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VIZUALIZATION OF PROTEIN RETENTION AND MIGRATION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The adsorption and elution of proteins in reversed-phase liquid chromatographic (RPLC) systems were investigated by visually observing the retention and migration of colored and non-colored proteins in glass columns, packed with RPLC matrices. This system permits direct observation and measurement of the retention characteristics of colored proteins under various isocratic and gradient conditions.

The system vividly illustrates that in organic-lean acidic eluents (*e.g.*, aq. 0.1% trifluoroacetic acid), proteins are adsorbed on the closest available hydrophobic surface of the support without displacing any noticeable amount of previously injected proteins. When the eluent contains a moderate amount of organic solvent (*e.g.*, aq. 20% acetonitrile), proteins displace previously injected proteins of lesser hydrophobicity but do not displace previously injected proteins of greater hydrophobicity. These observations support the view that proteins are adsorbed on RPLC matrices in a monolayer.

Observation of color-protein migration in glass columns during isocratic elution provides a practical means for determining very long retention times at very low mobile phase strength as well as the acceleration of protein migration during gradient elution. Our observations under such conditions agree with the retention relationships described by the chromatography theory for small molecules.

INTRODUCTION

The continuing growth of reversed-phase liquid chromatography (RPLC) for the isolation and analysis of peptides and proteins has motivated a great number of investigations concerning the physiochemical mechanisms and optimization of such separations[1–4]. RPLC of biopolymers typically involves elution from silicabased bonded phases by a gradient of increasing organic solvent content of an aqueous mobile phase at low pH. Although stationary phase characteristics, including those of the silica support (*i.e.*, chemical treatment, particle size, and pore structure) and of the hydrophobic matrix bonded to it (*i.e.*, alkyl or aryl ligand structure, bonded-phase density and surface coverage) are important, chromatographic performance is predominantly influenced by the organic and ionic makeup of the mobile phase[1–7].

Indeed, the most striking observation made when separating macromolecules

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by RPLC is the extraordinary dependence of retention on mobile phase strength. When systematically increasing the organic solvent percentage of mobile phase used for isocratic elution from RPLC columns, the retention times of proteins seem infinite up to a point, which is characteristic of the protein, where very small increments (*e.g.*, 1%) result in enormous decreases in retention[4]. Such observations have been explained by a model proposed by Armstrong and co-workers[8,9] in which macromolecules are precipitated at the head of a RPLC column in the presence of low-strength mobile phases and experience no significant migration until the mobile phase strength increases to some critical composition which induces dissolution and subsequent elution. These authors contend that the fundamental behavior of macromolecules in chromatographic systems differs from that of smaller molecules because conformational and chemical changes probably occur within macromolecules during the chromatographic process. Thus classical theory based on chromatography of small molecules is inappropriate to explain the chromatographic behavior of macromolecules.

However, Snyder and co-workers[10,11] have shown that the retention dependence of several macromolecules on solvent strength in the relatively narrow range where retention times can be measured practically is, in fact, adequately described by the so called "small-molecule" theory. This theory predicts a linear relationship between a sample's retention, in terms of the logarithm of its capacity factor (k'), with the volume fraction of organic solvent (φ) of mobile phase:

$$\log k' = \log K_w - S\,\varphi \tag{1}$$

where S is the slope of a plot of $\log k'$ *vs.* φ, and K_w is the capacity factor of the sample eluted by an organic-solvent-free mobile phase. This theory predicts that all sample molecules migrate through the column to some extent at all mobile phase strengths, even at very low strengths, where migration is unnoticeable by conventional methods. However, this theory assumes that the conformational and chemical nature of the sample molecules remain constant during the chromatographic process.

