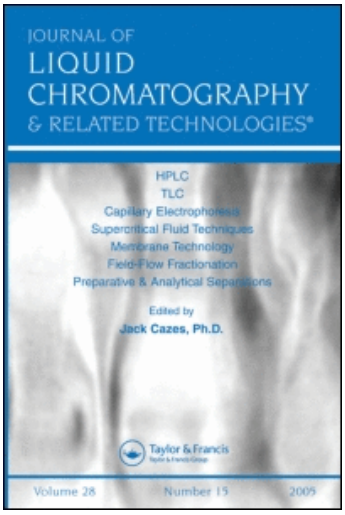

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STUDIES ON GRADIENT ELUTION CHROMATOGRAPHY FOR SEPARATION OF DNA AND OLIGONUCLEOTIDES

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STUDIES ON GRADIENT ELUTION CHROMATOGRAPHY FOR SEPARATION OF DNA AND OLIGONUCLEOTIDES

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ABSTRACT

Rate parameters and gradient correlations for nucleic acids are very important to engineering investigations of the separation of DNA products by gradient elution chromatography. Before carrying out computer simulations for gradient process, these rate parameters and gradient correlation must be determined from the experimental data. In this work, moment method was used to calculate the equilibrium adsorption constants (K_i) as a function of the ionic strength (I) of the mobile phase for different sizes of oligonucleotides from the isocratic elution data and later, these coefficient values were used in the computer simulation to predict the elution behavior. This analysis can eliminate extensive experimental trial and error and hence suitable for separation of oligonucleotides and DNA in a more effective manner.

INTRODUCTION

Separation and purification of nucleic acids are becoming increasingly important as the application is growing in gene therapy and genetic vaccination.[1,2] Recently, oligonucleotides have been used in the treatment and cure of diseases such as acquired immunodeficiency and heart diseases.[3,4] In addition to this, molecular applications and kinetic studies involving polymerase chain

reaction (PCR)[5,6] requires quantification and purification of the desired DNA products from non-specific background products. So, there is a need for the large scale separation and purification of oligonucleotides and DNA fragments of pharmaceutical grade.

Ion exchange chromatography using gradient elution with non-porous particles has been successfully used for the separation and purification of the nucleotides and restriction DNA fragments.[7-9] Determination of the gradient has always been a trial and error procedure. The major problem with trial and error is when it comes to the development, scale up, and optimization of the separation process. In order to carry out a complete engineering study of gradient elution chromatography, a mathematical model which more realistically simulates the chromatographic process is needed. Theoretical investigation in conjunction with the experimental data will provide insight for a systematic approach to solve the problems associated with the analytical scale as well as preparative scale separations.

Mathematical modeling and simulations for gradient elution of proteins have been extensively reported.[10-13] To establish a suitable model for the gradient elution of nucleic acid, it is required to determine the relationship between the equilibrium adsorption constant (K_i) and the ionic strength (I, salt concentration) of the mobile phase. It is likely that this relationship for the oligonucleotides and DNA is different from that of proteins. The separation using ion exchange chromatography is based on the net charge of the DNA fragments. Thus, the equilibrium adsorption constant varies with the nucleotide composition (% of A, T or G, C), as well as, the size of the oligonucleotides and DNA.

Besides equilibrium adsorption constant, effective separation of the oligonucleotides and DNA fragments is also very much dependent upon the transport parameters inside the column, such as, dispersion coefficient, mass transfer coefficients, and intraparticle diffusion. These rate parameters are specifically very important in preparative scale chromatography.

In this paper, we have investigated the gradient elution chromatography separation of oligonucleotides and DNA fragments, both experimentally and theoretically. A DEAE-NPR column was used to obtain the correlation of equilibrium adsorption constant of various DNA fragments with the ionic strength of the buffer. These correlations were used in the computer simulation to verify the residence times obtained by experiments.

GRADIENT ELUTION MODEL

In gradient elution processes, the ionic strength, pH, concentration and composition of the mobile phase (i.e. eluting buffer) are changed with time

according to a preset program. Since the retention strength of the feed components are affected by the equilibrium adsorption constants, retention times are functions of mobile phase ionic strength, pH or composition. In this investigation, only ionic strength of the mobile phase was varied with time for the elution, and keeping pH and buffer composition constant.

A rate model has been developed in the line of a model proposed by Luo and Hsu,[11] which takes into account the mass transport in the mobile phase and the surface adsorption kinetics. Because the particles are non-porous, the internal diffusion equation was eliminated from the model. Hence, the following differential equations and boundary conditions can be written for the mobile phase, eluting buffer and surface adsorption.

