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GRADIENT OPTIMIZATION PRINCIPLES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THE SEPARATION OF INFLUENZA VIRUS COMPONENTS

MICHAEL A. PHELAN
National Center for Drugs and Biologics, Bethesda, MD (U.S.A.)
and
KENNETH A, COHEN*
IBM Instruments, Inc., P.O. Box 332, *Orchard Park, Danbury, CT 06810 (U.S.A.)*

SUMMARY

Reversed-phase high-performance liquid chromatography is used to separate the major proteins of A/Bangkok/l/79 x 73 influenza virus. The purity of the isolated proteins, their yield and reactivity with monoclonal antibodies are discussed. The virus, in turn, is used as a probe to examine some theoretical gradient principles relating resolution, retention time and gradient time. Empirical compliance with these principles is generally shown.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is rapidly becoming a powerful technique for analytical and preparative applications in the biochemical area[1–3]. Due to the rigid, microparticulate, silica-based packings, HPLC improves resolution, reduces analysis time and permits higher sensitivity compared to some other methodologies[4]. This is particularly true for the separation of proteins and polypeptides many of which are still isolated by classical liquid chromatography or conventional electrophoresis. The full potential of applying HPLC to these biopolymers is not yet realized.

Reversed-phase HPLC separations of proteins generally require gradient elution[1]. A theoretical treatment of gradient elution principles has been developed and tested with aromatic derivatives as solutes[5,6]. This treatment identified the gradient parameters that are critical to chromatographic performance. Some of these parameters have been investigated phenomenologically using proteins as the test molecules[7–10]. In addition, the empirical application of some of the gradient principles has been studied within the full context of the postulated theory[11]. The latter work, however, employed an artificial admixture of proteins customarily selected for general biochemical studies.

Several studies of the influenza virus, its components and their roles in host infection and immune responses have depended upon isolated viral components[12–14]. Some methods developed for the separation of influenza virus components, although

effective, are complex and protracted[15,16] Other methods are effective with few strains of this virus, produce denatured materials or are sometimes limited by low yields[15]. Thus, a simple procedure which separates, for example, viral proteins rapidly and reliably may benefit further investigations with these components. Reversed-phase HPLC has been used to separate the proteins in murine leukemia virus[17]. A similar approach has been applied to influenza virus and is reported here.

The separation of the influenza virus proteins by HPLC permits the examination of certain gradient elution principles such as the relationships among resolution, gradient time and retention time. Although these principles hold for simulated mixtures of proteins and other compounds[11], their relations in natural biological samples have not generally been scrutinized. Our study examines not only the empirical compliance of gradient elution principles but also the utility of HPLC for the separation of influenza virus proteins.

CHROMATOGRAPHJC THEORY

For gradient elution, resolution can be expressed by the following equation:

$$R_s = (1/4)\ (\alpha - 1)\ (\bar{k}'/[\bar{k}' + 1])\ (N)^{1/2} \qquad (1)$$

where R_s = resolution, α = relative retention and N = number of theoretical plates'. Eqn. 1 is analogous to expressions that can be developed for resolution in the isocratic mode[4]. It should be noted, however, that in eqn. 1 for gradient elution, an average capacity factor, $\bar{k}'$, of an individual solute is used: $\bar{k}'$ in the gradient elution mode accounts for the change in the instantaneous capacity factor of a solute as the solute migrates through the column. This is a consequence of the variation in mobile phase composition during gradient elution. For a more comprehensive explanation of gradient elution theory the reader is referred to the work of Snyder', from which much of the following treatment is extracted.

As given in eqn. 1, resolution is proportionally related to the term $(\bar{k}'/[\bar{k}' + 1])$. This relation is illustrated in Fig. 1. in which the term $(\bar{k}'/[\bar{k}' + 1])$, or R_s, is plotted as a function of $\bar{k}'$. An asymptotic relationship results indicating that gradients should be designed such that $\bar{k}'$ of each solute falls within a compromise range of roughly 3–5. When $\bar{k}'$ is smaller, resolution is severely diminished; when $\bar{k}'$ is larger, resolution is

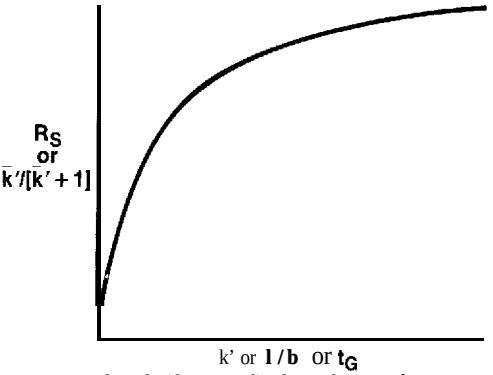

Fig. I. Predicted (theoretical) dependence of R_s on various gradient parameters.

increased but only slightly and at the expense of disproportionally longer analysis time. These observations are valid when the other terms (a, N) in eqn. 1 remain constant.

