Journal of Chromatography, 597 (1992) 83–92
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 815

Computer simulation of weak affinity chromatography

M. Wikström

Department of Physical Chemistry 2, University of Lund, S-221 00 Lund (Sweden)

S. Ohlson*

HyClone Laboratories, Inc., 1725 South HyClone Road, Logan, UT 84321 (USA)

ABSTRACT

Affinity chromatography is a selective purification technique in which the specific interactions between the bound ligand and the solutes (ligates) in the mobile phase are of high affinity [the equilibrium constant (K_L) is usually between 10^4 and 10^{15} l mol^{-1}]. By utilizing weaker affinities ($K_L < 10^4$ l mol^{-1}), fast and dynamic separations with high performance can be achieved exploiting the specificity introduced by the affinity ligand. As weak affinity chromatography (WAC) is a recent technique and the number of applications is limited, computer simulation of established mathematical models for chromatography was applied for the purpose of studying the basics of WAC and its applicability. The antigen–antibody system was selected owing to its general nature and permitted direct comparisons with experimental data. Important parameters such as particle size of support, kinetics, ligate concentration, bound ligand concentration and peak capacity were evaluated to determine their significance in WAC. A fit of the model to the experimental data from the antigen–antibody system generated information on the kinetics of the interaction and the number of active accessible sites.

INTRODUCTION

Affinity chromatography (AC) is a selective separation technique based on the specific interaction between complementary molecules [1]. Usually, pairs of biological molecules are utilized in the separation process, such as antibody–antigen, lectin–carbohydrate or nucleic acid–protein. In practice, a crude extract containing the substance or substances to be separated (ligates) is adsorbed specifically to an immobilized solute (ligand) which is bound in an active state to a stationary solid phase. When the contaminants have passed through the stationary phase, the desired substances are desorbed from the solid phase by various chemical (*e.g.*, pH, salt and counter ligands) or physical methods (*e.g.*, temperature).

The integration of AC into high-performance liquid chromatography (HPLC) in the late 1970s [2], termed high-performance liquid affinity chromatography (HPLAC), signified an optimization of the affinity technique, primarily with regard to speed and resolution. However, the high resolving power of HPLC was not experienced in various HPLAC applications, where plate numbers are hundreds per meter compared with an order of magnitude higher for HPLC. A recent update of HPLAC is available [3]. Traditional AC is basically an on–off procedure using high affinities between the bound ligand and the interacting ligates [the equilibrium constants (K_L) are usually between 10^4 and 10^{15} l mol^{-1}]. These systems are generally characterized by slow desorption kinetics of the adsorbed ligate from the immobilized ligand setting the overall performance of emerging peaks [4,5]. Therefore, in order to achieve chromatography within a reasonable time period, the eluting conditions have to be changed drastically to release the ligates (no affinity) in narrow zones. The major challenge we are facing is to realize a more "dynamic" form of affinity chromatography, where fast (isocratic) separations with high performance can be achieved while still taking

0021-9673/92/$05.00

advantage of the selectivity introduced by the affinity ligand.

In recent papers [6–10], we have addressed this area by using weak (readily reversible) biospecific recognition as the basis for chromatographic separations [weak affinity chromatography (WAC)]. We were able to achieve highly efficient separations of similar carbohydrate antigens under isocratic conditions, where the antigens were retarded differently under weak affinity (K_L = 10^2–10^4 l mol^{-1}) on columns containing selected monoclonal antibodies. By using WAC we can perform affinity chromatography where the features of HPLC can be fully exploited without jeopardizing the selectivity exhibited by the affinity ligand. However, as WAC is a recent technique and the number of applications is limited (for a review see ref. 9), we have now adopted computer simulation procedures to investigate the prospects for WAC, such as the influence of critical parameters on the separation ability and the design of affinity columns.

In this work we used a theoretical model to study the performance of WAC using selected analytical solutions to describe both linear and non-linear chromatography. Computer-generated chromatograms of critical parameters were obtained to assess the potential of WAC. The antigen–antibody system was selected as the model, where monoclonal antibodies interact weakly with their corresponding carbohydrate antigens. By these means we could make direct comparisons between the theoretical approach and experimental results and in addition use the model to calculate physico-chemical parameters of affinity chromatography.

THEORETICAL

General

The separation model is based on a column containing porous particles (solid phase) through which the mobile phase is passing. The ligates interact with the bound ligand on the solid phase, while the others flow through the column without being retarded. The gel bed is defined by its length (L, cm), circular cross-sectional area (A, cm^2) and void fraction, ε (the volume outside the gel particles per column volume). The gel particles are defined as spherical with a radius (R), density (ρ_p) and porosity (β) (the volume inside the gel particles per total volume of gel particles). In addition, we assume a second-order reversible reaction of ligate to ligand which can be considered to be a reasonable approximation:

$$\mathrm{Lt} + \mathrm{Ld} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{LtLd} \qquad (1)$$

where Lt, Ld and LtLd are the ligate, ligand and ligate–ligand complex, respectively, k_a is the rate constant for adsorption (l mol^{-1}s^{-1}) and k_d is the rate constant for desorption (s^{-1}).

