues are indicated by italics.

	Position																				
Source	*4*	*9*	*15*	*22*	*28*	*33*	*44*	*46*	*47*	*54*	*58*	*60*	*61*	*62*	*88*	*89*	*92*	*95*	*100*	*103*	*104*
Bovine	Glu	Ile	Ala	Lys	Thr	His	Pro	Phe	Ser	Asn	Thr	Gly	Glu	Glu	Lys	Gly	Glu	Ile	Lys	Asn	Glu
Horse	Glu	Ile	Ala	Lys	Thr	His	Pro	Phe	*Thr*	Asn	Thr	*Lys*	Glu	Glu	Lys	*Thr*	Glu	Ile	Lys	Asn	Glu
Dog	Glu	Ile	Ala	Lys	Thr	His	Pro	Phe	Ser	Asn	Thr	Gly	Glu	Glu	*Thr*	Gly	*Ala*	Ile	Lys	Asn	Glu
Rabbit	Glu	Ile	Ala	Lys	Thr	His	*Val*	Phe	Ser	Asn	Thr	Gly	Glu	*Asn*	Lys	*Asp*	*Ala*	Ile	Lys	Asn	Glu
Chicken	Glu	Ile	*Ser*	Lys	Thr	His	*Glu*	Phe	Ser	Asn	Thr	Gly	Glu	*Asp*	Lys	*Ser*	*Val*	Ile	*Asp*	*Ser*	*Lys*
Tuna	*Ala*	*Thr*	Ala	*Asn*	*Val*	*Trp*	*Glu*	*Tyr*	Ser	*Ser*	*Val*	*Asn*	*Asn*	*Asp*	Lys	Gly	Gln	*Val*	*Ser*	*Ser*	–

ganisms and consists of a single polypeptide chain of *ca.* 104 amino acid residues with the heme group attached through cysteine residues at positions 14 and 17. Studies were made on the separation of five mammalian cytochromes *c* whose sequences differ by between two and eight out of 104 amino acid residues, and one from tuna which is appreciably different from the mammalian proteins, for the purpose of examining the effects of experimental parameters and conditions.

EXPERIMENTAL

Reagents and materials

All six cytochromes *c* investigated were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. The differences between them in the sequences of amino acid residues are listed in Table I[20]. Acetonitrile (Wako, Osaka, Japan) and methanol (Nakarai, Kyoto, Japan) were of HPLC quality, and the other chemicals used were of reagent grade. Antioxidant- and peroxide-free tetrahydrofuran (THF) was obtained by passing commercial THF through an active aluminium oxide column. Water was purified by means of an ion-exchange column followed by single distillation in glassware.

Apparatus

A Perkin-Elmer Series 2/2 liquid chromatograph equipped with a Rheodyne 7105 sample injector was used in conjunction with a Perkin-Elmer LC-65T UV detector/oven. As packing materials, Nucleosil $7C_{18}$, Nucleosil $7C_8$ or Nucleosil 5CN (Macherey, Nagel & Co., Düren, G.F.R.) were used. The columns (10 cm × 4.6 mm I.D.) were packed by the modified viscosity method recommended by the manufacturer, using a Chemco slurry-packing apparatus Model 124 (Chemco, Osaka, Japan) at *ca.* 500 kg/cm^2.

Procedure

Cytochrome *c* was dissolved in distilled water to give a concentration of 1 mg/ml. The sample solutions were kept at −20°C when not in use. Amounts of sample injected were usually of the order of micrograms.

Experiments were carried out at room temperature unless otherwise stated. The flow-rate was 1.0 or 2.0 ml/min. The detector was operated at 210, 220 or 400 nm. The mobile phase was filtered through a 0.7-μm membrane filter and degassed prior to use. The recovery of cytochrome *c* eluted from the column was measured by the method of Lowry *et al.*[21].

RESULTS AND DISCUSSION

Reversed-phase materials

Three kinds of commercial packing material, *i.e.* silica gel with chemically bonded octadecyl (Nucleosil $7C_{18}$), octyl (Nucleosil $7C_8$) and nitrile (Nucleosil 5CN) groups, were used. The order of elution of the cytochromes *c* investigated on the C_{18} column under the conditions indicated in Fig. 1 was as follows: horse > rabbit > bovine = chicken = tuna > dog. Bovine, chicken and tuna cytochromes *c* could not be resolved on the C_{18} column although various conditions were examined.

