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RELATIONSHIP BETWEEN ISOCRATIC AND GRADIENT RETENTION TIMES IN THE HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF PROTEINS

THEORY AND EXPERIMENT

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SUMMARY

Using isocratic retention parameters, the gradient elution retention time for several proteins has been calculated. The gradient retention time calculation is based on fitting the isocratic retention data to an equation of the form: $\log k' = m \log (1/[Ca^{2+}]) + \log K$ and on applying well-established principles of gradient elution. A good correlation between the observed and calculated retention times for several test proteins was obtained at various total gradient times and column flow-rates.

Conversely, isocratic retention parameters characterizing protein retention can be calculated from gradient elution retention data. However, even with retention data of high quality, small errors are amplified by the log–log nature of the ion-exchange isocratic retention model employed.

Based on the close correlation between predicted and observed gradient retention times, no evidence for protein denaturation resulting from immobilization of the protein at high initial k' values at or near the column inlet was observed.

INTRODUCTION

For the separation of mixtures which contain components that elute over a wide range of mobile phase strengths, it is generally advantageous to employ gradient elution conditions. Given the above criterion and owing to the complex nature of samples derived from biological sources, it is not surprising that gradient elution has been widely used in protein chromatography. In addition to problems created by the complex sample matrix, it should also be pointed out that for any particular protein component its ion-exchange elution may occur over a relatively narrow range of salt concentration*, so that finding suitable isocratic conditions even for relatively pure components typically involves a certain amount of guesswork and trial and error.

* Presumably due to a multiple-site interaction of the protein with the stationary phase[1].

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The use of gradient elution provides a practical solution to the above problem since it is capable of rapidly sampling a wide range of elution conditions from which appropriate isocratic conditions may then be inferred.

The fundamental principles of gradient elution analysis are well-established. Several authors have derived mathematical expressions which relate isocratic and gradient elution conditions for certain conditions that have been shown to be capable of accurately predicting a number of chromatographic parameters (retention times, bandwidths, resolution, etc.). For a general accounting of the theory and application of the gradient elution technique in modern liquid chromatography, the reader is referred to the chapter by Snyder and Kirkland[2], the chapter by Snyder[3], and the book by Liteanu and Gocan[4]. Also noteworthy is the comprehensive review given by Jandera and Churacek[5]. Of particular interest in gradient elution theory as applied to ion-exchange is the early work of Freling[6] and the works of Koguchi *et al.*[7], Schwab *et al.*[8], Inczedy[9], and Jandera and Churacek[10,11]. Most of the theoretical studies of gradient elution done to date have been verified for ions and small molecules (mol.wt. <2000). The general extension of chromatographic theory developed for small molecules to include macromolecules is at this time the subject of some controversy[12]. In the case of high-performance reversed-phase, however, Larmann *et al.*[13] have shown that current theory is useful in the prediction of various chromatographic parameters for polystyrene samples of varying molecular weight. Also for reversed-phase, Stadalius *et al.*[14] have demonstrated that current gradient elution theory could be extended to include peptides and small proteins.

In the present paper, we report our studies relating isocratic and gradient elution conditions for a number of test proteins using high-performance ion-exchange chromatography (HPIEC). In the course of the study, we have employed one of the new generation of silica-based ion-exchange supports which has a surface chemistry specifically designed for use with proteins. Chromatographic conditions of temperature, pH and ionic strength were judiciously selected to avoid protein denaturation related problems for the proteins used in the study. The mathematical algorithms herein described were translated into Waterloo BASIC and are run on a IBM 3081D mainframe computer. Although at this time a complete optimization protocol for protein high-performance ion-exchange separations is not yet available, the use of a computer-assisted strategy has been found to greatly simplify the process of methods development in our laboratory.