The following kinetic equations can be derived:

$$dq/dt = k_a c_i(Q_{max} - q) - k_d q \qquad (2)$$

$$q^* = \frac{Q_{max} K_L c^*}{1 + K_L c^*} \qquad (3)$$

$$K_L = k_a/k_d$$

where q is the concentration of adsorbed ligate (moles per kg solid phase), q^* is the concentration of adsorbed ligate at equilibrium (moles per kg solid phase), t is the time (s), c_i is the ligate concentration in the gel particle (M), c^* is the concentration of free ligate at equilibrium (M), Q_{max} is the maximum accessible ligand sites (moles per kg solid phase) and K_L is the equilibrium constant (l mol^{-1}).

When $K_L c^* \ll 1$ (linear adsorption isotherm), eqns. 2 and 3 become

$$dq/dt = k_a(c - q/K_L Q_{max}) \qquad (4)$$

and

$$q^* = K_L Q_{max} c^* \qquad (5)$$

The chromatographic mass balance equation [11–13] is usually expressed as

$$u_0 dc/dz + \varepsilon dc/dt + (1 - \varepsilon) ds/dt = E_z d^2c/dz^2 \qquad (6)$$

$$s = \beta c_i + \rho_p q \qquad (7)$$

where u_0 is the linear flow-rate (cm s^{-1}), z is the distance (cm), c is the concentration of free ligate (M), c_i is the ligate concentration in the solid phase (M) and E_z is the axial dispersion coefficient (cm^2 s^{-1}).

The following equation is derived by doing a mass balance for the free ligate inside the gel particles (solid phase), c_i [11]:

$$\beta dc_i/dt + \rho_p dq/dt = D_i\, 1/r^2 d/dr\, (r^2 dc_i/dr) \qquad (8)$$

where D_i is the effective diffusivity of ligate (cm^2 s^{-1}) and r is the radial distance from center of the gel particle (solid phase).

The concentration of ligate in the pores and outside the particles, c_i and c, depends on the diffusion of ligate through the stagnant liquid film surrounding each particle [11–13]:

$$k_f(c - c_i\, I_{r=R}) = D_i\, dc_i/dr\, I_{r=R} \quad (9)$$

where k_f is the rate constant for diffusion through the liquid film (cm s^{-1}).

Further, at time $t = 0$ we have no adsorbed ligate, which gives the initial condition:

$$q(z,0) = 0 \quad (10)$$

In the following we shall consider the analytical solutions for the concentration of ligate at the outlet of the column as a function of time, *i.e.*, $c(L,t)$.

Linear condition

The assumption here is that we are in the linear part ($K_L c^* \ll 1$) of the adsorption isotherm, *i.e.*, eqns. 4 and 5 are valid. The boundary condition for injecting a short pulse of ligate with concentration c_0 during a time period t_0 is given by

$$c(0,t) = \begin{cases} c_0 & 0 \leqslant t \leqslant t_0 \\ 0 & t > t_0 \end{cases} \quad (11)$$

Kubin [14] and Kucera [15] derived in the Laplace domain a general solution to this system of equations (eqns. 4, 6, 7 and 9) with the associated boundary and initial conditions (eqns. 10 and 11). The inversion of these transformed solutions could not be obtained and instead the Laplace domain equations were used to find expressions for statistical moments.

The first moment, μ_1, give the retention time of the chromatographic peak [15]:

$$\mu_1 = \frac{\int_0^\infty c(L,t)t\,dt}{\int_0^\infty c(L,t)\,dt} =$$

$$L/u_0[\varepsilon_T + (1 - \varepsilon)\rho_p K_L Q_{max}] + t_0/2 \quad (12)$$

$$\varepsilon_T = \varepsilon + (1 - \varepsilon)\beta \quad (13)$$

The second moment, μ_2, is a measure of band spreading and is given by [16]:

$$\mu_2 = \frac{\int_0^\infty c(L,t)(t - \mu_1)^2 dt}{\int_0^\infty c(L,t)\,dt} =$$

$$2L/u_0\{E_z/u_0^2[\varepsilon_T + (1 - \varepsilon)\rho_p K_L Q_{max}]^2 + (1 - \varepsilon)\rho_p K_L Q_{max}/k_d + (1 - \varepsilon)[R^2/15(\beta + \rho_p K_L Q_{max})^2(1/D_i + 5/k_f R)]\} + t_0^2/12 \quad (14)$$

$$C(L,t) = 1/[\sqrt{(2\pi\mu_2)}]\, e^{-(t-\mu_1)^2/2\mu^2} \quad (15)$$

Non-linear conditions

Wade *et al.* [17] derived a valuable analytical solution (the impulse-input solution) to the theory of non-linear affinity chromatography.