Thus, resolution ingradient elution can be controlled through the manipulation of $\bar{k}'$. Manipulation of $\bar{k}'$ in turn can be achieved through variation of b, which is defined as the gradient steepness parameter: b is reciprocally related to k' as

$$\bar{k}' = 1/(1.15)\, b \tag{2}$$

Because of the inverse relationship between $\bar{k}'$ and b, the plot of resolution in Fig. 1 can be represented as a function of $(1/b)$ as well as $\bar{k}'$.

The gradient steepness parameter can also be expressed as

$$b = (\varphi' S V_m)/F \tag{3}$$

where φ' = volume fraction change of organic modifier per unit time in the gradient, S = solvent strength, V_m = mobile phase volume in the column and F = volumetric flow-rate[5,6]. Eqn. 3 can be instrumental in designing a gradient in reversed-phase HPLC to maximize resolution yet minimize analysis time. A regrouping of the parameters of eqn. 3 yields

$$b = (S V_m/F)\, \varphi' \tag{4}$$

If gradient elution is performed so that the term (SV_m/F) in eqn. 4 remains constant, then b becomes proportional to φ'. The term (SV_m/F) is easily made invariant by maintaining a constant volumetric flow-rate. When this is done, V_m remains essentially unchanged for a given column. There is considerable evidence that S also will remain virtually constant for a given solute and a given phase system particularly under the typical conditions employed in reversed-phase HPLC gradient elution[5,6,11,18]. It is possible to optimize gradient elution by varying other parameters. e.g., F and/or V_m, but these topics will not be addressed in this work.

Hence, within the constraints described above, b can be expressed as

$$b = C\varphi' \tag{5}$$

where C is simply a proportionality constant and defined as (SV_m/F). Eqn. 5 shows that b is directly proportional to φ'. By definition φ' is the rate of change of the volume fraction of organic modifier: φ' is dependent on t_G, the time period over which gradient elution is performed. If t_G is increased, and the gradient range is held constant, e.g. O-100 %, φ' will be decreased such that a longer gradient time, t_G, results in a decreased gradient rate, φ'. This, in turn, influences b directly according to eqn. 5 and b is decreased when t_G is made longer. Since b and t_G are inversely related, the plot of resolution in Fig. 1 can be represented as a function of t_G as illustrated. Hence, resolution as depicted in Fig. 1 is predicted to have an asymptotic dependence on the time, t_G, over which gradient elution is performed. As noted above, this dependence should hold true as long as N and α do not vary. Although relative retention can vary when gradient parameters, e.g. column dimensions, are altered, this variation de-

pends on the molecular nature of the solutes[19]. Such variation can be attributed to solutes with unequal values of b (ref. 5).

EXPERIMENTAL

Apparatus

The liquid chromatography unit consisted of an IBM Instruments LC/9533 ternary gradient liquid chrornatograph (IBM Instruments, Danbury, CT, U.S.A.), an IBM Instruments LC/9523 variable-wavelength detector and an IBM Instruments LC/9540 chromatography data integrator. The IBM LC/9533 includes a Rheodyne (Cotati, CA, U.S.A.) 7125 sample injector. An Aquapore RP-300 column (C03-10A, Rainin Instrument Co., Woburn, MA, U.S.A.), 25 x 0.46 cm, was used.

Mobile phase solvents

Acetonitrile and 2-propanol were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Trifluoroacetic acid (TFA) was 99+% pure (No. 67362, Pierce, Rockford, IL, U.S.A.) or 99 % pure (T6-220-0, Aldrich, Milwaukee, WI, U.S.A.). Water was glass distilled. All other mobile phase additives were either reagent grade or HPLC grade.

Procedure

Between 10 and 20 μl of sample were generally injected. Separations were performed at room temperature (*ca.* 24°C). Other operating conditions are cited in figure legends.