EXPERIMENTAL

Materials

Salt free, six-times crystallized, type II α-chymotrypsinogen A (bovine pancreas, No. C4879) and type VI cytochrome *c* (horse heart, No. C-N52) were purchased from Sigma (St. Louis, MO, U.S.A.). Hen egg white lysozyme (lot No. 7069) was purchased from Miles Labs. (PTY) (South Africa). Pancreatic trypsin inhibitor (bovine, No. 3212) was obtained from Worthington Biochemical (Freehold, NJ, U.S.A.). These proteins were used without further purification. All other reagents were ACS certified analytical reagent grade. Vydac 101TPB7.7 spherical silica (lot No. 180031) was used in the column packing preparation and was a gracious gift of The Separations Group (Hesperia, CA, U.S.A.). High-purity, HPLC grade water

was used throughout and was prepared in-house using a purification system from Mar Cor Medical Services (Harleysville, PA, U.S.A.).

Equipment

The chromatographic system employed consists of two Waters M6000A pumps, a Waters Model 660 solvent programmer, a Rheodyne Model 7125 injection valve equipped with a 20-μl sample loop, a Waters Lambda-Max Model 480 spectrophotometer and a Hewlett-Packard 3390A reporting integrator.

The chromatographic column packing was a weak cation exchanger that was prepared according to the procedure of Alpert[15]. This material has a hydrophilic poly (aspartic acid) coating bonded to a silica base which has a nominal pore size of 300 Å and an average particle size of 7 μm. A 250 × 4.6 mm I.D. column was packed to a constant pressure of 5000 p.s.i. using a Micromeretics Model 705 stirred-slurry column packer (Norcross, GA, U.S.A.). The column was thermostated in a water jacket using a circulating water bath to maintain the temperature at 30.0 ±0.2°C.

Buffers

Calcium acetate–acetic acid mobile phase buffers were prepared by the dilution of a concentrated stock solution prepared from calcium carbonate and glacial acetic acid. The pH of each buffer was adjusted to pH = 4.90 with glacial acetic acid at room temperature (21–24°C) using a glass electrode. The final calcium ion concentration was determined by titration with EDTA after dilution and pH adjustment.

All mobile phase buffers were filtered through a Millipore type HA (0.45 μm) filter and degassed by sparging with helium prior to use.

Sample preparation

Protein samples were prepared to contain *ca.* 1 mg/ml by dissolving the protein in the mobile phase for isocratic conditions and in the initial mobile phase for gradient conditions. In some cases a small amount of water was added to the sample solution to facilitate measurement of the unretained peak from the solvent perturbation. This was found not to affect the retention time of the protein component.

Gradient shape evaluation

The Waters 660 solvent programmer was carefully calibrated according to the manufacturer's specifications. To test the integrity of the gradient shape, the column was replaced with a small length of 0.009 in. I.D. stainless-steel tubing, gradients were run over the desired range employing a UV absorbing substance in the final buffer (*i.e.* 0.1 m*M* benzoic acid adjusted to pH = 8.0 with sodium hydroxide) and the absorbance change was monitored at 254 nm. As shown in Fig. 1, after calibration linear gradients were obtained, for various total gradient times.

Other instrumental parameters

The instrument dwell volume, V_D, was calculated from the flow-rate and the dwell time* which was determined by extrapolating the curves shown in Fig. 1 to the

* The dwell time is the time delay between the gradient initiation and the change in the mobile phase composition at the column inlet.

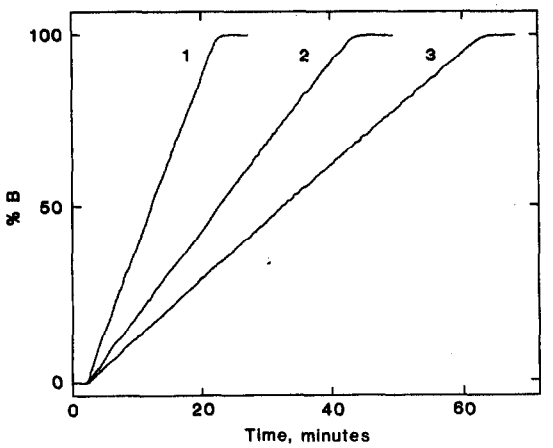

Fig. 1. Gradient linearity test for various total gradient times. (1) 20.38 min, (2) 40.46 min and (3) 60.69 min. Flow-rate was 0.98 ml/min.

initial baseline at zero benzoate concentration. For the actual determination, the scale used in Fig. 1 was expanded to permit accurate measurement. The column void volume, V_m, was determined from the time of the solvent pertubation and the flow-rate. The column flow-rate, F, was measured by weighing the effluent collected over a precise time interval. The total gradient time, t_G, was measured with a stopwatch.