In this model any influence from axial dispersion is neglected ($E_z = 0$) and non-linear chromatography (eqns. 2 and 3 are valid) is assumed. In addition, instead of using the rectangular boundary condition (eqn. 11), the injection pulse is described as a delta function:

$$c(0,t) = c_0^* \delta(t) \quad (16)$$

where c_0^* is the concentration of injected ligate multiplied by the width of the injection pulse (defined as a fraction of the column dead volume).

The impulse-input solution is considerably easier to evaluate as it contains only one Bessel integral compared with four for the Goldstein solution for zonal elution under non-linear chromatographic conditions [18].

Computer simulation

Theoretical chromatographic peaks were obtained by making a computer simulation of the analytical solutions of Kubin [14] and Kucera [15] for linear chromatography and the impulse-input solution for non-linear chromatography. The computer programs were written in Turbo-Pascal 3.01 (Borland International) and were processed on an IBM PC equipped with an Intel 8087 mathematical coprocessor. The algorithm for the Bessel integrals was kindly provided by Dr. P. Carr and Dr. J. Wade. Computed affinity chromatography was studied under various conditions and the resulting chromatograms were recorded on an HP 7475A plotter.

EXPERIMENTAL

Reagents

A glucose-containing tetrasaccharide, Glcα1–6Glcα1–4Glcα1–4Glc [termed $(Glc)_4$] was purified from human urine as described by Hallgren *et al.* [19]. Panose was obtained from BDH (Pool, UK). All other chemicals were of analytical-reagent grade.

Monoclonal antibody and affinity column

Hybridomas were produced [20] from Balb/cJ mice previously immunized with $(Glc)_4$ conjugated to keyhole limpet hemocyanin. A clone producing weak affinity monoclonal antibodies named 39.5 [21] was selected (IgG 2a) and was grown as ascites in Balb/cJ mice. The 39.5 was isolated by ammonium sulphate precipitation prior to chromatography on protein A-Sepharose according to the protocols of Gersten and Marchalonis [22]. The purified 39.5 monoclonal antibody was coupled *in situ* on to a SelectiSpher-10 Activated Tresyl HPLC column (100 × 5 mm I.D.) (HyClone, Lund, Sweden) as described by Ohlson *et al.* [7]. This column is named 39.5-HPLAC. For reference purposes, columns with no or irrelevant antibodies were produced using the same procedure as described above for 39.5.

Apparatus and chromatographic procedures

The chromatographic experiments were carried out on a Shimadzu (Kyoto, Japan) LC4A instrument at 1 ml min^{-1} at 30 atm (3.04 MPa) using 0.02 *M* sodium phosphate buffer–0.2 *M* sodium chloride (pH 7.5) as the mobile phase. A 10-μl volume of tritiated $(Glc)_4$ (1.5 ng; specific activity = 8.4 Ci $mmol^{-1}$) was injected at various temperatures and fractions (0.23–0.64 ml) were collected and counted in a liquid scintillation counter (LKB, Bromma, Sweden). All experiments were performed under carefully controlled thermostated conditions, where the HPLC columns and a 3-m stainless-steel tube (between pump and injector) were immersed in a controlled-temperature bath capable of regulating the temperature in the range 5–50°C. The tubing was included to ensure proper heating at the inlet of the column.

RESULTS AND DISCUSSION

We have recently studied (6–10) some of the features and benefits of weak affinity chromatography as applied to bound weak monoclonal antibodies (ligand) interacting with low-molecular-weight carbohydrate antigens (ligate). This system was selected because it is of a general character (antibody *versus* antigen) and ensures a one-to-one interaction between ligand and ligate. The latter is of importance for our mathematical models as they are based on single interactions with no cooperativity effects.

Traditional affinity chromatography is basically an extraction procedure in a column format where ligate is adsorbed at high affinity and eluted at no affinity. In WAC, interactions are weak ($K_L < 10^4$ l mol^{-1} with a practical range of 10^2–10^4 l mol^{-1}), allowing dynamic elution under isocratic or gradient conditions where ligates are separated according to their intrinsic affinities to the bound ligand. WAC is by nature a mild elution technique, thereby making it possible to preserve the biological activities of both ligand and ligates. HPLC columns were used for this study, where the 39.5 antibodies were coupled to 10-μm silica particles to maximize performance in terms of speed and resolution.