Virus sample

The recombinant whole live influenza virus, A/Bangkok/1/79 x 73 (X-73), grown in the allantoic cavity of hens' eggs, was purified by sucrose gradient centrifugation as previously described[15]. The virus was then dissociated in 8 *M* guanidine–HCl (Schwartz-Mann, Orangeburg, NJ, U.S.A.) and 2 m*M* dithiothreitol (DTT) to a final protein concentration of 3.9 mg/ml for chromatography.

Identification and quantitation

Column eluates were collected in 1.0-ml fractions using an LKB Model 2112 fraction collector (LKB, Gaithersburg, MD, U.S.A.) and dried using a vacuum centrifuge. The identity of the eluates was determined by discontinuous-slab sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described[15]. Molecular weight markers from 14,000 to 93,000 daltons (Bio-Rad Labs., Richmond, CA, U.S.A.) and whole virus were electrophoresed in each gel for reference. The proteins in each chromatographic fraction or whole virus were determined by densitometry of the Coomassie Blue-stained bands, generated in SDS-PAGE, using an Ortec Model 4310 visible and ultraviolet densitometer (Ortec, Oak Ridge, TN, U.S.A.)".

The protein concentration of virus and column eluates was measured as described earlier[15]. Briefly, samples were hydrolyzed in 5 N sodium hydroxide, neutralized with 5 N hydrochloric acid, diluted with 1 M borate buffer, pH 10.5 (containing 0.1 % Triton X-100, 0.1 % 2-mercaptoethanol and 1 % methanol), mixed with an

equal volume of borate buffer containing 0.08 % (w/v) o-phthalaldehyde (Pierce), and the fluorescence, excited at 340 nm, was measured at 455 nm. Calculations of sample concentration were based on the results of standards (BSA, Ref. 927, National Bureau of Standards, Gaithersburg, MD, U.S.A.) treated in the same manner.

Antibody reactions

The reactivity of chromatographic eluates with antibodies was assessed as already described[13], but expanded to include three mouse monoclonal antibodies directed against each of the three major viral proteins, hemagglutinin (HA), nucleoprotein (NP) and matrix (M)[20]. Eluates were flushed with nitrogen to remove acetonitrile and buffered to 0.1 A4 with a sodium hydrogen carbonate concentrated solution, pH 8.1.

Column regeneration

Pressure drop changes occurring with column usage or shifts in standard and sample retention times were rectified by flushing the RP-300 column with: (1) several gradient cycles from 0–100 % 2-propanol in 0.05% TFA (flow-rate, 1.0 ml/min; 33 min/gradient); (2) overnight cycling of these gradients at 0.2 or 0.3 ml/min; (3) flushing the column first with distilled water, then with 0.1% SDS followed again by distilled water or (4) soaking the column overnight in 0.1 % SDS.

RESULTS AND DISCUSSION

Virus dissolution

The dissociation of X-73 virus suspended in 8 *M* guanidine–HCl and 2 m*M* DTT depended on time as shown in Fig. 2. No increase in the area under the curve of the first ($\bar{t}_g$ = 20.32 min) of the three peaks was observed after *ca. 10* h. When DTT was omitted from the suspension mixture, the first peak never materialized (results not shown). It was assumed that one of the disulfide-linked subunits of HA, either HA, or HA,, had been released by DTT treatment. DTT itself was eluted by identical

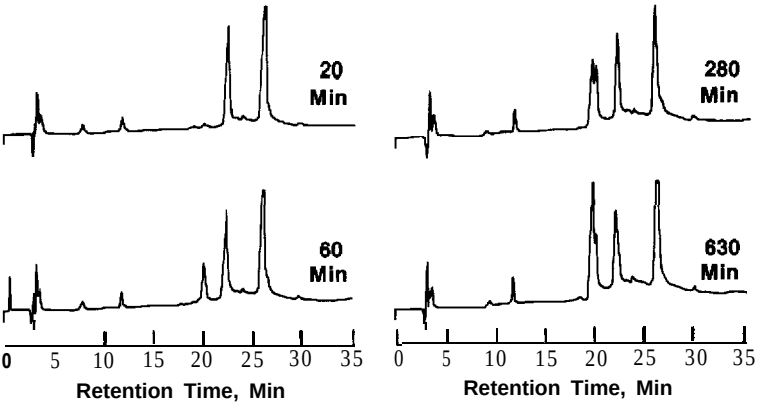

Fig. 2. Influence of solubilization time on the chromatographic profile of influenza X-73 virus dissolved in 8 *M* guanidine hydrochloride and 2 m*M* DTT. Conditions: 78 μg total viral protein; column, RP-300, 25 x 0.46 cm; linear gradient, 0.05 % TFA in water to 0.05 % TFA in acetonitrile over 33 min; 220 nm; 1.0 a.u.f.s.; flow-rate, 1.0 ml/min.