Most attempts to elucidate the retention–elution behavior of proteins in RPLC systems have relied largely upon measurements of the time required for their elution under various chromatographic conditions. This study attempts to verify the inferences made from such studies by directly observing the retention and elution of colored proteins in transparent columns, packed with RPLC matrices typically used for protein separations. Glass columns packed with a microparticulate silica-based octadecyl-bonded stationary phase were constructed and used to observe the chromatographic behavior of cytochrome *c* and hemoglobin, along with that of the non-colored proteins, ribonuclease and lysozyme, under isocratic and gradient elution conditions. In addition to permitting direct observation of how proteins initially interact with RPLC matrices, this system provided a practical means of measuring extremely large retention times (t_R) and capacity factors (k') during elution with low-strength mobile phases. These values are determined by directly measuring the linear velocity of the colored bands (u_x) and by estimating the linear velocity of the mobile phase (u) from column dead time (t_0) estimates and the column length (L):

$$k' = (u/u_x) + 1 = (t_0/Lu_x) + 1 \tag{2}$$

$$t_R = (L/u_x) \tag{3}$$

Direct observations of the migration of colored bands also permitted the measurement of the accelerated rate of movement through the glass column during gradient elution.

EXPERIMENTAL

Apparatus

The chromatographic system used in this study consisted of a Perkin-Elmer Series 4 pump, equipped with a Model 7125-S injection valve, a LC-95 variable-wavelength UV detector, and a Model R-100 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.).

Column

Borosilicate glass tubes of 2 mm I.D. and 6 mm O.D. were cut into 100-mm lengths and washed with 2-propanol, followed by acetone. One end of the glass tube was fitted with an internally threaded end-fitting held by a Vespel ferrule and a 1/4-in. nut. The end-fitting sealed a 2-μm porous stainless-steel frit and a PTFE O-ring (0.005 in. thickness) to the head of the glass column. The other end of the column was attached to a small funnel via a piece of PTFE tubing. The column was dry-packed with 10 μm HCODS reversed phase (Perkin-Elmer) by filling the funnel with packing material while applying a vacuum to the end fitting of the column. After packing, an end fitting was secured to the remaining end of the column in the manner described above. Acetone was then pumped through the column at a flow-rate of 0.2 ml/min which caused the packing bed to be compressed and to form a void at the head of the column. The end fitting at the column head was disassembled in order to fill the void with additional HCODS packing by gravity sedimentation from an acetone suspensoid. The fully packed column was washed with acetone, followed by acetonitrile at a flow-rate of 0.4 ml/min.

Reagents

Mobile phases were formulated with HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.), Gold-Label-grade trifluoroacetic acid (TFA) (Aldrich, Milwaukee, WI, U.S.A.), and distilled water, purified through a NanoPure III system (Barnstead/Sybron Boston, MA, U.S.A.). Mobile phases were degassed by helium sparging. The proteins (cytochrome *c*, hemoglobin, lysozyme, and ribonuclease A) were purchased from Sigma (St. Louis, MO, U.S.A.). Proteins were dissolved in aq. 0.1% TFA to achieve a concentration of 10 mg/ml.

Procedure

All mobile phases were blended from two solvent reservoirs, one containing pure acetonitrile and the other aq. 0.1% TFA. Flow-rates of 0.5 ml/min or less were used to keep the column backpressure to less than 6 MPa, which was the maximum the glass columns could tolerate. Flow-rate accuracy was periodically checked by timing volumetric collections of the column effluent. The column dead-time (t_0) was estimated from the retention times of repeated injections (via a 6-μl sample loop) of pure water, which were detected by a decrease in the background absorbance of 200 nm UV light. At this wavelength, gradient profiles were visualized by recording the

baseline shifts, caused by the addition of the less-absorbing acetonitrile to the more-absorbing aq. 0.1% TFA.

In an attempt to achieve an equilibrium distribution between the protein solutions and the stationary phase at the head of the column, 20- to 50-μl injections were made into the system, while the mobile phase flow-rate was at 0.1 ml/min. The migration of colored protein bands was measured in millimeters over time periods measured with a stopwatch. Photographs were taken with a 3-mm SLR camera through a 50-mm F2 S lens with a +4 close-up lens attachment. In order to avoid increasing the column temperature, photographs of the system were taken under ambient lighting conditions using 400 ASA film.