Parameter selection

Our strategy, when doing the computer simulations using the various mathematical models, has been to keep some of the basic parameters fixed, such as column and gel characteristics. The porosity was determined by injecting deuterium oxide on to the 39.5 HPLAC column and $(Glc)_4$ on to the reference columns. The two methods gave the same value, $\beta = 0.64$. The void volume was measured by injecting blue dextran (MW $\approx 2 \cdot 10^6$), which is not able to penetrate the pores of the gel particles ($\beta = 0$). The void volume was found to be $\varepsilon = 0.42$. In order to come into close proximity with the ligands inside the gel particles, the ligate has to diffuse through the liquid film surrounding each solid-phase particle and through the liquid of each pore. To describe the diffusion adequately we need to know the effective diffusion coefficient (D_i) as

defined by Horváth and Lin [23]. The ligate, $(Glc)_4$, was defined as a spherical molecule with a radius of 7.5 Å as deduced from molecular models. By using the Stokes–Einstein equation under the assumption of infinite dilution which is a good approximation ($c_0 = 2.1 \cdot 10^{-7}$ M) and the Horváth–Lin equation [23], D_i can be calculated at different temperatures. The liquid film mass transfer coefficient (k_f) can be calculated from the diffusion coefficient and is also dependent on particle size, flow-rate and void volume [23]. The density of the gel particles is $\rho_p = 2.42$ g cm^{-3} and chromatography was conducted at a linear flow-rate of 0.085 cm s^{-1} (1 ml min^{-1}).

In the following, with the example of generation of theoretical peak profiles, we discuss the impact of certain key parameters (*e.g.*, affinity and ligand concentration) on the performance of WAC, with special emphasis on the practical consequences of the technology. We shall also fit our model to the experimental chromatograms and compare the parameters obtained by different procedures.

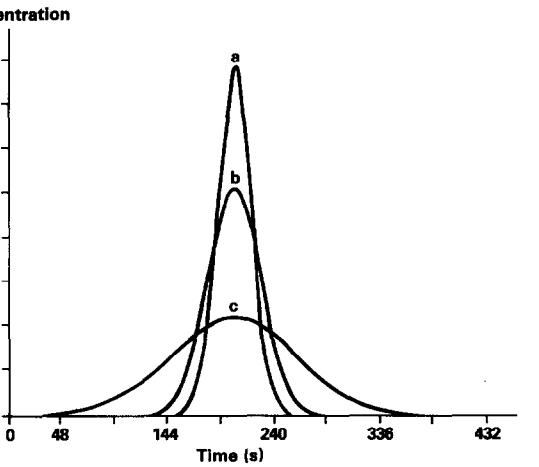

Fig. 1. Theoretical (Kubin–Kucera) chromatogram: dependence on particle size. Parameter selection: $c_0 = 1.4 \cdot 10^{-6}$ M; $\varepsilon = 0.42$; $\beta = 0.64$: $Q_{max} = 6.8 \cdot 10^{-4}$ mol kg^{-1} gel; $K_L = 10^3$ l mol^{-1}; $k_a = 10^3$ l mol^{-1} s^{-1}; time of injection = 0.6 s; $D_i = 10^{-6}$ cm^2 s^{-1}; $\rho_p = 2.42$ g cm^{-3}; $L = 10$ cm; $u_0 = 0.085$ cm s^{-1}; $k_f = 4 \cdot 10^{-2}$ cm s^{-1}; $E_z = 0$ cm^2 s^{-1}; R = (a) 5, (b) 25 and (c) 75 μm.

Simulation of WAC: dependence on particle size

In analytical HPLC, such as reversed-phase and ion-exchange chromatography, the solid phase is usually made of particles as small as possible to minimize any contributions from, *e.g.*, diffusional limitations on peak spreading. WAC shows a similar behavior where small particles improve the overall performance. The influence of particle size on peak spreading is shown in Fig. 1, where the default parameter values are given. In this example (Fig. 1) we have linear conditions ($K_L c_0 \ll 1$), weak affinity ($K_L = 10^3$ l mol^{-1}), and high loading of immobilized antibody ($Q_{max} = 6.8 \cdot 10^{-4}$ mol kg^{-1}). This set of parameters is not unrealistic for our system of immobilized monoclonal antibodies directed to a small carbohydrate antigen. The peak performance deteriorates in chromatography with larger particle sizes, as is expected when considering other modes of interactive chromatography. Particle size is not an important issue in attempts to do isocratic elutions with traditional high-affinity chromatography where it does not contribute significantly to peak spreading but rather is due to slow kinetics.

Simulation of WAC: influence of kinetics

The adsorption–desorption profile, expressed as kinetic rate constants, plays a significant role in determining the overall efficiency of an analytical affinity column. In attempts to achieve isocratic or gradient elution with moderate-to high-affinity chromatography, the performance will be poor owing to very slow desorption ($k_d \ll 1$ s^{-1}), which in practical terms renders the system not amenable to high-performance chromatography primarily because of loss of speed and resolution. Instead, the elution conditions have to be drastically changed to accomplish a complete release of ligates from the column, which is typical for the "on/off" procedure of high-affinity chromatography. As seen from Fig. 2, the influence of kinetics is illustrated at $K_L = 10^3$ l mol^{-1} varying the rate constants in the interval $k_d = 0.1$–10 s^{-1}. When k_d is low (<0.1 s^{-1}) the performance is essentially lost and no adequate chromatography can be achieved. It is interesting that reversed-phase chromatography shows often rapid kinetics [24] where high rates were observed ($k_d > 100$ s^{-1}) when small ligates were desorbed from the column. The kinetics of the ligate to ligand interaction are often the rate-limiting step in WAC and will usually determine the extent of peak broadening and shape.