TABLE I

VIRAL PROTEIN RETENTION DATA

Chromatographic and solubilization conditions as in Fig. 3.

Peak	*Number of assays*	*Mean retention time, $\bar{t}_g$ (min)*	*Standard deviation (min)*	*Relative standard deviation (%)*
III	7	26.20	0.09	0.3
II	7	22.50	0.16	0.7
I	5	20.32	0.15	0.7

gradients around $\bar{t}_g \approx 12$ min. The chromatographic profiles with respect to retention times, t_g, of each of three major peaks was highly reproducible as shown in Table I under the conditions listed therein.

Eluate identification

Analysis by SDS-PAGE of the fractions collected during many chromatographic procedures indicated that the three peaks contained HA_1, NP and M, respectively, but not exclusively. The SDS-PAGE and chromatogram presented in Fig. 3 showed that the second peak (t_g = 14.57 min in this case) was slightly contaminated with a higher-molecular-weight protein, possibly an oligomer of neuraminidase (NA). The third peak (t_g = 17.63) was noticeably contaminated with NP and some other high-molecular-weight moiety. The tailing observed across fractions in the SDS-PAGE, particularly in peaks II and III, was assumed to be attributable to plate

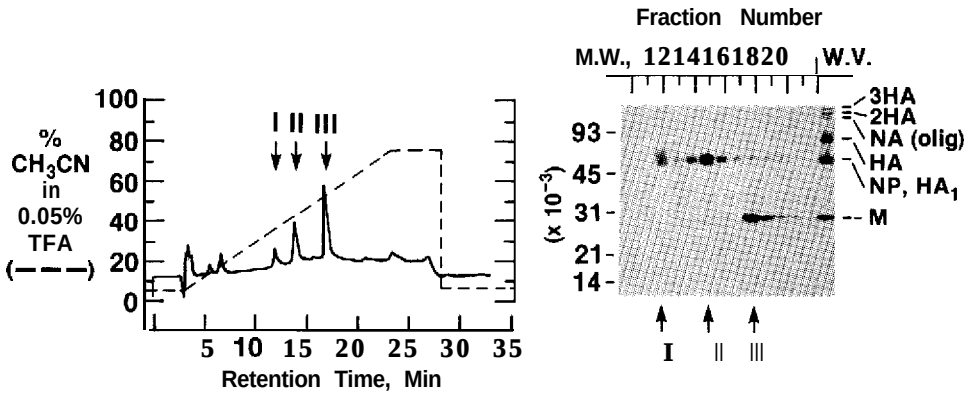

Fig. 3. Left: reversed-phase chromatography of whole influenza X-73 virus dissolved in 8 *M* guanidine hydrochloride and 2 m*M* DTT. Conditions: 78 μg total virus protein; RP-300 column; 220 nm: 1.0 a.u.f.s.; flow-rate. 1 ml/min; gradient 5 % acetonitrile in 0.05 % TFA to 75 % acetonitrile in 0.05 % TFA over 20 min as shown superimposed upon the chromatogram. Right: SDS polyacrylamide electrophoretic gel identification of the viral proteins in each elution peak recovered during a similar gradient. Molecular weight (M.W.) span determined by markers (not shown) entered on the left margin. Whole virus (W.V.) lane on right accompanied by protein identifications on the right margin [3HA = hemagglutinin trimer; 2HA = hemagglutinin dimer: NA (olig) = neuraminidase oligomer; HA = hemagglutinin monomer; NP = nucleoprotein; HA_1. subunit of hemagglutinin; M = matrix]. Peak identifications (I, II and III) correlate the PAGE lanes with the chromatographic peaks.

overloading and, to a lesser extent, the lack of precise eluate fractionation. Tailing of peak I would have been obscured by the tightly banded and densely staining NP, the characteristics of which were used, among other things, to distinguish NP from HA_1. The fate of HA_2 was not determined.