RESULTS AND DISCUSSION

System performance

Some difficulties were encountered with the glass-column system. The end fitting at the head of the column had a tendency to slip under pressure. The slippage was remedied by roughening the glass surface at each end of the column to provide sufficient friction for the Vespel ferrule. However, this treatment made the glass more susceptible to breakage at the ferrule–glass interface, especially at column back-pressures greater than 6 MPa. As a result, our experiments were limited by the lifetime of the columns. Although the column packing method used in this study was easily performed and reproduced, some inconsistencies in the packed bed structure were always evident by the distortion of chromatographic bands. Much of the band distortion arose from irregularities of the inside surface of the glass columns. Alternatives to the borosilicate glass used in this study for the fabrication of robust transparent columns with smooth internal surfaces are being explored. Despite these dif-

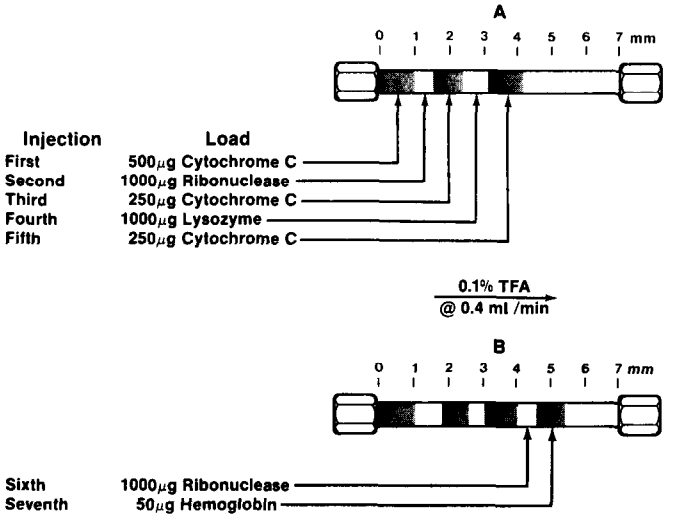

Fig. 1. Alternating injections of colored and non-colored proteins in low-strength mobile phase. Mobile phase: aq. 0.1% TFA; flow-rate: 0.4 ml/min. (A) Successive injections of proteins as shown; (B) additional injections of RNase followed by hemoglobin.

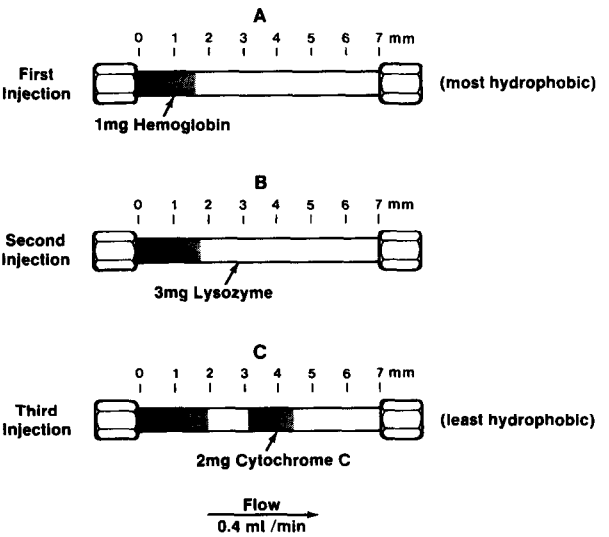

Fig. 2. Alternating injections of proteins in order of decreasing hydrophobicity in moderate-strength mobile phase. Mobile phase: aq. 20% acetonitrile in aq. 0.1% TFA; flow-rate: 0.4 ml/min. (A) First load: 1 mg hemoglobin; (B) second load: 3 mg lysozyme; (C) third load: 2 mg cytochrome *c*.

ficulties, the present system permitted several reliable observations and measurements to be made.

Protein adsorption

While the column was equilibrated with aq. 0.1% TFA, multiple injections of colored and non-colored proteins were made into the chromatographic system. The amount of colored protein injected was directly proportional to the length of the colored band produced in the column.