Simulation of WAC: effect of ligate concentration

The value of the parameter $K_L c_0$ reflects the

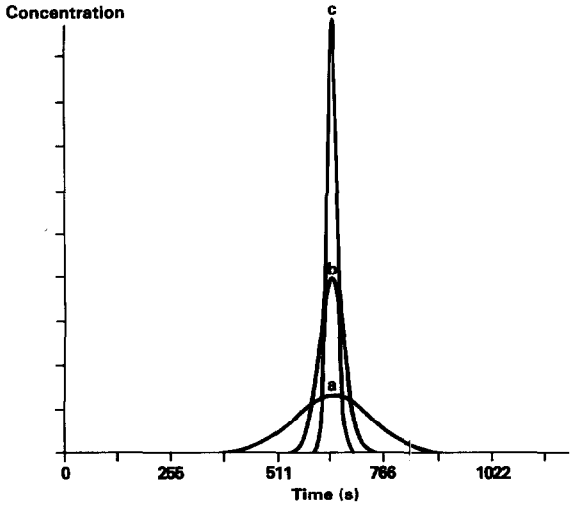

Fig. 2. Theoretical (Kubin–Kucera) chromatogram: influence of kinetics. Parameters as in Fig. 1 except $Q_{max} = 10^{-3}$ mol kg^{-1} gel; $L = 25$ cm; $R = 5 \cdot 10^{-4}$ cm; $E_z = 10^{-6}$ cm^2 s^{-1}; $k_a =$ (a) 10^2, (b) 10^3 and (c) 10^4 l mol^{-1} s^{-1}.

deviations from linearity in the adsorption isotherm, and in general when $K_L c_0$ is $\ll 1$ we are in the linear range. When the overload term, $K_L c_0$, is increased the number of ligand sites will not be sufficient to retard all ligate molecules properly. This means that a significant portion will be eluted earlier and as a result we obtain asymmetric and less retarded peaks. As is evident from the condition $K_L c_0 \ll 1$ for linearity, we see that under weak affinity the injected concentration c_0 can be considerably higher than in traditional high-affinity chromatography without jeopardizing the criteria for linearity. Fig. 3 illustrates the effect of ligate concentration on peak performance when running WAC. The typical features of a decrease in retention and tailing of peaks are seen and it is obvious how important the amount of injected ligate is on the performance, especially for applications in analytical affinity chromatography. Wade *et al.* [17] have studied these phenomenon in great detail for lectin–carbohydrate interactions in medium-affinity chromatography.

Simulation of WAC: comparison of high and low affinity

It can be argued that high-affinity chromatography, at least theoretically, can be performed in an isocratic mode with reasonable retention times

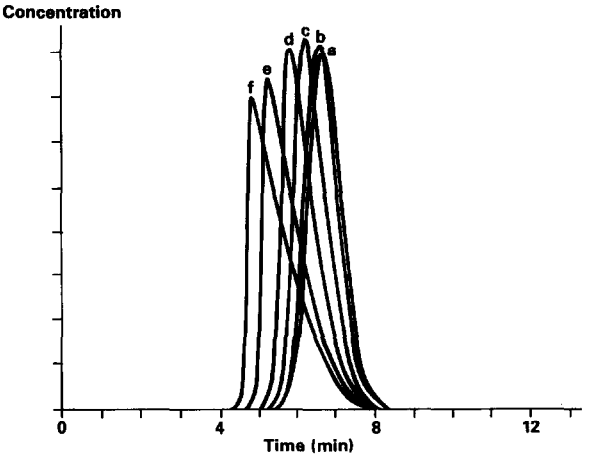

Fig. 3. Theoretical (impulse-input) chromatogram: effect of ligate concentration. Parameters as in Fig. 1 except $Q_{max} = 3.9 \cdot 10^{-4}$ mol kg^{-1} gel; $K_L = 5.2 \cdot 10^3$ l mol^{-1}; $k_a = 1.1 \cdot 10^4$ l mol^{-1} s^{-1}; $K_L c_0 =$ (a) 0, (b) 1, (c) 5, (d) 10, (e) 20 and (f) 30.