Mobile Phase

$$\frac{\partial C_i}{\partial t} = -v\frac{\partial C_i}{\partial z} + D_L\frac{\partial^2 C_i}{\partial z^2} - \left(\frac{1-\varepsilon}{\varepsilon}\right)\frac{3k_f}{R}\left(C_i - \frac{C_{p,i_s}}{K_i}\right) \tag{1}$$

Eluting Buffer

$$\frac{\partial C_b}{\partial t} = -v\frac{\partial C_b}{\partial z} + D_L\frac{\partial^2 C_b}{\partial z^2} \tag{2}$$

Particle Surface Concentration

$$\frac{2C_{p,i_s}}{\partial t} = \frac{3k_f}{R}\left[C_i - \frac{C_{p,i_s}}{K_i}\right] \tag{3}$$

Initial and Boundary Conditions

$$C_i(z > 0, t = 0) = C_{p,i_s}, (z > 0, t = 0) = 0 \tag{4}$$

$$C_i\ (\text{at } z = 0) = C_o(t_0 \bullet t \bullet 0) \tag{5}$$

$$C_i\ (\text{at } z = 0) = 0\ (t > t_0) \tag{6}$$

and

$$\left.\frac{\partial C_i}{\partial z}\right|_{z=L} = 0 \tag{7}$$

Where, C_i = Concentration of the ith solute in the mobile phase, M.

C_b = Concentration of the salt in the eluting buffer, M.

$C_{p,is}$ = Concentration of ith solute on the surface of the particle, M.

z = Distance along the axial coordinate, cm.

t = Time, min.

v = Superficial velocity, cm/min.

D_L = Axial dispersion coefficient, cm^2/min.

k_f = External mass transfer coefficient, cm/min.

K_i = Equilibrium adsorption coefficient for the *i*th solute, dimensionless.

R = Radius of the particles, cm.

ε = Bed porosity, dimensionless.

L = Length of the column, cm.

C_{io} = Concentration of *i*th solute in the sample mixture, M.

The partial differential equations (1-3) along with the initial and boundary conditions (4-7) were solved using finite difference method.

EXPERIMENTAL

Ten oligonucleotides of sizes ranging from 100 bp to 1000 bp with 100 bp interval and 50% A+T content (Sigma Chemicals, St. Louis, MO) were analyzed using an HPLC system (SSI instruments, Inc., State College, PA) which consists of SSI 220B series pump, a 500 UV/Vis variable wavelength detector and a manual injector valve equipped with 20-µL loop. The system is connected to an IBM PC and a software PEAK SIMPLE was used to integrate the peaks. A polymer based TSK gel DEAE-NPR ion-exchange column of dimensions 3.5 cm x 4.6 mm ID (Pharmacia, Uppsala, Sweden) was used for the separation of all the oligonucleotides.

The mobile phase was prepared with HPLC grade water as follows: reservoir A contained 25 mM NaCl and 25 mM Tris-HCl (pH 9.1) and reservoir B contained 1 M NaCl and 25 mM Tris-HCl (pH 9.1). Buffers from reservoir A

and B were mixed inside the gradient pump to create the mobile phase of desired ionic strength. For isocratic runs, the column was pre-equilibrated with 0.3 M buffer, and then at the time of sample injection, a step input of the desired ionic strength was introduced. For linear gradient runs, the column was also pre-equilibrated with 0.3 M ionic strength buffer. However, just after the sample injection, the ionic strength was raised to 0.48 M in 0.01 min. and subsequently, the linear gradient of the ionic strength was introduced. All the chromatographic analyses were carried out at ambient temperature (20°C).

RESULTS AND DISCUSSION

The residence times of various species under isocratic elution was determined experimentally. All the peaks in the chromatograms were close to Gaussian and, hence, one approximation could be made based on the work by Hsu and Chen[14] that the adsorption-desorption equilibrium constants for various species can be determined from the absolute first moment, which is:

$$m_1 = \frac{L}{v}\left[1+\frac{\varepsilon_p}{\alpha}\left(1+\frac{K_i}{\varepsilon_p}\right)\right] \tag{8}$$

where m_1 = first moment, L = length of the column, v = interstitial velocity of the mobile phase inside the column, ε_p = porosity of the particles = 0 because the particles are non-porous, $\alpha = \varepsilon/(1-\varepsilon)$ where ε is the void fraction for the column, and K_i = adsorption-desorption equilibrium constant which is dimensionless. Equation 8 can be modified and written as follows:

$$K_i = \left(\frac{\tau_i \times Q}{V\times(1-\varepsilon)} - \frac{\varepsilon}{1-\varepsilon}\right) \tag{9}$$

where τ_i is the residence time of ith species in minutes and is equal to its first moment m_1, Q is the flow rate of the mobile phase through the column in ml/min. (in this case Q = 1 mL/min. for all the analysis), V is the volume of the column, and ε is the bed porosity of the column = 0.3 in this case. K_i values are calculated for each τ_i (for ith species, where i = 1, 2, 3,...) which means that we have K_i for each ionic strength (I in moles/l).