When the glass RPLC column was equilibrated with aq. 0.1% TFA, a 500-μg load of cytochrome *c* formed a 2-cm long band at the head of the column. When followed by a 2-mg load of ribonuclease A (RNase) and then a 250-μg load of cytochrome *c*, the respective non-colored and colored bands gave the column a striped appearance (Fig. 1A). An additional load of 1 mg of lysozyme, followed by 250 μg of cytochrome *c*, also resulted in a striped appearance (Fig. 1A) as did alternating loads of 1 mg RNase and 50 μg hemoglobin (Fig. 1B). Regardless of the sequence of loading, the four proteins which differed in hydrophobicity (hemoglobin > lysozyme > cytochrome *c* > RNase), migrated down the column and were adsorbed on the closest unoccupied surfaces, without displacing previously loaded proteins.

When the column was equilibrated with aq. 20% acetonitrile in aq. 0.1% TFA, injctions of colored and non-colored proteins resulted in a striped appearance (Fig. 2) only when the loading sequence was from most hydrophobic to least hydrophobic (*i.e.*, hemoglobin first, followed by lysozyme, and cytochrome *c* last). When this sequence was reversed, injections of lysozyme apparently displaced the previously loaded cytochrome *c* molecules from the head of the column (Fig. 3A and B). A final load of hemoglobin apparently displaced the previously adsorbed proteins at the head of the column (Figure 3C).

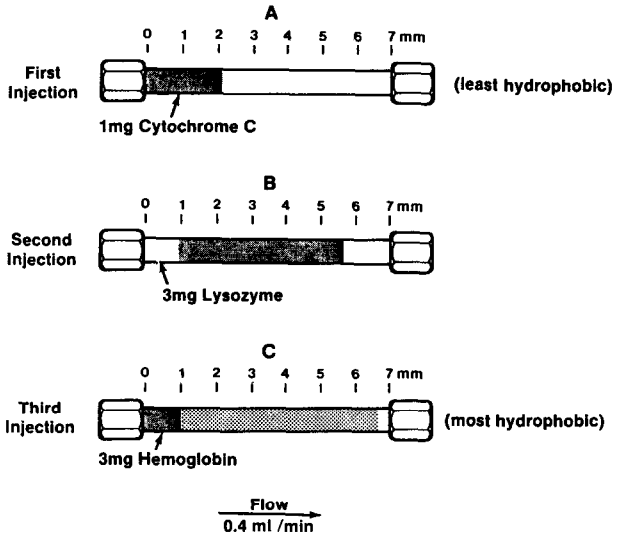

Fig. 3. Alternating injections of proteins in order of increasing hydrophobicity in moderate-strength mobile phase. Mobile phase conditions same as in Fig. 2. (A) first load: 1 mg cytochrome *c*; (B) second load: 3 mg lysozyme; (C) third load: 3 mg hemoglobin.

In the absence of organic solvent, the striped bands were formed, apparently because the rates of desorption of previously injected proteins under these condition were so low that equilibration of additional proteins could not occur until the injected sample had passed further into the column. The presence of aq. 20% acetonitrile in the mobile phase apparently increased the desorption rates and permitted competition between different protein molecules for hydrophobic sites on the stationary phase.

Protein elution

The above loading experiments indicate that proteins are not quite as stagnant in RPLC systems in the presence of moderate-strength mobile phases as previously assumed[4,8,9]. Such assumptions were based on retention time measurements by con-

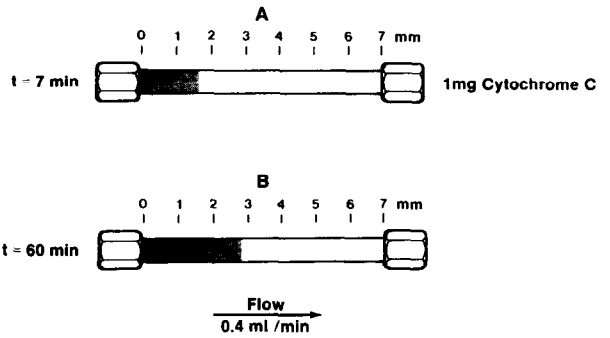

Fig. 4. Isocratic elution of cytochrome *c* by moderate strength mobile phase. Mobile phase conditions same as in Fig. 2. Injection: 1 mg cytochrome *c*. (A) 7 min after injection; (B) 60 min after injection.