(< 1 h) as long as the active bound ligand (Q_{max}) is kept low. It is possible, as illustrated in Fig. 4, to achieve this at the price of poor performance. It is also interesting to note how the theory predicts the appearance of non-retarded material, similarly to what has been described previously as the split peak phenomenon [25]. However, by running at weak affinity (low K_L and high Q_{max}), with the same

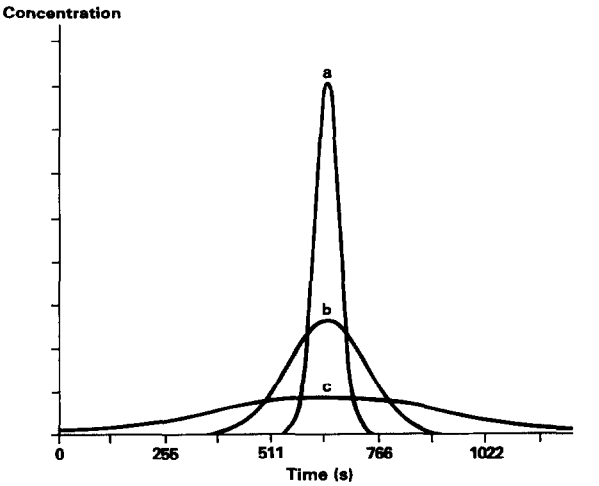

Fig. 4. Theoretical (Kubin–Kucera) chromatogram: comparison high *versus* low affinity. Parameters as in Fig. 1 except $Q_{max} = 10^{-3}$ mol kg^{-1} gel; $L = 25$ cm; $K_L =$ (a) 10^3, (b) 10^4 and (c) 10^5 l mol^{-1}.

retention (Fig. 4) peaks are sharpened and high performance is restored. Fig. 4 is a good illustration of the fundamental difference between high- and low-affinity chromatography and it shows clearly the inability of HPLAC using high affinities to become an ideal tool for dynamic affinity chromatography.

Simulation of WAC: dependence on bound ligand (Q_{max})

By doing a thorough theoretical analysis of affinity chromatography, it is clear that WAC can be realized if certain conditions are fulfilled. The introduction of high-performance affinity adsorbents, effective methods for immobilization of ligand, new methods for elution control such as temperature and various tools to produce ligands of predetermined specificities have all contributed to give opportunities to study the weak-affinity approach in the laboratory. A key factor to consider in WAC is the design of the ligand. To be successful in WAC we need first to select an appropriate ligand which carries both the desired weak-affinity and also carries handles for efficient binding. Second, large amounts of ligand (mM) have to be immobilized with maintained activity and be as homogeneous as possible. With larger ligands (*e.g.*, with antibodies) it is difficult to achieve a large number of interacting binding sites as the active site usually only represents a small fraction of the total molecule. Third, the ligates should interact freely with the ligand under a minimum of steric restrictions. Nevertheless, we have in previous studies [6–8,10] been able to bind monoclonal antibodies at high active concentrations (20–25 mg ml^{-1} gel), enabling us to study affinities at $K_L = 10^3$ l mol^{-1}. Fig. 5 shows the elution behaviour at different levels of active accessible ligand concentration (Q_{max}). There is, of course, a practical upper limit for the amount of ligand that can be bound. This is usually determined by the accessible surface area of the solid phase and the ability of the ligate to interact with the immobilized ligand.

Simulation of WAC: peak capacity

A striking feature of WAC is the capacity to resolve completely similar solutes present in a crude extract. Theoretically there is no limit on how weak the interactions can be to function as a specific

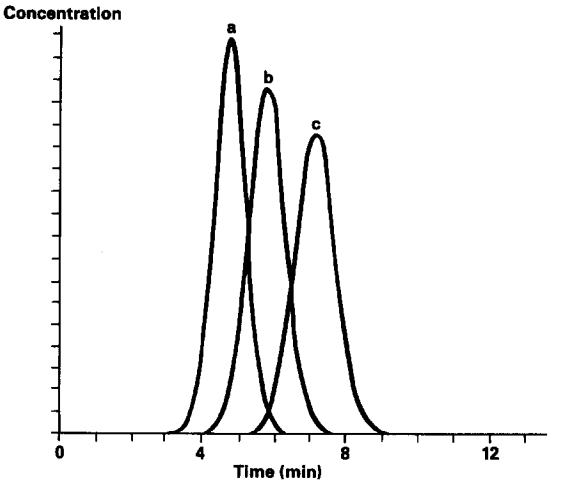

Fig. 5. Theoretical (Kubin–Kucera) chromatogram: dependence of bound ligand (Q_{max}). Parameters as in Fig. 3 except Q_{max} = (a) $2.3 \cdot 10^{-4}$, (b) $3.0 \cdot 10^{-4}$ and (c) $3.9 \cdot 10^{-4}$ mol kg^{-1} gel.

recognition system. However, practical considerations for the design of a WAC column set the limit on the size of column and amount of ligand, cooperativity effects between ligand and ligate and selection of parameters for elution. In previous studies [6–8, 10], a maximum Q_{max} of 50–100 mg monoclonal antibodies per milliliter of gel could be reached with a column format of 25 cm × 0.5 cm I.D. The consumption of antibodies is considerable, up to 500 mg per column. A support has to be selected to allow the maximum surface area to be available for both the ligand and the ligate. The matrix particles should be small (5–50 μm diameter) and rigid enough to allow fast separations with high performance.