Calculations

Isocratic retention model. Based on an earlier treatment of polyelectrolyte retention[1], Kopaciewicz *et al.*[16] have proposed a retention model useful for protein HPIEC of the form:

$$k' = Kx^{-m} \tag{1}$$

which can be written in logarithmic form as:

$$\log k' = m \log(1/x) + \log K \tag{2}$$

where k' is the capacity factor, x is the concentration of eluting ion and m and K are constants. Equations of this general form have also been suggested by other authors[10] as useful retention models for adsorption and ion-exchange chromatography. Experimentally, the parameters m and K are evaluated from the slope and intercept of a plot of $\log k'$ with $\log (1/x)$ (see eqn. 2) using isocratic conditions.

Relation of gradient composition to gradient volume. For linear gradients, the mobile phase concentration at the column inlet is given by

$$x_t = x_i + (x_f - x_i)\left(\frac{t}{t_G}\right) \tag{3}$$

where x_i is the initial buffer concentration; x_f is the final concentration; x_t is the concentration of eluting ion at the column inlet at time t; t is the time during gradient

corrected for instrumental dwell time*; t_G is the total gradient time. Since $V = Ft$ and $V_G = Ft_G$

$$x_t = x_i + (x_f - x_i)\left(\frac{V}{V_G}\right) \tag{4}$$

where F is the flow-rate in ml/min and V and V_G are the corrected gradient volume at time, t, and the total gradient volume respectively.

Relation of isocratic retention to gradient conditions. The fundamental relationship for gradient elution is given by

$$\int_0^{V_g} \frac{dV}{V_a} = 1 \tag{5}$$

where V_g is the corrected retention volume ($V_g = V_R - V_m - V_D$) at elution of the component band at its peak maximum, dV is the differential volume of mobile phase passing through the band center during its migration and V_a is the instantaneous value of the corrected volume at any given time.

V_a can be written as

$$V_a = V_m k_a \tag{6}$$

where k_a is the instantaneous k' for the band and V_m is the column void volume.

Calculation of gradient elution time from isocratic parameters. Using eqns. 1, 4 and 6, eqn. 5 can be rewritten as

$$\int_0^{V_g} \left\{[x_i] + ([x_f] - [x_i])\left(\frac{V}{V_G}\right)\right\}^m dV = KV_m \tag{7}$$

Integration of eqn. 7 and appropriate algebraic manipulation gives

$$V_g = \left\{\left[x_i^{m+1} + \frac{V_m K(m+1)(x_f - x_i)}{V_G}\right]^{\left(\frac{1}{m+1}\right)} - x_i\right\}\frac{V_G}{(x_f - x_i)} \tag{8}$$

The observed retention time of the component, t_R, can be calculated from eqn. 8 using

$$t_R = (V_g + V_m)/F + V_D/F \tag{9}$$

which corrects for column void volume and instrumental dwell volume**. Eqn. 8 has

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* Calculated by subtracting the instrumental dwell time, t_D, from the actual gradient run time.

** Our mathematical treatment assumes that no elution occurs during the instrumental dwell time. Using a modified method of calculation, however, it can be shown that this assumption is valid for the conditions employed in this study since the initial k' for each protein was sufficiently high that isocratic elution during the instrumental dwell time can be neglected.

previously been derived and has been shown to be capable of accurately predicting gradient retention of small molecules for adsorption chromatography on a silica column[11].

Calculation of isocratic retention parameters from two gradient runs. At least in principle, it should be possible to calculate the isocratic retention parameters m and K (see eqn. 1) from gradient retention data using two gradient runs with two different total gradient volumes, V_{G1} and V_{G2}. The corrected component elution volumes, V_{G1} and V_{G2}, can be calculated using eqn. 9 from the corresponding measured retention times, t_{R1} and t_{R2}. Eqn. 8 can be rearranged to yield two equations in two unknowns (*i.e.* m and K):

$$V_{G1}\left[x_i + (x_f - x_i)\frac{V_{g1}}{V_{G1}}\right]^{m+1} - V_{G1}(x_i)^{m+1} = V_m K(m+1)(x_f - x_i) \qquad (10a)$$

$$V_{G2}\left[x_i + (x_f - x_i)\frac{V_{g2}}{V_{G2}}\right]^{m+1} - V_{G2}(x_i)^{m+1} = V_m K(m+1)(x_f - x_i) \qquad (10b)$$