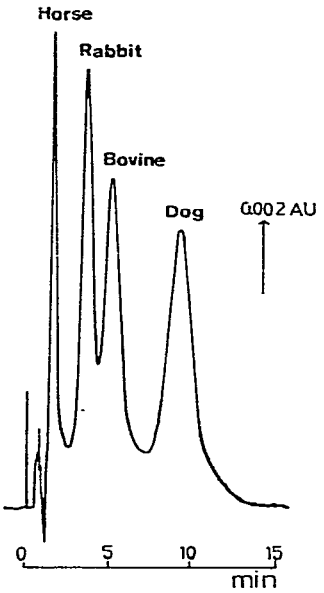

Fig. 1. Separation of cytochromes *c* on a C_{18} column. Conditions: mobile phase, mixture of 69% of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate, and 31% of acetonitrile; flow-rate, 2.0 ml/min; detection, absorption at 210 nm; sample size, total 6 μg in the ratio of 3:3:6:8 in the order of elution.

Five kinds of cytochromes *c* were successfully separated on the C_8 column as shown in Fig. 2, but bovine and chicken were again not separable. Cytochrome *c* from tuna was eluted between bovine and dog, the order of elution of the other cytochromes *c* being the same as for the C_{18} column. The content of acetonitrile in the

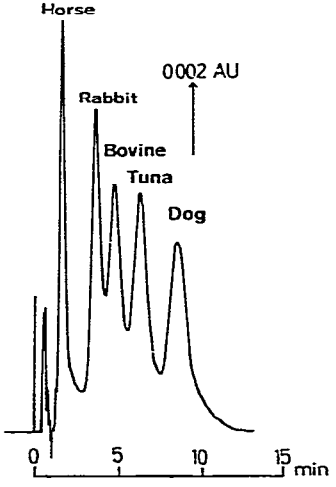

Fig. 2. Separation of cytochromes *c* on a C_8 column. Contitions: mobile phase, mixture of 72.5% of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate, and 27.5% of acetonitrile; flow-rate, 2.0 ml/min; detection, absorption at 220 nm; sample size, total 11 μg (2:2:2:2:3).

mobile phase was reduced to 27.5% on the C_8 column to obtain suitable retention times.

The order of elution on the CN column (Fig. 3A) was as follows: horse > rabbit = bovine = chicken > dog > tuna. It is noticeable that the order of elution of dog and tuna on the CN column was the reverse of that observed on the C_{18} and C_8 columns. The mobile phase containing a higher concentration of phosphate buffer (0.1 *M*) gave better resolution on the CN column than that employed for the C_{18} and C_8 columns.

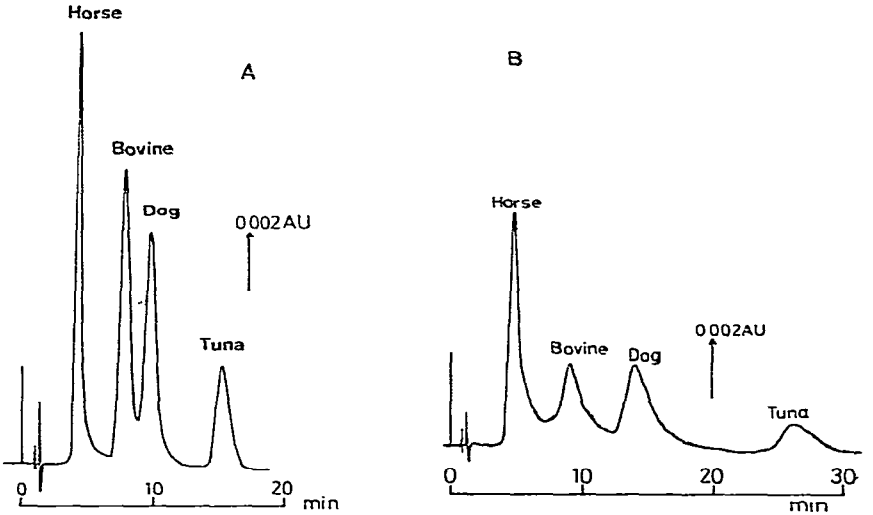

Fig. 3. Separation of cytochromes *c* on a CN column at different pH values. Conditions: mobile phase, 77.5% of 0.1 *M* phosphate buffer (A, pH 2.0; B, pH 3.0) containing 0.05 *M* sodium sulphate and 22.5% of acetonitrile; flow-rate, 1.0 ml/min; detection, absorption at 400 nm; sample size, total 10 μg (1:1:1:1).