Analysis of Oligonucleotides (10 DNA Fragments Ranging from 100 to 1000bp)

Using Eqn. 9, K values were calculated for the DNA markers using their residence times from the chromatograms. Figure 1 shows the K values as a function of ionic strength of the buffer for 5 oligonucleotides of sizes ranging

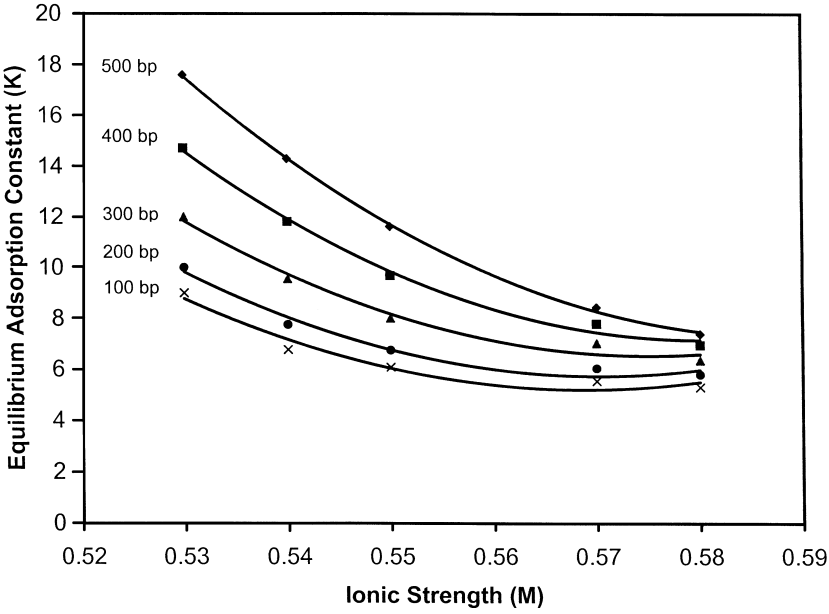

Figure 1. Equilibrium adsorption constant (K) as a function of ionic strength (I) for various sizes of oligonucleotides. Solid lines are second order polynomials obtained by curve-fitting the experimental points.

from 100 bp to 500 bp. It was found that K followed a second order polynomial curve with respect to the ionic strength:

$$K_i = \alpha + \beta I + \gamma I^2 \qquad (10)$$

The values of α, β, γ were determined by curve-fitting using Microsoft Excel spreadsheet. These constants are presented in Table 1. Gu et al.[13] have reviewed a variety of relationships for proteins as well as other molecules. A common relationship between K and I for proteins is of power-law nature. However, polynomial relationship between K and solvent strength similar to Eqn. 10 has been reported by Marengo et al.[15] for different sizes of aldehydes in reverse-phase HPLC analysis.

Using the K values, gradient elution model was solved to simulate the chromatogram peaks for ionic strength = 0.55 M, D_L for oligonucleotide = 0 due to their high molecular weight, D_L for buffer = 0.06 cm^2/min, and k_f = 0.3 cm/min. The values of D_L and k_f should be chosen carefully from empirical correlations or should be determined experimentally for preparative scale systems.

Table 1

Coefficients in the *K* vs. Ionic Strength (*I*) Relationship* for Various Sizes of Oligonucleotides

Oligonucleotides	α	β	γ	R^2
100 bp	775.45	-2708.7	2381.3	0.9588
200 bp	830.86	-2894.4	2538.3	0.9797
300 bp	872.66	-3012.6	2619.6	0.9838
400 bp	1005.5	-3441.8	2966.4	0.9949
500 bp	1053.6	-3557.0	3023	0.9994

* Equation 10.

The experimental HPLC chromatogram under isocratic condition for $I = 0.55$ M is shown in Figure 2. It was found that the residence time in the simulated chromatogram were within 2.0% of the actual residence time in the column (Table 2). This indicates that moment method as well as the polynomial correlation for the *K* vs. ionic strength (*I*) can be successfully used. Also, the mathematical model can be used to simulate the chromatogram for the oligonucleotides.