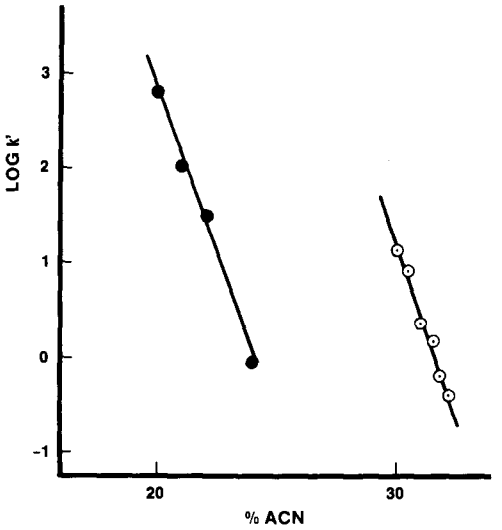

Fig. 5. Dependence of cytochrome *c* retention on acetonitrile content of the mobile phase. (●) k' Values calculated from direct measurements of rate of linear migration in glass column; (⊙) data of Terabe *et al.*[12].

ventional means which are impractical for retention times greater than a few hours. Direct observation of a colored cytochrome *c* band, injected into the glass column and eluted with aq. 20% acetonitrile in aq. 0.1% TFA, revealed the slow but definite movement of the protein. It took about 1 h for the band to move 1 cm (Fig. 4). On the basis of eqn. 2, this velocity represented a k' value of 650. On the basis of eqn. 3, the retention time of cytochrome *c* under these conditions was estimated to be 570 min or about 9.5 h. Cytochrome *c* retention determinations were repeated for isocratic mobile phases containing aq. 21%, 22%, and 24% acetonitrile. Additional measurements were not made because of the column failures described previously. However, the few data points obtained did show a linear relationship between log k' and mobile phase strength (Fig. 5) as predicted by eqn. 1. The resulting line of best fit had a slope of -0.93 with a standard error of estimate of 0.33 and a y intercept of 21.78. A linear relationship for log k' of cytochrome *c* *vs.* mobile phase strength was also reported by Terabe *et al.*[12]. Their data, extracted from that publication and replotted in Fig. 5, yields a line of best fit with a slope of -0.72, a standard error of estimate of 2.53, and a y intercept of 18.94. The differences in regression results between the two sets of data are presumably due to differences between the two chromatographic systems.

In addition to permitting the determination of very large retention values under isocratic conditions, the accelerated migration of colored proteins during gradient elution is easily observed in the glass HCODS column. Fig. 6 shows the sequence of events that occurs during gradient elution of the column, loaded with alternating colored and non-colored proteins, shown in Fig. 1B. At about 19.3 min, when the average acetonitrile concentration within the column was about 21%, the cytochrome *c* bands began to streak or "bleed" (Fig. 6B). At 21 min (acetonitrile = 25.5%), the cytochrome *c* molecules apparently skipped over the lysozyme and hemoglobin bands