A few initial injections of cytochrome *c* gave no appreciable peaks until the total injections amounted to *ca.* 10 μg, when the newly packed columns were used. Moreover, initially recorded peaks were unusually broad and unsymmetrical, and several additional injections were required in order to obtain satisfactory peak shapes. This observation suggests that the packing materials used have some active sites where cytochrome *c* is irreversibly adsorbed. The recovery of cytochrome *c* from the column was more than 80%, as determined by the method of Lowry *et al.*[21], after the column was conditioned by several injections of cytochrome *c*.

The capacity factors of the six cytochromes *c* as a function of the packing the naphthalene peak eluted with the methanol–water solvent system, although the efficiency of the columns for cytochromes *c* was rather low. No significant differences in the resolution were noticed between elution on the column mentioned above and elution on more efficient columns having more than 5000 theoretical plates for naphthalene.

The capacity factors of the six cytochromes *c* as a function of the packing material are shown in Fig. 4. Bovine and chicken cytochromes *c* could not be resolved on any of the three columns, as shown in Fig. 4. Their separation can be effected on an NH_2 column (Nucleosil $5NH_2$, 15 cm × 4.6 mm I.D.) with 0.005 *M* phosphate

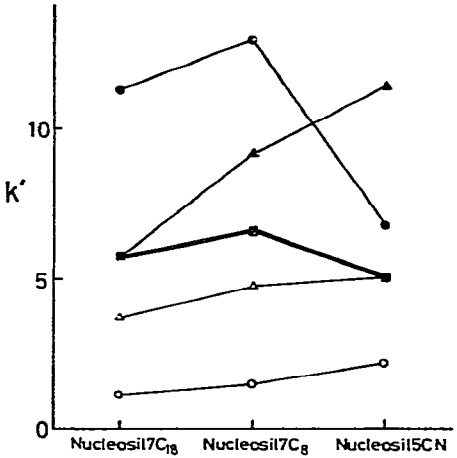

Fig. 4. Plot of capacity factor (k') of cytochrome *c* *vs.* packing material. (○) Horse, (△) rabbit, (■) bovine and chicken, (▲) tuna and (●) dog. Conditions are indicated in Figs. 1–3.

buffer (pH 6.0) containing 0.05 *M* sodium sulphate as the mobile phase, although these conditions may not be regarded as those for RP-HPLC. The order of elution was as follows: tuna > horse = chicken > rabbit = bovine = dog.

Mobile phase

Effect of organic modifier. When 30% of THF was employed instead of 31% of acetonitrile under the conditions indicated in Fig. 1, tuna cytochrome *c* was eluted last from the C_{18} column and the other cytochromes *c* were not resolved, except for horse cytochrome *c* which was eluted first. In contrast, the same order of elution was observed on the nitrile column for the following two solvent systems: one consisting of 22.5% of acetonitrile and 77.5% of 0.1 *M* phosphate buffer (pH 3.0) containing 0.005 *M* sodium sulphate, and one consisting of 40% of methanol and 60% of the buffer. A much higher content of methanol than acetonitrile was required to obtain comparable retention times. The solvent system containing methanol gave broader peaks than the acetonitrile system.

The dependence of the capacity factor on the composition of the mobile phase was investigated for four peptides of different molecular weights on the C_{18} column. The mobile phases with slightly different compositions were prepared by means of the solvent programmer of the liquid chromatograph in order to obtain reproducible compositions. The plot of the logarithm of the capacity factor against the content of acetonitrile gave straight lines with different slopes, as shown in Fig. 5. The larger the peptide molecule, the steeper the slope of the line in Fig. 5. This result is consistent with observations[6,8] that the retention times of peptides are highly dependent on the amount of the organic modifier present. In addition, it is interesting that the plot of molecular weights of peptides against the slopes of the plots in Fig. 5 gives an almost straight line.