Simultaneous solution of eqns. 10a and 10b with the elimination of K gives

$$V_{G1}\left[\frac{x_i + (x_f - x_i)\frac{V_{g1}}{V_{G1}}}{x_i}\right]^{m+1} - V_{G1} - V_{G2}\left[\frac{x_i + (x_f - x_i)\frac{V_{g2}}{V_{G2}}}{x_i}\right]^{m+1} + V_{G2} = 0 \qquad (11)$$

Note that this equation (although one equation in one unknown) is of the general form $a^d + b^d = c$ (where a, b, c and d are constants) which cannot be solved algebraically except in special cases. The values of m in eqn. 11 can be obtained, however, by numerical approximation. In practice this is done using a BASIC computer program which utilizes the *regula falsi* algorithm for finding equation roots[17]. Once m is obtained, K can be found using eqn. 10a or eqn. 10b.

A method for the calculation of the isocratic retention parameters, m and K, from gradient retention data has previously been given and verified to be applicable to the adsorption chromatography of small molecules[11]. Compared to this method, the method described above (eqn. 11) is more general insofar as it also applies for the case where $x_i \neq 0$. Although the assumption that $x_i = 0$ would lead to a less complicated mathematical formulation than given by eqn. 11, for ion-exchange applications this condition is generally not fulfilled. It can also be noted that the possibility of calculating isocratic retention parameters from gradient retention data in reversed-phase systems has been described[13,14]. For reversed-phase, however, the form of the retention–composition equation is different than that given by eqn. 1.

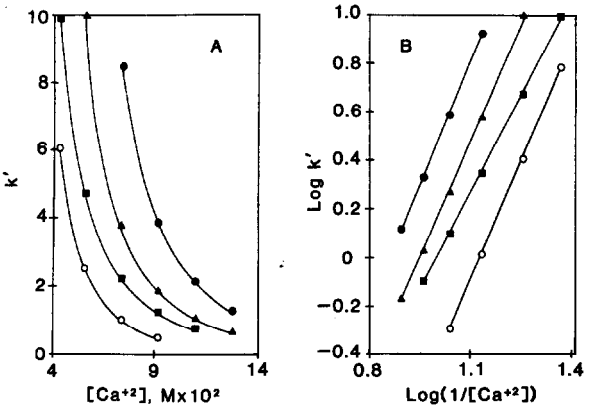

Fig. 2. (A) Relationship of the capacity factor, k', to $[Ca^{2+}]$ in a calcium acetate–acetic acid buffer (pH = 4.90) for several proteins using isocratic conditions. (○) α-Chymotrypsinogen A, (■) trypsin inhibitor, (▲) cytochrome *c* and (●) lysozyme. (B) Isocratic retention data for several test proteins plotted using the retention formalism of eqn. 2 (see text) for the proteins in A.

RESULTS

Using isocratic conditions the capacity factor for each protein was measured as a function of calcium ion concentration. As shown in Fig. 2A when the calcium ion concentration is increased, k' decreases. In Fig. 2B the data of Fig. 2A were replotted using the retention formalism of eqn. 2. A linear correlation was observed for each protein in the k' range of *ca.* 1–10 which is in the k' range of interest for gradient elution. Using linear regression the slope and intercept characterizing the retention of each protein were calculated from the data in Fig. 2B and are given in Table I. The gradient elution retention time of each of these proteins was then measured using linear gradients in which the total gradient times and flow-rates were varied. A typical chromatogram for the gradient elution separation of the test protein mixture using the column which was prepared in-house is shown in Fig. 3. As summarized in Table II the observed gradient retention time for each protein was within *ca.*

TABLE I

LINEAR REGRESSION PARAMETERS CHARACTERIZING THE HPIEC ISOCRATIC RETENTION OF SEVERAL TEST PROTEINS

Slope = m, intercept = log K (see eqn. 2). The parameters below were determined at a flow-rate of 0.98 ml/min; error estimates represent the 90% confidence limit.