It is expected that a linear gradient of the ionic strength is required to obtain a clear separation between all the oligonucleotide products. As an example shown in Figure 3, a particular gradient program was used and a reasonable degree of separation between the oligonucleotides was achieved. However, the residence times obtained by computer simulation for 300, 400, and 500 bp oligonucleotides were about 25 to 30% less than the residence times obtained

Table 2

Comparison Between Experimental and Simulated Residence Times of Oligonucleotides at Ionic Strength 0.55 M

Oligonucleotides	Res. Time (min.) Experimental	Res. Time (min.) Simulated
100 bp	5.63	5.63
200 bp	5.94	5.99
300 bp	6.53	6.64
400 bp	7.31	7.43
500 bp	8.21	8.31

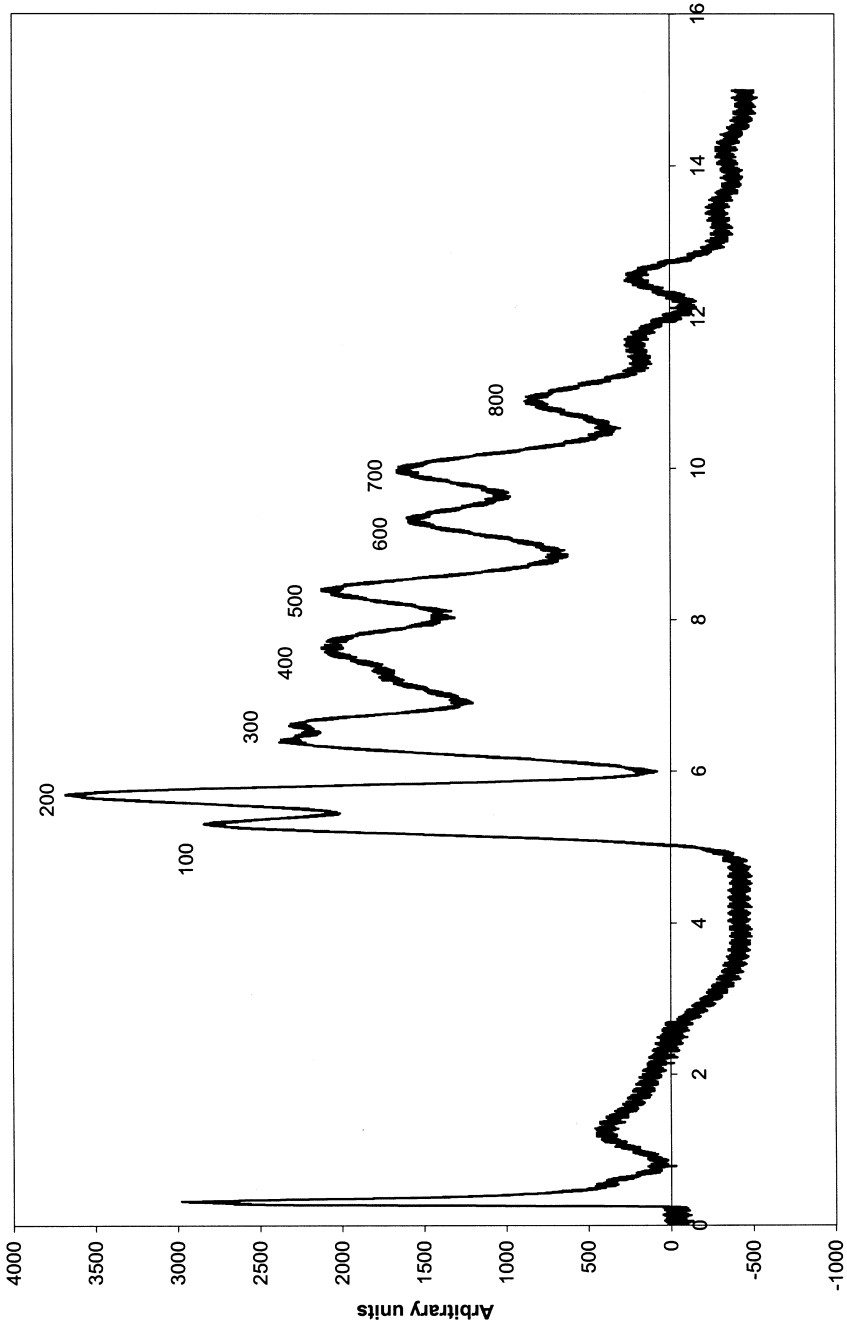

Figure 2. Chromatogram of 100bp ladder under isocratic elution with ionic strength = 0.55 M.