Effect of pH. As the packing materials employed are based on silica gel, the pH of the mobile phase is limited to the range 2–8. The acidic mobile phases (pH 2–3) were preferable to the neutral in reducing peak tailings for cytochrome *c*. No signifi-

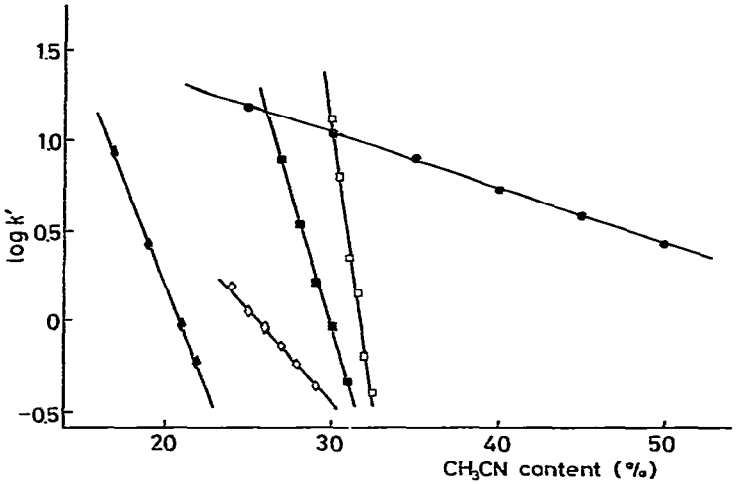

Fig. 5. Dependence of k' of cytochrome *c* on the content of acetonitrile in the mobile phase. (●) Benzene, (◇) [Gly^4,Phe^5]-LH-RH^8, (◆) ACTH-(1-26)-NH$_2$^{8}, (■) porcine insulin^8 and (□) bovine cytochrome *c*. Conditions: column, Nucleosil 7C$_{18}$ 10 cm × 4.6 mm I.D.; mobile phase, mixture of acetonitrile and 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate; flow-rate, 1.0 ml/min.

cant differences in the chromatograms were recognized between pH 2.0 and 3.0 on the C_{18} and C_8 columns. However, alterations in retention behaviour and peak widths were noticed on the CN column between pH 2.0 and 3.0, as shown in Fig. 3.

Effect of salt concentration. The effect of the concentration of sodium sulphate was shown in Fig. 6. The capacity factor decreased with an increase in salt concentration. Although the separation was not appreciably influenced in the tested range of

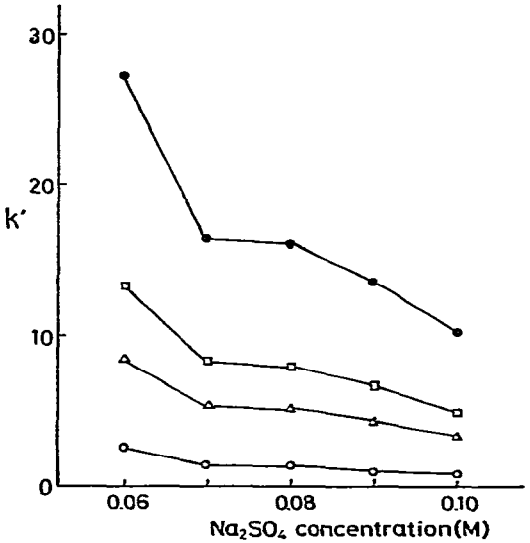

Fig. 6. Dependence of k' of cytochrome *c* on the salt concentration. (○) Horse, (△) rabbit, (□) bovine and (●) dog. Conditions are the same as shown in Fig. 1 except for the concentration of sodium sulphate.

salt concentration, the addition of some salt was found desirable in order to obtain symmetrical peaks.

Effect of additives. Capacity factors of cytochromes *c* on the C_{18} column increased with an increasing amount of EDTA disodium salt added to the mobile phase in the range 0.001–0.003 *M*, but otherwise no appreciable effect was observed. No significant change in capacity factors or peak symmetry was noticed when an ion-pairing agent, 0.005 *M* sodium 1-butanesulphonate or 0.0001–0.0004 *M* L-arginine, was added to the mobile phase.

The peak symmetry and resolution of cytochromes *c* on the C_{18} column were much improved by the addition of horse cytochrome *c* in the concentration of 3.3 mg per litre of the buffer ($2.6 \cdot 10^{-7}$ *M*), as shown in Fig. 7. The addition of cytochrome *c* in higher concentration resulted in an extra peak at the retention time of horse cytochrome *c*.