Protein	*Slope*	*Intercept*	*Correlation coefficient*
α-Chymotrypsinogen A	3.27 ± 0.08	−3.69 ± 0.09	0.9999
Trypsin inhibitor	2.69 ± 0.08	−2.69 ± 0.10	0.9997
Cytochrome *c*	3.24 ± 0.13	−3.08 ± 0.14	0.9996
Lysozyme	3.39 ± 0.17	−2.92 ± 0.17	0.9997

TABLE II

COMPARISON OF OBSERVED RETENTION TIMES IN GRADIENT ELUTION WITH RETENTION TIMES CALCULATED FROM ISOCRATIC PARAMETERS

Calcium acetate–acetic acid buffers, pH 4.90, were employed. Buffer A = 0.0196 M Ca^{2+}, buffer B = 0.1996 M Ca^{2+}. V_m = 3.02 ml, V_D = 1.77 ml.

Protein	F	t_G	t_R *(obs.)*	t_R *(calc.)*	*Error (%)*
α-Chymotrypsinogen A	0.49	20.38	18.81	18.56	−1.3
	0.49	40.46	24.18	23.98	−0.8
	0.49	60.69	28.74	28.55	−0.7
	0.98	20.38	12.08	12.02	−0.5
	0.98	40.46	16.34	16.31	−0.2
	0.98	60.69	19.94	19.88	−0.3
	1.92	20.38	8.22	8.27	+0.6
	1.92	40.46	11.58	11.62	+0.3
	1.92	60.69	14.37	14.36	−0.1
Trypsin inhibitor	0.49	20.38	*	–	–
	0.49	40.46	27.96	27.93	−0.1
	0.49	60.69	33.43	33.50	+0.2
	0.98	20.38	13.99	14.01	+0.1
	0.98	40.46	19.09	19.19	+0.5
	0.98	60.69	23.34	23.44	+0.4
	1.92	20.38	9.64	9.73	+0.9
	1.92	40.46	13.61	13.70	+0.7
	1.92	60.69	16.85	16.90	+0.3
Cytochrome *c*	0.49	20.38	*	–	–
	0.49	40.46	30.70	30.78	+0.3
	0.49	60.69	37.56	37.81	+0.7
	0.98	20.38	15.36	15.44	+0.5
	0.98	40.46	21.84	22.06	+1.0
	0.98	60.69	27.46	27.70	+0.9
	1.92	20.38	11.02	11.18	+1.5
	1.92	40.46	16.32	16.51	+1.2
	1.92	60.69	20.89	21.00	+0.5
Lysozyme	0.49	20.38	*	–	–
	0.49	40.46	35.10	35.37	+0.8
	0.49	60.69	43.72	44.20	+1.1
	0.98	20.38	17.66	17.76	+0.6
	0.98	40.46	25.81	26.10	+1.1
	0.98	60.69	32.96	33.32	+1.1
	1.92	20.38	12.98	13.22	+1.8
	1.92	40.46	19.76	20.07	+1.6
	1.92	60.69	25.71	25.96	+1.0

* Eluted after gradient ended.

± 1% of the retention time which had been calculated from isocratic retention parameters.

Using various permutations of the observed gradient retention times given in Table II, the parameters characterizing isocratic retention (*i.e.* m and log K of eqn. 2) were calculated for each possible two gradient run pair at a particular flow-rate. These calculated isocratic parameters are summarized in Table III. The average val-