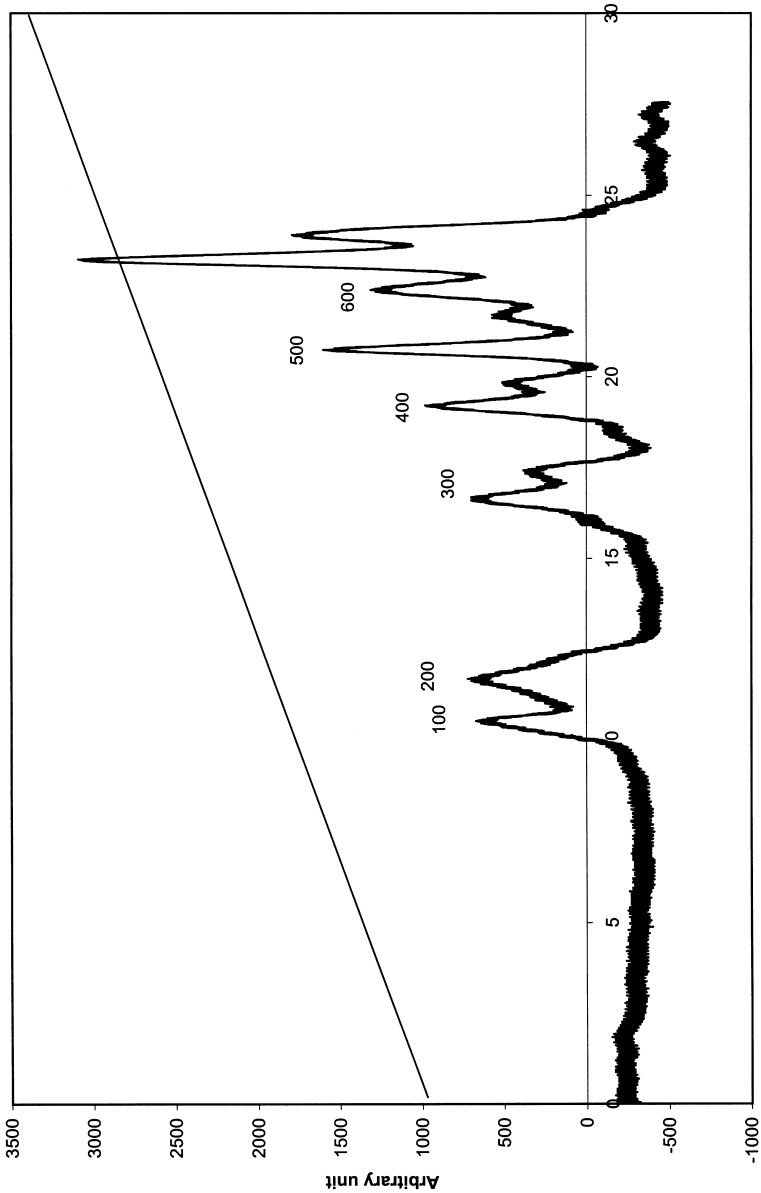

Figure 3. Chromatogram of 100bp ladder under gradient elution. The gradient program was: at $t = 0$ min., $I = 0.3$ M; $t = 0.01$ min., $I = 0.48$ M; from $t = 0.01$ to 30.0 min., I varies from 0.48 M to 0.60 M linearly.

by experiment, whereas, the residence times for 100 and 200 bp showed a reasonable match.

The mismatch in theoretical and experimental residence times for 300, 400, and 500 bp oligonucleotides could be due to the fact that the *K* values are obtained from the isocratic runs. Since the buffer moves much faster than the oligonucleotides in the HPLC column, the latter have sufficient time to achieve the equilibrium for ion-exchange during an isocratic run. However, during a gradient run, when the condition inside the column continuously changes, it is possible that it takes more time for equilibration and hence, the solutes slow down. It is also possible that the structure of the oligonucleotide continuously changes as it moves through the column due to the change in the ionic strength. The slow down is more apparent for the higher base-pair oligonucleotides because the equilibration time may vary with the total charge and the length of the solute molecules. Although, this might be the explanation for the slow down of the oligonucleotides, the exact cause for this dynamic behavior inside the column is currently under investigation.

In summary, this research provided a basic framework for the theoretical and experimental investigation of the gradient elution chromatography for the separation of oligonucleotides of different sizes. The equilibrium adsorption constants were obtained by using the first moment and the relationship of this constant with the ionic strength of the mobile phase was established for different oligonucleotides. Although, the theoretical solution matches well with the experimental data for isocratic runs, there is a mismatch for higher base-pair oligonucleotides when gradient was used and this is attributed to the slow down of the large molecular weight solutes due to the slow equilibration during a gradient run.

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