A matter of concern in affinity chromatography is the appearance of non-specific adsorption, which is generally defined as the amount of non-desired interactions between the ligate or other solutes in the crude sample and the support. The general observation that non-specific adsorption of solutes to the support media is often of weak affinity does not support the opposite statement that WAC is a non-specific chromatographic method. The specificity in WAC is due to the sum of many serial weak binding events. In Fig. 6 we have simulated the capacity of a WAC system to resolve ligates which are separated to >90% (peak capacity). To simulate specificity with as weak interactions as possible we

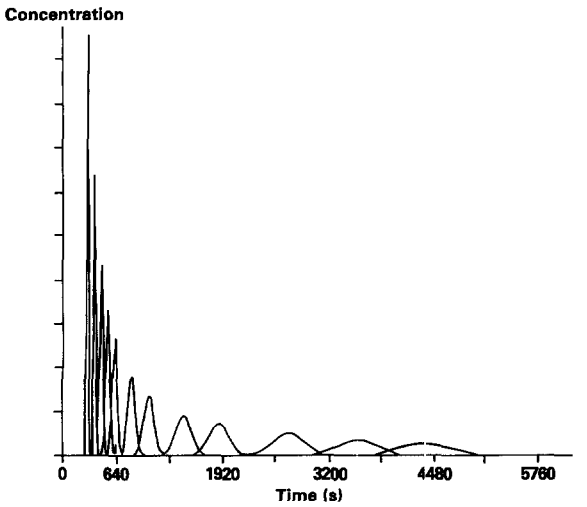

Fig. 6. Theoretical (Kubin–Kucera) chromatogram: peak capacity. Parameters as in Fig. 1 except $Q_{max} = 10^{-3}$ mol kg^{-1} gel; L = 25 cm; $E_z = 10^{-6}$ cm^2 s^{-1}; and K_L varied between $2 \cdot 10^2$ and 10^4 l mol^{-1}.

selected a long (25 cm) column with high Q_{max} (75 mg antibody per milliliter of gel). In addition, we assumed a linear adsorption isotherm. A suitable separation "window" is defined from capacity factor $k' = 1$ at *ca.* 8 min to $k' = 22$ at *ca.* 90 min. This will allow ample time for non-retarded or weakly interacting species ($K_L < 5 \cdot 10^2$ l mol^{-1}) to pass through the column. As shown in Fig. 6, the peak capacity is *ca.* 10, which means that ten similar ligates varying in affinity from $K_L = 6 \cdot 10^2$ to $1 \cdot 10^4$ l mol^{-1} can be separated isocratically on this column. Most interestingly, we can theoretically separate substances by affinity chromatography at very weak affinities of $K_L = 10^2$–10^3 l mol^{-1}.

Fitting of model to experimental data

A number of zonal and frontal elutions were accomplished to study the retention of $(Glc)_4$ on a 39.5 HPLAC column at various temperatures as described above and in more detail in a previous paper [7]. Affinity was regulated by temperature and was determined at each temperature by frontal analysis [26]. Frontal analysis was performed in the temperature range 10–50°C and affinities were estimated to be $K_L = 0.8 \cdot 10^3$–$16 \cdot 10^3$ l mol^{-1} (data not shown). As we are in the linear range ($K_L c_0 = 2 \cdot 10^{-4}$–$3 \cdot 10^{-3}$), the Kubin–Kucera solution coincides with the impulse-input solution. Therefore, the Kubin–Kucera model was used in a least-squares fit to our normalized experimental data to obtain an estimate of the kinetics and Q_{max}. This was done on a Macintosh IIcx computer using the curve-fitting program MULTIFIT 2.0 (DayComputing).

The influence from extra-column effects was estimated by injecting deuterium oxide ($\sigma^2_{ext} = 17$ s^2) with no column attached. The contribution of mass transfer effects was evaluated with the aid of D_i and k_f at the various temperatures. Any dispersion effects were neglected. The data fit, showed in Fig. 7, indicated that 67% of the ligand sites were active and accessible, *i.e.*, $Q_{max} = 3.9 \cdot 10^{-4}$ mol kg^{-1} gel (59 mg g^{-1} gel). In other words, we were able to bind successfully large amounts of monoclonal antibody in an active state and as a result we were able to monitor the weak affinities at 45 and 50°C. The association rate constant (k_a) was in the range $0.4 \cdot 10^3$–$2.7 \cdot 10^3$ l mol^{-1} s^{-1} and is apparently much less than that in high-affinity reactions where the on-reaction is often diffusion controlled and amounts