The HA oligomers observed in whole virus may have been dissociated in the dissolution mixture and reduced to HA_1 and HA_2. Previous evidence[21] had shown that a portion of the HA oligomers was not dissociable by SDS and 2-mercaptoethanol. Thus. if covalent linkage other than reducible disulfide bonds existed between any oligomers prior to chromatography, these oligomers may have been trapped in the column matrix. HA_2, on the other hand, noted for the sequence of hydrophobic amino acids at its carboxyl terminus, had been shown to form aggregates even in the presence of guanidine–HCl and DTT[21]. Aggregated HA, may also have been trapped on the column matrix by virtue of its size or hydrophobicity and monomeric HA, by its hydrophobic residues.

Eluate recovery

The recovery of total X-73 proteins in chromatographic eluates was ea. 20 % (± 7) as shown in Table 11. The recovery of specific proteins ranged from 32 % for HA, to 13 % for NP based upon the relative concentration of each protein in the whole virus. Despite the wide deviation from the means, notably in HA_2, it was concluded that recovery values were less than quantitative. In all cases, the whole virus samples in 8 *M* guanidine–HCl and 2 m*M* DTT had been aged at least 24 h to maximize dissociation. It was not clear whether the dissolution conditions, the mobile phase conditions and/or the column were responsible for these low yields.

The degree of contamination in chromatographic eluates was also determined (Table II). The trace of high-molecular-weight protein associated with NP could not

TABLE II

RECOVERY OF X-73 PROTEINS IN REVERSED-PHASE CHROMATOGRAPHY

Conditions for chromatography and virus solubilization as in Fig. 3.

Peak	*% of total injected viral protein**	*% of specified injected viral protein***	*Eluate identification****
I	*6.7 (± 3.6)*	*32*	HA_1
II	*5.6 (± 1.6)*	*13*	NP (Trace of high-molecular-weight moiety)
III	6.X (± 2.3)	22	M (88–89 %)
		2	NP (8–12 %)
			Other (O-3 %)
Total	≈ 20 (± 7)	–	–

* Determined as total protein in each peak from three chromatographic assays.

** Determined from the proportion of the major proteins in X-73 whole virus found by densitometric analysis of the Coomassie Blue-stained proteins generated in SDS-PAGE such that HA = 32 % (HA, = 21 % and HA, = 1 1 %), NP = 42 %, and M = 28 %.

*** Detcrmincd by SDS-PAGE analysis of chromatographic eluates and by densitometric analysis of Coomassie Blue-stained proteins generated in SDS-PAGE of these eluates.

be measured. The degree of contamination of M depended on the particular fraction analyzed in peak III. Thus, fraction 18 (Fig. 3 right) contained 89 % M and 8 % NP and 3 % of some higher-molecular-weight moiety, while fraction 19 contained 88% M and 12% NP.

Eluate-antibody reactivity

Preliminary examination indicated that only HPLC-isolated X-73 M was recognized by monoclonal antibodies directed against it. Neither HA, nor NP reacted even at concentrations sixty times greater than those required to produce the enzyme-linked immunosorbent assay (ELISA) endpoint. The ELISA endpoint for HPLC-separated matrix was cu. 90 ng. Since the established endpoint for this assay was ca. 25 ng for M purified by other methods, it appeared that HPLC-separated matrix was about 30 % as reactive as controls. This indicated that some degree of higher-order protein structure of M had been preserved throughout the HPLC separation procedure or had been reconstituted thereafter. Since the error in ELISA is about two-fold, additional testing is currently underway.

Column regeneration

The pressure required to maintain a flow-rate of 1 ml/min with 0.05% TFA increased from 60 bars for the new RP-300 column to levels as high as 90 bars after repeated chromatographic procedures. Furthermore, not only a standard but also a well-characterized influenza protein mixture yielded chromatographic peaks with retention times shifted as much as 24 % (depending on the peak) toward zero. At first, continuous overnight cycling of 0–100 % 2-propanol in 0.05 % TFA gradients (33 min at 0.2 or 0.3 ml/min) reduced the pressure to 61 bars and re-established characteristic retention times for the standard and the protein mixture. When 2-propanol cycling eventually failed to reduce column back-pressure, either flushing the column rapidly with 0.1 % SDS (5 ml/min for 10 min) or soaking the column overnight in 0.1% SDS reduced the pressure to 65 bars with 0.05 % TFA, nearly normal levels. It was felt, in view of the low recoveries of injected viral proteins, that some of the viral materials were trapped on the column during each gradient elution. This eventually caused the observed pressure changes and possibly the shifts in retention times. It should be noted that column regeneration was performed routinely as described above, prior to the collection of the results appearing in all figures and tables presented in this report.