TABLE III

ISOCRATIC RETENTION PARAMETERS CALCULATED FROM GRADIENT ELUTION RETENTION TIME DATA

Protein	F	t_{G1}	t_{G2}	t_{R1}	t_{R2}	*Calculated*	
						Slope	*Intercept*
α-Chymotrypsinogen A	0.49	20.38	40.46	18.81	24.18	2.98	−3.32
	0.49	40.46	60.69	24.18	28.74	3.11	−3.48
	0.49	20.38	60.69	18.81	28.74	3.03	−3.38
	0.98	20.38	40.46	12.08	16.34	3.13	−3.52
	0.98	40.46	60.69	16.34	19.94	3.31	−3.74
	0.98	20.38	60.69	12.08	19.94	3.22	−3.62
	1.92	20.38	40.46	8.22	11.58	3.37	−3.82
	1.92	40.46	60.69	11.58	14.37	3.44	−3.92
	1.92	20.38	60.69	8.22	14.37	3.39	−3.85
Trypsin inhibitor	0.49	20.38	40.46	*	27.96	–	–
	0.49	40.46	60.69	27.96	33.43	2.57	−2.56
	0.49	20.38	60.69	*	33.43	–	–
	0.98	20.38	40.46	13.99	19.09	2.62	−2.61
	0.98	40.46	60.69	19.09	23.34	2.74	−2.76
	0.98	20.38	60.69	13.99	23.34	2.66	−2.66
	1.92	20.38	40.46	9.64	13.61	2.78	−2.81
	1.92	40.46	60.69	13.61	16.85	2.83	−2.88
	1.92	20.38	60.69	9.64	16.85	2.79	−2.83
Cytochrome *c*	0.49	20.38	40.46	*	30.70	–	–
	0.49	40.46	60.69	30.70	37.56	3.07	−2.90
	0.49	20.38	60.69	*	37.56	–	–
	0.98	20.38	40.46	15.36	21.84	3.15	−2.99
	0.98	40.46	60.69	21.84	27.46	3.34	−3.21
	0.98	20.38	60.69	15.36	27.46	3.21	−3.07
	1.92	20.38	40.46	11.02	16.32	3.39	−3.28
	1.92	40.46	60.69	16.32	20.89	3.52	−3.43
	1.92	20.38	60.69	11.02	20.89	3.42	−3.31
Lysozyme	0.49	20.38	40.46	*	35.10	–	–
	0.49	40.46	60.69	35.10	43.72	3.27	−2.82
	0.49	20.38	60.69	*	43.72	–	–
	0.98	20.38	40.46	17.66	25.81	3.25	−2.79
	0.98	40.46	60.69	25.81	32.96	3.44	−3.00
	0.98	20.38	60.69	17.66	32.96	3.32	−2.86
	1.92	20.38	40.46	12.98	19.76	3.52	−3.09
	1.92	40.46	60.69	19.76	25.71	3.70	−3.30
	1.92	20.38	60.69	12.98	25.71	3.58	−3.16

* Peak eluted after end of gradient time.

ues of the isocratic slopes and intercepts which had been derived from gradient retention times were then obtained from the data in Table III for each test protein. As can be seen in Table IV, the average values obtained using the gradient method were found to be in relatively good agreement with the observed isocratic retention parameters.

The deviation of the calculated parameters from their respective average for

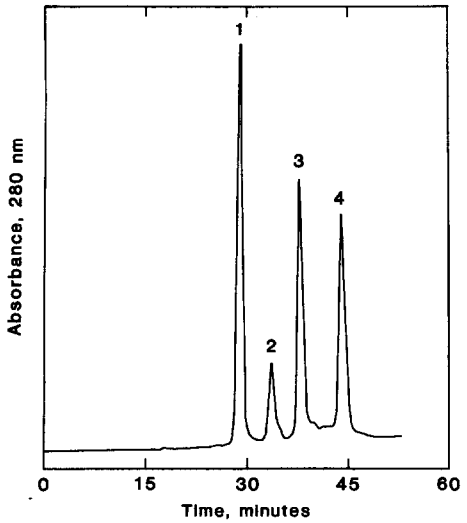

Fig. 3. Typical high-performance ion-exchange chromatogram obtained using gradient elution conditions for several proteins. Peaks: 1 = α-chymotrypsinogen A; 2 = trypsin inhibitor; 3 = cytochrome *c*; 4 = lysozyme. Chromatographic conditions: flow-rate, 0.49 ml/min; mobile phase, calcium acetate–acetic acid buffer (pH = 4.90) containing 20 m*M* Ca^{2+} and 200 m*M* Ca^{2+}; linear gradient from 20 to 200 m*M* Ca^{2+} in 60 min.