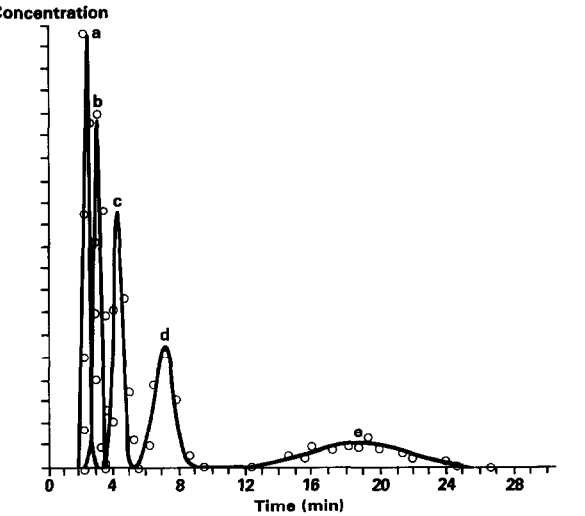

Fig. 7. Fitting of mathematical model to experimental data. The experimental points are given at temperatures of (e) 10, (d) 25, (c) 37, (b) 45 and (a) 50°C together with the theoretical data-fit curve. Parameters as in Fig. 3 are valid for each curve except: K_L = (e) $16 \cdot 10^3$, (d) $5.2 \cdot 10^3$, (c) $2.5 \cdot 10^3$, (b) $1.3 \cdot 10^3$ and (a) $0.8 \cdot 10^3$ l mol^{-1}; k_a = (e) $1.0 \cdot 10^3$, (d) $2.7 \cdot 10^3$, (c) $2.0 \cdot 10^3$, (b) $1.2 \cdot 10^3$ and (a) $0.4 \cdot 10^3$ l mol^{-1} s^{-1}; D_i = (e) $0.7 \cdot 10^{-6}$, (d) $1.0 \cdot 10^{-6}$, (c) $1.4 \cdot 10^{-6}$, (b) $1.7 \cdot 10^{-6}$ and (a) $1.8 \cdot 10^{-6}$ cm^2 s^{-1}; k_f = (e) $3.0 \cdot 10^{-2}$, (d) $4.1 \cdot 10^{-2}$, (c) $5.0 \cdot 10^{-2}$, (b) $5.6 \cdot 10^{-2}$ and (a) $6.0 \cdot 10^{-2}$ cm s^{-1}.

to *ca.* 10^6 l mol^{-1} s^{-1}. The desorption rate constant (k_d) was 0.5 s^{-1} at $K_L = 0.8 \cdot 10^3$ l mol^{-1} and decreased to 0.06 s^{-1} at a moderate affinity of $K_L = 1.6 \cdot 10^4$ l mol^{-1}. It might be concluded that in high-affinity chromatography the speed of reaction is characterized by high on-rates (in many instances diffusion is the rate-limiting step) and very low off-rates, whereas a weak-affinity system shows moderate to high on/off rate-constants.

CONCLUSIONS

The approach to use weak interactions in affinity chromatography is new and only a few applications have been reported, mainly in the area of carbohydrate antigens binding to various monoclonal antibodies [6–8,10]. In this study, we attempted to use computer simulation as a tool to provide a more adequate knowledge of the potential of WAC. Well established mathematical models to describe both linear and non-linear chromatography were applied to the weak-affinity approach and valuable information was extracted. Important characteristics such as kinetic constants were obtained by fitting theoretical curves to experimental data.

This study has demonstrated that a number of parameters, including amount of ligand, selection of support media and kinetics, are critical to the proper design of a successful WAC system. In addition, the choice of injection and elution conditions establishing the state of linearity is of significant importance in performing weak-affinity. In this context, it is worth mentioning that temperature, long forgotten as a means for elution in traditional affinity chromatography, could be a valuable tool for WAC. Basically there is no theoretical limit on how weak-affinity can be to be useful for WAC. However, a practical limit is set by the appearance of non-specific adsorption and difficulties in ligand design and attachment. Generally we feel that the practical limit will be in the range $K_L = 10$–100 l mol^{-1}.

The rapid developments in gene technology and peptide synthesis will provide us with the necessary tools to perform a successful WAC to separate and analyze almost any kind of molecule. In the near future, WAC may find applications in analytical affinity chromatography for accurate monitoring directly from crude samples of closely similar solutes such as steroid hormones, pesticides and drug metabolites. It should also be of interest to use WAC for preparative purposes, especially as the mild elution techniques can enhance the recovery of fragile biomolecules. Finally, WAC can become an important research tool to understand more fully the nature of weak interactions in biological processes. For example, it may offer a means of defining the specific targets on cellular surfaces for various activator and inhibitor molecules.

ACKNOWLEDGEMENTS

The authors thank Dr. Peter Carr and Dr. Jim Wade for providing the algorithm for the Bessel integrals.

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