Gradient theory examination

X-73 virus treated as above for 90 min was used to examine the compliance of this protein mixture with the gradient elution principles postulated earlier. As noted, the contents of the peaks of Fig. 3 were confirmed by SDS-PAGE to contain mainly the proteins HA,, NP and M. The apparent molecular weights were approximately 55,000, 55,000 and 28,000, respectively. The effect of gradient time on separation and retention time is illustrated in Fig. 4.

These chromatograms were obtained by gradient elution with acetonitrile (5-75 %) over various gradient times. As required by the postulates of the gradient theory[5], the sample was injected at the moment the gradient reached the top of the column. The delay volume of the chromatographic system, therefore, was taken into account and was determined to be ea. 3 ml. As shown in Fig. 4, when t_G was in-

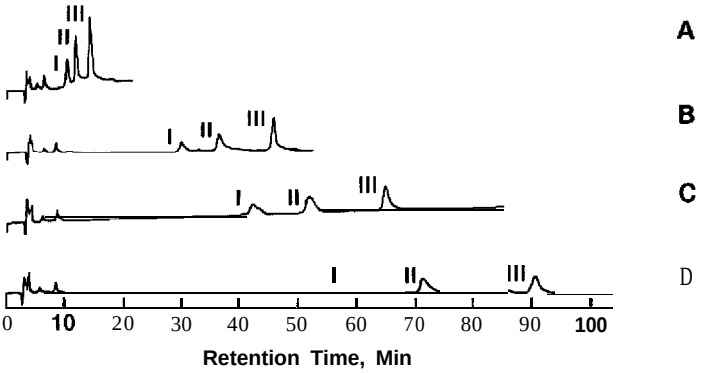

Fig. 4. Influence of gradient time, ı,, on the separation of viral proteins. Conditions as in Fig. 3. A: $t_G = 15$ min; $\varphi' = 0.047$. B: $t_G = 60$ min; $\varphi' = 0.012$. C: $t_G = 90$ min; $\varphi' = 0.008$. D: $t_G = 130$ min; $\varphi' = 0.005$.

creased, the analysis time was increased as well. There also appeared to be some gain in separation when t_G was increased from 15 to 60 min. However, the gain in separation seemed marginal when gradient time was increased further.

Data taken from these experiments were plotted in Fig. 5. The t_g of the most retained peak (III) was again shown to increase with an increase in gradient time. A similar increase was observed for peaks I and II (not shown). The resolution between peaks I and II and between II and III was also plotted as a function of t_G. Roughly asymptotic relationships resulted similar to those predicted by the theoretical treatment developed above. The resolution values in Fig. 5 were calculated using the well-known equation

$$R_s = \frac{\Delta t_R}{2(\sigma_1 + \sigma_2)} \tag{6}$$

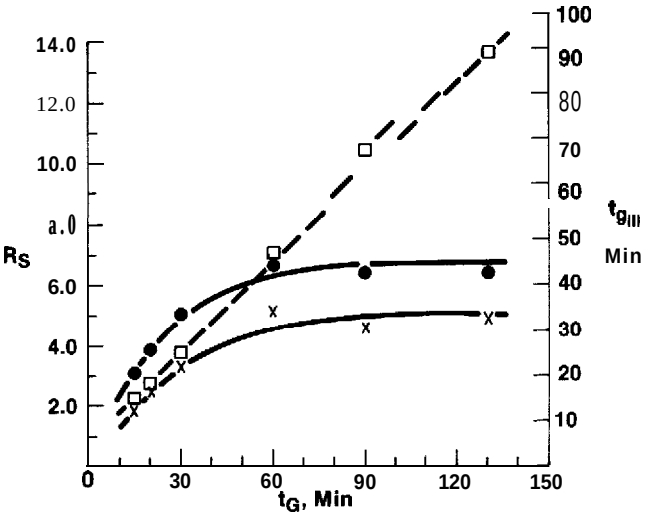

Fig. 5. Influence of gradient time, t_G, on the resolution and retention time of X-73 viral proteins of Fig. 4. × = Resolution between I and II; ● = resolution between II and III: □ = retention time, t_{gIII} of III.

where Δt_R is the difference in retention times of two peaks and σ_1 and σ_2 are the time standard deviations of peaks 1 and 2, respectively.