TABLE IV

COMPARISON OF OBSERVED HPIEC ISOCRATIC RETENTION PARAMETERS AND THOSE CALCULATED FROM GRADIENT ELUTION RETENTION DATA

Protein	*Slope****	*Intercept****
α-Chymotrypsinogen A	3.22 ± 0.10	−3.63 ± 0.13
Calculated*	3.22 ± 0.15	−3.63 ± 0.19
Calculated**	3.27 ± 0.08	−3.69 ± 0.09
Observed		
Trypsin inhibitor	2.71 ± 0.07	−2.73 ± 0.09
Calculated*	2.67 ± 0.10	−2.68 ± 0.13
Calculated**	2.69 ± 0.08	−2.69 ± 0.10
Observed		
Cytochrome *c*	3.30 ± 0.12	−3.17 ± 0.14
Calculated*	3.23 ± 0.16	−3.09 ± 0.19
Calculated**	3.24 ± 0.13	−3.08 ± 0.14
Observed		
Lysozyme	3.44 ± 0.12	−3.00 ± 0.14
Calculated*	3.34 ± 0.16	−2.88 ± 0.18
Calculated**	3.39 ± 0.17	−2.92 ± 0.17
Observed		

* Average values for all flow-rates.
** Determined at a flow-rate of 0.98 ml/min.
*** Error estimates represent 90% confidence limit.

any two gradient pair (±6%), however, was larger than had been expected given the small error (±1%) in the observed retention data used in the calculation. This error propagation can generally be attributed to the log–log nature of the isocratic retention model itself. Although in theory it would be possible to derive isocratic parameters using only two gradient runs, we suggest that values be obtained from at least three gradient runs and averaged to overcome this problem. As a check of the gradient calculation algorithm, similar calculations were made using the calculated gradient retention times given in Table II instead of the observed retention times. For each individual gradient pair combination, it was found that the original isocratic parameters could be recalculated very accurately (*i.e.* less than 0.1% relative error) demonstrating the theoretical validity of the algorithm with hypothetical error-free data.

It should also be pointed out that in Table III a slight trend in the calculated parameters was observed which could be correlated with changes in the column flow-rate. In a separate control experiment, it was found that the measured isocratic k' for a particular protein decreased slightly (*ca.* 2%) as the column flow-rate was increased over the range of 0.5 to 2.0 ml/min. Although the exact source of this change has not been identified, we believe it to be instrumental in origin and attribute it to a slight mismatch in the solvent compressibility settings on the 6000A pumps. This slight change in k' with flow-rate is believed to be responsible for the observed flow-rate trend in the parameters calculated from gradients at different flow-rates. For the sake of comparison, the average values of calculated parameters obtained at the same flow-rate used in the isocratic determination were tabulated and are shown in Table IV. Using data from only three gradient runs, accurate estimates of the isocratic slope and intercept were found which had uncertainties comparable to those experimentally determined.

DISCUSSION

One potential complication in protein ion-exchange chromatography could arise if the interaction of the protein with either the mobile phase or the stationary phase was such that it could cause significant changes in the protein surface morphology. For example, one may envision that there is some process that is intrinsically denaturing related to the sorption of a protein to the ion-exchange surface which could cause even stable proteins under mild conditions of pH and ionic strength to unfold on the stationary phase surface. The comparison of retention under gradient and isocratic elution conditions provides a way to test this proposition.

Although isocratic and gradient elution can be related mathematically, the dynamics of each process are quite different from the standpoint of how the solute interacts with the stationary phase. In greater elution the protein may be viewed as being initially more or less immobilized at or near the column inlet at the beginning of the run, which is not the case for isocratic conditions.

In previous studies[18,19] we had shown that large changes in ion-exchange retention occurred concomitant with protein denaturation. One might expect that if a protein was being denatured during binding to the stationary phase that this would be reflected differently in its chromatographic retention under isocratic and gradient conditions due to differences in the dynamics of solute binding. Based on the close

correlation between the measured isocratic retention parameters and those calculated using a wide variety of gradient conditions, no evidence for spontaneous protein denaturation due to the interaction of the protein with the stationary phase was observed within the limits of resolution of the chromatographic measurements. It should be stated, however, that this conclusion may not be generalized for proteins which have a large degree of structural flexibility or that are only marginally stable under the chromatographic conditions employed.

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