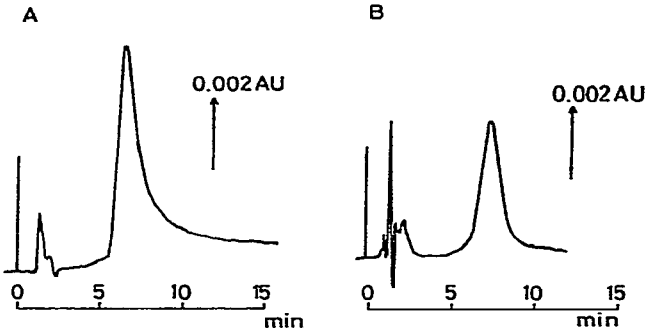

Fig. 7. Effect of cytochrome *c* added to the mobile phase. (A) No cytochrome *c* added, (B) $2.6 \cdot 10^{-7}$ *M* horse cytochrome *c* added to the buffer. Conditions: column, Nucleosil $7C_{18}$; mobile phase, mixture of 68.5% of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate and 31.5% of acetonitrile; flow-rate, 1.0 ml/min; detection, absorption at 220 nm; sample, bovine cytochrome *c* (A) 4 μg, (B) 2 μg.

Effect of temperature

The temperature dependence of retention in RP-HPLC is receiving increasing attention (*e.g.*, ref. 22). Plots of the logarithm of the capacity factor against the reciprocal of the temperature usually show a linear relationship, and this was in fact observed for cytochromes *c* on the CN column although the slopes of the plots were different for different cytochromes *c*. On the C_{18} column, however, no such dependence was obtained, as shown in Fig. 8. The reason for this observation is not clear. Increasing temperature was less effective for improving the separation of cytochrome *c* both on the CN and the C_{18} columns.

Relative order of elution of cytochrome c

The separation of closely related peptides by RP-HPLC has generally been explained in terms of the difference in hydrophobicity of the side-chains of the constituent amino acid residues[8,14,23]. This interpretation is invalid in some instances, however, and conformational and hydrophobic properties of the whole molecule and its size should be taken into account[8,14,16,23].

All the amino acid residues different among five cytochromes *c* shown in Table

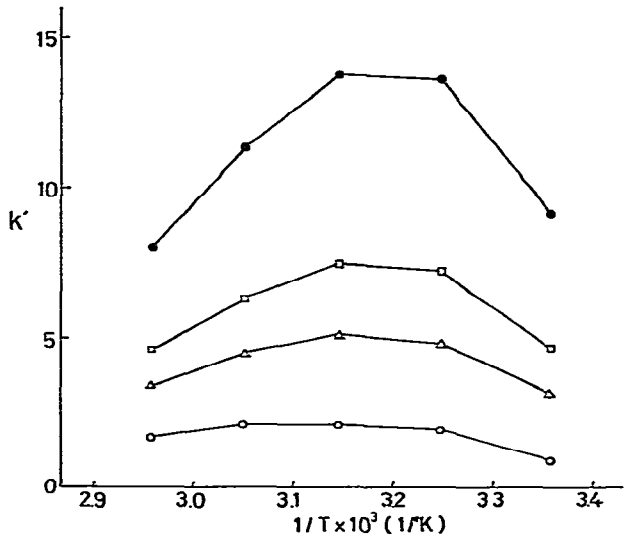

Fig. 8. Dependence of k' of cytochrome *c* on the temperature. (○) Horse, (△) rabbit, (□) bovine and (●) dog. Conditions: column, Nucleosil $7C_{18}$; mobile phase, mixture of 69 % of 0.005 *M* phosphate buffer (pH 3.0) containing 0.1 *M* sodium sulphate and 31 % of acetonitrile.

I (except for tuna) are situated on the outside of the molecule[24]. This means that the side-chains of these amino acid residues can interact with the stationary phase and, therefore, the alteration of the amino acid residue at these positions can affect the hydrophobic interaction of cytochrome *c* with the hydrocarbonaceous stationary phase.

The relative retention values of the six cytochromes *c* do not correspond with the relative hydrophobicities calculated from the relative lipophilicities[25] of the side-chains of amino acid residues at the structurally variable positions. This suggests that the minor conformational change of the molecule supposedly produced by the alteration of the amino acid residue is more dominant in determining the retention of cytochrome *c* than the change in hydrophobicity of the side-chain at the relevant position.

CONCLUSION

Five kinds of cytochrome *c*, *i.e.*, bovine, dog, horse, rabbit and tuna, have been separated by employing appropriate reversed-phase, chromatographic conditions. However, the efficiency of the columns was much lower than for small molecules. This low efficiency can be attributed partly to the low diffusitivity of cytochrome *c* in the mobile phase and partly to the irreversible adsorption of cytochrome *c* on the stationary phase. It is desirable to develop new packing materials for the separation of proteins by RP-HPLC.

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