The standard deviations of the peaks in Fig. 5 were approximated by the tangent method[4]. Rigorously, this method yields an accurate value of σ only when a peak is symmetrical, but the peaks in Fig. 5 were clearly asymmetrical. Hence, it is possible that the asymmetry affected the accuracy of the calculation of resolution values. This in turn may have caused some of the deviation of the points from the asymptotic line fits in Fig. 5.

Because of the excellent symmetry of peaks X and Y in Fig. 6, they were selected as probes to examine further the compliance with the gradient theory. Although their identities were not confirmed, it was felt that these peaks, derived from X-73 virus. were degradation products. probably peptides. resulting from ageing (more than 1 month) of this solubilized sample. Throughout the various gradients shown in Fig. 6, the symmetry of peaks X and Y remained. The areas under the peaks held constant as well. These findings indicated that each of the two peaks was essentially pure.

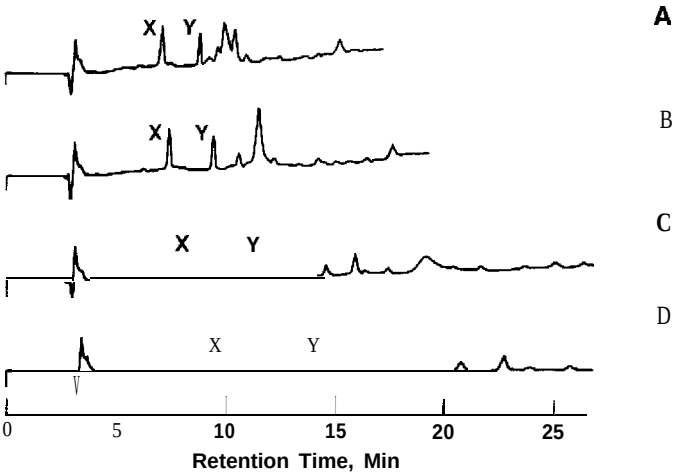

Fig. 6. Influence of gradient time, t_G, on the separation of select products of solubilized influenza X-73 virus. Conditions: column. RP-300. 25 × 0.46 cm: solvent A. 0.05% TFA in water; solvent B. 0.05 % TFA in acetonitrile, linear gradient. 5 % B → 75 % B in t_G minutes: 220 nm; 1.0 a.u.f.s.: flow-rate. 1.0 mlimin. A: t_G = 12 min; $\varphi' = 0.060$. B: t_G = 15 min: $\varphi' = 0.048$. C: $t_G = 30$ min; $\varphi' = 0.024$. **D: t_G = 60** min; $\varphi' = 0.012$.

Gradient relationships based on the empirical results of Fig. 6 have been plotted in Fig. 7. The retention time of peak Y increased when the gradient time was increased. Similar results were obtained for peak X (not shown). As predicted by the gradient theory, resolution between peaks X and Y were asymptotically dependent on t_G. In addition, the height of peak Y decreased as t_G increased, also in accordance with gradient theory predictions[5]. Hence, in view of the data of Figs. 4-7, we felt that these X-73 components conform to the postulates of gradient elution theory developed earlier.