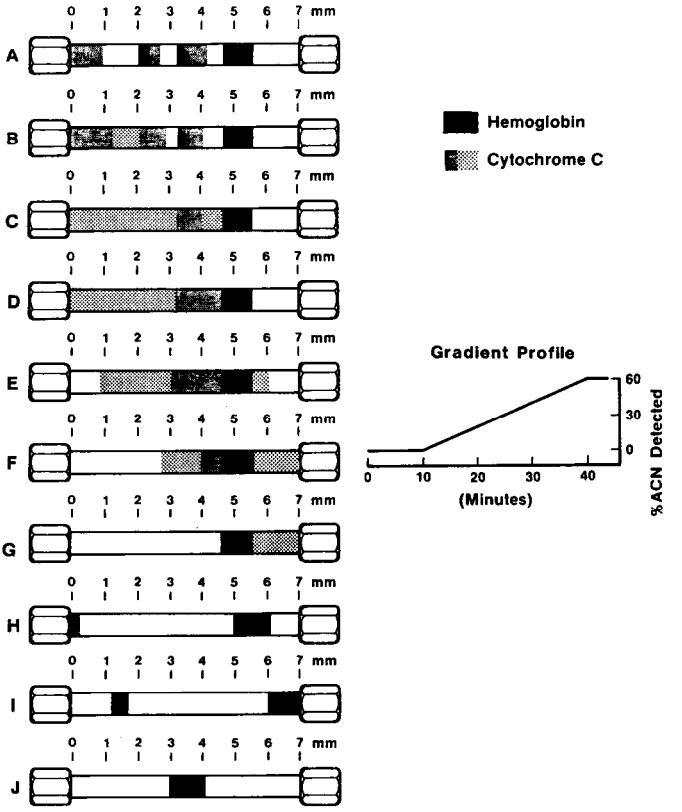

Fig. 6. Gradient elution of column loaded with alternating injections of proteins. Mobile Phase: 30 min linear gradient from 0 to 60% acetonitrile in aq. 0.1% TFA. Flow-rate: 0.4 ml/min. (A) Column appearance after loading completed; (B) after 19 min; (C) after 20 min; (D) after 21 min; (E) after 22 min; (F) after 23 min; (G) after 24 min; (H) after 26 min; (I) after 27 min; (J) after 28 min.

to form a band at the 5.3-cm mark in Fig. 6D. This band then formed a streak toward the end of the column (Fig. 6E) by 22 min (acetonitrile = 28%). By 24 min (acetonitrile = 33%) all cytochrome *c* had been eluted from the column (Fig. 6G). At this time, the hemoglobin band started to move and a colored contaminant of the hemoglobin sample emerged from the end fitting at the column head (Fig. 6G). At 26 min (acetonitrile = 37.7%) the movement of the hemoglobin and contaminant band accelerated (Fig. 6H) until both were eluted (Fig. 6I and J).

The fractional migrations of cytochrome *c*, hemoglobin, and the hemoglobin contaminant during the above gradient elution are represented by the closed symbols in Fig. 7. The fractional migrations during gradient elution for individual protein samples are represented by the open symbols in Fig. 7. Notice that the migration of the cytochrome *c* band in the column loaded as shown in Fig. 6A experienced a greater acceleration around 21 min where it apparently skipped over the lysozyme and hemoglobin bands (Fig. 6C and D). This is consistant with previous observations, in which cytochrome *c* could not displace these more hydrophobic proteins under isocratic conditions. In general, observations of the migration of cytochrome

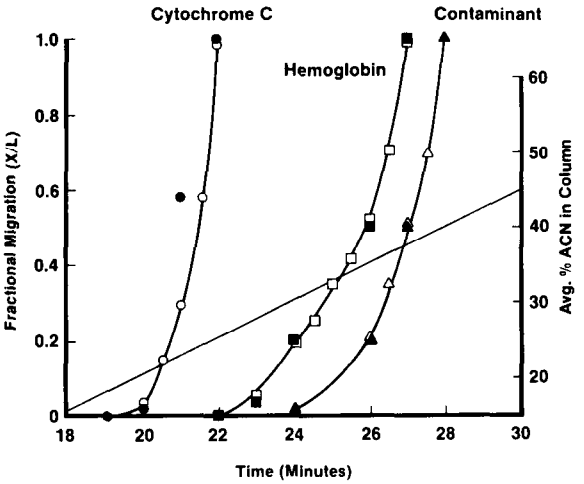

Fig. 7. Fractional migration of proteins during gradient elution. Mobil phase gradient conditions same as in Fig. 6. Closed symbols, data taken from Fig. 6; open symbols, data obtained from gradient elution of single protein load (200 μg).

c, hemoglobin, and the hemoglobin contaminant during gradient elution of the HCODS-packed glass columns are consistent with the predictions of classical gradient theory[10–13].

CONCLUSIONS

In addition to revealing phenomena that are undetectable by other means, direct observation of the chromatographic behavior of colored molecules in transparent columns permits practical measurements of very long retention values as well as elution accelerations during gradient elution. Observations of the RPLC of cytochrome *c* and hemoglobin made under the various chromatographic conditions of this study generally agree with the relationships between retention and solvent strength predicted by classical chromatographic theory.

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