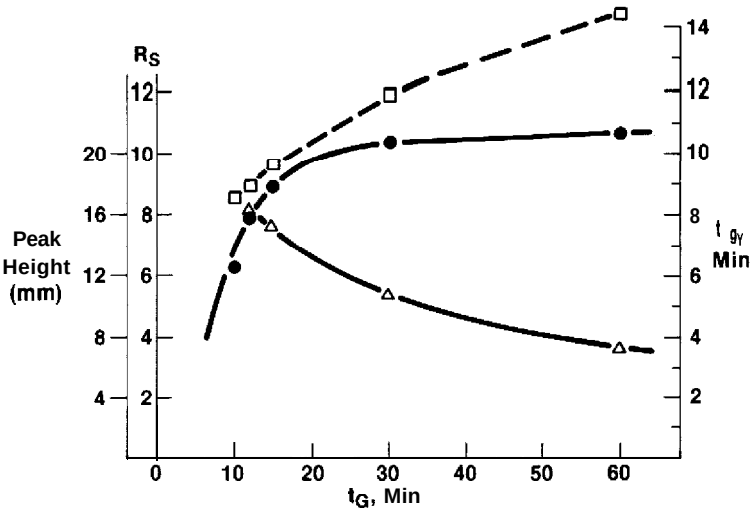

Fig. 7. Influence of gradient time. t_G, on the resolution, retention time and peak height of the viral products X and Y of Fig. 6. ● = Resolution between X and Y: □ = retention time. t_{g_Y}, of Y; △ = peak height of Y.

CONCLUSIONS

Dissolution of X-73 in 8 A4 guanidine hydrochloride and 2 mM DTT and reversed-phase HPLC using a gradient of acetonitrile in 0.05 % TFA separated the major proteins of this influenza virus. Despite time-dependent dissolution, the separation of these proteins which include hemagglutinin, nucleoprotein and matrix was highly reproducible, giving relative standard deviations of retention time less than 1 %. Although the total protein recovered was only cu. 20 %, the purity of specific proteins after a column passage is greater than or equal to 88%. Only X-73 matrix (M) recovered following chromatography is still reactive with monoclonal antibodies directed against it suggesting that the higher order structure of M is preserved at least to some degree. Resolution of certain viral components, including the major proteins, is asymptotically dependent on gradient time. This dependence is similar to that predicted by theoretical gradient principles.

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REFERENCES

1 M. T. W. Hearn, F. E. Regnier and C. T. Wehr, *Amer. Lab.*, 10 (1982) 18.
2 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
3 M. T. W. Hearn, *J. Liquid Chromatogr.*, 3 (1980) 125.5.
4 L. R. Snyder and J. J. Kirkland (Editors), *Introduction to Modern Liquid Chromatography*, Wiley. New York. 1979.

5 L. R. Snyder, in Cs. Horváth (Editor), ***High-Performance Liquid Chromatography: Advances and Perspectives,*** Vol. 1, Academic Press, New York, 1980, pp. 208-316.
6 J. W. Dolan, J. R. Cant and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
7 J. L. Meek and Z. L. Rossetti, ***J. Chromatogr., 211*** (1981) 15.
8 B. N. Jones, R. V. Lewis, S. Paabo, K. Kojima, S. Kimura and S. Stein, ***J. Liquid Chromatogr., 3 (1980)*** 1313.
9 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
10 W. S. Hancock and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 71.
11 K. A. Cohen, ***Ph.D. Thesis,*** Northeastern University, Boston, 1982.
12 J. R. Lamb, D. D. Eckels, M. Phelan, P. Lake and J. N. Woody, *J. Immun.*, 128 (1982) 1428.
13 B. R. Murphy, M. A. Phelan, D. L. Nelson, R. Yarchoan, E. L. Tierney, D. W. Alling and R. M. Chanock, ***J. Clin. Micro.,*** 13 (1981) 554.
14 F. A. Ennis, M. A. Phelan, R. E. Mayner and A. Meagar, *Interferons*, Academic Press, London, 1982, pp. 279-285.
15 M. A. Phelan, R. E. Mayner, D. J. Bucher and F. A. Ennis, *J. Biol.* ***Stand.,*** 8 (1980) 233.
16 D. J. Bucher, S. S.-L. Li, J. M. Kehoe and E. D. Kilbourne, *Proc.* ***Nut.*** *Acad.* ***Sci. U.S., 73 (1976) 238.***
17 L. E. Henderson, R. Sowder and S. Oroszlan, in T.-Y. Liu, A. N. Schechter, R. L. Heinrikson and P. G. Condliffe (Editors), ***Chemical Synthesis and Sequencing*** *of Peptides* ***and Proteins,*** Elsevier, Amsterdam, 1981, pp. 251–260.
18 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J.* ***Chromatogr., 205 (1981) 13.***
19 R. van der Zee and G. W. Welling, ***J. Chromatogr., 244 (1982) 134.***
20 K. L. van Wyke, V. S. Hinshaw, W. J. Bean, Jr. and R. G. Webster, *J. Virol.*, ***3.5 (1980) 24.***
21 W. G. Laver, ***Virology, 45 (1971) 275.***